# ANIMAL REPRODUCTION

Official journal of the Brazilian College of Animal Reproduction

**v.14, n.3** **July/September** **2017**


# Contents

**Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Cabo de Santo Agostinho, PE, Brazil, August 17th to 19th, 2017, and 33rd Meeting of the Association of Embryo Transfer in Europe (AETE); Bath, United Kingdom, September 8th and 9th, 2017**



**Conferences papers**

## 33rd Meeting of the Association of Embryo Transfer in Europe (AETE)

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From the SBTE President

Dear colleagues

It is with great joy that I welcome you to the 31th Annual Meeting of the Brazilian Embryo Technology Society (SBTE-2017) at the Sheraton Reserva do Paiva Resort & Convention, Cabo de Santo Agostinho – Pernambuco, Brazil. In addition, it is a great honor to present these proceedings, our third joint publication with the Association of Embryo Technology in Europe (AETE). We thank all AETE directors, members and authors for contributing for the scientific quality of these proceedings. I reinforce my strong believe that this partnership will last long and be very fruitful for both societies.

My special thanks to our Scientific Committee (Drs. Paula Papa, Felipe Perecin and José Eduardo Santos), who put together a fantastic group of speakers this year, generating an outstanding program covering cutting edge basic and applied topics related to embryo production and fertility. Many thanks also to all speakers for their time and generosity in sharing their knowledge with us. I hope you all enjoy the talks and respective papers, through which our society hopes to expand our vision about the biological processes and new technologies relevant to embryo production and fertility control. And I do hope that these opportunities can not only increase our knowledge, but also inspire thinking and questioning, that can contribute to evolve the strategies for embryo production and fertility control towards real economical and social gains.

Many thanks also to the authors of the abstracts for choosing our meeting to present their work, as well as to the students and researchers participating in our competitions. The Poster Session and the Student Competition are major events in our program that have greatly contributed for the formation of new scientists and exchange of ideas. Very special thanks to the coordinators and reviewers of the abstract reviewing process. Their efforts are priceless.

The scientific quality of our meeting requires investment. We immensely thank our public (CNPq, CAPES, FAPESP) and private sponsors, particularly the partner companies of the SBTE condominium, for providing funds without which this meeting would not be possible.

Finally but not less importantly, I heartily thank all SBTE directors and secretariat that made this meeting happen (This team of hard workers and great friends is really incredible. It has been much more than a privilege.), and all SBTE members who have supported our society financially and ideologically through the last 31 years.

Very best wishes,

**José Buratini**
SBTE President (2016-2017)

**Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Cabo de Santo Agostinho, PE, Brazil, August 17th to 19th, 2017, and 33rd Meeting of the Association of Embryo Transfer in Europe (AETE); Bath, United Kingdom, September 8th and 9th, 2017**

From the President of AETE

Dear Colleagues,

We are pleased to announce the 33rd Annual Scientific Meeting of the European Embryo Transfer Association (AETE), which will be held in the World Heritage City of Bath, southwest England, from the 8th to the 9th of September 2017.

This is the third year of a joint publication between AETE and SBTE in a special issue of Animal Reproduction and I would like to thank José Buratini Jn, President of SBTE, Felipe Perecin, Co-Chair of the SBTE scientific committee, Urban Besenfelder, AETE Secretary and the Editorial Board of Animal Reproduction. This collaboration has already gone further by sharing scientific and practical knowledge from both societies through a vice versa participation of an active member from each society with a plenary talk in the annual meetings every year. This year Dr. Jo Leroy will represent AETE to the SBTE annual meeting in Cabo de Santo Agostinho, while Dr. Jose Buratini Jnr. will be represent SBTE to the AETE annual meeting in Bath, U.K. Looking forward to both events.

The scientific program put together by the board of AETE is very inspiring covering most interests of our members. Dr. Jose Buratini Jn, Universidade Estadual Paulista (UNESP) will talk about "Follicular Environment and Oocyte Maturation", while Dr. Ann Van Soom from University of Gent, Belgium as a leader of the Cost Action-Epiconcept, will give an update on "Cost-Epiconcept: What we have learned and application for the future". Dr. Martin Sheldon, from Swansea University Medical School, U.K., will give an overview on "Uterine infection and immunity" followed by Dr. Heiner Bollwein from University of Zurich, Switzerland on "Effects of nutritional programming on sexual development in bulls". We will have 14 short oral communications including the student competition and two workshops. The first workshop will be managed by Jan Detterer, AI- and ET-Center Georgsheil, Germany and Pasqualino Loi, University of Teramo, Italy on "Micromanipulation", while the second will be leaded by Erik Mullaart, CRV, The Netherlands on "Selection and treatment of animals for embryo production". This year we have over 80 accepted abstracts to be presented as posters.

It is a great honor for all board members and for me personally to present Prof. Cesare Galli as the 2017 AETE Medallist. Prof. Galli is founder and Managing Director of Avantea srl, Cremona, Italy and also an Associated Professor of Animal Reproduction at the University of Bologna, Italy. He developed numerous scientific interests in reproductive biotechnologies from *in vitro* embryo production to embryo manipulation and animal cloning by somatic cell nuclear transfer, in a range of different species such as cattle, horse and pig. His scientific contributions are recognized internationally. Prof. Galli has been President of the AETE from 1997 to 2000.

As part of this year's AETE meeting in Bath the LOC have organised a Pre-conference "Practitioner Repro Day" on Thursday 7th of September. The day consists of a series of workshops in the morning, Flushing, OPU, Embryology and Embryo freezing followed by IVF and lectures by Dr. John Hasler in reproductive technologies and genomics in the afternoon.

Every annual meeting of our society depends on the effort of the local organizing committee, this year headed by Brian Graham, E.G.G. Technologies International, U.K. and his colleagues and the support of our sponsors (until 26th of July 2017): Main: [VETOQUINOL]; General: [*EGG TECH*]; Exhibitors: [*BCF Technology Ltd*] [*ECM*] [*ICP Reproduction/Bodinco BV*] [*IMV Technologies*] [*IVF*] [*IVF Technologies*] [*IVF Biosciences*] [*Professional Embryo Transfer*]; Supporters: [*Calier*] [*CryoLogic Ltd*] to whom we are deeply grateful.

Last but not least a great thank to all AETE Board members for their support through the year turning this meeting into a great event for all our members.

We look forward to seeing you in Bath.

**Dimitrios Rizos**, PhD
AETE President

**Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Cabo de Santo Agostinho, PE, Brazil, August 17th to 19th, 2017, and 33rd Meeting of the Association of Embryo Transfer in Europe (AETE); Bath, United Kingdom, September 8th and 9th, 2017**

From the Chairs of the Scientific Committee

Greetings and welcome to the 31st Annual Meeting of the Brazilian Society for Embryo Technology (SBTE). This is our third joint proceedings with the Association of Embryo Technology in Europe (AETE) that held the 33rd Annual Meeting on September 8 and 9 in Bath, United Kingdom. We express our appreciation to the AETE Board for this opportunity of continuing scientific collaboration and would like to highlight the commitment of Dr. Urban Besenfelder, who shared the editorial duties and responsibilities of these joint proceedings.

This special issue of Animal Reproduction contains the full papers from invited speakers of both societies' annual meetings. For the SBTE meeting held on August 17 to 19 in the city of Cabo de Santo Agostinho, Brazil, the program included an opening session, three plenary sessions and four concurrent basic and applied sessions featuring nineteen world-class invited speakers. We thank all the speakers for their commitment and effort in preparing the scientific review articles, their oral presentations during the annual meeting, and for their kind willingness to comply with our deadlines.

The opening session included an update on embryo production activity worldwide by Daniel Salamone, the current vice-president of the International Embryo Transfer Society. We organized a program that covered different aspects of the ever-progressing field of embryo-related biology and reproductive biotechnologies. The presentations encompassed applied aspects and new knowledge, covering themes from the effect of maternal metabolic disorders on reproduction to the effects of cellular metabolic disruption during *in vitro* culture of gametes and embryos. The program included topics related to the integration of big-data to understanding of animal reproduction, animal welfare, follicle development, cryopreservation, fetal programming, sperm selection techniques, and reproductive programs to enhance fertility in cattle.

The program was complemented by 2 high-quality independently coordinated workshops. We are thankful to Marcelo Nogueira and Edmundo Vilela who dedicated time and resources to elaborate on topics on cattle fertility and wide-ranging discussions on development and application of reproductive technologies in cattle production to the SBTE annual meeting.

We received 279 abstracts. We would like to express our gratitude to the coordinators of the abstracts' sessions, to the reviewers of abstracts, and to the colleagues who evaluated the abstracts for the competitions and awards. A team of reviewers listed in these proceedings carefully reviewed all manuscripts published herein. Thank you all for your time and effort dedicated to the review process.

We are also very thankful to all members of the SBTE board. They worked tirelessly to make this meeting possible.

On behalf the SBTE and the AETE we acknowledge the dedication of Maria Helena Chaves da Silva that made editorial corrections and formatting of manuscripts. We also express our gratitude to the editorial board and staff of the Animal Reproduction Journal for their support and collaboration in organizing and publishing the joint proceedings of the annual meetings of the Brazilian Society for Embryo Technology and the Association of Embryo Technology in Europe.

Finally, we thank you all for attending the annual meeting.

**Felipe Perecin**
**Paula de Carvalho Papa**
**José Eduardo Santos**
Co-Chairs of the SBTE Scientific Committee (2016-2017)

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# The role of L-carnitine during oocyte *in vitro* maturation: essential co-factor?

**Kylie R. Dunning[1,2,3,4], Rebecca L. Robker[3]**

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## Abstract

*In vitro* maturation (IVM) of oocytes is a promising technology for both the treatment of human infertility and in animal production as a means of improving genetic gain. However, IVM derived oocytes remain inferior to those matured *in vivo* with reduced developmental potential. The environment in which an oocyte matures *in vitro* is vastly different to *in vivo* where maturation takes place within the ovarian follicle. The *in vitro* environment differs in oxygen concentration, exposure to light, and metabolite composition of culture media *vs*. follicle fluid, to name a few. Human follicle fluid contains the metabolite L-carnitine and has shown to be associated with human fertility. L-carnitine has known biological functions as an essential co-factor for beta-oxidation, regulating ATP production from lipids, and as a potent antioxidant. Importantly, it appears that cumulus cells and the oocyte lack the machinery to synthesize L-carnitine *de novo*. The inability for local production of L-carnitine during IVM and its importance in human fertility warrants investigation of its affects during IVM. The potential to improve oocyte quality by inclusion of L-carnitine in the culture media thus increasing the capacity for beta-oxidation and/or antioxidant activity of the culture media is receiving increased attention. This review summarizes studies to date investigating the developmental importance of L-carnitine during IVM and the mechanisms by which improved developmental potential is elicited. Overall, the inclusion of L-carnitine during IVM of several species results in improved oocyte quality with increased development to blastocyst. This is likely due to the antioxidant capacity of L-carnitine and its ability to increase ATP production from intracellular lipid stores.

**Keywords:** antioxidant, beta-oxidation, *in vitro* maturation, L-carnitine, oocyte,

## Introduction

The final stages of oocyte maturation *in vivo* occur once folliculogenesis has reached the antral stage. The periovulatory antral follicle is capable of responding to the LH-surge from the pituitary which initiates the ovulatory cascade. The LH-surge acts on the granulosa cells of the follicle which transmit the ovulatory signal to the cumulus oocyte complex (COC) via secretion of EGF-like peptides. This initiates nuclear and cytoplasmic maturation in the oocyte and the cumulus cells to undergo expansion via the production of vast amounts of extracellular matrix. Following this, ovulation ensues with the COC then picked up by the fimbria of the oviduct and transported into the oviduct where fertilization can occur. Following fertilization, meiosis is completed and the one-cell zygote develops to the blastocyst stage whilst transcending through the oviduct to the uterus where the blastocyst embryo implants and pregnancy commences.

The ability of a zygote to successfully complete preimplantation embryo development, implant in the uterus and develop into a healthy offspring is dependent on the quality, or developmental competence, of the oocyte. The environment in which the oocyte matures *in vivo* is modified by maternal diet and metabolic status, which can negatively affect oocyte quality and the health of the offspring (Lane *et al*., 2014). Similarly, *in vitro* maturation of oocytes occurs in a sub-optimal environment drastically different from that which occurs *in vivo*. For *in vitro* maturation the COC is removed from the follicle prior to the LH-surge and the final stages of maturation occur *in vitro* in the absence of the follicle and follicular fluid.

Oocyte *in vitro* maturation (IVM) is a potentially useful assisted reproductive technology both clinically and in animal production (Gilchrist and Thompson, 2007). Developmental potential of IVM oocytes following fertilization is however, lower than their *in vivo* matured counterparts (Rizos *et al*., 2002; Gilchrist and Thompson, 2007). Oocytes matured *in vitro* have lower rates of development to the blastocyst stage and have increased rates of miscarriage (Buckett *et al*., 2008). The direct mechanisms responsible for the poorer oocyte quality are the topic of continued investigation and not as yet, clear. As discussed above, the environment in which the oocyte matures *in vitro* is dramatically different to *in vivo* maturation. *In vitro* maturation differs in the exposure to light, oxygen concentration and different metabolite composition of culture media *vs*. follicular fluid, among many other factors.

The composition of the IVM culture media is known to affect COC metabolism and metabolic rate (reviewed in Brown *et al*., 2017). Further, oocyte developmental competence is affected by metabolism with generation of sufficient levels of ATP required for oocyte growth, meiosis, fertilization and the early stages

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of preimplantation embryo development (Van Blerkom *et al*., 1995). The metabolism of carbohydrates, glucose, pyruvate and lactate for the production of ATP, have been well characterized in the COC (Sutton *et al*., 2003; Brown *et al*., 2017). Metabolism of fatty acids via beta-oxidation is a potent source of energy, several fold more energy dense than glucose, and the developmental importance of this form of metabolism for the COC is gaining recognition. The use of oocyte intracellular stores for energy production during IVM is of particular interest in species with high levels of stored lipid such as bovine, sheep and porcine (reviewed in Dunning *et al*., 2014b). Even in species with comparatively low levels of oocyte lipid (mouse and human; Dunning *et al*., 2014b), beta-oxidation during oocyte maturation appears essential for subsequent developmental competence (Dunning *et al*., 2010, 2011, 2014b) Beta-oxidation of fatty acids requires the essential co-factor L-carnitine to shuttle activated fatty acids from the cytosol into mitochondria. The addition of L-carnitine to the *in vitro* culture of cell lines in known to increase beta-oxidation (Nada *et al*., 1995; Huynh *et al*., 2014). L-carnitine also acts as a powerful antioxidant shown in other *in vitro* systems to quench reactive oxygen species (ROS) and in turn protect cells from oxidant injury (Ye *et al*., 2010). Oocytes matured *in vitro* are exposed to increased levels of ROS (reviewed in Combelles *et al*., 2009), thus inclusion of L-carnitine may be of benefit. The role of L-carnitine during IVM has gained interest in recent years as evidenced by a growing body of literature. This review will highlight studies utilizing L-carnitine during IVM, the resultant impact on oocyte quality and the mechanism(s) by which it acts.

## Carnitine and fertility

Carnitine is a naturally occurring quaternary amine with the L-stereoisomer (3R)-3-hydroxy-4-(trimethylazaniumyl)butanoate having potent bioactivity. Synthesis of carnitine from lysine and methionine occurs mainly in the liver with highest levels found in skeletal muscle and heart (Vaz and Wanders, 2002). Dietary sources high in carnitine include meat and dairy (Rebouche, 1992). L-carnitine is essential for the transport of activated long chain fatty acids from the cytoplasm into mitochondria where, via beta-oxidation, ATP can be generated. Carnitine is also responsible for the transfer of peroxisomal beta-oxidation metabolites to the mitochondria for completion of oxidation to $CO_2$ and $H_2O$ via the TCA cycle (reviewed in Vaz and Wanders, 2002). Other functions of carnitine include the maintenance of the acyl-CoA:CoA ratio and as a means of storing cellular energy in the form of acetylcarnitine (Vaz and Wanders, 2002). Additionally, L-carnitine has been used during *in vitro* culture and animal studies as a scavenger of free radicals, in turn protecting antioxidant enzymes from oxidant injury (Bremer, 1983; Sener *et al*., 2004; Gulcin, 2006; Kolodziejczyk *et al*., 2011).

Carnitine is present in human follicular fluid (Dunning and Robker, 2012; Montjean *et al*., 2012; Valckx *et al*., 2012; Varnagy *et al*., 2013; Zhao *et al*., 2015; Giorgi *et al*., 2016). While follicular fluid from these studies was sampled following ovulation induction, we can infer from the chemical properties of carnitine and its presence in serum, that carnitine would be present during oocyte maturation *in vivo*. In relation to oocyte quality and human fertility, it was shown that decreased carnitine (total and free) and acylcarnitine metabolites in follicular fluid was associated with increased numbers of oocytes (>9) and embryos (>6) from women undergoing IVF (Varnagy *et al*., 2013). We can speculate that decreased levels of carnitine and acylcarnitines were associated with improved reproductive potential as a result of their increased utilization via beta-oxidation during oocyte maturation (Varnagy *et al*., 2013). A similar association was not seen in in a separate study, however, only total carnitine was measured and compared with pregnant and non-pregnant cycles (Montjean *et al*., 2012). Replication of the association between carnitine (total and free) and the acylcarnitine metabolites with oocyte quality and fertility outcomes, in particular live birth, would be of interest.

Current evidence suggests that cumulus cells and oocytes do not have the capacity to biosynthesize L-carnitine from precursor amino acids as they lack the metabolic machinery (Montjean *et al*., 2012). This study examined human cumulus cells and oocytes at the end of maturation and only mRNA for two of the enzymes in the biosynthesis pathway. Whether regulation of these genes or the presence of the enzymes themselves occurs earlier in maturation of COCs is yet to be determined.

The potential developmental importance of carnitine and its acylcarnitine derivatives for oocyte quality following *in vivo* maturation of human oocytes and the apparent inability of COCs to synthesize carnitine points to the importance of investigating the role of carnitine during *in vitro* maturation. There is a growing number of studies that have investigated the role of L-carnitine during IVM in various species including mouse (Dunning *et al*., 2010, 2011; Paczkowski *et al*., 2014), bovine (Ferguson and Leese, 2006; Phongnimitr *et al*., 2013; Giorgi *et al*., 2016; Sovernigo *et al*., 2017), porcine (Hashimoto, 2009; Somfai *et al*., 2011; Wu *et al*., 2011; You *et al*., 2012) and sheep (Reader *et al*., 2015; Mishra *et al*., 2016a). The effects of L-carnitine on oocyte quality and the associated mechanism(s) are detailed in the following sections.

## Importance of beta-oxidation and its modulation by L-carnitine during IVM

There is a growing body of literature describing the importance of fatty acid beta-oxidation as a source of energy during oocyte IVM (Ferguson and Leese, 2006; Sturmey *et al*., 2006; Downs *et al*., 2009; Dunning *et al*., 2010; Dunning and Robker, 2012; Valsangkar and Downs, 2013; Dunning *et al*., 2014b; Paczkowski *et al*., 2014). This is not surprising when one considers the energy dense nature of fatty acids with the potential to produce 106 ATP molecules from a

single fatty acid, greater than 3.5-fold the capacity of a single glucose molecule.

The metabolism of fatty acids for the production of ATP occurs within mitochondria. This requires entry of activated fatty acids into the mitochondrial matrix, a process catalyzed by carnitine palmitoyl-transferase 1 (CPT1) and also requiring carnitine. CPT2 then removes carnitine from the fatty acid which can then enter the mitochondrial matrix for catabolism via beta-oxidation (McGarry and Brown, 1997).

Beta-oxidation appears to be important for oocyte nuclear maturation; meiotic maturation to metaphase II and extrusion of the first polar body (Downs *et al*., 2009; Valsangkar and Downs, 2013; Paczkowski *et al*., 2014). Further, it appears that beta-oxidation is important in the acquisition of oocyte developmental competence (reviewed in McKeegan and Sturmey, 2011; Dunning *et al*., 2014b). Of particular interest here are studies demonstrating a reduction in beta-oxidation in both the oocyte and cumulus cell compartments of the COC when maturation occurs *in vitro* (Lee *et al*., 2011; Dunning *et al*., 2014a), demonstrating the importance of the ovarian follicular environment in appropriate regulation and/or the supply of metabolic factors that support this metabolic pathway (including supply of carnitine). The expression of genes involved in the beta-oxidation pathway are dysregulated in non-human primate cumulus cells and mouse COCs that have undergone maturation *in vitro* compared to *in vivo* maturation (Lee *et al*., 2011; Dunning *et al*., 2014a). In functional assays, both mouse and cat oocytes have decreased levels of beta-oxidation compared to those matured *in vivo* (Spindler *et al*., 2000; Dunning *et al*., 2014a). Specifically, we have shown that the rate of beta-oxidation in mouse COCs is significantly reduced, more than fifty percent, during IVM (Dunning *et al*., 2014a).

The importance of beta-oxidation is further exemplified in studies using inhibitors of this pathway during *in vitro* maturation. These studies demonstrate a requirement for beta-oxidation in nuclear maturation (Downs *et al*., 2009; Valsangkar and Downs, 2013; Paczkowski *et al*., 2014; Sanchez-Lazo *et al*., 2014), as described above, and in the acquisition of oocyte developmental competence in mouse, bovine and porcine oocytes (Ferguson and Leese, 2006; Sturmey *et al*., 2006; Dunning *et al*., 2010; Paczkowski *et al*., 2014).

We and others have shown that supplementation of IVM culture media with L-carnitine significantly increases beta-oxidation in mouse COCs (Dunning *et al*., 2010; Valsangkar and Downs, 2013). The inclusion of L-carnitine during maturation also results in a significant increase in oocytes reaching metaphase II in mouse (Dunning *et al*., 2011) and porcine (Somfai *et al*., 2011) and significantly more blastocyst embryos following fertilization (Dunning et al., 2010). The inclusion of L-carnitine has also been shown to increase oocyte mitochondrial activity in mouse (Wu *et al*., 2012), bovine (Hashimoto, 2009), and porcine (Somfai *et al*., 2011) and decrease intracellular lipid stores in porcine oocytes (Somfai *et al*., 2011). Further, our work has demonstrated the ability for L-carnitine to facilitate metabolism of intracellular lipid stores in preimplantation mouse and bovine embryos, significantly improving development (Dunning *et al*., 2010; Sutton-McDowall *et al*., 2012). Collectively, these studies illustrate the ability of L-carnitine to upregulate beta-oxidation during IVM and utilize intracellular lipid stores. The beneficial effects of L-carnitine supplementation on oocyte quality can at least be partially ascribed to its effects on stimulating fatty acid beta-oxidation. The role of supplying exogenous fatty acid to IVM for utilization via beta-oxidation, at least in the mouse, appears detrimental (Paczkowski *et al*., 2014). This may be due to the concentration or type of fatty acid used in this study. Elucidation of the oocytes preferential fatty acid type(s) and concentration and the detrimental effects of excess lipid in relation to oocyte developmental competence, is ongoing in a number of models and agriculturally important species (reviewed in Dunning *et al*., 2014b).

**Anti-oxidant capacity of L-carnitine during IVM**

Reactive oxygen species (ROS) are naturally formed during cellular metabolism, but kept in check by enzymatic and non-enzymatic antioxidants. If the production of ROS outweighs the cells antioxidant capacity this leads to oxidative stress (cellular damage via lipid peroxidation, DNA and protein damage). The process of *in vitro* culture itself generates increased oxidative stress, in addition to that generated from cellular metabolism, by exposure of cells to light, increased oxygen concentration and atmospheric conditions during handling (reviewed in Combelles *et al*., 2009). Specifically, *in vitro* maturation of oocytes has been shown to result in excessive production of ROS compared to *in vivo* matured oocytes (Banwell *et al*., 2007; Zeng *et al*., 2014). Further, the *in vitro* matured oocytes may be less equipped to attenuate deleterious effects of increased ROS compared to those matured *in vivo* (Uppangala *et al*., 2015). Collectively, this may contribute to the poorer developmental competence of IVM derived oocytes.

*In vitro* assays have demonstrated the ability of L-carnitine to act as an effective scavenger of superoxide anions, hydrogen peroxide and inhibit lipid peroxidation (Gulcin, 2006). The anti-oxidant property of L-carnitine is supported by *in vitro* studies demonstrating its capacity to prevent oxidant injury in various somatic cells (Silva-Adaya *et al*., 2008; Ribas *et al*., 2010). Thus, investigation of L-carnitines capacity to scavenge free radicles during IVM and its resultant effect on oocyte quality warrants investigation.

There are a number of studies demonstrating a link between inclusion of L-carnitine during IVM on reduced intracellular ROS and increased antioxidant enzymes within the oocyte of a number of species including mouse (Zare *et al*., 2015), bovine (Sovernigo *et al*., 2017), porcine (Somfai *et al*., 2011; You *et al*., 2012) and sheep (Mishra *et al*., 2016b). IVM of mouse oocytes in the presence of carnitine decreased ROS and increased the antioxidant glutathione (GSH) within

oocytes and was associated with significantly increased rates of maturation to MII, cleavage and blastocyst embryos, but no change in blastocyst cell number (Zare *et al*., 2015). Similarly, the inclusion of L-carnitine during bovine IVM, in addition to reduced oxidative stress, resulted in significantly more blastocyst embryos with these embryos containing significantly increased number of cells (Sovernigo *et al*., 2017). Decreased ROS and increased GSH within porcine oocytes matured with L-carnitine also resulted in significantly improved oocyte developmental competence with increased rates of blastocysts following parthenogenetic activation (Wu *et al*., 2011; You *et al*., 2012) or somatic cell nuclear transfer (You *et al*., 2012).

Challenging the *in vitro* maturation environment by inclusion of hydrogen peroxide ($H_2O_2$) or TNFα during oocyte maturation *in vitro* leads to a significant reduction in the number of mature oocytes and the number of blastocyst embryos post fertilization in both sheep and pig (Yazaki *et al*., 2013, Mishra *et al*., 2016a, b). The inclusion of L-carnitine has shown to reverse the effects of these exogenous insults on nuclear maturation (Yazaki *et al*., 2013; Mishra *et al*., 2016a) and capacity of the fertilized zygote to develop to blastocyst (Yazaki *et al*., 2013; Mishra *et al*., 2016a, b). The ability of L-carnitine to attenuate the detrimental effects of $H_2O_2$ during IVM has been shown to be associated with a reduction in ROS and increase in intracellular GSH within oocytes (Mishra *et al*., 2016b). To investigate the impact of increased ROS in human follicular fluid on oocyte quality following maturation, one study matured mouse oocytes in the presence of control follicular fluid and follicular fluid from women with endometriosis and suffering infertility (Giorgi *et al*., 2016). Oocyte maturation in the presence of follicular fluid from women with endometriosis resulted in a significant increase in meiotic spindle abnormalities that were reversed to control levels when 0.6 mg/ml L-carnitine was included in the maturation media (Giorgi *et al*., 2016).

Decreased oxidative stress and improved oocyte developmental competence in response to L-carnitine has been associated with increased expression of cell cycle machinery in mouse oocytes (Zare *et al*., 2015); however the activity of these factors are regulated at the level of phosphorylation which was not investigated, likely due to the number of oocytes required to perform such experiments being prohibitive. A direct causal link between L-carnitine treatment and cell cycle machinery via carnitine's anti-oxidant capacity seems unlikely. Similarly, in porcine oocytes decreased ROS and improved developmental competence was associated with increased expression of genes involved in nuclear reprogramming (You *et al*., 2012) but again, a causal link between these pathways was not determined.

## Effects of L-carnitine during IVM on the cryotolerance of oocytes

Oocyte cryopreservation would preserve fertility for women undergoing treatment for cancer and be useful in avoiding the effects of aging by preserving oocytes earlier in life. While human and mouse MII oocytes have been successfully used following cryopreservation, MII oocytes are susceptible to cryoinjury including spindle damage (Gomes *et al*., 2008; Huang *et al*., 2008). Cryopreservation of GV oocytes would be advantageous as they are not susceptible to damage inflicted by cryopreservation due to the chromosomes being at interphase, and utilizing oocytes at this stage would avoid the need for ovarian hyperstimulation and its associated risks. However, cryopreserved GV oocytes are developmentally compromised with significantly reduced number of blastocysts post-thawing and fertilization (Aono *et al*., 2003, 2005). Cryopreservation of oocytes in agriculturally important species also has advantages with the ability to bank oocytes from genetically important animals. However, cryopreserved bovine oocytes have poorer developmental competence on thawing. Supplementation of IVM and/or vitrification media with L-carnitine has resulted improvements in oocyte quality in mouse GV oocytes (Moawad *et al*., 2013, 2014) and bovine MII oocytes following cryopreservation and thawing (Spricigo *et al*., 2017). Specifically, the inclusion of L-carnitine during IVM and vitrification of mouse GV oocytes significantly increased the number of blastocyst embryos, comparable to the number of blastocysts developed from oocytes that were not vitrified or matured *in vivo* and vitrified (Moawad *et al*., 2013, 2014). L-carnitine treatment during IVM and vitrification was associated with improved meiotic spindle formation and distribution and increased activity of mitochondria (Moawad *et al*., 2014).

Conflicting evidence is seen in studies investigating the effects of L-carnitine on bovine oocyte developmental competence post vitrification/thawing. L-carnitine inclusion during bovine IVM has been shown to result in significantly increased numbers of blastocysts following cryopreservation and thawing of oocytes, which was associated with redistribution of lipid within oocytes (Chankitisakul *et al*., 2013). In contrast, Phongnimitr *et al*. (2013) showed that L-carnitine supplementation during IVM significantly improved subsequent development to blastocyst, but this effect was lost following vitrification. Similarly, inclusion of L-carnitine during IVM of prepubertal bovine oocytes had no beneficial effect on oocyte developmental competence post thawing (Spricigo *et al*., 2017). Thus, further research is required to determine the effects of L-carnitine on bovine oocyte cryotolerance.

## Summary and future directions

There is mounting evidence in the literature that L-carnitine has beneficial effects during the *in vitro* maturation of oocytes. The mechanism by which L-carnitine improves oocyte quality is likely to be via both increased beta-oxidation and reducing oxidative stress via is ability to scavenge free radicals. Future studies could interrogate the relative contribution of these

actions to improved oocyte developmental competence.

Differential effects of L-carnitine in various studies may be a result of different animal strains/breeds used and different laboratory practices, including culture media composition or the use of fully grown oocytes or oocytes collected from smaller follicles or prepubertal animals.

Based on the studies discussed here, future improvements in IVM culture media may benefit from the inclusion of L-carnitine. However, as promotion of fatty acid beta-oxidation impacts glucose metabolism in the COC, it is important to consider the appropriate balance between these metabolic pathways (Paczkowski *et al*., 2014), and utilization of *in vivo* maturation as the *gold standard* would assist in optimization.

As yet, there are no studies demonstrating impact of L-carnitine during IVM on offspring health. Use of L-carnitine during mouse preimplantation embryo development *in vitro* demonstrates safety (Truong *et al*., 2016). However, the effect of L-carnitine supplementation specifically during IVM on offspring health is yet to be studied in any species. Demonstration of safety in several mouse strains and animal models would be advisable before implementation in the fertility clinic.

## References


**Aono N, Naganuma T, Abe Y, Hara K, Sasada H, Sato E, Yoshida H**. 2003. Successful production of blastocysts following ultrarapid vitrification with step-wise equilibriation of germinal vesicle-stage mouse oocytes. *J Reprod Dev*, 49:501-506.

**Aono N, Abe Y, Hara K, Sasada H, Sato E, Yoshida H**. 2005. Production of live offspring from mouse germinal vesicle-stage oocytes vitrified by a modified stepwise method, SWEID. *Fertil Steril*, 84(suppl. 2):1078-1082.

**Banwell KM, Lane M, Russell DL, Kind KL, Thompson JG**. 2007. Oxygen concentration during mouse oocyte in vitro maturation affects embryo and fetal development. *Hum Reprod*, 22:2768-2775.

**Bremer J**. 1983. Carnitine--metabolism and functions. *Physiol Rev*, 63:1420-1480.

**Brown HM, Dunning KR, Sutton-McDowall M, Gilchrist RB, Thompson JG, Russell DL**. 2017. Failure to launch: aberrant cumulus gene expression during oocyte in vitro maturation. *Reproduction*, 153:R109-R120.

**Buckett WM, Chian RC, Dean NL, Sylvestre C, Holzer HE, Tan SL**. 2008. Pregnancy loss in pregnancies conceived after in vitro oocyte maturation, conventional in vitro fertilization, and intracytoplasmic sperm injection. *Fertil Steril*, 90:546-550.

**Chankitisakul V, Somfai T, Inaba Y, Techakumphu M, Nagai T**. 2013. Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine in vitro matured oocytes. *Theriogenology*, 79:590-598.

**Combelles CM, Gupta S, Agarwal A**. 2009. Could oxidative stress influence the in-vitro maturation of oocytes? *Reprod Biomed Online*, 18:864-880.

**Downs SM, Mosey JL, Klinger J**. 2009. Fatty acid oxidation and meiotic resumption in mouse oocytes. *Mol Reprod Dev*, 76:844-853.

**Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL**. 2010. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol Reprod*, 83:909-918.

**Dunning KR, Akison LK, Russell DL, Norman RJ, Robker RL**. 2011. Increased beta-oxidation and improved oocyte developmental competence in response to L-carnitine during ovarian in vitro follicle development in mice. *Biol Reprod*, 85:548-555.

**Dunning KR, Robker RL**. 2012. Promoting lipid utilization with l-carnitine to improve oocyte quality. *Anim Reprod Sci*, 134:69-75.

**Dunning KR, Anastasi MR, Zhang VJ, Russell DL, Robker RL**. 2014a. Regulation of fatty acid oxidation in mouse cumulus-oocyte complexes during maturation and modulation by PPAR agonists. *PloS One*, 9:e87327.

**Dunning KR, Russell DL, Robker RL**. 2014b. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. *Reproduction*, 148:R15-27.

**Ferguson EM, Leese HJ**. 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Mol Reprod Dev*, 73:1195-1201.

**Gilchrist RB, Thompson JG**. 2007. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology*, 67:6-15.

**Giorgi VS, Da Broi MG, Paz CC, Ferriani RA, Navarro PA**. 2016. N-acetyl-cysteine and L-carnitine prevent meiotic oocyte damage induced by follicular fluid from infertile women with mild endometriosis. *Reprod Sci*, 23:342-351.

**Gomes CM, Silva CA, Acevedo N, Baracat E, Serafini P, Smith GD**. 2008. Influence of vitrification on mouse metaphase II oocyte spindle dynamics and chromatin alignment. *Fertil Steril*, 90:1396-1404.

**Gulcin I**. 2006. Antioxidant and antiradical activities of L-carnitine. *Life Sci*, 78:803-811.

**Hashimoto S**. 2009. Application of in vitro maturation to assisted reproductive technology. *J Reprod Dev*, 55:1-10.

**Huang JY, Chen HY, Park JY, Tan SL, Chian RC**. 2008. Comparison of spindle and chromosome configuration in in vitro- and in vivo-matured mouse oocytes after vitrification. *Fertil Steril*, 90:1424-1432.

**Huynh FK, Green MF, Koves TR, Hirschey MD**. 2014. Measurement of fatty acid oxidation rates in animal tissues and cell lines. *Methods Enzymol*, 542:391-405.

**Kolodziejczyk J, Saluk-Juszczak J, Wachowicz B**. 2011. L-Carnitine protects plasma components against oxidative alterations. *Nutrition*, 27:693-699.

**Lane M, Robker RL, Robertson SA**. 2014. Parenting from before conception. *Science*, 345:756-760.

**Lee YS, VandeVoort CA, Gaughan JP, Midic U, Obradovic Z, Latham KE**. 2011. Extensive effects of in vitro oocyte maturation on rhesus monkey cumulus cell transcriptome. *Am J Physiol Endocrinol Metab*,

301:E196-209.
**McGarry JD, Brown NF**. 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem*, 244:1-14.
**McKeegan PJ, Sturmey RG**. 2011. The role of fatty acids in oocyte and early embryo development. *Reprod Fertil Dev*, 24:59-67.
**Mishra A, Reddy IJ, Gupta P, Mondal S**. 2016a. Developmental regulation and modulation of apoptotic genes expression in sheep oocytes and embryos cultured in vitro with L-carnitine. *Reprod Domest Anim*, 51:1020-1029.
**Mishra A, Reddy IJ, Gupta PS, Mondal S**. 2016b. L-carnitine mediated reduction in oxidative stress and alteration in transcript level of antioxidant enzymes in sheep embryos produced in vitro. *Reprod Domest Anim*, 51:311-321.
**Moawad AR, Tan SL, Xu B, Chen HY, Taketo T**. 2013. L-carnitine supplementation during vitrification of mouse oocytes at the germinal vesicle stage improves preimplantation development following maturation and fertilization in vitro. *Biol Reprod*, 88:104. doi: 10.1095/biolreprod.112.107433.
**Moawad AR, Xu B, Tan SL, Taketo T**. 2014. L-carnitine supplementation during vitrification of mouse germinal vesicle stage-oocytes and their subsequent in vitro maturation improves meiotic spindle configuration and mitochondrial distribution in metaphase II oocytes. *Hum Reprod*, 29:2256-2268.
**Montjean D, Entezami F, Lichtblau I, Belloc S, Gurgan T, Menezo Y**. 2012. Carnitine content in the follicular fluid and expression of the enzymes involved in beta oxidation in oocytes and cumulus cells. *J Assist Reprod Genet*, 29:1221-1225.
**Nada MA, Rhead WJ, Sprecher H, Schulz H, Roe CR**. 1995. Evidence for intermediate channeling in mitochondrial beta-oxidation. *J Biol Chem*, 270:530-535.
**Paczkowski M, Schoolcraft WB, Krisher RL**. 2014. Fatty acid metabolism during maturation affects glucose uptake and is essential to oocyte competence. *Reproduction*, 148:429-439.
**Phongnimitr T, Liang Y, Srirattana K, Panyawai K, Sripunya N, Treetampinich C, Parnpai R**. 2013. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. *Anim Sci J*, 84:719-725.
**Reader KL, Cox NR, Stanton JA, Juengel JL**. 2015. Effects of acetyl-L-carnitine on lamb oocyte blastocyst rate, ultrastructure, and mitochondrial DNA copy number. *Theriogenology*, 83:1484-1492.
**Rebouche CJ**. 1992. Carnitine function and requirements during the life cycle. *FASEB J*, 6:3379-3386.
**Ribas GS, Manfredini V, de Marco MG, Vieira RB, Wayhs CY, Vanzin CS, Biancini GB, Wajner M, Vargas CR**. 2010. Prevention by L-carnitine of DNA damage induced by propionic and L-methylmalonic acids in human peripheral leukocytes in vitro. *Mutat Res*, 702:123-128.
**Rizos D, Ward F, Duffy P, Boland MP, Lonergan P**. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev*, 61:234-248.
**Sanchez-Lazo L, Brisard D, Elis S, Maillard V, Uzbekov R, Labas V, Desmarchais A, Papillier P, Monget P, Uzbekova S**. 2014. Fatty acid synthesis and oxidation in cumulus cells support oocyte maturation in bovine. *Mol Endocrinol*, 28:1502-1521.
**Sener G, Paskaloglu K, Satiroglu H, Alican I, Kacmaz A, Sakarcan A**. 2004. L-carnitine ameliorates oxidative damage due to chronic renal failure in rats. *J Cardiovasc Pharmacol*, 43:698-705.
**Silva-Adaya D, Perez-De La Cruz V, Herrera-Mundo MN, Mendoza-Macedo K, Villeda-Hernandez J, Binienda Z, Ali SF, Santamaria A**. 2008. Excitotoxic damage, disrupted energy metabolism, and oxidative stress in the rat brain: antioxidant and neuroprotective effects of L-carnitine. *J Neurochem*, 105:677-689.
**Somfai T, Kaneda M, Akagi S, Watanabe S, Haraguchi S, Mizutani E, Dang-Nguyen TQ, Geshi M, Kikuchi K, Nagai T**. 2011. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod Fertil Dev*, 23:912-920.
**Sovernigo TC, Adona PR, Monzani PS, Guemra S, Barros F, Lopes FG, Leal C**. 2017. Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. *Reprod Domest Anim*. doi: 10.1111/rda.12946.
**Spindler RE, Pukazhenthi BS, Wildt DE**. 2000. Oocyte metabolism predicts the development of cat embryos to blastocyst in vitro. *Mol Reprod Dev*, 56:163-171.
**Spricigo JF, Morato R, Arcarons N, Yeste M, Dode MA, Lopez-Bejar M, Mogas T**. 2017. Assessment of the effect of adding L-carnitine and/or resveratrol to maturation medium before vitrification on in vitro-matured calf oocytes. *Theriogenology*, 89:47-57.
**Sturmey RG, O'Toole PJ, Leese HJ**. 2006. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. *Reproduction*, 132:829-837.
**Sutton ML, Gilchrist RB, Thompson JG**. 2003. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update*, 9:35-48.
**Sutton-McDowall ML, Feil D, Robker RL, Thompson JG, Dunning KR**. 2012. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. *Theriogenology*, 77:1632-1641.
**Truong TT, Soh YM, Gardner DK**. 2016. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod*, 31:1445-1454.
**Uppangala S, Dhiman S, Salian SR, Singh VJ, Kalthur G, Adiga SK**. 2015. In vitro matured oocytes are more susceptible than in vivo matured oocytes to mock ICSI induced functional and genetic changes.

*PloS One*, 10:e0119735.

**Valckx SD, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, Bols PE, Leroy JL**. 2012. BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. *Hum Reprod*, 27:3531-3539.

**Valsangkar D, Downs SM**. 2013. A requirement for fatty acid oxidation in the hormone-induced meiotic maturation of mouse oocytes. *Biol Reprod*, 89:43. doi: 10.1095/biolreprod.113.109058.

**Van Blerkom J, Davis PW, Lee J**. 1995. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod*, 10:415-424.

**Varnagy A, Bene J, Sulyok E, Kovacs GL, Bodis J, Melegh B**. 2013. Acylcarnitine esters profiling of serum and follicular fluid in patients undergoing in vitro fertilization. *Reprod Biol Endocrinol*, 11:67. doi: 10.1186/1477-7827-11-67.

**Vaz FM, Wanders RJ**. 2002. Carnitine biosynthesis in mammals. *Biochem J*, 361:417-429.

**Wu GQ, Jia BY, Li JJ, Fu XW, Zhou GB, Hou YP, Zhu SE**. 2011. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pigs. *Theriogenology*, 76:785-793.

**Wu LL, Russell DL, Norman RJ, Robker RL**. 2012. Endoplasmic reticulum (ER) stress in cumulus-oocyte complexes impairs pentraxin-3 secretion, mitochondrial membrane potential (DeltaPsi m), and embryo development. *Mol Endocrinol*, 26:562-573.

**Yazaki T, Hiradate Y, Hoshino Y, Tanemura K, Sato E**. 2013. L-carnitine improves hydrogen peroxide-induced impairment of nuclear maturation in porcine oocytes. *Anim Sci J*, 84:395-402.

**Ye J, Li J, Yu Y, Wei Q, Deng W, Yu L**. 2010. L-carnitine attenuates oxidant injury in HK-2 cells via ROS-mitochondria pathway. *Regul Pept*, 161:58-66.

**You J, Lee J, Hyun SH, Lee E**. 2012. L-carnitine treatment during oocyte maturation improves in vitro development of cloned pig embryos by influencing intracellular glutathione synthesis and embryonic gene expression. *Theriogenology*, 78:235-243.

**Zare Z, Masteri Farahani R, Salehi M, Piryaei A, Ghaffari Novin M, Fadaei Fathabadi F, Mohammadi M, Dehghani-Mohammadabadi M**. 2015. Effect of L-carnitine supplementation on maturation and early embryo development of immature mouse oocytes selected by brilliant cresyle blue staining. *J Assist Reprod Genet*, 32:635-643.

**Zeng HT, Richani D, Sutton-McDowall ML, Ren Z, Smitz JE, Stokes Y, Gilchrist RB, Thompson JG**. 2014. Prematuration with cyclic adenosine monophosphate modulators alters cumulus cell and oocyte metabolism and enhances developmental competence of in vitro-matured mouse oocytes. *Biol Reprod*, 91:47.

**Zhao H, Zhao Y, Li T, Li M, Li J, Li R, Liu P, Yu Y, Qiao J**. 2015. Metabolism alteration in follicular niche: The nexus among intermediary metabolism, mitochondrial function, and classic polycystic ovary syndrome. *Free Radic Biol Med*, 86:295-307.

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# Brazilian embryo industry in context: pitfalls, lessons, and expectations for the future

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## Abstract

The aim of this review is to provide a brief description of the current situation of the embryo industry in Brazil, in the context of the national and international livestock production scenario. Total number of embryos produced (375,894) declined in 2015 in comparison with the previous years. *B. taurus* dairy breeds and crossbreds accounted for 51.8% of the total embryo production. Moreover, the percentage of frozen-tawed embryos transferred reached 22.8% in 2015, the highest value in a decade. A greater proportion of embryos were produced *in vitro*, both in dairy (97.2%) and beef (90.2%) breeds. The use of embryo technologies in Brazil has remarkably increased 726.5% in the past 20 years, but still represents only 0.33% of the number of cows and heifers at reproductive age. Nonetheless, embryo transfer (ET) accounted for an estimated 19.7% of all purebred calves born and registered by the Brazilian Zebu Cattle Breeders Association in the period 2005-2015, highlighting the importance of ET for animal breeding and genetic improvement of the herd. In the world´s context, Brazil is the largest producer of bovine *in vitro* produced (IVP) embryos, but is ranked only 11th based on an index of intensity of use of embryo technologies, below Canada, USA and various European countries. This scenario demonstrates a potential for further increases in embryo production in Brazil, mainly associated with the expected adoption of new technologies by a large proportion of dairy and beef farms; the use of embryo technologies for large scale production of crossbreds; and to eventual increases in the international embryo import/export activity.

**Keywords**: cattle production, embryo technologies, statistics.

## Introduction

The Brazilian embryo industry underwent remarkable changes in the past 15 years, most of them associated to the adoption of *in vitro* technologies. In cattle, there was a >5-fold increase in embryo production, and *in vitro* fertilization/culture has almost fully replaced superovulation (MOET) as the technique of choice for embryo production. Development of IVP in Brazil was addressed in previous studies (Viana *et al*., 2010, 2012; Sartori *et al*., 2016), with a main focus on the technical aspects of the technology. The use of embryo transfer, however, affects overall cattle production both by increasing genetic progress of animal breeding programs and by providing new alternative methods to produce crossbred animals.

Nevertheless, changes in dairy and beef production systems, induced by the pressure to increase productivity, reduce costs, or improve animal welfare will probably drive further demands for the development of embryo technologies. Thus, to understand the past contribution and the prospective expectations of Brazilian embryo industry, records and numbers must be analyzed in a context. In this study we present a brief description of the current situation of embryo production in Brazil, as well as some proposed indexes to characterize activities related to embryo technologies in the national and international scenario.

## A summary of changes in the Brazilian embryo industry during the past decade

Data of Brazilian embryo production in 2015, discriminated by genetic group (*Bos taurus* and *Bos indicus*), industry (dairy and beef) and technology used (*in vivo* derived – IVD or *in vitro* produced - IVP) is shown in Table 1. A decline in the number of embryos produced was observed relative to the previous year (375,894 in 2015 *vs*. 391,805 in 2014; -4.1%), similar to what has been observed since 2013, which was probably related to the Brazilian economic recession during this period. This retraction in the activity of the embryo industry ceased the trend of growth in Brazilian numbers observed during the last decade, determined mainly by a significant increase in the use of *in vitro* embryo production (IVEP) in dairy breeds (Fig. 1).

The increase in dairy IVEP was primarily supported by the commercial availability of sex-sorted semen (Pontes *et al*., 2010) and caused important changes in the features of the Brazilian embryo market. The most significant one was a shift towards the use of *B. taurus* breeds for IVEP (Fig. 2). In 2005, zebu beef breeds (mostly Nelore) accounted for 93.8% of all embryos produced, whereas dairy *B. taurus* breeds and crossbreds became the most important market for the embryo industry in 2015, corresponding to 51.8% of the total number of embryos. The majority of embryos are still transferred fresh, but the percentage of frozen-thawed embryos has grown in the past few years, reaching 22.8% in 2015, the highest in a decade, likely due to improvements in cryopreservation strategies, such as direct transfer (Sartori *et al*., 2016). Data from

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2005-2015 also demonstrates that there was no change in the use of *in vitro* fertilization/culture as the technique of choice for embryo production, both in dairy (97.2%) and beef (90.2%) breeds.

Table 1. Bovine embryo production in Brazil in 2015, according to genetic group (*Bos taurus* or *Bos indicus*), industry (dairy or beef), and technology used (*in vivo* derived - IVD or *in vitro* produced - IVP).

| Group | IVD | IVP |
|---|---|---|
| *B. indicus* dairy | 172 | 13,481 |
| *B. taurus* dairy | 5,759 | 188,853 |
| Subtotal dairy | 5,931 | 202,334 |
| *B. indicus* beef | 2,042 | 81,636 |
| *B. taurus* beef | 14,382 | 69,569 |
| Subtotal beef | 16,424 | 151,205 |
| Total | 22,355 | 353,539 |

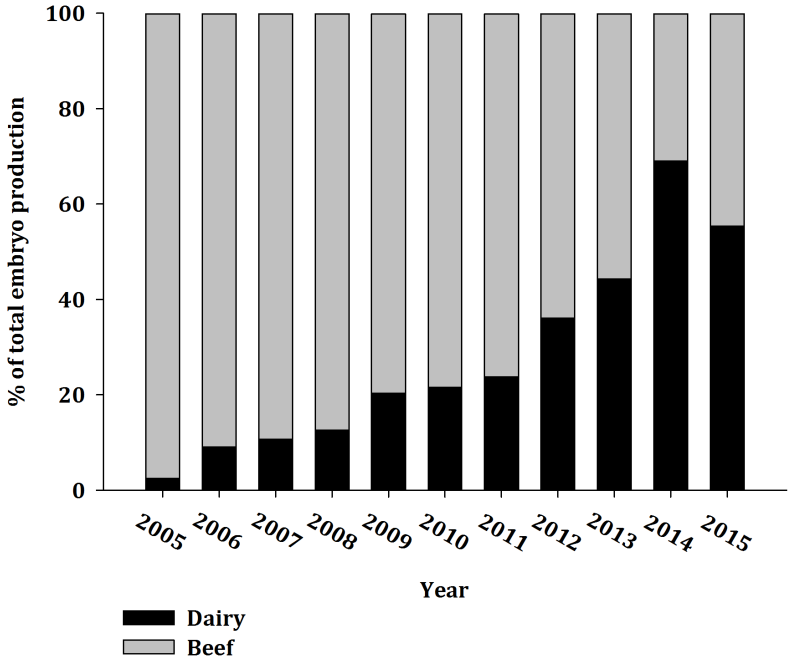


Figure 1. Percentage of Brazilian total embryo production from dairy and beef breeds, from 2005 to 2015.

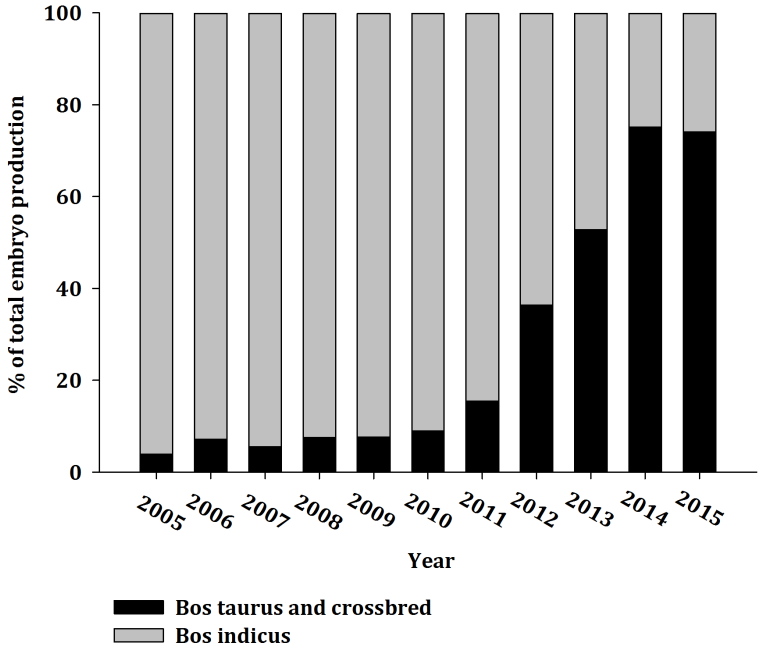


Figure 2. Percentage of Brazilian total embryo production from *B. taurus* and *B. indicus* breeds, from 2005 to 2015.

**The relevance of embryo technologies**

The success of commercial IVEP initiated an exponential growth in Brazilian embryo production and, since 2010, the country reports >300,000 embryos per year. Despite the scarce number of studies evaluating the economical aspects of IVEP in Brazil, the number of embryos produced undoubtly illustrates the impact of the adoption of *in vitro* technologies for the embryo industry. Likewise, the growth of IVEP was associated with the parallel development of a chain of suppliers of veterinary services, hormones, media, disposables, equipments, recipients, among others.

Interestingly, if embryo production is analyzed in face of the Brazilian cattle herd size, an apparent paradox is evident. Table 2 shows the evolution of cattle population in the period 1995-2015 and the estimated relative use of artificial isemination (AI) and embryo transfer (ET). These projections demonstrate that the use of embryo technologies increased by a remarkable rate of 726.5% in the past 20 years; yet, these technologies still reach only 0.33% of the cows and heifers suitable for reproduction, a very low proportion even considering the low use of other reproductive technologies such as AI (13.3%). In dairy breeds, the increase in the use of ET was even greater (2,261.7%), but the relative use is also very limited (0.48%). Therefore, we pose a question: how relevant were embryo technologies for the Brazilian livestock production?

Table 2. Estimated use of artificial insemination (AI) and embryo transfer (ET) as a percentage of the Brazilian cattle herd, 1995-2015.

| | 1995 | 2000 | 2005 | 2010 | 2015 |
|---|---|---|---|---|---|
| Herd size[1] | 161,227,938 | 169,875,524 | 207,156,696 | 209,541,109 | 215,199,488 |
| Heifers and cows[2] | 42,886,632 | 45,186,889 | 55,103,681 | 55,810,800 | 57,243,064 |
| Semen straws sales | 4,180,971 | 5,769,348 | 7,028,308 | 9,637,337 | 13,700,000 |
| Use of AI (%)[3] | 5.4 | 7.1 | 7.1 | 9.6 | 13.3 |
| Embryos produced | 34,076 | 72,050 | 259,252 | 303,237 | 375,894 |
| Use of ET (%)[4] | 0.04 | 0.08 | 0.24 | 0.27 | 0.33 |

[1]Total number of heads (Instituto Brasileiro de Geografia e Estatística - IBGE, 2016). [2]Number of females at reproductive age, based on projections by the Brazilian Artificial Insemination Association (ASBIA). [3]Proportion relative to the number of females at reproductive age. Estimated considering 1.8 straws per successful pregnancy. [4]Proportion relative to the number of females at reproductive age. Estimated considering 2.0 ET per successful pregnancy in a given recipient.

To address this question, it is important to depict the use of embryo technologies amongst different livestock sectors and production strata. Since the early 2000's, when commercial IVEP began its rise in Brazil, until very recently, most embryos were produced from zebu breeds. As a consequence, these breeds represent the most comprehensive database available. In Table 3, we estimate the percentage of calves born by ET (conceived by IVP or IVD embryos) relative to all birth records (RGN) reported by the Brazilian Association of Zebu Cattle Breeders (Associação Brasileira dos Criadores de Zebu - ABCZ, 2017) in the period 2005-2015.

Table 3. Estimated percentage of birth records (RGN) from embryo transfers based on records of the Brazilian Zebu Cattle Breeders Association, from 2005 to 2015*.

| Breed | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gir | 18.8 | 34.9 | 41.2 | 47.5 | 71.0 | 67.1 | 54.3 | 52.1 | 40.2 | 32.9 | 39.0 | 39.8 |
| Guzerá | 18.9 | 25.3 | 31.1 | 34.7 | 30.9 | 28.5 | 27.1 | 30.3 | 23.5 | 13.7 | 10.8 | 22.7 |
| Indubrasil | 21.8 | 22.8 | 7.8 | 0.5 | 6.8 | 51.1 | 29.0 | 17.8 | 24.0 | 2.3 | 27.8 | 16.3 |
| Nelore | 20.7 | 21.8 | 21.1 | 22.7 | 21.0 | 19.5 | 22.2 | 21.7 | 19.6 | 9.1 | 9.0 | 17.8 |
| Sindi | 0.0 | 0.1 | 13.2 | 19.6 | 29.4 | 26.8 | 21.6 | 28.6 | 21.5 | 25.4 | 28.8 | 16.5 |
| Tababuã | 4.5 | 6.5 | 7.8 | 6.9 | 6.0 | 8.3 | 7.3 | 6.9 | 3.0 | 2.8 | 5.4 | 5.5 |
| Cangaian | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 7.7 |
| Brahman | 63.6 | 78.0 | 73.9 | 55.6 | 46.4 | 48.3 | 50.1 | 40.5 | 29.8 | 9.4 | 8.7 | 45.5 |
| Total | 21.5 | 24.1 | 23.7 | 25.1 | 25.0 | 23.7 | 25.1 | 24.0 | 20.3 | 10.2 | 10.1 | 19.7 |

*Based on a projected 40% pregnancy rate relative to the total number of embryos transferred in the previous year.

The results of this projection suggest an entirely different scenario compared to the overall ET data, with ET accounting for an impressive 19.7% of all purebred calves registered by ABCZ. Because zebu breeds are part of most Brazilian beef and dairy herds (either as purebred or crossbreds), any genetic progress in *B. indicus* profoundly affects national livestock productivity indexes. Such impact is likely to have been

boosted by the intensive use of embryo technologies in the past 10-15 years. Results from the Girolando (Gir x Holstein crosses) breeding program, for example, support this theory. Over 600 of the top 1,000 Girolando cows, ranked according to genetic merit for milk production (Silva *et al*., 2016), were generated by ET (IVP or IVD embryos).

In Table 3 we provide data to demonstrate that the use of ET was not uniform amongst zebu breeds. In absolute numbers, Nelore accounted for most embryos (76.8%) produced between 2005 and 2015. Nonetheless, the relative contribution of ET for Nelore calf production (17.8%) was lesser (although more stable) than for some other breeds. On average, ET was used more intensively in Brahman and Gir females (45.5 and 39.8%, respectively), but in both cases these numbers fluctuated, with peaks in 2006 for the former (78.0%) and 2009 for the latter (71.0%). In these particular two breeds, such oscillations were probably associated with the transitory use of ET to increase the number of animals rather than for genetic selection, due to the repressed demand for potential oocyte donors.

**The Brazilian embryo industry in the world´s context**

The remarkable increase in embryo production caused by the use of *in vitro* technologies also changed Brazil´s position in the world rank. In the late 1990´s, the Brazilian embryo industry was already very active and the country ranked among the TOP 5 in bovine embryo production (Thibier, 2000). After the rise of commercial IVEP, the participation of Brazil in the world´s total numbers increased two-fold (from 14.3% in 1999 to 33.9% in 2012) and the country became the largest producer of bovine IVP embryos (Table 4). Interestingly, in the early days of IVEP (up to 2005), the Brazilian embryo market diverged from the trends observed in other countries, particularly from those with relevant embryo production. Therefore, the commercial success of IVEP in Brazil seemed to be a result of particularities of the Brazilian internal market, such as the high economic values and a greater oocyte yield of zebu breeds.

Table 4. Brazilian share in the world´s production of bovine embryos *in vivo*, *in vitro*, and total, from 2005 to 2015.

| | *In vivo* | | | *In vitro* | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| | Brazil | World | % | Brazil | World | % | Brazil | World | % |
| 2015 | 22,355 | 660,221 | 3.4 | 353,539 | 612,709 | 57.7 | 375,894 | 1,272,930 | 29.5 |
| 2014 | 43,337 | 614,464 | 7.1 | 348,468 | 590,359 | 59.0 | 391,805 | 1,204,823 | 32.5 |
| 2013 | 50,455 | 729,246 | 6.9 | 366,517 | 546,628 | 67.1 | 416,972 | 1,275,874 | 32.7 |
| 2012 | 52,719 | 699,585 | 7.5 | 334,913 | 443,533 | 75.5 | 387,632 | 1,143,118 | 33.9 |
| 2011 | 32,646 | 732,862 | 4.5 | 318,116 | 453,471 | 70.2 | 350,762 | 1,186,333 | 29.6 |
| 2010 | 38,974 | 732,227 | 5.3 | 264,263 | 450,549 | 58.7 | 303,237 | 1,182,776 | 25.6 |
| 2009 | 42,397 | 704,230 | 6.0 | 255,993 | 378,244 | 67.7 | 298,390 | 1,082,474 | 27.6 |
| 2008 | 69,527 | 746,250 | 9.3 | 220,425 | 330,953 | 66.6 | 289,952 | 1,077,203 | 26.9 |
| 2007 | 57,368 | 763,467 | 7.5 | 212,441 | 434,581 | 48.9 | 269,809 | 1,198,048 | 22.5 |
| 2006 | 83,741 | 777,747 | 10.8 | 204,402 | 441,364 | 46.3 | 288,143 | 1,219,111 | 23.6 |
| 2005 | 122,210 | 789,972 | 15.5 | 137,042 | 330,647 | 41.4 | 259,252 | 1,120,619 | 23.1 |

The recent trends in both Brazilian and international numbers, however, are changing this perception. The use of IVEP has increased consistently in the past 5 years in Europe and North America, considering either absolute numbers (+71.5 and +337.4%; Europe and North America, respectively) or the proportion of total embryo production (+41.3 and +214.5%; respectively), and may possibly replace MOET as the main source of embryos in a near future (Stroud, 2012; Perry, 2013, 2014, 2015, 2016). Conversely, production of embryos from *B. taurus* and other dairy breeds is increasing in Brazil, as shown in Fig. 1 and 2. Altogether, the general trend is that features of the embryo industry will become more similar worldwide, in spite of regional differences (Table 5). This will probably contribute to an increase in the demand for international trade of embryos and also stimulates a greater participation of large animal breeding companies in the embryo industry, as we currently see for cattle semen.

Table 5. World production of bovine embryos in 2015, according to region, industry (dairy or beef), and technology used (*in vivo* derived - IVD or *in vitro* produced - IVP).

| Region | IVD | | | | IVP | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | Dairy | Beef | Subtotal | % | Dairy | Beef | Subtotal | % | |
| Africa | 402 | 5,132 | 5,534 | 59.7 | 0 | 3,733 | 3,733 | 40.3 | 9,267 |
| Asia | 16,057 | 89,628 | 105,685 | 91.8 | 1,162 | 8,276 | 9,438 | 8.2 | 115,123 |
| Europe | 104,174 | 23,806 | 127,980 | 90.3 | 12,840 | 940 | 13,780 | 9.7 | 141,760 |
| North America | 127,613 | 232,407 | 360,020 | 62.9 | 105,198 | 106,848 | 212,046 | 37.1 | 572,066 |
| Oceania | 2,178 | 9,009 | 11,187 | 74.2 | 3,892 | 0 | 3,892 | 25.8 | 15,079 |
| South America | 8,636 | 41,179 | 49,815 | 11.9 | 208,752 | 161,068 | 369,820 | 88.1 | 419,635 |
| Total | 259,060 | 401,161 | 660,221 | 51.9 | 331,844 | 280,865 | 612,709 | 48.1 | 1,272,930 |

Adapted from Perry (2016).

The United States of America is currently the global leader in total embryo production (IVD plus IVP; Table 6) and may overtake Brazil´s leadership in the production of embryos *in vitro*, as this technology replaces MOET, i.e., without any further increase in total embryo production. Taking into account the ratio between embryo production and cattle population, the Netherlands is the country that most intensively uses embryo technologies, followed by Canada. According to this criteria, Brazil ranks only 11th. Nevertheless, considering that Brazil has the largest commercial cattle herd in the world, livestock production is of great importance for the country´s economy, and that there is an ongoing pressure to increase cattle productivity, we believe that there is room for a sustainable increase in embryo production in the near future, which may eventually change positions on this ranking.

Table 6. Ranking of countries based on their proportion of embryos produced relative to size of the cattle herd, based on data from the year 2014.

| Country | Embryos produced | Cattle population[1] | Intensity of use of ET (%) | Rank |
|---|---|---|---|---|
| The Netherlands | 38,637 | 4,169,000 | 0.93 | 1st |
| Canada | 87,113 | 12,220,000 | 0.71 | 2nd |
| Luxembourg | 1,282 | 198,780 | 0.64 | 3rd |
| USA | 506,626 | 88,526,000 | 0.57 | 4th |
| Finland | 3,617 | 914,439 | 0.40 | 5th |
| Italy | 19,355 | 5,756,072 | 0.34 | 6th |
| Denmark | 4,428 | 1,563,535 | 0.28 | 7th |
| Switzerland | 3,397 | 1,562,801 | 0.22 | 8th |
| Belgium | 5,138 | 2,477,236 | 0.21 | 9th |
| France | 38,422 | 19,095,797 | 0.20 | 10th |
| Brazil | 391,805 | 212,366,132 | 0.18 | 11th |
| Panama | 2,708 | 1,625,200 | 0.17 | 12th |
| Germany | 20,913 | 12,742,190 | 0.16 | 13th |
| Hungary | 675 | 782,000 | 0.09 | 14th |
| Spain | 4,900 | 6,078,700 | 0.08 | 15th |

[1]Food Agriculture Organization (FAO; 2014).

## Conclusions

Predicting the development of the embryo industry is always a complex task, due to the dynamic nature of livestock production and the continuous advance in the 'state of the art' of embryo technologies. Nonetheless, the numbers and trends observed in embryo production in the past decade points to some possible scenarios for the industry in the near future:

1. Despite the current retraction in activity, ET is still used in a low percentage of females and herds in Brazil and, therefore, there is a great potential to increase embryo production as more farms adopt these technologies to increase production and productivity, which was also predicted for AI (Baruselli *et al*., 2012). A new cycle of expansion in the use of ET, however, is more likely to occur associated to the supply of crossbred calves for dairy and beef farms, instead of animal breeding programs;
2. As first observed in Brazil, IVEP is likely to become the technique of choice for ET worldwide. This will reduce regional differences in embryo production systems, as well as reduce costs and increase the scale of embryo production, probably boosting national and international trading of embryos. Consequently, the international embryo market may become more similar to the semen market;
3. In both scenarios described above, the development of successful cryopreservation strategies is key to further expand the embryo industry, as along with an agreement of sanitary protocols and regulations for the export/import of IVP embryos.

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## References

**Associação Brasileira dos Criadores de Zebu (ABCZ)**. 2017. Estatísticas. Available on: http://www.abcz.org.br/conteudos/tecnica/estatistica_1939-2016.xls.

**Baruselli PS, Sales JNS, Sala RV, Vieira LM, Sa Filho MF**. 2012. History evolution and perspectives of timed artificial insemination programs in Brazil. *Anim Reprod*, 9:139-152.

**Food Agriculture Organization (FAO)**. 2014. Cattle inventory. Available on: http://www.fao.org/faostat/en/#data/QA.

**Instituto Brasileiro de Geografia e Estatística (IBGE)**. 2016. Indicadores de produção. Available on: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria.

**Perry G**. 2013. 2012 statistics of embryo collection and transfer in domestic farm animals. *Embryo Transfer Newsl*, 31(4):24-46.

**Perry G**. 2014. 2013 statistics of embryo collection and transfer in domestic farm animals. *Embryo Transfer Newsl*, 32(4):14-24.

**Perry G**. 2015. 2014 statistics of embryo collection and transfer in domestic farm animals. *Embryo Transfer Newsl*, 33(4):9-18.

**Perry G**. 2016. 2015 statistics of embryo collection and transfer in domestic farm animals. *Embryo Transfer Newsl*, 34(4):10-24.

**Pontes JH, Silva KC, Basso AC, Rigo AG, Ferreira CR, Santos GM, Sanches BV, Porcionato JP, Vieira PH, Faifer FS, Sterza FA, Schenk JL, Seneda MM**. 2010. Large-scale in vitro embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and *indicus-taurus* dairy cows using sexed sperm. *Theriogenology*, 74:1349-1355.

**Sartori R, Prata AB, Figueiredo ACS, Sanches BV, Pontes GCS, Viana JHM, Pontes JH, Vasconcelos JLM, Pereira MHC, Dode MAN, Monteiro Jr PLJ, Baruselli PS**. 2016. Update and overview on assisted reproductive technologies (ARTs) in Brazil. *Anim Reprod*, 13:300-312.

**Silva MVGB, Martins MF, Cembranelli MAR, Paiva LC, Panetto JCC, Alves BRC, Campos MM, Carvalho BC, Machado MA, Faza DRLR**. 2016. Girolando breed genetic improvement program. Sire Summary. Progeny Test Results. June 2016. Juiz de Fora: Embrapa Dairy Cattle. 50 pp. (Embrapa Dairy Cattle. Documents, 190).

**Stroud B**. 2012. The year 2011 worldwide statistics of embryo transfer in domestic farm animals.. *Embryo Transfer Newsl*, 30(4):16-26.

**Thibier M**. 2000. The IETS statistics of embryo transfers in livestock in the world for the year 1999: a new record for bovine in vivo-derived embryos transferred. *Embryo Transfer Newsl*, 18(4):24-28.

**Viana JHM, Siqueira LGB, Palhao MP, Camargo LSA**. 2010. Use of in vitro fertilization technique in the last decade and its effect on Brazilian embryo industry and animal production. *Acta Sci Vet*, 38:s661-s674.

**Viana JHM, Siqueira LGB, Palhao MP, Camargo LSA**. 2012. Features and perspectives of the Brazilian in vitro embryo industry. *Anim Reprod*, 9:12-18.

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# *In vitro* production of bovine embryos: cumulus/granulosa cell gene expression patterns point to early atresia as beneficial for oocyte competence

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## Abstract

*In vitro* production (IVP) of bovine embryos has become widespread technology implemented in cattle breeding and production. Here, we review novel data on cumulus/granulosa cell gene expression, as determined by RNAseq on cellular material from pooled follicular fluids at the single animal level, and relate these finding to previous data on oocyte developmental competence and ultrastructure. The cumulus/granulosa cell gene expression patterns indicate that early follicular atresia is associated with increased blastocyst yield and this hypothesis is supported by previous data on oocyte competence and ultrastructure.

**Keywords**: IVP, biomarkers, transcriptomics, granulosa cells, atresia, cattle, oocyte competence.

## Introduction

In the Western World, there are increasing governmental and public demands for environmentally friendly dairy products derived under conditions securing animal health and welfare. These demands are reflected in innovation programs and proposed laws specifying production methods, but also in the increasing demands from e.g. Danish consumers for organic products resulting in a 60% higher price for the primary organic milk.

Breeding can significantly improve feed-conversion efficiency and resilience and lower methane-emission in cattle (Colditz and Hine, 2016; Negussie *et al*., 2017). Since the beginning of 2000, novel technologies have made it possible to accelerate the rates of genetic gain in domestic cattle by genomic selection of calves (Blasco and Toro, 2014; Daetwyler *et al*., 2014). The association between phenotypic traits and genomic markers are getting more and more reliable and includes increasing focus on resilience and feed-efficiency. Denmark is one of the leading countries with regards to correlation of phenotypes for production and health to genomic markers according to the Nordic total merit (NTM) in the form of single nucleotide polymorphisms (SNPs) using the Illumina BeadChip. Likewise, Denmark and other countries put great emphasis in registration of methane-emission in dairy cows with the prospect of identifying genomic markers for low emission (Sousa *et al*., 2017).

Ultrasound-guided ovum pick-up (OPU) and *in vitro* production (IVP) of embryos allows for a significant improved utilization of the female gene pool as multiple embryos of a specific gender can be produced from elite females. If these technologies are used on very young heifers and combined with genomic selection of the embryos, performed on a small cell biopsy before the transfer to recipients, the gain of a significant shortening of the generation interval is added (Kasinathan *et al*., 2015). The combined application of OPU, IVP and genomic selection has until recently posed technical challenges with respect to embryo handling and DNA amplification. However, great progress has been achieved by e.g. the EmbryoGene consortium in Canada with respect to optimization of methodologies, and the combination of OPU, IVP and genomic selection of embryos is believed to hold great promises in cattle breeding (Saadi *et al*., 2014).

At present, there is no commercial use of OPU/IVP in Denmark, and the application of the technologies for scientific purposes has been on hold until 2014. These restrictions have been due to the fact that bovine IVP has, over time, been hampered by impaired embryo quality resulting in the large offspring syndrome (LOS; Behboodi *et al*., 1995; Kruip and Den Daas, 1997; Van Wagtendonk-de Leeuw *et al*., 2000). Over the past years, improved formulations of the media for oocyte maturation and embryo culture have resulted in improved embryo quality, even though increased early embryo loss may still be seen over the first trimester of pregnancy (Alberto *et al*., 2013). Aberrant epigenetic programming, with respect to e.g. DNA methylation, is a potential factor underlying these losses (Hori *et al*., 2010; Chen *et al*., 2015). Nevertheless, IVP has become widely accepted for commercial production of bovine embryos in South and North America and parts of Europe with Denmark lacking behind.

Great advances have been done with respect to the development of media for bovine IVP, which to a great extent has eliminated LOS. However, less attention has been paid to the variable yield of competent oocytes between donor cows (Tamassia *et al*., 2003) as well as to the fact that the basal efficiency of IVP is still relatively low with only 35-45 % of

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cumulus-oocyte complexes (COCs) of good morphology resulting in blastocysts (Mayes and Sirard, 2001; Sirard *et al*., 2006; Muñoz *et al*., 2014).

Transcriptomics can help in identifying biomarkers for oocyte competence (Uyar *et al*., 2013). In cattle, many studies have exploited the power of Next-Generation Sequencing (NGS) technologies to identify follicular biomarkers (Orozco-Lucero and Sirard, 2014). Cumulus and granulosa cells are intimately coupled to the oocyte through paracrine and intercellular communication systems and play major roles in the development of oocyte competence (Macaulay *et al*., 2015). Hence, these cellular compartments may reflect the quality of the oocyte and represent assessable targets for analyses, as they are aspirated together with the COCs.

Previous studies have dissected how the granulosa cell profile varies between follicles with different characteristics, for example between follicles at different developmental stages (Girard *et al*., 2015), between follicles of different sizes (Hatzirodos *et al*., 2014b) and between healthy and atretic follicles (Hatzirodos *et al*., 2014a). None of these studies, however, have been conducted at the single animal level to give information on the particular animal's quality as a potential oocyte donor. The aim of the present manuscript is to review our recent attempt to analyse the transcriptome of the collective cumulus/granulosa cell transcriptome of individual oocyte donor cows in order to dissect potential gene expression patterns associated with high competence of the donor for IVP (Mazzoni *et al*., 2017). These data are subsequently combined with previous data from our group on oocyte ultrastructure during follicular dominance and atresia in order to propose a mechanistic understanding of oocyte IVP competence (for review, see (Hyttel *et al*., 1997).

## Expression of candidate genes associated with IVP outcome at the single cow level

In order to find associations between the collective cumulus/granulosa cell transcriptome and IVP competences within individual donor cows, COCs and follicular fluids were collected from 67 individual cows and processed for IVP including *in vitro* maturation, fertilization and culture (Mazzoni *et al*., 2017; Fig. 1). On day eight after fertilization, embryos from all animals were scored with regard to three parameters: the blastocyst rate was computed for each animal as the number of blastocysts over the total number of inseminated COCs; a kinetic score was obtained by visual classification of each blastocyst as non-expanded, expanded or hatching/hatched; and a morphology score was obtained by visual classification of each blastocyst as poor, good or excellent.

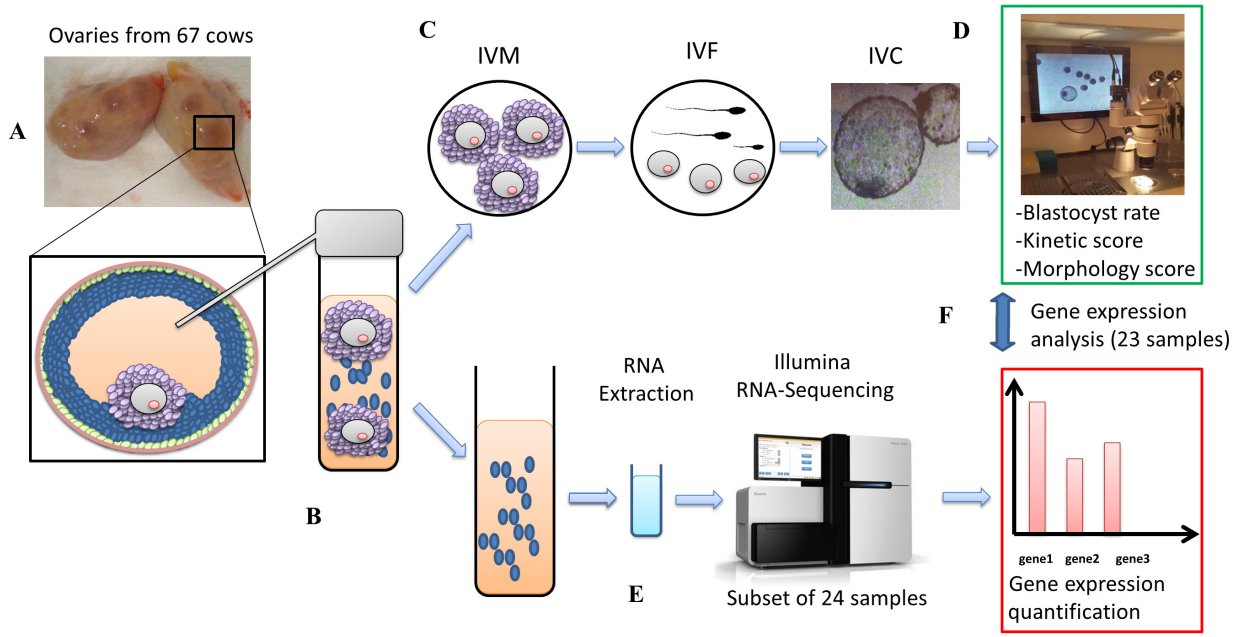


Figure 1. Experimental set-up used in Mazzoni *et al*. (2017). A) Ovaries from 67 Danish cows were collected after slaughter and each pair of ovaries kept separate. B) The COCs from each animal were aspirated with a vacuum pump and kept separate. C) The COCs were processed for *in vitro* production (IVP) including *in vitro* maturation (IVM), insemination (IVF) and culture (IVC) until the blastocyst (BL) stage (day 8). D) IVP parameters for each animal (BL rate, kinetics and morphology) were evaluated at day 8. E) RNA was extracted from the follicular cells contained in the aspirated fluids. A subset of 24 samples was selected and sequenced; F) RNA sequencing analysis was performed to identify genes associated with IVP performances.

Centrifuged cell pellets (cumulus/granulosa cells) from the follicular fluids were processed for RNA sequencing and bioinformatic analyses (Mazzoni *et al*., 2017). For the RNA analysis, only Holstein first- or multiple-lactation cows were used and the 24 samples with higher RNA quality (RNA integrity number) were

selected. One sample was excluded during the quality control procedure. Consequently, the final association between gene expression patterns and IVP outcome was performed on a selected group of 23 cows.

As referenced above the cumulus/granulosa transcriptome is associated with follicular status. COCs aspirated for IVP originate from follicles of a very heterogeneous background and will include dominant and subordinate follicles on ascending and descending slopes of the follicular waves (Forde *et al*., 2011). Interestingly, we found that two of the most important genes related to follicular development, i.e. the FSH receptor gene and the P450 aromatase gene, were expressed on average constantly across all the animals. This fact indicated that there were no systematic differences in follicular status between the animals. Hence, we consider genes that under these circumstances are correlated with IVP outcome as good candidate genes for understanding the molecular background for oocyte competence and potentially predicting the individual cows IVP outcome by identification of assessable biomarkers. The three IVP parameters were scored independently of each other. Hence, it is our hypothesis that genes significantly correlated with all three parameters are the most significant with respect to oocyte competence and potential biomarker discoveries.

The expression of seven genes was noted to be significantly associated with all three blastocyst parameters: Expression of STC1 and Mx1 were positively correlated, while expression of BEX2, RGN, HEY2, TXNDC11 and TNFAIP6 were negatively correlated with a good IVP outcome. Most of these genes have previously been found to be involved in the control of follicular development and oocyte developmental potential.

STC1 is highly expressed in both *in vivo* and *in vitro* matured oocytes (Mamo *et al*., 2011). The STC1 protein is secreted and exerts paracrine control of granulosa cell development, and it is expected to play a critical role in feedback loops between cumulus/granulosa and oocytes (Luo *et al*., 2004). A pro-apoptotic function of STC1 has previously been reported (Law *et al*., 2008; Guo *et al*., 2013).Thus, the increase of STC1 expression in cows with good IVP outcome could be associated with the presence of early atresia. Mx1 is involved in interferon signalling together with IRF and IFNAR, which were both identified as being correlated only to blastocyst rate, indicating that in particular Mx1, but also the other two genes, are of significance for oocyte competence in accordance with observations in other species including man (Lédée *et al*., 2008). Moreover, Mx1 has been reported as inducing cell death and apoptosis (Mibayashi *et al*., 2002). Hence, upregulation of Mx1 may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

BEX2 has previously been reported to be upregulated in large follicles as compared with their smaller counterparts (Hatzirodos *et al*., 2014b). BEX2 acts as an inhibitor of apoptosis in mitochondria and may, thus, prevent follicular atresia. Hence, downregulation of BEX2 may be associated with increased apoptosis and early atresia and, hence, improved IVP outcome (Fig. 2A). RGN has previously been found as highly expressed during follicular dominance and, interestingly, as acting to increase granulosa cell survival (Li *et al*., 2016). Again, downregulation of RGN may be associated with early atresia and, hence, improved IVP outcome (Fig. 2A). HEY2 encodes a transcriptional repressor, which is a downstream target of the Notch cell signalling. Interestingly, the expression of NOTCH2 was significantly correlated with low blastocyst rate although no significant correlations with kinetics and morphology was noted. It may be speculated that downregulation of HEY2 and NOTCH2, and of notch signalling as such, may induce apoptosis as it has been demonstrated in mice (Zhang *et al*., 2011). Again, downregulation of HEY2 may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

TXNDC11 encodes a protein with the thioredoxin domain that might act as a redox regulator. TXNDC11 expression has never been associated to oocyte competence in granulosa cells, although other thioredoxin proteins have been associated with the control of ovarian follicular atresia through scavenging action on reactive oxygen species (ROS; Townson and Combelles, 2012). ROS represent one of the major contributors to oxidative damage (Cadenas and Packer, 1999; Patel *et al*., 1999; Turrens, 2003; Townson and Combelles, 2012) and cell death (Ott *et al*., 2007). We speculate that a lower expression of TXNDC11 could lead to an increase in the concentration of ROS and consequently promote atresia. Again, downregulation of TXNDC11 may be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

The expression of TNFAIP6 in granulosa cells has been correlated with decreased bovine oocyte competence after ovarian stimulation (Gilbert *et al*., 2012). The TNFAIP6 protein is an important component of the extracellular matrix (ECM) thanks to its hyaluronal-binding LINK domain. The ECM promotes cell survival and proliferation of granulosa cells during the follicle development in cattle (Woodruff and Shea, 2007; Salilew-Wondim *et al*., 2014; Ploutarchou *et al*., 2015). Again, downregulation of TNFAIP6 may be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

An important consideration during the selection of candidate genes encoding potential biomarkers for a particular cow's competence for IVP is the subcellular localization of their protein products. Genes whose protein products are secreted into the extracellular space (including the follicular fluid and eventually blood plasma) can potentially be measured in these fluids and used as biomarkers of IVP traits. Among the candidate genes described above, only STC1 encodes a protein, which is secreted.

**Gene expression patterns in relation to follicular size and atresia**

The functional analysis of the seven candidate

genes pointed to their potential involvement in follicular atresia. Previously, gene expression in cattle has been reported in healthy *vs*. early atretic antral follicles (Hatzirodos *et al*., 2014a) and in small vs. medium and large follicles (Hatzirodos *et al*., 2014b). A comparative analysis revealed that 65% of the genes identified as differentially expressed in early atretic follicles vs. healthy follicles (Hatzirodos *et al*., 2014a) showed the same trend (being up- or downregulated) in our study and this percentage increased to 90% considering the top 25% of the genes positively correlated with good IVP outcome in our study (Fig. 2B). Conversely, 84% of the genes identified as differentially expressed in medium and large follicles *vs*. small follicles (Hatzirodos *et al*., 2014b) showed the opposite trend in our study and this percentage increased to 92% when considering the top 25% genes correlated to blastocyst rate in our study. Taken together, the gene expression patterns in our study combined with the data on atresia and follicle size indicates that good IVP outcome is positively correlated with early atresia and negatively correlated with follicle size. Hence, very interestingly, this relationship points to small early atretic follicles as yielding the most competent oocytes for IVP.

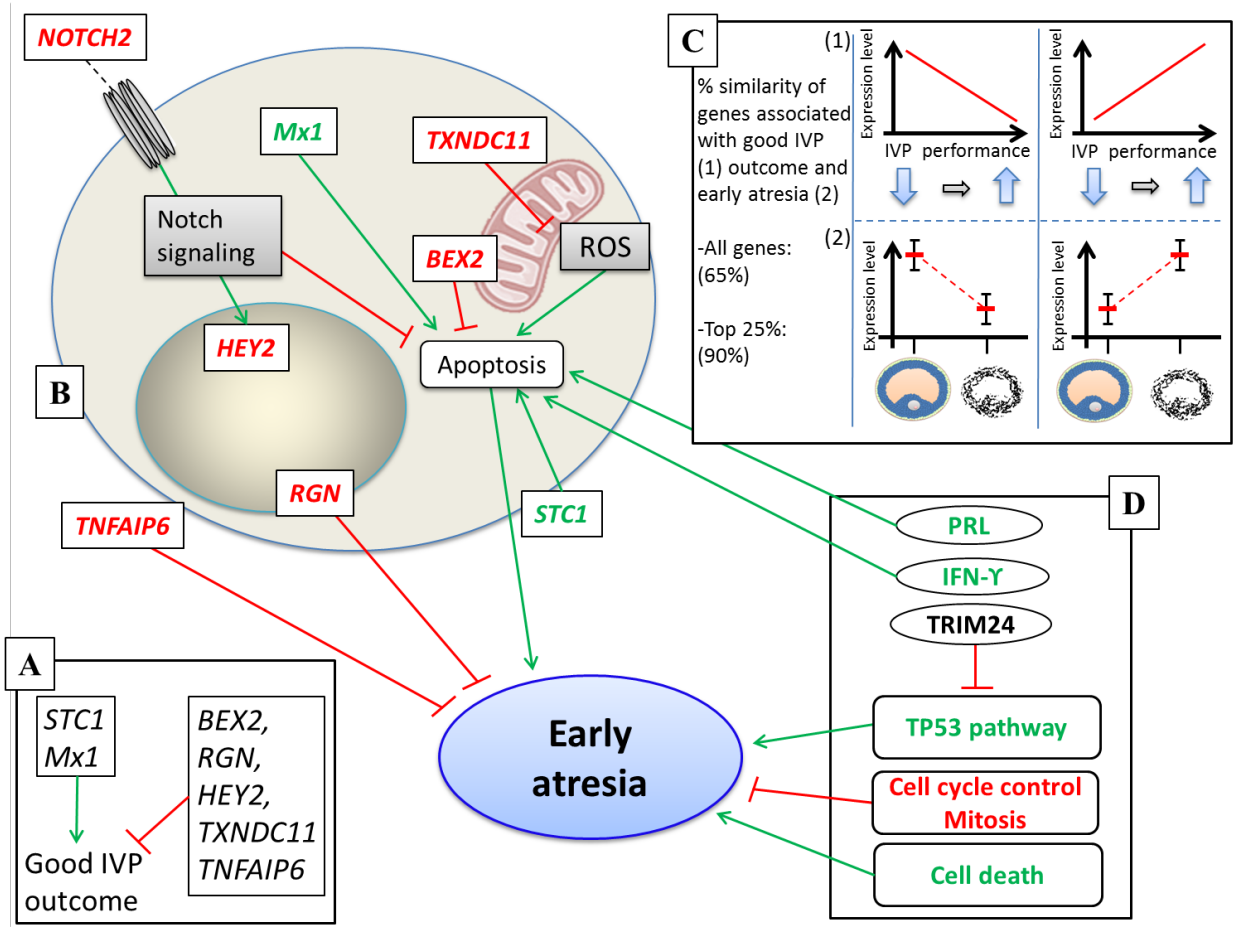


Figure 2. Summary of the evidences that lead to the hypothesis that early atresia is correlated with good IVP outcome. The figure summarizes all the bioinformatics evidences that lead to the atresia hypothesis. A) The expression level of 7 genes were significantly correlated with all parameters of a good IVP outcome: Blastocyst rate, kinetic score and morphology score. B) Schematic representation of a granulosa cell with the candidate genes and the mechanisms that support the positive correlation between early atresia and good IVP outcome. C) Representation of the comparison between the expression profiles in our study (1) and the expression profiles of atretic versus healthy follicles from Hatzirodos *et al*. (2014a; 2). D) Enriched pathways and upstream regulators associated with IVP outcome. Text color code: Green = positive correlation with good IVP outcome; red = negative correlation with good IVP outcome. Shape code: Rectangles = candidate genes; ovals = upstream-regulators; rounded rectangles = biological processes and pathways, grey-filled rectangles = molecules or molecular signalling. Arrow code: Green arrows = activation; red lines = inhibition.

### Pathway enrichment and upstream regulator analysis associated with follicular atresia

Functional pathway enrichment analysis can identify biological functions and pathways overrepresented in the set of genes associated with a certain trait of interest. Functional analysis with Ingenuity® Pathway suite (IPA®) predicts the activation state (activated or inhibited) of the biological processes and of the main upstream regulators for the genes associated with a specific trait of interest. Functional enrichment was performed to extract biological insight

related to the IVP process from the gene expression profiles and provided new evidences sustaining the relationship between small early atretic follicles and good IVP outcome. Hence, several biological pathways or processes were identified as being of significance for the IVP outcome. In details, processes for the control of cell proliferation and development (mitosis, cell cycle control) were negatively correlated with good IVP outcome while cell death process and the TP53 pathway were positively correlated with good IVP outcome (Fig. 2C).

The upstream regulator analysis was performed to identify key proteins responsible for the control of the expression of the genes that we found associated with good IVP outcome. Therefore, even if the expression of the upstream regulators was not identified in our dataset as being absolutely differentially expressed they indirectly represent potential candidate proteins for IVP outcome. The important upstream regulators of genes associated with good IVP outcome were TRIM24, PRL and IFN-ϒ (Fig. 2C).

TRIM24 was identified as an upstream repressor of TP53 promoting the degradation of this protein. Hence, TRIM24 is thought to prevent TP53-induced apoptosis and, thus, atresia. The TP53 pathway is specifically interesting as it is upregulated at apoptosis (Fridman and Lowe, 2003) and has been identified as being activated when bovine growing follicles enter the plateau phase of the follicular wave and initiate atresia (Nivet *et al*., 2013). Hence, upregulation of the TP53 pathway may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2C).

PRL or prolactin was predicted as being activated in cows with good IVP outcome by the upstream regulator analysis with IPA® The activation of the PRL pathway has previously been reported as positively correlated with oocyte competence (Nivet *et al*., 2013) and with the occurrence of atresia (Lebedeva *et al*., 1998). In the rat, PRL administration has on the one hand resulted in an increased number of atretic follicles *in vivo* (Besnard *et al*., 2001) and, on the other hand, in a decrease in the abundance of granulosa cells in late stages of cell death *in vitro* (Lebedeva *et al*., 1998; Heleil *et al*., 2010) combined with an increase in embryo development to the morula and blastocyst stages (Kuz'mina *et al*., 2001). Again, upregulation of the PRL pathway may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2C).

The expression of IFN-γ was not observed in our granulosa cell samples. However, the upstream regulator analysis with IPA® predicted IFN-γ to be activated. Within the ovary, IFN-γ is only synthesized by immune cells (Best *et al*., 1995). The protein enhances apoptosis and it has been found exclusively in atretic follicles in human (Best *et al*., 1995; Best and Hill, 2000). Again, upregulation of the IFN-γ pathway may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2C).

Interestingly, we found that activation of the immune system was negatively correlated with good IVP outcome. We speculate that immune system activation is related to late atresia whereas the early atresia has not yet activated this type of response. This is partially confirmed by previous studies in human, where immune cells and, in particular, macrophages are abundantly recruited within the follicles at an advanced stage of atresia (Petrovská *et al*., 1996; Takaya *et al*., 1997; Gaytan *et al*., 1998). Again, this speculation supports the notion that early atresia, but not late atresia, is positively correlated with good IVP outcome.

However, macrophages have also been found to be present in healthy follicles and their abundance increases during follicle growth (Wu *et al*., 2004). It has been suggested that macrophages promote granulosa proliferation (Fukumatsu *et al*., 1992) or atresia by regulating the balance between cellular proliferation and apoptosis through the secretion of factors like TNFα (Kaipia *et al*., 1996; Wu *et al*., 2004) or IFN-γ (Mazzoni *et al*., 2017) as previously described. These mechanisms are still debated and must be addressed in future studies.

**Oocyte ultrastructure and early atresia**

The theory that early atresia is associated with good IVP outcome has been addressed earlier (Moor and Trounson, 1977; Wurth and Kruip, 1992; Feng *et al*., 2007) and it has directly been demonstrated that embryo yield is positively correlated with early atresia whereas late atresia has a negative impact (De Wit *et al*., 2000). Accordingly, the developmental potential of oocytes has earlier been reported as being positively correlated with granulosa cell apoptosis, which is widely used to identify atretic follicles (Feng *et al*., 2007; Heleil *et al*., 2010).

Along this line, previous ultrastructural studies performed in our lab also point to potential underlying explanations of the positive correlation between good IVP outcome and early atresia. Hence, studies of the ultrastructure of oocytes from dominant follicles approaching ovulation has clearly demonstrated that initial cumulus cell expansion and gradual retraction of the cumulus cell processes, attached to the oocyte through the zona pellucida, are initiated even prior to the LH peak (Assey *et al*., 1994). These somatic cell modulations are associated with changes in the oocyte nucleus, i.e. the germinal vesicle, which develops undulations of the nuclear envelope, likewise prior to the LH peak. After the LH peak these processes culminate in resumption of meiosis and progress of cytoplasmic oocyte maturation over a 24 h period leading to ovulation. We also found that the above-described sequence of processes can be observed in oocytes in the subordinate follicles of the follicular wave, i.e. follicles representing early atresia. Hence, in early atretic follicles, the oocyte apparently undergoes processes that mimic those seen in the dominant follicle approaching ovulation. Seen in this light, it is not surprising that oocytes harvested from early atretic follicles may be better qualified for entering final maturation *in vitro* as they may be "primed" for the process, whereas oocytes from healthy growing follicles are totally locked in meiosis and may experience problems in an immediate resumption of this process

when placed at IVM. Interestingly, coasting has been demonstrated to increase IVP embryo yield (Nivet *et al*., 2013); an effect that is likely also to be based upon initiation of early atresia in the follicular pool.

One approach to deal with the problem that oocytes may not be "primed" for IVM directly upon aspiration has been to induce a temporary arrest of oocyte maturation (Lonergan *et al*., 2003; Donnay *et al*., 2004; Vigneron *et al*., 2004). Years later, this concept was launched again through a specially designed medium, referred to as simulated physiological oocyte maturation (Albuz *et al*., 2010). The results have been varying, and a modified second version is now being tested (Gilchrist *et al*., 2015), illustrating that a practical solution to such a complex challenge is not always so easy. A dissection of this phenomenon is presently being concluded in another branch of the Brazilian-Danish GIFT project (Razza *et al*., 2016).

## Conclusions

Cumulus/granulosa cell gene expression patterns indicate that early atresia is associated with increased blastocyst yield and this hypothesis is supported by previous data on oocyte competence and ultrastructure.

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## References

**Alberto ML, Meirelles FV, Perecin F, Ambrósio CE, Favaron PO, Franciolli AL, Mess AM, Santos, JM, Rici RE, Bertolini M**. 2013. Development of bovine embryos derived from reproductive techniques. *Reprod Fertil Dev*, 25:907-917.

**Albuz F, Sasseville M, Lane M, Armstrong, D, Thompson J, Gilchrist R**. 2010. Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum Reprod,* 25:2999-3011.

**Assey R, Hyttel P, Greve T, Purwantara B**. 1994. Oocyte morphology in dominant and subordinate follicles. *Mol Reprod Dev*, 37:335-344.

**Behboodi E, Anderson G, BonDurant R, Cargill S, Kreuscher B, Medrano J, Murray J**. 1995. Birth of large calves that developed from in vitro-derived bovine embryos. *Theriogenology*, 44:227-232.

**Besnard N, Horne E, Whitehead S**. 2001. Prolactin and lipopolysaccharide treatment increased apoptosis and atresia in rat ovarian follicles. *Acta Physiol,* 172:17-25.

**Best CL, Griffin PM, Hill JA**. 1995. Interferon gamma inhibits luteinized human granulosa cell steroid production in vitro. *Am J Obstet Gynecol*, 172:1505-1510.

**Best CL, Hill JA**. 2000. Cytokines in ovarian function. *In*: Hill JA (Ed.). *Cytokines in Human Reproduction*. New York: Wiley-Liss. pp. 43-77.

**Blasco A, Toro MA**. 2014. A short critical history of the application of genomics to animal breeding. *Livest Sci*, 166:4-9.

**Cadenas E, Packer L** (Ed.). 1999. *Understanding the Process of Aging: The Roles of Mitochondria: Free Radicals, and Antioxidants*. Boca Raton, FL: CRC Press. 384 pp.

**Chen Z, Hagen DE, Elsik CG, Ji T, Morris CJ, Moon LE, Rivera RM**. 2015. Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction. *Proc Nat Acad Sci,* 112:4618-4623.

**Colditz IG, Hine BC**. 2016. Resilience in farm animals: biology, management, breeding and implications for animal welfare. *Anim Prod Sci,* 56:1961-1983.

**Daetwyler HD, Capitan A, Pausch H, Stothard P, Van Binsbergen R, Brøndum RF, Liao X, Djari A, Rodriguez SC, Grohs C**. 2014. Whole-genome sequencing of 234 bulls facilitates mapping of monogenic and complex traits in cattle. *Nat Genet,* 46:858-865.

**De Wit A, Wurth Y, Kruip T**. 2000. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. *J Anim Sci,* 78:1277-1283.

**Donnay I, Faerge I, Grøndahl C, Verhaeghe B, Sayoud H, Ponderato N, Galli C, Lazzari G**. 2004. Effect of prematuration, meiosis activating sterol and enriched maturation medium on the nuclear maturation and competence to development of calf oocytes. *Theriogenology*, 62:1093-1107.

**Feng W-G, Sui H-S, Han Z-B, Chang Z-L, Zhou P, Liu D-J, Bao S, Tan J-H**. 2007. Effects of follicular atresia and size on the developmental competence of bovine oocytes: a study using the well-in-drop culture system. *Theriogenology*, 67:1339-1350.

**Forde N, Beltman M, Lonergan P, Diskin M, Roche J, Crowe M**. 2011. Oestrous cycles in *Bos taurus* cattle. *Anim Reprod Sci,* 124:163-169.

**Fridman JS, Lowe SW**. 2003. Control of apoptosis by p53. *Oncogene*, 22:9030-9040.

**Fukumatsu Y, Katabuchi H, Naito M, Takeya M, Takahashi K, Okamura H**. 1992. Effect of macrophages on proliferation of granulosa cells in the ovary in rats. *J Reprod Fertil,* 96:241-249.

**Gaytan F, Morales C, Bellido C, Aguilar E, Sanchez-Criado J**. 1998. Ovarian follicle macrophages: is follicular atresia in the immature rat a macrophage-mediated event? *Biol Reprod,* 58:52-59.

**Gilbert I, Robert C, Vigneault C, Blondin P, Sirard M-A**. 2012. Impact of the LH surge on granulosa cell transcript levels as markers of oocyte developmental competence in cattle. *Reproduction*, 143:735-747.

**Gilchrist R, Zeng H, Wang X, Richani D, Smitz J,**

**Thompson J**. 2015. Reevaluation and evolution of the simulated physiological oocyte maturation system. *Theriogenology*, 84:656-657.

**Girard A, Dufort I, Douville G, Sirard M-A**. 2015. Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. *Reprod Biol Endocrinol,* 13:17. doi: 10.1186/s12958-015-0010-7.

**Guo F, Li Y, Wang J, Li Y, Li Y, Li G**. 2013. Stanniocalcin1 (STC1) inhibits cell proliferation and invasion of cervical cancer cells. *PloS One*, 8:e53989.

**Hatzirodos N, Hummitzsch K, Irving-Rodgers HF, Harland ML, Morris SE, Rodgers RJ**. 2014a. Transcriptome profiling of granulosa cells from bovine ovarian follicles during atresia. *BMC Genomics*, 15, 40. doi: 10.1186/1471-2164-15-40.

**Hatzirodos N, Irving-Rodgers HF, Hummitzsch K, Harland ML, Morris SE, Rodgers RJ**. 2014b. Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes. *BMC Genomics*, 15:24. doi: 10.1186/1471-2164-15-24.

**Heleil B, Kuzmina T, Alm H, Scotti O, Tuchscherer A, Torner H**. 2010. Involvement of granulosa cells in realization of prolactin effects on the developmental competence of bovine oocytes matured in vitro. *J Am Sci,* 6:796-805

**Hori N, Nagai M, Hirayama M, Hirai T, Matsuda K, Hayashi M, Tanaka T, Ozawa T, Horike S-I**. 2010. Aberrant CpG methylation of the imprinting control region KvDMR1 detected in assisted reproductive technology-produced calves and pathogenesis of large offspring syndrome. *Anim Reprod Sci,* 122:303-312.

**Hyttel P, Fair T, Callesen H, Greve T**. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology*, 47:23-32.

**Kaipia A, Chun S-Y, Eisenhauer K, Hsueh A**. 1996. Tumor necrosis factor-alpha and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology*, 137:4864-4870.

**Kasinathan P, Wei H, Xiang T, Molina JA, Metzger J, Broek D, Kasinathan S, Faber DC, Allan MF**. 2015. Acceleration of genetic gain in cattle by reduction of generation interval. *Sci Rep,* 5:8674. doi: 10.1038/srep08674.

**Kruip TA, Den Daas J**. 1997. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. *Theriogenology*, 47:43-52.

**Kuz'mina T, Lebedeva IY, Torner H, Alm H**. 2001. Effects of prolactin in different culture systems on the maturation of bovine oocytes and their capacity for subsequent development. *Russ J Dev Biol,*, 32:112-118.

**Law A, Lai K, Lui W, Wan H, Wong CK**. 2008. Histone deacetylase inhibitor-induced cellular apoptosis involves stanniocalcin-1 activation. *Exp Cell Res*, 314:2975-2984.

**Lebedeva IY, Denisenko VY, Lebedev V, Kuzmina T**. 1998. Prolactin in follicular fluid and intracellular store calcium in follicular cells are related to morphological signs of ovarian follicle atresia in cows: work in progress. *Theriogenology*, 49:509-519.

**Lédée N, Lombroso R, Lombardelli L, Selva J, Dubanchet S, Chaouat G, Frankenne F, Foidart J-M, Maggi E, Romagnani S**. 2008. Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor. *Hum Reprod,* 23:2001-2009.

**Li P, Meng J, Liu W, Smith GW, Yao J, Lyu L**. 2016. Transcriptome analysis of bovine ovarian follicles at predeviation and onset of deviation stages of a follicular wave. *Int J Genomics*, 2016:9pp.

**Lonergan P, Gutiérrez-Adán A, Rizos D, Pintado B, De La Fuente J, Boland MP**. 2003. Relative messenger RNA abundance in bovine oocytes collected in vitro or in vivo before and 20 hr after the preovulatory luteinizing hormone surge. *Mol Reprod Dev,* 66:297-305.

**Luo C-W, Kawamura K, Klein C, Hsueh AJ**. 2004. Paracrine regulation of ovarian granulosa cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors. *Mol Endocrinol,* 18:2085-2096.

**Macaulay AD, Gilbert I, Scantland S, Fournier E, Ashkar F, Bastien A, Saadi HAS, Gagné D, Sirard M-A, Khandjian ÉW, Richard FJ, Hytte P, Robert C**. 2015. Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation. *Biol Reprod*, 94:16. doi: 10.1095/biolreprod.114.127571.

**Mamo S, Carter F, Lonergan P, Leal CL, Al Naib A, McGettigan P, Mehta JP, Evans AC, Fair T**. 2011. Sequential analysis of global gene expression profiles in immature and in vitro matured bovine oocytes: potential molecular markers of oocyte maturation. *BMC Genomics*, 12:151. doi: 10.1186/1471-2164-12-151.

**Mayes M, Sirard M**. 2001. The influence of cumulus-oocyte complex morphology and meiotic inhibitors on the kinetics of nuclear maturation in cattle. *Theriogenology*, 55:911-922.

**Mazzoni G, Salleh SM, Freude K, Pedersen HS, Stroebech L, Callesen H, Hyttel P, Kadarmideen HN**. 2017. Identification of potential biomarkers in donor cows for in vitro embryo production by granulosa cell transcriptomics. *PloS One*, 12:e0175464.

**Mibayashi M, Nakade K, Nagata K**. 2002. Promoted cell death of cells expressing human MxA by influenza virus infection. *Microbiol Immunol,* 46:29-36.

**Moor R, Trounson A**. 1977. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. *J Reprod Fertil,* 49:101-109.

**Muñoz M, Uyar A, Correia E, Díez C, Fernandez-Gonzalez A, Caamaño J, Martínez-Bello D, Trigal B, Humblot P, Ponsart C**. 2014. Prediction of pregnancy viability in bovine in vitro-produced embryos and recipient plasma with Fourier transform infrared spectroscopy. *J Dairy Sci,* 97:5497-5507.

**Negussie E, Lehtinen J, Mäntysaari P, Bayat A, Liinamo A-E, Mäntysaari E, Lidauer M**. 2017. Non-invasive individual methane measurement in dairy cows. *Animal*, 11:890-899.

**Nivet A-L, Vigneault C, Blondin P, Sirard M-A**. 2013. Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. *Reproduction*, 145:555-565.

**Orozco-Lucero E, Sirard M**. 2014. Molecular markers of fertility in cattle oocytes and embryos: progress and challenges. *Anim Reprod,* 11:183-194.

**Ott M, Gogvadze V, Orrenius S, Zhivotovsky B**. 2007. Mitochondria, oxidative stress and cell death. *Apoptosis*, 12:913-922.

**Patel RP, Cornwell T, Darley-Usmar VM**. 1999. The biochemistry of nitric oxide and peroxynitrite: implications. *In*: Cadenas E, Packer L (Ed.). *Understanding the Process of Aging: The Roles of Mitochondria: Free Radicals, and Antioxidants*. Boca Raton, FL: CRC Press. pp. 39-56.

**Petrovská M, Dimitrov DG, Michael SD**. 1996. Quantitative changes in macrophage distribution in normal mouse ovary over the course of the estrous cycle examined with an image analysis system. *Am J Reprod Immunol,* 36:175-183.

**Ploutarchou P, Melo P, Day AJ, Milner CM, Williams SA**. 2015. Molecular analysis of the cumulus matrix: insights from mice with O-glycan-deficient oocytes. *Reproduction*, 149:533-543.

**Razza E, Pedersen H, Stroebech L, Machado M, Nogueira M, Kadarmideen H, Callesen H, Hyttel P**. 2016. 192 Prematuration of bovine cumulus-oocyte complexes with cyclic adenosine monophosphate modulators affects both oocyte and blastocyst ultrastructure. *Reprod Fertil Dev*, 28:227. (abstract).

**Saadi HAS, Vigneault C, Sargolzaei M, Gagné D, Fournier É, de Montera B, Chesnais J, Blondin P, Robert C**. 2014. Impact of whole-genome amplification on the reliability of pre-transfer cattle embryo breeding value estimates. *BMC Genomics*, 15:889.

**Salilew-Wondim D, Ahmad I, Gebremedhn S, Sahadevan S, Hossain MM, Rings F, Hoelker M, Tholen E, Neuhoff C, Looft C**. 2014. The expression pattern of microRNAs in granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle. *PloS One*, 9:e106795.

**Sirard M-A, Richard F, Blondin P, Robert C**. 2006. Contribution of the oocyte to embryo quality. *Theriogenology*, 65:126-136.

**Sousa RV, Silva Cardoso CR, Butzke G, Dode MAN, Rumpf R, Franco MM**. 2017. Biopsy of bovine embryos produced in vivo and in vitro does not affect pregnancy rates. *Theriogenology*, 90:25-31.

**Takaya R, Fukaya T, Sasano H, Suzuki T, Tamura M, Yajima A**. 1997. Macrophages in normal cycling human ovaries; immunohistochemical localization and characterization. *Hum Reprod,* 12:1508-1512.

**Tamassia M, Heyman Y, Lavergne Y, Richard C, Gelin V, Renard J, Chastant-Maillard S**. 2003. Evidence of oocyte donor cow effect over oocyte production and embryo development in vitro. *Reproduction*, 126:629-637.

**Townson DH, Combelles CM**. 2012. Ovarian follicular atresia. *In*: Darwis A. (Ed.). *Basic Gynecology - Some Related Issues*. Rijeka, Croatia: In Tech. pp. 43-76. Available on: www.intechopen.com/ /books/basic-gynecology-some-related-issues/ovarian-follicular-atresia.

**Turrens JF**. 2003. Mitochondrial formation of reactive oxygen species. *J Physiol,* 552:335-344.

**Uyar A, Torrealday S, Seli E**. 2013. Cumulus and granulosa cell markers of oocyte and embryo quality. *Fertil Steril,* 99:979-997.

**Van Wagtendonk-de Leeuw A, Mullaart E, De Roos A, Merton J, Den Daas J, Kemp B, De Ruigh L**. 2000. Effects of different reproduction techniques: AI, MOET or IVP, on health and welfare of bovine offspring. *Theriogenology*, 53:575-597.

**Vigneron C, Perreau C, Dupont J, Uzbekova S, Prigent C, Mermillod P**. 2004. Several signaling pathways are involved in the control of cattle oocyte maturation. *Mol Reprod Dev,* 69:466-474.

**Woodruff TK, Shea LD**. 2007. The role of the extracellular matrix in ovarian follicle development. *Reprod Sci,* 14:6-10.

**Wu R, Van der Hoek KH, Ryan NK, Norman RJ, Robker RL**. 2004. Macrophage contributions to ovarian function. *Hum Reprod Update*, 10:119-133.

**Wurth Y, Kruip TA**. 1992. Bovine embryo production in vitro after selection of the follicles and oocytes. *In*: Proceedings of the 12th International Congress on Animal Reproduction. The Hague, The Netherlands: ICAR. pp. 387-389.

**Zhang C-P, Yang J-L., Zhang J, Li L, Huang L, Ji S-Y, Hu Z-Y, Gao F, Liu Y-X**. 2011. Notch signaling is involved in ovarian follicle development by regulating granulosa cell proliferation. *Endocrinology*, 152:2437-2447.

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# Postnatal consequences of assisted reproductive technologies in cattle

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## Abstract

Experiments in mice and clinical observations in humans are indicative that adult phenotype can be altered in offspring derived from embryos that were subjected to culture or that were produced by the combination of *in vitro* oocyte maturation, fertilization and embryonic development (*in vitro* production; IVP). The most commonly observed changes are in body and organ size, growth rate, cardiovascular function and regulation of glucose homeostasis. In cattle, IVP is associated with increased birth weight and neonatal death loss but little is known about the long-term consequences of embryo technologies. Recently, postnatal characteristics were compared between females born as a result of artificial insemination, IVP using conventional semen, IVP using reverse X-sorted semen, and multiple ovulation – embryo transfer. Females born following IVP using reverse X-sorted semen produced less milk, milk fat, and milk protein than females in the other three groups, which were similar to each other. These results point out the importance of 1) examining other data sets to examine long-term impact of assisted reproductive technologies on adult physiology and performance and 2) evaluating whether use of sexed semen alters the adult phenotype when used for artificial insemination.

**Keywords**: dairy cattle, *in vitro* fertilization, milk yield, sexed semen.

## Introduction: The promise and perils of assisted reproductive technologies

Embryo technologies, whether involving superovulation (multiple ovulation – embryo transfer; MOET) or *in vitro* production (IVP), create multiple opportunities for those involved in production of dairy and beef cattle. Both MOET and IVP were developed primarily as a method for increasing the number of offspring from genetically-valuable females. Rates of genetic gain can be increased by combining genomic testing and embryo transfer (Pryce *et al*., 2010; Thomasen *et al*., 2016; Kaniyattam *et al*., 2017). Use of embryo transfer in a commercial dairy can be profitable when costs of embryo production are low and the excess heifers sold from the herd are valuable (Kaniyattam *et al*., 2017). Embryo transfer can also improve fertility as compared to artificial insemination (AI) for subpopulations of cows with low fertility – those exposed to heat stress and designated as repeat breeding females (Fig. 1). In addition, embryo transfer allows rapid change in the breed composition of a herd (Wheeler *et al*., 2006) and can allow production of crossbred animals without the need to maintain large numbers of purebred females. Moreover, IVP is an efficient method for using sexed semen (Wheeler *et al*., 2006; Pontes *et al*., 2010; Rasmussen *et al*., 2013; Pellegrino *et al*., 2016) and multiple embryos can be produced using a single straw of semen.

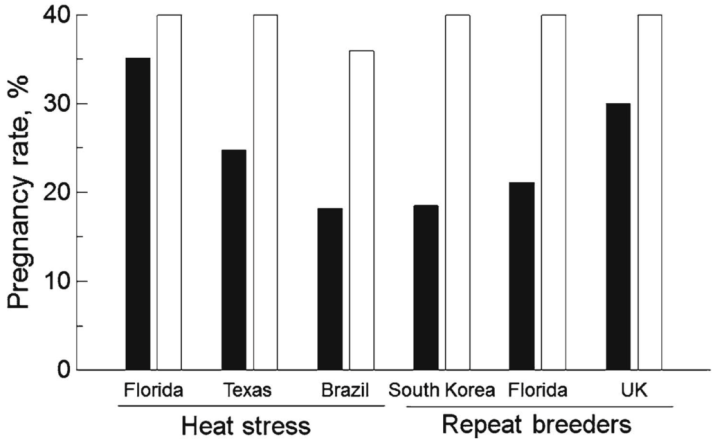


Figure 1. One of the promises of embryo transfer – improved pregnancy rates for cows that are heat-stressed or classified as repeat-breeders (third or more consecutive attempt to establish pregnancy). The figure is reproduced from Hansen (2014) with permission of Springer. Shown are examples of studies in lactating dairy cows in which pregnancy rates were improved by embryo transfer (open bar) as compared to artificial insemination (filled bar). Data from heat stress experiments are from Block *et al*. (2010; Florida), Stewart *et al*. (2011; Texas) and Vasconcelos *et al*. (2011; Brazil). Data from repeat breeder studies are from Son *et al*. (2007; South Korea), Block *et al*. (2010; Florida) and Canu *et al*. (2010; UK).

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Despite the benefits that accrue from embryo technologies, some of the promise is not always realized because of aberrant embryonic development. It is well known that the IVP embryo can differ from the embryo produced *in vivo* in terms of morphology, gene expression and freezability (Hansen, 2006, 2014). Even superovulation can cause changes in embryonic developmental competence, gene expression and DNA methylation (Markert-Velker *et al*., 2010; Gad *et al*., 2011). One consequence of embryo production *in vitro* is that pregnancy rates are often lower than for MOET (Pontes *et al*., 2009; Siqueira *et al*., 2009). Moreover, pregnancy failure for pregnancies established following transfer of an IVP embryo can be elevated (Stewart *et al*., 2011) although this is not always seen (Pereira *et al*., 2016). Calves derived from IVP embryos have also been reported to experience increased neonatal mortality (van Wagtendonk-de Leeuw *et al*., 1998; Bonilla *et al*., 2014). Increased perinatal deaths can be ascribed in large part to abnormal offspring syndrome (Farin *et al*., 2010) which is often manifested as calves born with abnormally large body size. An example of a fetus derived from an IVP embryo that experienced excessive somatic growth is shown in Fig. 2.

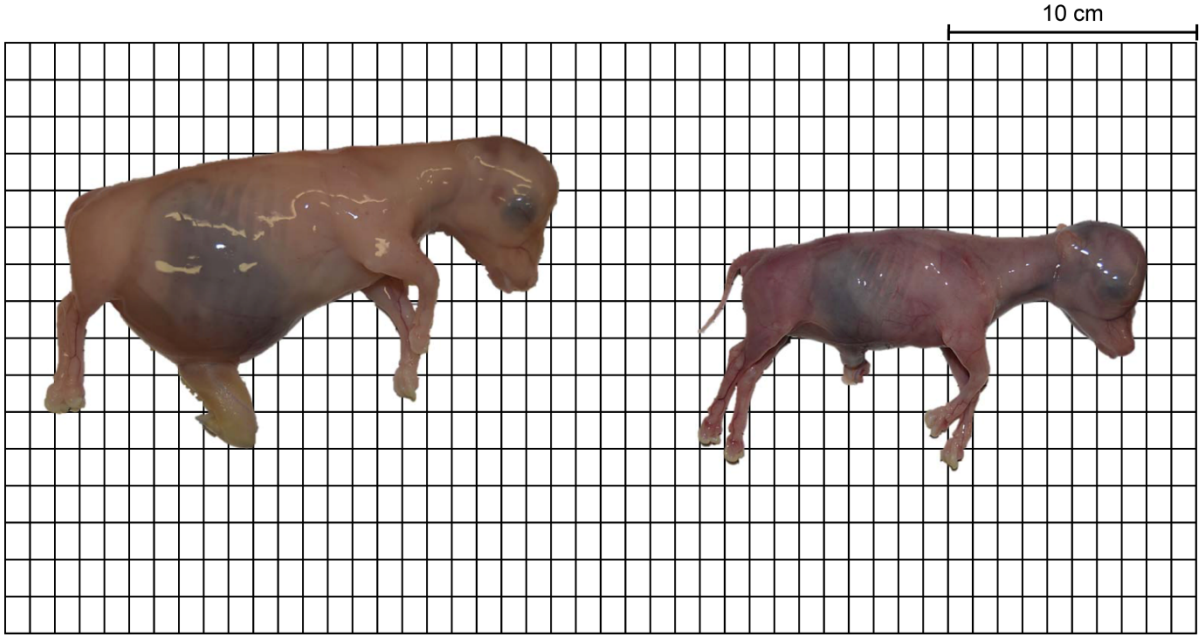


Figure 2. Excessive fetal growth at day 86 of gestation associated with *in vitro* embryo production. The fetus on the left was produced *in vitro* and the embryo on the right was produced by AI. The image is reproduced from Siqueira *et al*. (2017b) with permission of Oxford University Press.

**Evidence from other species that culture of embryos can affect postnatal phenotype**

Alterations in the function of the IVP embryo mean that one or more of the processes of oocyte maturation, sperm capacitation, fertilization, or embryonic development are not optimal. To be sure, the environment in which these events occur *in vitro* does not reproduce in all respects the environment in which these events occur *in vivo*. It is known from studies in various mammalian species that alterations in maternal environment during periods of oocyte maturation (Watkins *et al*., 2008; Turner and Robker, 2015) and preimplantation embryonic development (Fleming *et al*., 2015; Hansen, 2015; Hansen *et al*., 2016) can cause a change in developmental processes that have consequences for the resulting offspring in the postnatal period. This phenomenon, referred to as developmental programming, is likely to result either from epigenetic changes in the embryo that persist into later life or to a change in specific developmental processes that affect derived tissues or organs. In many cases, the change in phenotype caused by environmental cues is different for male offspring than for female offspring (Hansen *et al*., 2016). Effects of environment on the sire can also have impacts on his offspring after birth (Rando and Simmons, 2015; Schagdarsurengin and Steger, 2016).

Given the importance of parental environment for shaping postnatal phenotype, it is reasonable to question whether either IVP, which causes large perturbations in the environment of the gametes and embryo, or MOET, which is associated with ovulation of follicles not normally destined to ovulate and an abnormal endocrine environment, can change the phenotype of the animals produced using such technologies. This question has been addressed in the human, for embryos produced *in vitro*, and in the mouse, for embryos either produced and cultured *in vitro* or produced *in vivo* and cultured *in vitro*.

Studies in the human are difficult to interpret because of possible biases associated with the choice by a patient to seek IVP treatment. However, epidemiological studies have been conducted in which inclusion criteria were developed to minimize bias between children born from IVP vs control children. Increased frequency of hypertension in pre-adolescent and adolescent children born using IVP has been frequently observed (Scherrer *et al*., 2015). There are also data from the Netherlands that children and adolescents born as a result of IVP tend to be fatter than controls (Ceelen *et al*., 2007). In contrast,

educational performance was not associated with IVP (Wagenaar *et al*., 2008).

Controlled experiments in the mouse also show that production of embryos *in vitro* can cause alterations in postnatal phenotype and that some perturbations occur in a sex-specific context. For example, mice derived from embryos that developed *in vitro* from the two-cell stage exhibited altered behavior in the Morris water maze task, with male offspring being more affected than females (Ecker *et al*., 2004). In another study, Watkins *et al*. (2007) compared offspring derived from three procedures: transfer of embryos cultured from the two-cell to blastocyst stages of development, transfer of embryos flushed from the reproductive tract at the blastocyst stage, and natural mating. Among other endpoints studied, postnatal growth was generally similar between groups but mice derived from cultured embryos had higher systolic blood pressure than other groups at 21 weeks of age. Subsequently, Calle *et al*. (2012) examined male offspring derived from embryos cultured from the one-cell to blastocyst stages of development. Males derived from cultured embryos were less fertile than males derived from embryos produced *in vivo* as reflected by epididymal sperm count, sperm motility and the percent of females pregnant after breeding. In addition, male offspring, but not female offspring, experienced reduced clearance of an administered dose of glucose.

Specific components of culture medium used to produce mouse embryos *in vitro* have also been examined for their programming effects. In one experiment, addition of serum to the medium of embryos cultured from the one-cell to blastocyst stages resulted in offspring with altered behavioral responses in males and females, increased adult weight in females at 31 weeks of age and later and increased liver and heart size in both sexes at 20 month of age (Fernández-Gonzalez *et al*., 2004). Using *in vitro* fertilization and embryo culture, Donjacour *et al*. (2014) evaluated effect of embryo culture medium on properties of the resultant offspring. Culture media evaluated were KSOM and Whitten medium. Offspring derived from embryos cultured in KSOM were similar to offspring derived from controls (either from transfer of flushed blastocysts or produced from natural mating). In contrast, male offspring from embryos cultured in Whitten medium experienced increased body weight during the first 19 weeks of life, enlarged left heart and reduced response to a glucose tolerance test compared to male offspring from other treatments. There were no differences between groups for female offspring.

Finally, Rexhaj *et al*. (2015) evaluated whether addition of melatonin to embryo culture medium would reduce developmental abnormalities associated with *in vitro* fertilization and embryo culture. Results indicated that *in vitro* fertilization and embryo culture caused various vascular abnormalities in offspring and associated changes in DNA methylation and that addition of melatonin to the culture medium prevented these changes.

The phenotype expressed by mice derived by IVP may depend upon the pre- and postnatal environment. Thus, for example, Strata *et al*. (2015) observed no difference in behavior between offspring derived from *in vitro* fertilization and embryo culture vs those produced by natural mating when the prenatal diet was a typical rodent diet and the postnatal diet was designed to mimic a moderately high-fat diet. In contrast, feeding a low-protein diet prenatally and a high far diet postnatally was associated with differences in behavior between the mice derived from IVP *vs*. natural mating. Similarly, Cerny *et al*. (2017) observed no differences in glucose tolerance, insulin sensitivity, or insulin stimulation of muscle blood flow *in vivo* between male mice derived by IVP *vs*. those derived *in vivo* (females were not observed). However, when offspring were fed a high fat diet, those produced *in vitro* were fatter, experienced fasting hyperinsulinemia and hyperglycemia, and had reduced utilization of glucose in response to insulin treatment.

Transgenerational effects of embryo culture have also been reported. Mahsoudi *et al*. (2007) examined properties of mice derived from cultured embryos as compared to those derived from embryos that developed *in vivo* (F0). In addition, mice of the next two generations (F1 and F2) were also evaluated. There were few differences between mice in the F0 generation. However, mice derived from cultured embryos had lower weight at weaning and higher thyroid weight and epididymis weight at maturity. Differences in weaning weight persisted in the F1 generation and, additionally, F1 mice descended from cultured embryos had higher brain, pituitary and kidney weights and lower prostate weights. There were no differences between groups in body weight in the F2 generation and other measurements were not taken. Calle *et al*. (2012) also examined the F1 and F2 descendants of males offspring derived from cultured embryos. The F1 and F2 male offspring of males derived by embryo culture had reduced clearance of glucose in the glucose tolerance test and increased liver size but there was no effect on female descendants.

## First evidence that an assisted reproductive technology can affect adult phenotype in cattle

Studies in mice and humans are indicative that the entire process of IVP or, for mice, embryo culture, can change the adult phenotype of the offspring. However, results also indicate the degree to which phenotype is modified can depend on characteristics of the culture conditions, the sex of the offspring, and the environmental conditions offspring experience in postnatal life.

We recently analyzed a dataset from a registered Holstein dairy in north Florida that makes extensive use of embryo transfer using both IVP and MOET (Siqueira *et al*., 2017a). Included in the study were females born from June, 2012 to April, 2014. This resulted in records for 3,465 AI females born alive (2,037 with production records for first lactation), 249 heifer calves born alive following MOET (183 with lactation records), 345 heifer calves born alive following IVP with fertilization using conventional

semen (IVP-conv; 218 with lactation records) and 685 heifers born alive from IVP with fertilization using reverse X-sorted semen (IVP-sexed; 430 with lactation records).

As is true for studies on the consequences of human IVP, animals were not randomly assigned to reproductive technique. Rather, the managers of the farm assigned animals to the appropriate reproductive technique based on the breeding and management objectives of the farm. One difference between groups was for genomic estimates of predicted transmitting abilities (PTA) for economically important traits. For example, the genomic PTA for milk averaged 203 kg for AI, 290 kg for IVP-conv calves, 284 for IVP-sexed calves and 235 for MOET calves. Accordingly, lactational performance of the offspring was adjusted by using genomic PTA for milk, fat or protein as a covariate.

Heifer birth weight was significantly affected by reproductive technique but differences between groups were numerically small. In particular, calves born by IVP-conv (39.4 ± 0.3 kg) and IVP-sexed (39.0 ± 0.2) were larger than AI calves (38.5 ± 0.1 kg); values for MOET were intermediate and not different from any group (38.7 ± 0.4 kg). Weaning weight and average daily gain from birth to weaning did not differ between groups. However, weight at first breeding was highest for IVP-sexed (355 ± 2 kg), intermediate for IVP-conv (351 ± 3 kg) and MOET (346 ± 3 kg) and lowest for AI (344 ± 0.8 kg). Effects of treatment on average daily gain from weaning to breeding paralleled results for weight at breeding.

There was no significant difference between groups for age at first calving or interval from calving to conception in the first lactation. There were, however, significant effects of reproductive technique on lactational performance during first lactation (Table 1). In particular, IVF-sexed females produced less milk, fat and protein than cows of other groups. The difference in average projected actual milk yield between cows produced using IVF-sexed and cows produced by AI was 321 kg.

Table 1. Effects of technique used to produce a pregnancy on first-lactation milk yield of the resultant offspring after adjusting for genomic predicted transmitting ability for yield[1,2,3].

| | AI | IVP-conv | IVP-sexed | MOET | P-value |
|---|---|---|---|---|---|
| Projected actual milk yield, 305 days (kg) | 11038 ± 31$^{a}$ | 10946 ± 100$^{ab}$ | 10717 ± 76$^{b}$ | 10891 ± 149$^{ab}$ | 0.0014 |
| Projected actual fat yield, 305 days (kg) | 388.3 ± 1.2$^{a}$ | 385.6 ± 3.9$^{ab}$ | 377.1 ± 3.0$^{b}$ | 384.7 ± 5.8$^{ab}$ | 0.0072 |
| Projected actual protein yield, 305 days (kg) | 334.6 ± 1.0$^{a}$ | 336.5 ± 3.3$^{a}$ | 327.1 ± 2.5$^{b}$ | 331.2 ± 4.8$^{ab}$ | 0.0318 |

[1]Abbreviations used are as follows: AI: artificial insemination; IVP-conv: *in vitro* embryo production with conventional semen; IVP-sexed: *in vitro* embryo production with reverse X-sorted semen; MOET: multiple ovulation and embryo transfer; PTA: predicted transmitting ability; DPR: daughter pregnancy rate. [2]The P-values are for the main effect of reproductive technique. Within a row, means without a common superscript differ at $P < 0.05$. [3]Data are from Siqueira *et al*. (2017a).

**Implications of bovine data**

Caution must be taken when interpreting these data. Most importantly, neither dams nor sires of the animals studied were assigned randomly to treatment. Differences in genomic estimates of lactational performance were corrected for but other differences could have existed. Also, while to the best of our knowledge, calves were managed similarly after birth regardless of reproductive technique, one cannot discount some unknown difference in management. For example, fetal sex can affect milk yield in the existing lactation (Hinde *et al*., 2014) and it is conceivable that sex ratio of pregnancies in first lactation varied between groups. Given that culture system affected the nature of developmental programming in mice (Fernández-Gonzalez *et al*., 2004; Donjacour *et al*., 2014; Rexhaj *et al*., 2015), the effect of being born using a specific reproductive technology might depend on the particular details of the techniques employed. Accordingly, general conclusions should not be derived until the performance of animals derived from IVP or MOET is examined in a wide range of settings.

With these caveats in mind, the results of Siqueira et al. (2017a) indicate that IVP was only associated with adverse lactational outcomes when sexed semen produced using reverse sorting was used for fertilization. The fact that milk yield of IVP-conv and MOET cows was not different from that of AI cows may mean that the physiological processes controlling milk synthesis and secretion are less amenable to programming errors than for traits measured in mice and humans, i.e., cardiovascular function, growth rate, and regulation of glucose metabolism.

An important question to address is the mechanism by which use of reverse-sorted semen caused altered postnatal phenotype. The sorting process damages spermatozoa so that fertilization rate *in vitro* is compromised (Wheeler *et al*., 2006; Rasmussen *et al*., 2013). Perhaps, reverse sorting reduces non-nuclear components of sperm that are delivered to the oocyte at fertilization, for example sperm-borne miRNA (Liu *et al*., 2012). Also, a possible delay in fertilization due to damaged sperm could conceivably result in oocyte aging, which has negative consequences for the oocyte and subsequent embryo (Agung *et al*., 2006; Koyama *et al*., 2014). A compelling question is whether use of sexed semen can alter adult phenotype only when included as part of IVP with reverse-sorting or whether similar effects would be manifest using IVP with semen sorted before cryopreservation. Indeed, it is possible that alterations in adult capacity for milk yield could be caused by use of sex-sorted semen for AI. There is one report that incidence of stillbirths is increased among

bull calves born to females inseminated with X-sorted sperm (DeJarnette *et al*., 2009). There is also a report from a smaller study that incidence of stillbirth is higher among females born following AI with X-sorted sperm (Healy *et al*., 2013). The adult performance of calves born using AI with sexed semen remains to be determined and should be assessed.

## Future directions

Observations that assisted reproductive technologies can result in alterations in the resultant offspring are part of a larger and still-emerging story that postnatal phenotype of mammalsis can be modified by the microenvironment of the embryo during the preimplantation period (Fleming *et al*., 2015; Hansen, 2015; Hansen *et al*., 2016) and of the gametes from which it was derived (Watkins *et al*., 2008; Turner and Robker, 2015; Rando and Simmons, 2015; Schagdarsurengin and Steger, 2016). Thus, production efficiency of an animal may depend not only on its genotype and the environment in which it is raised but also on changes to its developmental program early in gestation. Understanding the nature of developmental programming may lead to novel interventions to improve efficiency of livestock production.

## References

**Agung B, Otoi T, Wongsrikeao P, Taniguchi M, Shimizu R, Watari H, Nagai T**. 2006. Effect of maturation culture period of oocytes on the sex ratio of in vitro fertilized bovine embryos. *J Reprod Dev*, 52:123-127.

**Block J, Bonilla L, Hansen PJ**. 2010. Efficacy of in vitro embryo transfer in lactating dairy cows using fresh or vitrified embryos produced in a novel embryo culture medium. *J Dairy Sci,* 93:5234-2542.

**Bonilla L, Block J, Denicol AC, Hansen PJ**. 2014. Consequences of transfer of an in vitro-produced embryo for the dam and resultant calf. *J Dairy Sci,*97:229-239.

**Calle A, Miranda A, Fernandez-Gonzalez R, Pericuesta E, Laguna R, Gutierrez-Adan A**. 2012. Male mice produced by in vitro culture have reduced fertility and transmit organomegaly and glucose intolerance to their male offspring. *Biol Reprod,* 87:34. doi: 10.1095/biolreprod.112.100743.

**Canu S, Boland M, Lloyd GM, Newman M, Christie MF, May PJ, Christley RM, Smith RF, Dobson H**. 2010. Predisposition to repeat breeding in UK cattle and success of artificial insemination alone or in combination with embryo transfer. *Vet Rec,* 167:44-51.

**Ceelen M, van Weissenbruch MM, Roos JC, Vermeiden JP, van Leeuwen FE, Delemarre-van de Waal HA**. 2007. Body composition in children and adolescents born after in vitro fertilization or spontaneous conception. *J Clin Endocrinol Metab*, 92:3417-3423.

**Cerny D, Sartori C, Rimoldi SF, Meister T, Soria R, Bouillet E, Scherrer U, Rexhaj E**. 2017. Assisted reproductive technologies predispose to insulin resistance and obesity in male mice challenged with a high fat diet. *Endocrinology*, 158:1152-1159.

**DeJarnette JM, Nebel RL, Marshall CE**. 2009. Evaluating the success of sex-sorted semen in US dairy herds from on farm records. *Theriogenology*, 71:49-58.

**Donjacour A, Liu X, Lin W, Simbulan R, Rinaudo PF**. 2014. In vitro fertilization affects growth and glucose metabolism in a sex-specific manner in an outbred mouse model. *Biol Reprod*, 90:80. doi: 10.1095/biolreprod.113.113134.

**Ecker DJ, Stein P, Xu Z, Williams CJ, Kopf GS, Bilker WB, Abel T, Schultz RM**. 2004. Long-term effects of culture of preimplantation mouse embryos on behavior. *Proc Natl Acad Sci USA*, 101:1595-600.

**Farin CE, Farmer WT, Farin PW**. 2010. Pregnancy recognition and abnormal offspring syndrome in cattle. *Reprod Fertil Dev*, 22:75-87.

**Fernández-Gonzalez R, Moreira P, Bilbao A, Jiménez A, Pérez-Crespo M, Ramírez MA, Rodríguez De Fonseca F, Pintado B, Gutiérrez-Adán A**. 2004. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proc Natl Acad Sci USA*, 101:5880-5885.

**Fleming TP, Watkins AJ, Sun C, Velazquez MA, Smyth NR, Eckert JJ**. 2015. Do little embryos make big decisions? How maternal dietary protein restriction can permanently change an embryo's potential, affecting adult health. *Reprod Fertil Dev*, 27:684-692.

**Gad A, Besenfelder U, Rings F, Ghanem N, Salilew-Wondim D, Hossain MM, Tesfaye D, Lonergan P, Becker A, Cinar U, Schellander K, Havlicek V, Hölker M.** 2011. Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and human assisted reproduction. *Hum Reprod*, 26:1693-707.

**Hansen PJ.** 2006. Realizing the promise of IVF in cattle--an overview. *Theriogenology*, 65:119-125.

**Hansen PJ**. 2014. Current and future assisted reproductive technologies for mammalian farm animals. *Adv Exp Med Biol,* 752:1-22.

**Hansen, PJ**. 2015. Developmental programming in the preimplantation period: can it be exploited to enhance postnatal function in cattle? *Anim Reprod,* 12:428-436.

**Hansen PJ, Dobbs KB, Denicol AC, Siqueira LG**. 2016. Sex and the preimplantation embryo: implications of sexual dimorphism in the preimplantation period for maternal programming of embryonic development. *Cell Tissue Res*, 363:237-247.

**Healy AA, House JK, Thomson PC**. 2013. Artificial insemination field data on the use of sexed and conventional semen in nulliparous Holstein heifers. *J Dairy Sci*, 96:1905-1914.

**Hinde K, Carpenter AJ, Clay JS, Bradford BJ**. 2014**.** Holsteins favor heifers, not bulls: biased milk production programmed during pregnancy as a function of fetal sex. *PLoS One,* 9:e86169.

**Kaniyamattam K, Block J, Hansen PJ, De Vries A**. 2017. Comparison between an exclusive in vitro-produced embryo transfer system and artificial

insemination for genetic, technical, and financial herd performance. *J Dairy Sci*, 100: 5729-5745.

**Koyama K, Kang SS, Huang W, Yanagawa Y, Takahashi Y, Nagano M**. 2014. Aging-related changes in in vitro-matured bovine oocytes: oxidative stress, mitochondrial activity and ATP content after nuclear maturation. *J Reprod Dev*, 60:136-142.

**Liu W-M, Pang RTK, Chiu PCN, Wong BPC, Lao K, Lao, KF, Yeung WSB**. 2012. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc Natl Acad Sci USA*, 109:490-494.

**Mahsoudi B, Li A, O'Neill C**. 2007. Assessment of the long-term and transgenerational consequences of perturbing preimplantation embryo development in mice. *Biol Reprod,* 77:889-896.

**Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC, Mann MRW**. 2010. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum Mol Genet*, 19:36-51.

**Pellegrino CA, Morotti F, Untura RM, Pontes JH, Pellegrino MF, Campolina JP, Seneda MM, Barbosa FA, Henry M**. 2016. Use of sexed sorted semen for fixed-time artificial insemination or fixed-time embryo transfer of in vitro-produced embryos in cattle. *Theriogenology*, 86:888-893.

**Pereira MH, Wiltbank MC, Vasconcelos JL**. 2016. Expression of estrus improves fertility and decreases pregnancy losses in lactating dairy cows that receive artificial insemination or embryo transfer. *J Dairy Sci*, 99:2237-2247.

**Pontes JH, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, Barreiros TR, Oliveira JA, Hasler JF, Seneda MM.** 2009. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (*Bos indicus*) donor cows. *Theriogenology*, 71:690-697.

**Pontes JH, Silva KC, Basso AC, Rigo AG, Ferreira CR, Santos GM, Sanches BV, Porcionato JP, Vieira PH, Faifer FS, Sterza FA, Schenk JL, Seneda MM**. 2010. Large-scale in vitro embryo production and pregnancy rates from *Bos taurus*, *Bos indicus,* and *indicus-taurus* dairy cows using sexed sperm. *Theriogenology*, 74:1349-1355.

**Pryce JE, Goddard ME, Raadsma HW, Hayes BJ**. 2010. Deterministic models of breeding scheme designs that incorporate genomic selection. *J Dairy Sci*, 93:5455-5466

**Rando OJ, Simmons RA.** 2015. I'm eating for two: parental dietary effects on offspring metabolism. *Cell*, 161:93-105.

**Rasmussen S, Block J, Seidel GE Jr, Brink Z, McSweeney K, Farin PW, Bonilla L, Hansen PJ**. 2013. Pregnancy rates of lactating cows after transfer of in vitro produced embryos using X-sorted sperm. *Theriogenology*. 79:453-461.

**Rexhaj E, Pireva A, Paoloni-Giacobino A, Allemann Y, Cerny D, Dessen P, Sartori C, Scherrer U, Rimoldi SF**. 2015. Prevention of vascular dysfunction and arterial hypertension in mice generated by assisted reproductive technologies by addition of melatonin to culture media. *Am J Physiol Heart Circ Physiol*, 309:H1151-1156.

**Schagdarsurengin U, Steger K**. 2016. Epigenetics in male reproduction: effect of paternal diet on sperm quality and offspring health. *Nat Rev Urol,* 13:584-595.

**Scherrer U, Rexhaj E, Allemann Y, Sartori C, Rimoldi SF**. 2015. Cardiovascular dysfunction in children conceived by assisted reproductive technologies. *Eur Heart J*, 36:1583-1589.

**Siqueira LG, Torres CA, Souza ED, Monteiro PL Jr, Arashiro EK, Camargo LS, Fernandes CA, Viana JH**. 2009. Pregnancy rates and corpus luteum-related factors affecting pregnancy establishment in bovine recipients synchronized for fixed-time embryo transfer. *Theriogenology*, 72:949-958.

**Siqueira LGB, Dikmen S, Ortega MS, Hansen PJ**. 2017a. Postnatal phenotype of dairy cows is altered by in vitro embryo production using reverse X-sorted semen. *J Dairy Sci*, 100:5899-5908.

**Siqueira, LGB, Tribulo P, Chen Z, Denicol AC, Ortega MS,Negrón-Pérez WM, Kannampuzha-Francis J, Pohler KG, Rivera RM, Hansen PJ**. 2017b. Colony stimulating factor 2 acts from Days 5 to 7 of development to modify programming of the bovine conceptus at day 86 of gestation. *Biol Reprod*, 96:743-757.

**Son DS, Choe CY, Cho SR, Choi SH, Kim HJ, Hur TY, Jung YG, Kang HG, Kim IM.** 2007. A CIDR-based timed embryo transfer protocol increases the pregnancy rate of lactating repeat breeder dairy cows. *J Reprod Dev*, 53:1313-1318.

**Stewart BM, Block J, Morelli P, Navarette AE, Amstalden M, Bonilla L, Hansen PJ, Bilby TR**. 2011. Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced in vitro with sex-sorted semen. *J Dairy Sci*, 94:3437-3445.

**Strata F, Giritharan G, Sebastiano FD, Piane LD, Kao CN, Donjacour A, Rinaudo P.** 2015. Behavior and brain gene expression changes in mice exposed to preimplantation and prenatal stress. *Reprod Sci,* 22:23-30.

**Thomasen JR, Willam A, Egger-Danner C, Sørensen AC**. 2016. Reproductive technologies combine well with genomic selection in dairy breeding programs. *J Dairy Sci*, 99:1331-1340.

**Turner N, Robker RL**. 2015. Developmental programming of obesity and insulin resistance: does mitochondrial dysfunction in oocytes play a role? *Mol Hum Reprod,* 21:23-30.

**van Wagtendonk-de Leeuw AM, Aerts BJ, den Daas JH**. 1998. Abnormal offspring following in vitro production of bovine preimplantation embryos: a field study. *Theriogenology*, 49:883-894.

**Vasconcelos JL, Jardina DT, Sá Filho OG, Aragon FL, Veras MB**. 2011. Comparison of progesterone-based protocols with gonadotropin-releasing hormone or estradiol benzoate for timed artificial insemination or embryo transfer in lactating dairy cows. *Theriogenology*, 75:1153-1160.

**Wagenaar K, Ceelen M, van Weissenbruch MM, Knol DL, Delemarre-van de Waal HA, Huisman J**.

2008. School functioning in 8- to 18-year-old children born after in vitro fertilization. *Eur J Pediatr*, 167:1289-1295.

**Watkins AJ, Platt D, Papenbrock T, Wilkins A, Eckert JJ, Kwong WY, Osmond C, Hanson M, Fleming TP**. 2007. Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. *Proc Natl Acad Sci USA,* 104:5449-5454.

**Watkins AJ, Wilkins A, Cunningham C, Perry VH, Seet MJ, Osmond C, Eckert JJ, Torrens C, Cagampang FR, Cleal J, Gray WP, Hanson MA, Fleming TP**. 2008. Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. *J Physiol*, 586:2231-2244.

**Wheeler MB, Rutledge JJ, Fischer-Brown A, VanEtten T, Malusky S, Beebe DJ**. 2006. Application of sexed semen technology to in vitro embryo production in cattle. *Theriogenology*, 65:219-227.

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# Negative energy balance and metabolic stress in relation to oocyte and embryo quality: an update on possible pathways reducing fertility in dairy cows

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## Abstract

A negative energy balance in metabolically compromised high producing dairy cows has been shown to influence oocyte and embryo quality. However, the possible involved pathways needed more attention to better understand specific deleterious effects. Oocyte maturation is the first process to be scrutinized. Because many possible metabolic factors might directly impact oocyte quality, systematic *in vitro* approaches were used to investigate the effects of oocyte maturation under elevated NEFA concentrations. Blastocysts originating from NEFA-exposed oocytes showed a lower cell number, an increased apoptotic cell index, signs of glucose intolerance, sensitive to oxidative stress and mitochondrial dysfunction. Defining these embryos' transcriptome and epigenome signatures revealed changes in DNA methylation patterns. Long-term exposure of developing murine follicles to elevated NEFA concentrations showed to impair oocyte developmental competence even more. While little is known on how the oviductal micro-environment can change as a consequence of a negative energy balance, a validated *in vitro* bovine oviduct model offered some valuable insights on how NEFAs disturb oviductal cell physiology. NEFA exposure reduces cell proliferation, cell migration, sperm binding capacity and monolayer integrity. In addition, oviductal cells seem to play an active role in regulating luminal NEFA-concentrations through increased permeability, intracellular lipid accumulation and fatty acid metabolism. This might favour early embryo development. The establishment of a successful pregnancy largely depends on the ability of the embryo to interact with a properly prepared endometrium. Because suboptimal physiological conditions influence oocyte maturation and embryo development to the extent that epigenetic consequences are unavoidable, the question arises if these changes hamper embryo implantation and subsequent development. Gene expression studies on epithelial endometrial cells brought in contact with *in vitro* embryos cultured for 4 days under suboptimal conditions reveal that the embryo-endometrial signaling is affected. Transfer of bovine embryos derived from compromised oocytes showed disturbed embryo development following recovery at day 14 with a negative impact on IFNt secretion and therefore suggesting carry-over effects from suboptimal culture conditions. The current paper will document the most important recent findings and comment on extrapolation possibilities from *in vitro* studies to field conditions in daily dairy practice. In addition, the possibility of remediating approaches will be discussed to see how this knowledge might generate insights on possible alleviating strategies.

**Keywords**: embryo, follicle, maternal metabolic disorders, oocyte, peri-conception, subfertility.

## Introduction

Mammals should reproduce to be able to lactate. Scientists, advising nutritionists and veterinarians are now aware of the fact that dairy cows should reproduce within a limited time span after calving to obtain the best economical results. Dairy cows are, from an economical point of view, the best producers during and just after peak lactation. Only fertile cows will have several of these efficient periods in their productive live span. It is not always easy to convince our dairy farmers of the central role "dairy cow fertility" plays in generating a substantial income, in sustainability and in the environmental impact of their dairy business. The fertility "story" is difficult to tell because it is not easy to measure reproductive performance of an individual cow or a dairy herd (in contrast to for example milk production performances) in a sound way (for review: Leblanc *et al*., 2010). Many heavily confounded fertility indices have been used (and are still routinely reproduced) to advice farmers, leading to no or wrong management decisions. Furthermore, this resulted in difficulties benchmarking farms or in judging the real gain of improved management strategies on fertility. When we discuss fertility with farmers it is not always clear whether we focus on the inherent reproductive ability of the cow's body (physiology) and/or on the reproductive performance of that specific cow kept under specific management conditions. It is very important to know the difference, as it will determine whether a cow and/or a cow's management should be "treated". Optimal reproduction and the generation of healthy offspring is the result of a very long chain of finely tuned physiological processes. Everything starts with the formation of the primordial germ cells in the embryo early during pregnancy and should lead to the successful ovulation and fertilization of a top quality oocyte, finally resulting in an ongoing pregnancy culminating in successful birth of a healthy offspring. Numerous factors affect this intricate long play of reproductive physiology and will determine the cow's reproductive efficiency. Apart from the genetic trait effects, the nurture effects on reproductive

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capacities start *in utero*, and show to be also important during the calf and heifer rearing period (Gonzales-Recio *et al*., 2012; Lohakare *et al*. 2012; Sinclair *et al*., 2016). Many studies focused already on the effect of the conditions during the dry and the transition period on reproduction. Dry matter intake, feed composition, social stress, housing conditions have all been associated with reproductive outcome (Wathes *et al*., 2012. Furthermore, awareness grows on the direct link between metabolic diseases in the dairy cow and the concomitant subfertility issue (Leroy *et al*., 2008). Numerous studies come up with conflicting results, but the most important generally agreed conclusion is that the metabolic health of the cow directly impacts on fertility. This is because the somatotropic axis affects the fertility axis at several levels, disturbing the endocrine interplay of female reproduction and thereby hampering timely ovulation. Furthermore, more and more data appear on how maternal metabolism directly affects oocyte and embryo quality due to changes in their micro-environment. For some years now, our laboratory focused on the pivotal role of the oocyte and the pre-implantation embryo in the link between maternal metabolic health and fertility. This review aims to provide an overview on the most recent advances and on the new insights this research has yielded by using the research model as presented in Fig. 1.

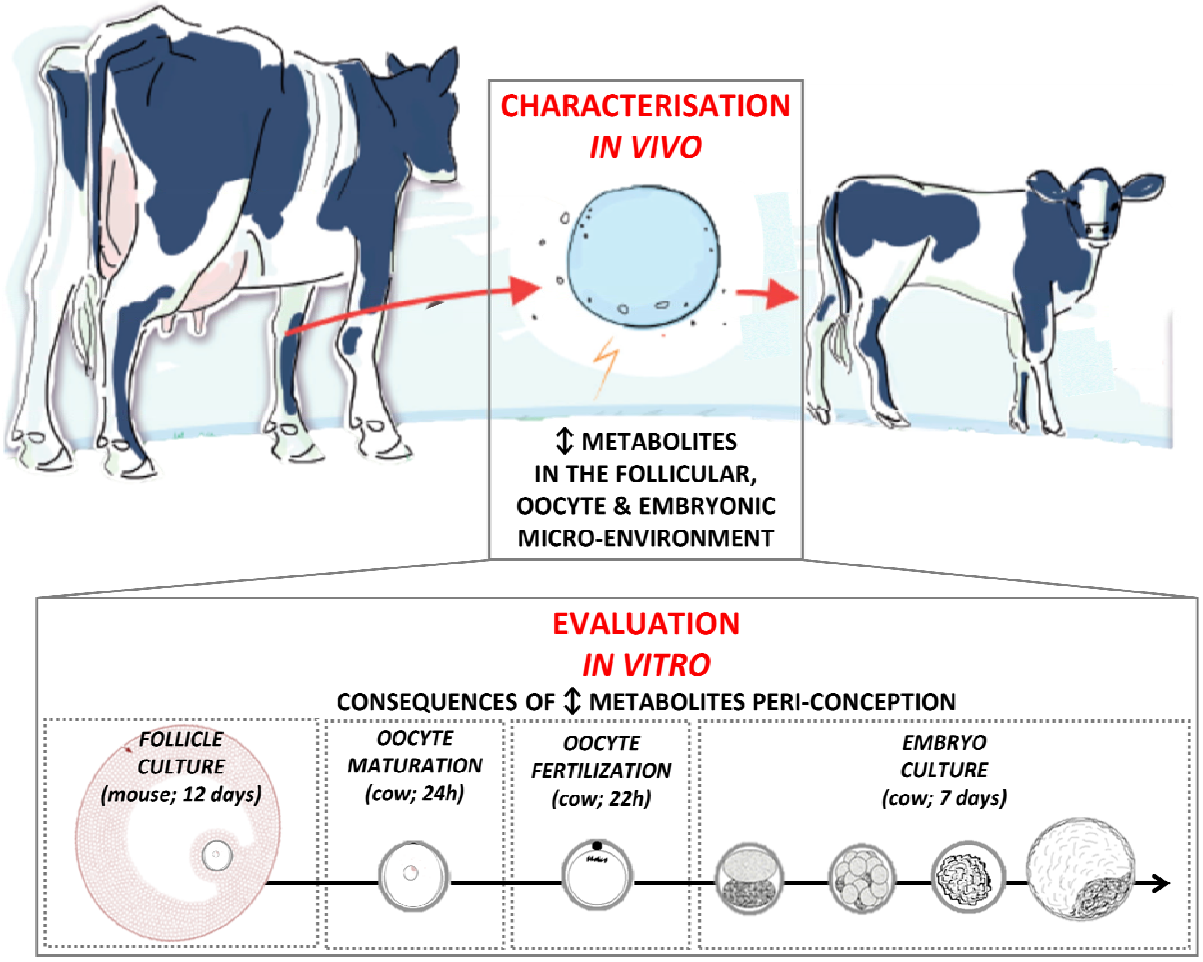


Figure 1. Overview of the general research model from which all data, described in this review, originate.

## How sensitive is the oocyte during the process of maturation?

The oocyte and embryo are of questionable quality in females suffering lipolytic disorders. Sartori *et al*. (2002) and Leroy *et al*. (2005) showed that the proportion of viable embryos is drastically reduced in lactating cows compared to non-lactating cows or heifers. More than 40% of the dairy cow conceptuses are lost within two weeks post-insemination (Leroy *et al*., 2008). Based on the known metabolic changes in the blood during periods of metabolic stress, several 'candidate' metabolic factors have been proposed to directly impact on fertility. Meticulously designed lab animal studies are, however, needed to distinguish whether the observed effect is exerted at the level the oocyte, zygote, embryo or sperm cell, or rather a combination of the latter. Hereto, when using *in vitro* oocyte maturation models, which allow to mimic metabolic disorders at the level of the oocyte, metabolite concentrations should be based on intra-follicular rather than on serum concentrations (Leroy *et al*., 2004). One of the best-studied examples of metabolic changes in follicular fluid is the negative

energy balance (NEB) condition in the high-yielding dairy cow (Leroy *et al*., 2008). These insights provide the basis for translation of metabolic disorders from the *in vivo* follicular environment towards our *in vitro* wells. Based on the known intra-follicular metabolite changes, during NEB periods of metabolic stress, several "candidate" factors have been extensively tested using our bovine and murine *in vitro* oocyte maturation models.

For example, effects of high free fatty acid (NEFA) concentrations during the final phase of oocyte maturation have been tested. Using a bovine *in vitro* model, we examined the effect of oocyte maturation in the presence of patho-physiologically relevant high concentrations of the three most important NEFAs (palmitic, stearic and oleic acid). We learned that final oocyte maturation under elevated NEFA concentrations (425 µm) results in blastocysts with a significantly lower cell number and increased apoptotic cell index (Van Hoeck *et al*., 2011). Surprisingly, such embryos displayed glucose intolerant characteristics, were triggered by oxidative stress regulating mechanisms and showed signs of mitochondrial dysfunction. Gene expression and functional data suggest that the resulting embryos have altered metabolic strategies, which might explain the aberrant energy metabolism and suggests a mechanism of metabolic dysregulation appearing in the pre-implantation embryo as a consequence of elevated NEFA concentrations peri-conception (Van Hoeck *et al*., 2013). All details of the latter studies are compiled in Table 1.

Furthermore, it was questioned whether glucose availability in the oocyte's micro-environment could intensify the observed effect of the high NEFA exposure. A lipolytic state in maternal metabolic disorders may also be accompanied by hypoglycemia during a NEB status in dairy cows (Leroy *et al*., 2008). Therefore, the effects of high and low glucose concentrations (9.9 and 2.8 mM, respectively) in the presence of elevated NEFAs (420 µm) during oocyte maturation were tested on oocyte and embryo quality, metabolism and developmental competence. Interestingly, in case of limited glucose availability, the effect of the high NEFA exposure on cumulus cell expansion and oocyte development was more prominent then when NEFAs were added in combination with high glucose concentrations. However, both conditions resulted in surviving embryos of reduced quality regarding to blastomere apoptosis and cell allocation (De Bie *et al*., 2017).

Step by step, based on the above described data, awareness grows that altered metabolic conditions during oocyte maturation alters transcriptome and epigenome signatures of the resulting blastocysts. Indeed, oocyte maturation under elevated NEFA concentrations significantly up-regulated the gene expression of DNMT3A both in the matured oocyte and in the day 7.5 blastocysts after routine fertilization and culture (Van Hoeck *et al*., 2013). The DNMT3A gene encodes a "de novo DNA methyltransferase", whose regulation is essential for the proper establishment of epigenetic marks (Van Hoeck *et al*., 2013). Further focus on elevated NEFA concentrations during oocyte *in vitro* maturation reveal influenced DNA methylation patterns in the resultant blastocysts, although to a relatively limited extent (Desmet *et al*., 2016b). Key cellular pathways affected by NEFA exposure were similar after the integration of gene expression and methylation patterns, with particular reference to lipid and carbohydrate metabolism, cell death, immune response and metabolic disorders (Desmet *et al*., 2016b). Thereby, metabolic perturbations, induced in the oocyte, may not simply result in suboptimal conception rates, but can also result in persisting affects during fetal development or become visible after birth as stated by Vickers (2014).

Finally, it is important to keep in mind that before entering the oviduct, the oocyte has spent several months to years in the ovary. Most of the *in vitro* studies performed so far, describe effects of suboptimal nutrient environments during the final oocyte maturation period under suboptimal metabolite conditions for merely 24 h. The physiological relevance of the highly defined 24 h *in vitro* maturation exposure model is therefore debatable. Therefore, a murine model was used to study the effect of long-term elevated NEFA concentrations on the follicle as a whole. This model includes the individual culture of early secondary pre-antral follicles up until day 13, when the *in vitro* follicles reach the antral stage, followed by oocyte isolation, *in vitro* fertilization and embryo culture. The follicle culture has been validated as a functional follicular unit, much resembling the *in vivo* situation (Cortvrindt and Smitz, 1998). In this model, *in vitro* cultured murine follicles exposed to elevated NEFA concentrations resulted in an altered follicular physiology, only moderately affecting follicular growth and antrum formation (Valckx *et al*., 2013). Importantly, when these follicles are exposed to elevated NEFA concentrations throughout follicle growth or only during the final maturation phase, it was shown that long term exposure (13 days) more severely impairs oocyte developmental competence, compared to short term NEFA exposure (only during the final phase of oocyte maturation; Valckx *et al*., 2015). These data strengthen our impression that not only final oocyte maturation, but also the preceding period of follicular and oocyte growth is pivotal for oocyte developmental competence. Indeed, 25 years ago, Britt (1992) already hypothesized that follicles developing and growing during the period of NEB early post-partum could be affected by the unfavorable metabolic changes and therefore may contain a developmentally incompetent oocyte at the time of ovulation after the voluntary waiting period. Only recently, Carvalho *et al*. (2014) studied 70 cows and showed that cows significantly losing body weight during the first three weeks of lactation displayed a dramatically lower number of viable and transferable good quality embryos after a superovulation treatment around 100 days post-partum. These results are obviously consistent with the hypothesis proposed by Britt and, up till the report of Valckx *et al*. (2015), a potential clarifying mechanism was lacking.

**What happens with the developing zygote during the first days after fertilization?**

In cattle, the early embryo resides in the oviduct for 4 days during which the embryo is highly sensitive to environmental changes (Latham and Schultz, 2001). Little is known about "whether and how" maternal metabolic disorders can modulate the embryonic micro-environment. Therefore, Jordaens *et al*. (2015) used a two-compartment polarized cell culture technique with hanging inserts (Miessen *et al*., 2011) as attempt to evaluate whether metabolic changes in the blood of mothers affect oviductal cell features and thereby potentially jeopardize the micro-environment of the early embryo. Results show that elevated NEFAs can affect *in vitro* bovine oviduct epithelial cell (BOEC) physiology by reducing cell proliferation, cell migration capacity, BOEC sperm binding capacity and monolayer integrity, in a cell polarity dependent manner (Jordaens *et al*., 2015). When studying these effects into closer detail (Jordaens *et al*., 2017), it was noticed that, when NEFAs were supplemented for 24 h in the basal (blood) compartment, their concentrations decreased in the basal compartment with a concomitant increase in the opposing apical chamber, indicating fatty acid transfer. Fascinatingly, when the 24 h NEFA-administration was performed at the apical side of the oviductal lumen, which will form the micro-environment of the embryo, no fatty acid transfer from apical to basal compartment could be observed, but intracellular lipid accumulation increased massively. Thus, apically administered NEFAs also induced anti-apoptotic and anti-oxidative pathways in the oviductal cells. These data suggest that BOECs may clear the micro-environment of the pre-implantation embryo from luminal NEFAs through increased monolayer permeability, intracellular lipid accumulation and fatty acid metabolism. Thereby, in order to 'safeguard the embryo, the oviduct is suggested to perform a gatekeeper function to modulate its micro-environment in favor of the early embryo by alleviating potential lipotoxic effects (Jordaens *et al*., 2017).

However, it remains debatable whether this protective/clearing effect is sufficient, to actually protect *in vivo* embryos passing the oviduct from females suffering metabolic disorders. Recently, our research group investigated whether and to which extent plasma NEFAs are reflected in oviduct fluid of healthy cattle using an *ex vivo* flushing method. Interestingly, oviductal fluid and plasma NEFA concentrations did not differ significantly and tended to be positively correlated. Thereby, we can conclude that a tight regulation of the embryonic milieu might not be assured in females suffering metabolic disorders (Jordaens *et al*., 2017). Of course, ongoing research is further studying how the oviductal environment is affected in maternal metabolic stressed conditions. This can become an important threat in females suffering metabolic disorders. Indeed, several *in vitro* embryo culture studies, in which embryos were exposed to distinct suboptimal metabolite conditions, show that a pre-implantation embryo is very sensitive to any perturbation in its micro-environment.

For example, our data show that bovine embryos exposed to elevated NEFA concentrations during the *in vitro* culture period have a lower developmental potential (Van Hoeck *et al*., 2012). Based on this information, a recent study furthermore examined underlying pathways in bovine embryos based on a genome-wide microarray analysis (Desmet *et al*., 2016b). Details of the latter studies are compiled in Table 1. Overall, expression of different genes was affected, with particular focus on oxidative metabolism, apoptosis, endoplasmic reticulum stress and lipid metabolism. Furthermore, vast epigenetic erasure and reprogramming events take place during early embryogenesis (Dean *et al*., 2001; Smith *et al*., 2012), making the embryo also susceptible for epigenetic alterations. More in-depth research indeed confirms this by showing changes in DNA methylation after elevated NEFA exposure during embryo culture. The latter methylation data cover similar pathways as described for the, earlier documented, transcriptomic alterations (Desmet *et al*., 2016b). This implies that suboptimal metabolite conditions can directly influence epigenetic reprogramming in the embryo and affect its genetic signature.

Furthermore, combined *in vivo* and *in vitro* studies were used to investigate the effect of dietary induced hyperlipidemia during embryo culture. Overall, addition of hyperlipidemic serum rich in saturated NEFAs during bovine *in vitro* embryo culture significantly reduced embryo development and quality (Leroy *et al*., 2010). Importantly, the extent of the observed effects seems to depend on the fatty acid types involved. Indeed, changing the predominant fatty acid to an unsaturated type in hyperlipidemic serum differentially influenced embryo development (Marei *et al*., 2016). More specifically, both saturated fat rich and unsaturated fat rich diets induced hyperlipidemia and increased total serum NEFAs by >33% in non-lactating cows, however, saturated serum was rich in palmitic acid while unsaturated serum was rich in alfa-linoleic acid (ALA). Addition of saturated hyperlipidemic serum during the *in vitro* embryo culture decreased blastocyst rates compared to control serum and surviving blastocysts had increased apoptosis. In contrast, unsaturated serum resulted in normal embryo development and quality with up-regulated DNMT3A indicating a regulatory role in DNA methylation compared to control serum (Marei *et al*., 2016).

Finally, it is important to consider that the oviduct not only provides the early embryonic environment, but also regulates the selection of spermatozoa, sperm storage, sperm motility and guidance of spermatozoa towards the egg (Holt and Fazeli, 2010). So far, we tested effects of direct NEFA exposure on follicle cells, oocytes, oviductal cells and embryos. However, information on effects of suboptimal metabolite conditions on the male gamete passing the female reproductive tract remained lacking. Therefore, we investigated to which extent sperm cells are influenced by altered metabolic conditions, such as elevated NEFAs. No obvious effect on the bovine fertilization process itself was noticed since cleavage

rates were not significantly affected after *in vitro* fertilization under high NEFA conditions. However, further embryonic development was hampered when fertilization occurred in the presence of high NEFAs. Interestingly, as shown in Table 1, elevated NEFAs had no influence on the fertilizing capacity of pre-exposed sperm, suggesting that NEFA-induced reduction in developmental competence is through alterations in oocyte quality but not through affecting sperm quality (Desmet *et al*., 2016a). More research is ongoing to investigate underlying mechanisms.

**The fate of the embryo upon arrival in the uterus.**

Years of intense research in our laboratory taught us that maternal metabolic changes can affect follicle health, oocyte development and even subsequent embryo quality and metabolism. This can help to explain why, in dairy cows, a great proportion of the embryos die before day 7 following insemination (Spencer, 2013). However, we also showed how bovine embryos may survive acute NEFA exposure probably due to an adaption in their metabolism (Van Hoeck *et al*., 2013, 2015). The latter does not inevitably guarantee that the embryo will implant successfully and lead to a full-term pregnancy. In cattle, embryonic loss peaks between days 7 and 16 post-mating during the period in which the uterus must develop receptivity in response to ovarian P4 to support conceptus growth and implantation (Spencer, 2013). Ayalon (1978) reported that critical period for embryonic death appears to be soon after the embryo enters the uterus. Indeed, upon arrival in the uterus a critical maternal-embryonic interplay needs to occur in order to fulfill: shedding of the zona pellucida, orientation, apposition, pre-attachment and adhesion of the blastocyst to the endometrium (Spencer *et al*., 2004). For the latter events, the differentiation fingerprint of both blastocyst and endometrial tissue will be of crucial importance. Suboptimal differentiation of the TE cell line, that becomes aligned against the endometrial epithelium, will compromise the success of the very first maternal-embryonic interactions (Armant, 2005). The embryo will then not be able to surpass the critical pre-implantation period in the uterus. Based on the latter insights, the question arises whether suboptimal metabolite conditions during oocyte and embryo culture can alter differentiation patterns in surviving embryos entering the uterus. Translation of recent cross-disciplinary insights might pave the way to understand how suboptimal maternal metabolite conditions could impact not only on the embryo till day 7-8, but also on embryo survival beyond this timing and first embryonic-maternal interactions. Recent cancer cell studies revealed that regulation of cell differentiation occurs via nutrient-sensing mechanisms (Folmes *et al*., 2012). Therefore, the earliest pre-implantation phases of *in vitro* embryo development were studied as 'window' for nutrient sensitive manipulations. Embryos were cultured during the first 4 days after fertilization under distinct nutrient conditions (control, high glucose, low amino acids) and were transferred, at morula stage, on a monolayer of epithelial endometrial cells (BEEC) till day 8 p.i.. Gene expression data show that under suboptimal metabolite conditions during the first 4 days of embryo culture nutrient sensing programs in embryos are prompted through mTOR mediated pathways. This might be the mechanism through which suboptimal metabolic environments during the early embryonic reprogramming impact on resultant blastocyst cell proliferation and differentiation programs (Van Hoeck *et al*., 2016). Fascinatingly, when BEEC were placed in contact with the three distinct groups of embryos, BEEC transcriptome profiles were differently regulated. Regulation of the latter genes might be critical for creating a receptive state towards the embryo. Even more, in BEEC exposed to embryos cultured under suboptimal conditions, the expression of two IFNt-responsive genes was down-regulated. Finally, a decreased integrin gene expression in blastocysts from nutrient-sensed embryos, and the subsequent down-regulated expression of cell adhesion factors in the allied BEEC, suggests that ligand-ligand interaction can be important for the first maternal-embryonic interactions. This further supports the importance of close contact between mother and embryo yet at this early timing, as recently stated by Sponchiado *et al*. (2017).

As stated above, there is a clear need for more studies focusing on post day 7 embryo development. In order to expand on this, we transferred morphologically equal day 7.5 blastocysts derived from NEFA-exposed or control oocytes to recipient cows and recovered the 14 day old embryos to investigate post-hatching development. Considering that the second week of development coincides, as explained above, with processes such as cell lineage specialization, embryo elongation and embryonic-maternal signaling (Berg *et al*., 2010), this *post* day 7 investigation is crucial. Preliminary data indicate that day 14 embryos derived from high NEFA-exposed oocytes were less developed after recovery compared to their control counterparts. Moreover, these embryos were metabolically compromised and had reduced IFNt secretion, a major signal of pregnancy recognition. This suggests that an insult during oocyte maturation may have long-lasting effects until the peri-implantation period, possibly affecting further development. Ongoing research focuses on the genome wide transcriptomic profiles using RNA sequencing techniques to discover potentially affected pathways.

**What did we learn so far that can be useful to improve fertility?**

Whether our *in vitro* models are suitable to withdraw/extrapolate conclusions towards the *in vivo* problem of the increased incidence in embryonic loss remains a matter for debate. By using such *in vitro* models, the awareness increased about whether and how suboptimal metabolite concentrations, such as elevated serum NEFA concentrations, are a potential threat around the period of conception (for an overview of peri-conception NEFA-toxicity; Table 1).

Table 1. The effects of elevated NEFAs during bovine *in vitro* oocyte maturation, fertilization or embryo culture.

| HIGH NEFA EXPOSURE DURING FINAL BOVINE *IN VITRO* OOCYTE MATURATION (24 H) | | | EVALUATED STAGE | | | |
|---|---|---|---|---|---|---|
| | | | Oocyte | 2-Cell Zygote | Morula | Blastocyst |
| Van Hoeck *et al*., 2011 | DEVELOPMENT | | | ≈ | | ↓ |
| | Quality | Cell Number | | | | ↓ |
| | | Apoptotic Cell Index | | | | ≈ |
| | Metabolism | Oxygen Consumption | | | | ↓ |
| | | Glucose Consumption | | | | ↓ |
| | | Pyruvate Consumption | | | | ↓ |
| | | Lactate Consumption | | | | ↑ |
| | Viability | Amino Acid Turn Over | | | | ↑ |
| Van Hoeck *et al*., 2013 | RESCUED DEVELOPMENT WHEN B-OXIDATION BLOCKED | | | | | ↑ |
| | Metabolism | Oxygen Consumption | | ≈ | | |
| | | Mitochondrial Membrane Potential | | ↗ | | |
| | ULTRASTRUCTURE | | ≈ | | | |
| Van Hoeck *et al*., 2015 | Transcriptome | # Differently Expressed Genes (DEG = P<0,05 & FC>1,5) | | | | 190 DEG 85↑ 105↓ Major pathways affected -lipid metabolism -carbohydrate metabolism |
| | Metabolism | Gluthation content | ↗ | | | |
| | | Lipid content | | | ↘ | |
| | Quality | Cryotolerance | | | | ≈ |
| Desmet *et al*., 2016b | Methylation Patterns | # Differently Methylated GENES (DMG = P<0,05 & FC>1,5) | | | | 268 DMG 148↑ 120↓ Major pathways affected - apoptosis - lipid metabolism pathways. - regulation of inflammation - gene transcription |

| HIGH NEFA EXPOSURE DURING FINAL BOVINE *IN VITRO* OOCYTE FERTILIZATION (22 H) | | | EVALUATED STAGE | | | |
|---|---|---|---|---|---|---|
| | | | **Oocyte** | **2-Cell Zygote** | **Morula** | **Blastocyst** |
| **Desmet *et al*., 2016a** | **DEVELOPMENT** | | | ↘ cleavage<br>↑ 2-cell block | | ↓ |
| | **DEVELOPMENT OUTGROWTH** | | | | | ↘<br>Outgrowth post day 9 |
| **HIGH NEFA EXPOSURE DURING FINAL BOVINE *IN VITRO* OOCYTE FERTILIZATION (7 days)** | | | **EVALUATED STAGE** | | | |
| | | | **Oocyte** | **2-Cell Zygote** | **Morula** | **Blastocyst** |
| **Desmet *et al*., 2016b** | **DEVELOPMENT** | | | ↓cleavage | | ↓ |
| | **Quality** | **Morphology** | | | | ↘ |
| | **Transcriptome** | **# Differently Expressed Genes**<br>**(DEG = P<0,05 & FC>1,5)** | | | | 311 DEG<br>206↑ 105↓<br>Major pathways affected<br>-cell morphology<br>-cell-to-cell signaling<br>-hematological system<br>-lipid metabolism<br>-small molecule biochemistry |
| | **Methylation Patterns** | **# Differently Methylated Genes**<br>**(DMG = P<0,05 & FC>1,5)** | | | | 1576 DMG<br>697↑ 879↓<br>Major pathways affected:<br>-cell death and survival<br>-lipid metabolism<br>-carbohydrate metabolism<br>-molecular transport<br>-embryonic development |

↓significantly reduced compared to control / ↘ trend for reduction compared to control ≈ no difference compared to control / ↗ trend for increase compared to control / ↑ significant increase compared to control

However, while most *in vitro* studies, designed to carefully mimic disturbed metabolic conditions in the well, show drastic effects on oocyte quality, *in vivo* dairy cow studies are less univocal (Matoba *et al*., 2012). Differences in study design, environmental conditions, evaluated end-point parameter, genetic

background and diet may explain this lack of uniformity.

Furthermore, when observing all data generated in our laboratory, one important question arises; is the damage observed during early embryonic culture a point of no return that will be imprinted in the embryo and will become visible later in life? Or are there compensating mechanisms that come into play during further embryo and fetal development that will erase all errors? Interestingly, the condition in which oocyte growth and maturation takes place is critical for its subsequent developmental capacity (Van Hoeck *et al*., 2011). However the micro-environment in which the embryo develops after fertilization is also important (Lazzari *et al*., 2010; Sermondade *et al*., 2012).

Therefore, we tested whether metabolically-compromised bovine oocytes matured under elevated pathophysiological NEFA concentrations (and altered glucose availability), could be rescued by adding rescuing compounds to the subsequent embryo culture medium. Remarkably, detrimental effects of high NEFA concentrations during oocyte maturation on embryo development could be alleviated, at least in part, by supplementation of additives, such as serum or insulin-transferrin-selenium, during culture. However, blastocysts were still inferior in quality, as evidenced by increased apoptosis, abnormal cell segregation patterns and altered metabolic behavior (Smits *et al*., 2016; De Bie *et al*., 2017).

Furthermore, also fatty acid saturation comes into play when considering potential interventions for recovery of metabolically compromised matured oocytes. Each type of NEFA and each ratio of saturated and unsaturated fatty acids seems to have its specific effects on the early stages in life, but also seems to be able, at least to some extent, to counteract effects of other NEFA types. However, to which extent such counteracting mechanisms are sufficient in order to sustain further development of the gamete (and subsequent embryo physiology) remains an intriguing issue. In this context, omega-3 fatty acids may have a promising role in protecting maturing oocytes from lipotoxic insults and we showed that it is possible to enhance oocyte quality by reducing cellular stress levels by e.g. ALA supplementation during oocyte maturation (Marei *et al*., 2017). Therefore, dietary supplementation with ALA in cows might be a promising strategy to resolve subfertility problems that are associated with reduced oocyte quality due to metabolic disorders associated with upregulated lipolysis during periods of negative energy balance.

## Conclusions. Significance for the dairy manager

Focusing on dairy cow fertility, pathological suboptimal metabolite conditions are proposed as a risk factor for disappointing reproductive performance. Years of expertise in animal *in vitro* embryo culture models contribute to the awareness that a normal maternal 'metabolic health' status is essential to safeguard successful ovulation, conception and further embryo development. In this context, high NEFA concentrations in the blood are known to alter the follicular and most probably also the oviductal micro-environment. The latter alterations in NEFA concentrations have been associated with a disappointing fertility outcome through disabled ovarian cell function, reduced oocyte's developmental competence and even embryonic transcriptome, metabolome and phenotypic fingerprints. Strategies should thus be based on alleviating suboptimal metabolite concentrations in the blood, and thereby aim to create optimal nutrient environments for the oocyte and embryo. Multiple "symptomatic" efforts have been done by designing dietary strategies to improve metabolically stressed condition of dairy cows. However, it is hard to come up with practical guidelines. It is generally accepted that the nutritional requirements for early resumption of ovarian activity and follicular growth are different from the nutritional conditions optimal for conception and early embryo growth. In this context, one should reflect on the believe that attempts to alleviate metabolic disorders, such as NEB status in the cow, are not always compatible with attempts to create optimal nutrient environments for the oocyte during final follicular growth and embryo during the first steps of development. Furthermore, besides focusing on the oocyte and early embryonic environment, one should consider the uterine environment which needs to prompt first maternal-embryonic interactions. Based on our first *in vitro* co-culture data, in which we detected different responses in BEEC allied with distinct types of 'nutrient-sensed' embryos, the emerging hypothesis raised that the endometrium is an active participant of successful pre-implantation embryo development. This reflection provides a new approach towards strategies that are highly needed to improve efficiency of fertility treatments.

## References

**Armant DR**. 2005. Blastocysts don't go it alone. Extrinsic signals fine-tune the intrinsic developmental program of trophoblast cells. *Dev Biol,* 280:260-280.

**Ayalon N**. 1978. A review of embryonic mortality in cattle. *J Reprod Fertil,* 54:483-493.

**Berg DK, van Leeuwen J, Beaumont S, Berg M, Pfeffer PL**. 2010. Embryo loss in cattle between days 7 and 16 of pregnancy. *Theriogenology*, 73:250-260.

**Britt JH**. 1992. Impacts of early postpartum metabolism on follicular development and fertility. *In*: Proceedings of the 24th Annual Convention of the American Association of Bovine Practitioners, 1991, Orlando, FL. Ashland, OH: AABP. pp. 39-43.

**Carvalho PD, Souza AH, Amundson MC, Hackbart KS, Fuenzalida MJ, Herlihy MM, Ayres H, Dresch AR, Vieira LM, Guenther JN, Grummer RR, Fricke PM, Shaver RD, Wilbank MC**. 2014. Relationships between fertility and postpartum changes in body condition and body weight in lactating dairy cows. *J Dairy Sci*, 97:3666-3683.

**Cortvrindt R, Smitz J**. 1998. Early preantral mouse follicle in vitro maturation: oocyte growth, meiotic maturation and granulosa-cell proliferation.

*Theriogenology*, 49:845-859.

**Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W**. 2001. Conservation of methylation reprogramming in mammalian development: Aberrant reprogramming in cloned embryos. *Proc Natl Acad Sci*, 98:13734-13738.

**De Bie J, Marei WF, Maillo V, Jordaens L, Gutierrez-Adan A, Bols PE, Leroy JL**. 2017. Differential effects of high and low glucose concentrations during lipolysis-like conditions on bovine in vitro oocyte quality, metabolism and subsequent embryo development. *Reprod Fertil Dev*. doi: 10.1071/RD16474.

**Desmet KLJ, Marei WF, Merckx E, Bols PEJ, Leroy JLMR**. 2016a. Effect of non-esterified fatty acids during sperm capacitation or IVF on developmental competence of bovine oocytes. *Anim Reprod*, 13:643.

**Desmet KLJ, Van Hoeck V, Gagné D, Fournier E, Thakur A, O'Doherty AM, Walsh CP, Sirard MA, Bols PEJ, Leroy JLMR**. 2016b. Exposure of bovine oocytes and embryos to elevated non-esterified fatty acid concentrations: integration of epigenetic and transcriptomic signatures in resultant blastocysts. *BMC Genomics*, 17:1004. doi: 10.1186/s12864-016-3366-y

**Folmes CDL, Dzeja PP, Nelson TJ, Terzic A**. 2012. Metabolic plasticity in stem cell homeostasis and differentiation. *Cell Stem Cell*, 11:596-606.

**Gonzalez-Recio O, Ugarte E, Bach A**. 2012. Transgenerational effect of maternal lactation during pregnancy: a Holstein cow model. *PLOS One,* 7:1-7.

**Holt WV, Fazeli A**. 2010. The oviduct as a complex mediator of mammalian sperm function and selection. *Mol Reprod Dev*, 77:934-943.

**Jordaens L, Arias-Alvarez M, Pintelon I, Thys S, Valckx S, Dezhkam Y, Bols PEJ, Leroy JLMR**. 2015. Elevated non-esterified fatty acid concentrations hamper bovine oviductal epithelial cell physiology in three different in vitro culture systems. *Theriogenology,* 84:899-910.

**Jordaens L, Van Hoeck V, Maillo V, Gutierrez-Adan A, Marei WFA, Vlaeminck B, Thys S, Sturmey RG, Bols PEJ, Leroy JLMR**. 2017. Maternal metabolic stress may affect oviduct gatekeeper function. *Reproduction*. doi: 10.1530/REP-16-0569.

**Latham KE, Schultz RM**. 2001. Embryonic genome activation. *Front Biosci,* 6:748-759.

**Lazzari G, Colleoni S, Lagutina I, Crotti G, Turini P, Tessaro I, Brunetti D, Duchi R, Galli C**. 2010. Short-term and long-term effects of embryo culture in the surrogate sheep oviduct versus in vitro culture for different domestic species. *Theriogenology*, 73:748-757.

**Leblanc S**. 2010. Assessing the association of the level of milk production with reproductive performance in dairy cattle. *J Reprod Dev*, 56:S1-S7.

**Leroy JL, Vanholder T, Delanghe JR, Opsomer G, van Soom A, Bols PE, Dewulf J, de Kruif A**. 2004. Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* 62:1131-1143.

**Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G**. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction*, 130:485-495.

**Leroy JLMR, Van Soom A, Opsomer G, Goovaerts IG, Bols PE**. 2008. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II. Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. *Reprod Domest Anim* 43: 623-632.

**Leroy JL, Van Hoeck V, Clemente M, Rizos D, Gutierrez-Adan A, Van Soom A, Uytterhoeven M, Bols PE**. 2010. The effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality. *Hum Reprod*, 25:768-778.

**Lohakare JD, Südekum KH, Pattanaik AK**. 2012. Nutrition-induced changes of growth from birth to first calving and its impact on mammary development and first-lactation milk yield in dairy heifers: a review. *Asian-Australas J Anim Sci,* 25:1338-1350.

**Marei WF, Alvarez MA, Van Hoeck V, Gutierrez-Adan A, Bols PE, Leroy JL**. 2016. Effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality depends on the type of major fatty acid in the diet. *Reprod Fertil Dev*, doi: 10.1071/RD16297.

**Marei WFA, De Bie J, Mohey-Elsaeed O, Wydooghe E, Bols PEJ, Leroy JLMR**. 2017. Alpha-linolenic acid protects the developmental capacity of bovine cumulus-oocyte complexes matured under lipotoxic conditions in vitro. *Biol Reprod*, doi10.1093/biolre/iox046.

**Matoba S, O'Hara L, Carter F, Kelly AK, Fair T, Rizos D, Lonergan P**. 2012. The association between metabolic parameters and oocyte quality early and late postpartum in Holstein dairy cows. *J Dairy Sci,* 95:1257-1266.

**Miessen K, Sharbati S, Einspanier R, Schoen J**. 2011. Modelling the porcine oviduct epithelium: a polarized in vitro system suitable for long-term cultivation. *Theriogenology*, 76:900-910.

**Sartori R, Bergfelt R, Mertens SA, Guenther JN, Parish JJ, Wiltbank MC**. 2002. Fertilization and early embryonic developmentin heifers and lactating cows in summer and lactating and dry cowsin winter. *J Dairy Sci,* 85:2803-2812.

**Sermondade N, Delarouzière V, Ravel C, Berthaut I, Verstraete L, Mathieu E, Antoine JM, Mandelbaum J**. 2012. Characterization of a recurrent poor-quality embryo morphology phenotype and zygote transfer as a rescue strategy. *Reprod Biomed Online*, 24:403-409.

**Sinclair KD, Rutherford KMD, Wallace JM, Brameld JM, Stöger R, Alberio R, Sweetman D, Gardner DS, Perry VEA, Adam CL, Ashworth CJ, Robinson JE, Dwyer CM**. 2016. Epigenetics and developmental programming of welfare and production traits in farm animals. *Reprod Fertil Dev*, 28:1443-1478.

**Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnirke A, Regev A, Meissner A**. 2012. A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature*, 484:339-344.

**Smits A, De Bie J, Bols PEJ, Marei WF, Leroy JLMR**. 2016. Effect of embryo culture conditions on developmental potential of bovine oocytes matured under lipotoxic conditions. *In:* Proceedings of the 18th International Congress on Animal Reproduction, 2016,

Tours, France. Tours: ICAR. pp. PW552.]

**Spencer TE, Johnson GA, Bazer FW, Burghardt RC**. 2004. Implantation mechanisms: insights from the sheep *Reproduction*, 128:657-668.

**Spencer TE**. 2013. Early pregnancy: concepts, challenges and potential solutions. *Anim Front,* 3:48-55.

**Sponchiado M, Gomes NS, Fontes PK, Martis T, del Collado M, Pastore A, Pugliesi G, Nogueira MFG, Binelli M**. 2017. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. *PLOS One*. doi.org/10.1371/journal.pone.0175954.

**Valckx S, Van Hoeck V, Arias-Alvarez M, Maillo V, Lopez-Cardona AP, Gutierrez-Adan A, Berth M, Cortvrindt R, Bols P, Leroy J**. 2014. Elevated non-esterified fatty acid concentrations during in vitro murine follicle growth alter follicular physiology and reduce oocyte developmental competence. *Fertil Steril*, 102:1769-1776 e1.

**Valckx SDM, Jordaens L, Cortvrindt R, Bols PEJ, Leroy JLMR**. 2015. The effect of short versus long term elevated non-esterified fatty acid concentrations during murine in vitro follicle growth on oocyte developmental competence. *Reprod Fertil Dev,* 27:184-184.

**Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PEJ, Leroy JLMR**. 2011. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *PLOS One*, 6:23183.

**Van Hoeck V, De Bie J, Andries S, Merckx E, Bols PEJ, Leroy JLMR**. 2012. Elevated concentrations of saturated NEFA during bovine in vitro embryo culture compromise pre-implantation embryo development. *In*: 28th Scientific Meeting European Embryo Transfer Association, 2012, Saint-Malo, France. Saint Malo: AETE.

**Van Hoeck V, Leroy JLMR, Arias-Álvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, Bols PEJ, Leese HJ, Sturmey RGS**. 2013. Oocyte developmental failure in response to elevated non-esterified fatty acid concentrations: mechanistic insights. *Reproduction*, 145:33-44.

**Van Hoeck V, Rizos D, Gutierrez-Adan A, Pintelon I, Jorssen E, Dufort I, Sirard MA, Verlaet A, Hermans N, Bols PEJ, Leroy JLMR**. 2015. The interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations. *Reprod Fertil Dev*, 27:372-384.

**Van Hoeck V, Marei W, Bols PEJ, Leroy JLMR**. 2016. Maternal impact of metabolic diseases: Effect of nutrient sensing pathways on developmental and differentiation programs in the bovine embryo. *Anim Reprod*, 13:667, 2016.

**Vickers MH**. 2014. Early life nutrition, epigenetics and programming of later life disease. *Nutrients*, 6:2165-2178.

**Wathes DC**. 2012. Mechanisms linking metabolic status and disease with reproductive outcome in the dairy cow. *Reprod Domest Anim* 47:304-312.

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# Application of integrative genomics and systems biology to conventional and *in vitro* reproductive traits in cattle

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## Abstract

Assisted reproductive technologies (ARTs) have a strong impact on breeding especially when coupled with genomic selection (GS). The routine implementation of *in vitro* production (IVP) and GS of embryos before embryo transfer (ET) in breeding companies is not yet possible. Improvement of oocyte donor and embryo recipient quality is needed to make realistic a commercialization of these procedures in the near future. A better understanding of both biological mechanisms and molecular markers associated to IVP-ET related traits is necessary to improve the prediction of donor and recipient cow quality for IVP procedures. The huge amount of data generated from high throughput technologies has a tremendous impact in the search for biomarkers of complex traits. This paper reviews integrative genomics and systems biology approaches as applied to both *Bos indicus* and *Bos taurus* cattle reproduction by both conventional and ARTs such as OPU-IVP. The integration of systems biology information across different biological layers generates a complete view of the different molecular networks that control complex traits and can provide a strong contribution to the understanding of traits related to ARTs.

**Keywords**: systems biology, IVP, reproduction, cattle, biomarkers, data integration.

## Introduction

Breeding for more efficient animals is becoming of increasing importance, and new and faster breeding methods are needed. Assisted reproductive technologies (artificial insemination (AI), ovum pick-up (OPU), IVP and ET) have significantly contributed to animal breeding programs. Similarly, genomics has significantly increased both speed of genetic gain and selection accuracy (Kadarmideen *et al*., 2015). However, the greatest benefits of those tools can only be expected when they are combined, allowing animals to be selected accurately early in life and with a more precise estimation of their breeding value.

The combined GS-IVP-ET procedures are not routinely implemented yet. While it is possible to collect embryo biopsies for DNA genotyping without affecting the pregnancy rate of the embryos (Saadi *et al*., 2014), the IVP-ET efficiency is still relatively low and remains the bottleneck in this process. A possible way to increase the efficiency of IVP-ET procedures is identification and selection of high-quality oocyte donors and embryo recipients. In order to successfully predict donor and recipient cow quality in IVP procedures, a better understanding of the molecular mechanisms responsible for these traits is needed (Salilew-Wondim *et al*., 2010).

The literature provides a complex picture of the molecules associated with oocyte competence at different molecular level: proteomics, transcriptomics (Jiang *et al*., 2010; Gilbert *et al*., 2012; Nivet *et al*., 2013) and metabolomics (Matoba *et al*., 2014). Furthermore, numerous studies focused on the characterization of the expression profiles of granulosa cells from follicles at different developmental stages (Hatzirodos *et al*., 2014b; Girard *et al*., 2015) and physiological condition (Hatzirodos *et al*., 2014a). Research in recipient cows on transcriptomic profiles has usually been applied to the endometrial tissue, and this has increased the knowledge about molecular mechanisms responsible for good or inadequate uterine environment for pregnancy recognition and implantation (Salilew-Wondim *et al*., 2010; Forde and Lonergan, 2012; Ponsuksili *et al*., 2012; Minten *et al*., 2013; Killeen *et al*., 2014). In the same way endometrial gene expressions have been characterized both during the estrous cycle and early pregnancy before implantation (Bauersachs et al., 2005; Mitko et al., 2008; Bauersachs et al., 2009; Mansouri-Attia et al., 2009; Ponsuksili et al., 2012) as well as after implantation (Bauersachs *et al*., 2006; Binelli *et al*., 2015).

Considering the huge amount of data generated by use of modern high-throughput technologies at all levels of biological systems (e.g. genome-wide, transcriptome-wide, metabolome-wide or proteome-wide measurements), systems biology analysis is the most promising approach to provide a holistic view of important biological mechanisms and of molecular

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markers associated with complex traits, such as those related to IVP-ET procedures. Systems biology is a holistic approach to analyze and decipher complex biological systems through computational and mathematical modeling of the whole set of molecular networks within an organism. Systems biology aims to decipher systems structures (the network of gene interactions and biochemical pathways), but also the dynamic changes of these network under different conditions (Kitano, 2002; Chuang *et al*., 2010; Kadarmideen *et al*., 2011; Kadarmideen, 2014). A huge part of the computational methods in systems biology is based on network approaches that identify group of genes or molecules with common behavior assuming that clustered molecules are functionally related (Brazhnik *et al*., 2002). As reviewed in (Mazzoni *et al*., 2015), several approaches are available to build gene interaction or association networks: Co-expression/regulatory patterns (Horvath, 2011), Bayesian networks (Friedman *et al*., 2000), Random Forest Tree approaches (Breiman, 2001) or Artificial Neural Networks (ANN) (McCulloch and Pitts, 1943; Cookson et al., 2009).

Integrative systems biology is based on analyses of networks of different types of molecules trying to integrate information at different biological levels (Dixon et al., 2007; Mazzoni et al., 2015; Suravajhala et al., 2016; Wang and Michoel, 2016). The analyses of a specific outcome (blastocyst rate and quality or endometrial receptive competence) with systems biology methods together with the integration of systems biology approaches at different biological levels is of vital importance for identification of biomarkers for incredibly complex traits like IVP and ET outcome.

While above we discussed about ARTs, it is very important to apply integrative systems biology approaches also to conventional cattle reproduction via GS and genomic breeding. Fundamental to this are Genome Wide Association Studies (GWAS). A very important proportion of the world beef production trade comes from tropical and subtropical regions. *Bos indicus,* from Brahman breed in Australia, and Nellore in Brazil, and their crosses are the most important breeds used in tropical areas. Candidate genes and biological information to be included in genomic selection of very young donors are essential to increase the productivity in tropical and sub-tropical areas (Simianer, 2016). The identification of candidate genes and metabolic pathways associated with reproductive traits such as antral follicular population and early pregnancy in Nellore heifers has become of great importance with the increasing use of ARTs technologies. In this context, genome wide association studies followed by functional analysis such as Medical Subject Heading (MeSH) terms enrichment can give a strong contribution.

In this mini-review we describe (i) the importance of the application of integrative systems biology approaches in the analysis of ARTs related traits, (ii) the two integrative systems biology experimental designs from the GIFT project consortium (www.gift.ku.dk) aimed to identify biomarkers for the selection of superior oocyte donor and embryo recipient cows, and (iii) a GWAS for the identification of candidate genes and metabolic pathways for early puberty and reproductive traits in Nellore heifers.

## Integrative systems biology analyses and application to IVP related traits in cattle

To correctly predict donor and recipient quality we need a list of biomarkers and biological processes to be included in the prediction methods for the selection of the best donor or recipient cows. This implies gathering enough information about specific molecules and biological processes associated to the IVP-ET performance of the animals.

The definition of a biomarker is strictly dependent on the field of study. The features of a biomarker are described by Austin Bradford Hill's guidelines (Aronson, 2005) and here adapted to the context of IVP related traits: strength (a strong association between marker and IVP-ET outcome), consistency (the association identified in the cow reference set should persist in cow populations from different farms and different physiological status of the cows), specificity (the biomarker should be associated with the specific IVP-ET related trait), plausibility (the biomarker should be part of meaningful biological mechanisms), coherence (the biomarker and the biological functions performed should be consistent with previous knowledge), analogy (previous findings for the same association make the biomarkers more valuable). Furthermore, in order to be routinely applied in IVP-ET procedures, the biomarker should be easily accessible and the costs of its measurement affordable. According to the guidelines the search for a biomarker is not an easy task. Until now, the search of biomarkers for IVP related traits have been based mainly on transcriptomic data for two reasons: first, it allows amplification of small samples (Orozco-Lucero and Sirard, 2014) and second, it provides information about the biology of a trait. Many studies have focused on functional enrichment and analysis of the differentially expressed genes to give a biological context of the biomarker. However, systems biology approaches like co-expression analysis were rarely adopted. The possibility to analyze transcriptomic data in a more holistic way opens for a better understanding of the biological mechanisms and allows the identification of key genes that would be impossible to identify with traditional “reductionist” approaches (Kitano, 2002; Chuang *et al*., 2010; Kadarmideen, 2014). For example, co-expression network can be used to identify regulatory genes that are key genes responsible for the control of the expression of a set of genes expected to be involved in the same biological process (Zhao *et al*., 2010). Thus, regulatory genes are candidate genes to be used for biomarker development.

The mRNA expression data are used as a surrogate for protein expression. However, it is known that the correlation between mRNA and protein levels is moderate due to complex regulation mechanisms

occurring after transcription (Lu *et al*., 2007; Schwanhäusser *et al*., 2011). A perfect systems biology experiment could avoid this issue including use of all the biological levels and integration of the information to create a complete overview. The integration of different biological levels does not imply necessarily to measure all biological levels in a sample set. A huge amount of multi-omics data (transcriptomics, genomics, proteomics, metabolomics, interactomics etc.) is available in public databases. A possibility would be the integration of experimental data with data from publicly available repositories (Mazzoni *et al*., 2015), for example STRING v.10 database (Szklarczyk *et al*., 2015) and Gene Mania (Montojo *et al*., 2010).

Measurements of the expression pattern of a specific set of mRNA molecules from follicular cells or endometrial biopsies could be used directly to select the best cows but they cannot be implemented in routine breeding procedures (Ponsuksili *et al*., 2010). Furthermore, expression profiles are subject to continuous variation due to environmental and physiological status of the cows. Most of the molecules associated with IVP traits are not consistent (as required by the Austin Bradford Hill's guidelines). This means that when the cows are tested for prediction they should be in the same biological status and environmental condition as the reference set used to identify these biomarkers. Integration of the transcriptomic level with the genomic level could overcome this issue. The approach is called expression QTL (eQTL) mapping and allows for the identification of genomic variants (e.g. SNPs) that are correlated with the expression level of a specific transcript (Dixon *et al*., 2007; Cookson *et al*., 2009; Wang and Michoel, 2016). The eQTL mapping integrates variation at the level of RNA expression with the variation at the DNA level. The advantage of eQTL mapping is that it identifies DNA variants that can be used in breeding (Ponsuksili *et al*., 2010), for example to select for ARTs related traits.

## Co-expression network analysis of granulosa cells in donor cows

In order to identify biomarkers to predict the quality of a donor cow and to perform an eQTL analysis, it is necessary to collect data at the single animal level. An integrative systems biology analysis at the single animal level is the ideal way to obtain a more complete understanding of the biological processes associated with oocyte competence in follicular cells and to identify genetic variants to be included in breeding procedures. On this idea, we based the experimental design of our transcriptomic donor cow analysis (Mazzoni *et al*., 2017). Briefly, all antral follicles present in each pair of ovaries were collected and the IVP performances for each animal were evaluated. The mural granulosa cells and a small amount of granulosa cells from the cumulus layer were collected as a byproduct of the follicle aspiration, and the total RNA was extracted and 24 samples sequenced. The RNA-Seq data were analyzed with a bioinformatics pipeline to quantify the expression of the entire set of known genes. Therefore we generated the average ovarian expression profile of granulosa cells and the respective IVP performances of the entire set of cumulus-oocyte-complexes.

We identified 51 differentially expressed genes associated to IVP performances and seven candidate genes associated with all IVP parameters analyzed (Mazzoni *et al*., 2017). In the same study, the functional enrichment of the differentially expressed genes and the comparison with previous findings in the literature confirmed the positive association between the IVP outcome and the presence of early atresia as previously observed (Moor and Trounson, 1977; Wurth and Kruip, 1992; De Wit et al., 2000; Feng et al., 2007; Heleil et al., 2010).

A systems biology analysis of the same dataset would provide more information about the biological mechanisms controlling atresia and consequently result in more plausible biomarkers. Thus, we are working on a co-expression network analysis of the same dataset using the Weighted Gene Co-expression Network Analysis (WGCNA) R package. WGCNA identifies groups of co-expressed genes called “modules” that are expected to be involved in the same biological process (Zhao *et al*., 2010). Furthermore, WGCNA provides the ability to select only the modules that are correlated with a trait of interest. Briefly, we identified modules associated with IVP performances, and these data were then integrated with information about protein-protein association provided by STRING v.10 (Szklarczyk *et al*., 2015). The functional enrichment based on STRING information was performed (i) to understand the biology behind the IVP performance and (ii) to select the biologically meaningful modules that are the most suitable for selection of new candidate genes. We analyzed the selected modules to identify central genes (hub genes) and regulatory genes integrating information from other sources like Ingenuity® Pathway Analysis.

The preliminary results confirmed that the systems biology is a good approach to study IVP related traits in donor cows. The atresia mechanism was confirmed to be positively correlated with IVP performances. Moreover, the analysis provided a more detailed description of the molecular mechanisms that link atresia with IVP performances which are new evidences to support the candidate genes.

## Co-expression analysis of endometrial biopsies

Similarly to the donor cow analysis, we structured the experimental design of the recipient cow analysis for the embryo implantation to collect information for each single animal. Briefly, endometrial biopsies from the uterine horn ipsilateral to the corpus luteum were sampled from experimental cows on day 6-8 in the estrous cycle, and RNA was extracted and sequenced. On day 6-8 in the following cycle *in vitro* produced blastocysts were transferred to the animals and the pregnancy status was determined at slaughter on day 26-47. In the pregnant animals, embryo/fetuses were fixed and their quality evaluated according to

external characteristics and histology.

While in the donor cow the IVP performances were measured as continuous traits, in the analysis of the recipient cows the trait was a factor with two outcomes (pregnant and not pregnant). Thus, it is possible to study the differential wiring between two biological networks (one for each phenotypic group). In other words, we can generate one network for the group of pregnant cows and one for the group of non-pregnant cows by using WGCNA. With statistical approaches it is then possible to identify genes (nodes) that are differentially wired comparing the two networks. Therefore, we can identify gene groups that must be co-expressed and activated together to generate an ideal receptive condition of the endometrium during ET. The functional enrichment and the integration with publicly available data could shed light on the mechanisms and the differences between receptive and non-receptive animals. Thus, we expect to identify novel biomarkers and information about biological mechanisms that can be used for prediction of good quality recipient cows.

**Expression QTL mapping in donor and recipient cows**

The eQTL mapping is an example of integrative systems biology analysis. An eQTL is a genomic region associated with transcript expression levels, and it can be close to the transcription start site (cis-eQTL) or acting on a larger distance or on another chromosome (trans-eQTL; (Mazzoni et al., 2015; Wang and Michoel, 2016). Mapping of eQTL can be used to link genetic variants to a specific trait (Buchner and Nadeau, 2015; Mazzoni *et al*., 2015; Suravajhala *et al*., 2016). Cis-regulated genes can also be involved in controlling a trait of interest, for example when the gene controlled by the eQTL is differentially expressed between two specific conditions of a target phenotype (Ponsuksili *et al*., 2010; Nica and Dermitzakis, 2013).

In the context of oocyte donor or embryo recipient cow selection, eQTLs can be a useful breeding tool (Ponsuksili et al., 2010) and they represent genomic biomarkers to include in animal selection (Westra and Franke, 2014). Furthermore, eQTL approaches require smaller sample size to obtain good detection power (Kadarmideen, 2008). In the two GIFT consortium projects previously described, both transcriptomic and genomic data were collected for eQTL mapping. The hub genes and the regulatory genes identified with co-expression analyses will be included in eQTL analysis. The eQTLs together with biological information obtained from integrative systems biology studies could be adopted in animal selection processes through the inclusion in GS methodologies utilizing functional information, e.g. sgBLUP (systems genomic BLUP; (Kadarmideen, 2014) and BLUPGA (BLUP approach given the Genetic Architecture; (Zhang et al., 2014).

**GWAS and functional enrichment analyses of early pregnancy of Nellore heifers**

Functional enrichment applied to standard analysis such as GWAS can give a strong contribution to the identification of candidate genes and metabolic pathways associated with conventional reproductive techniques. This is even more relevant if applied to Nellore cattle, one of the most important *Bos indicus* breeds for beef production in the world. We performed a GWAS followed by MeSH (Medical Subject Headings) enrichment to analyze pregnancy and antral follicular population in Nellore heifers (*Bos indicus)*. Briefly, ovarian ultrasound (7.5 MHz transrectal linear transducer, Mindray M5Vet, China) was performed to count visible follicles on Nellore heifers submitted to fixed-time artificial insemination. Nellore heifers (n = 1,255) from 3 different farms in Central-Western Brazil were exposed to breeding season at an average of around 16 month of age and were genotyped with GeneSeek GGP *Bos indicus* HD Technology (74,677 SNPs). After quality control with the software PREGSF90 (developed by Misztal I. et., al (2002), 64,753 SNP were included in the GWAS for both traits. The GWAS was performed using Gensel software (Fernando and Garrick, 2012) under Bayes B method.

(Co)variance components study was performed with single step analysis (Wang *et al*., 2012) under Bayesian method, and heritability estimates were 0.28 ± 0.07 (heifer pregnancy) and 0.49 ± 0.09 (follicular population), with a genetic correlation of -0,21 ± 0.29, meaning that in this sample of Nellore heifers, the number of antral follicles and heifer pregnancy may have antagonism.

SNP markers, located in genomic windows of approximately 1Mb that explained more than one per cent of genetic variance, were included in the functional enrichment. The MeSH enrichment was performed with MESHR (Morota *et al*., 2015; Tsuyuzaki *et al*., 2015). The functional analysis revealed 74 terms related to heifer pregnancy. These terms were related to: i) the metabolic pathways of fucose, that is involved in fertilization and in particular in the interaction between spermatozoa and oviduct and fertilization, ii) to Munc18 that plays a role in pituitary hormone secretion and iii) hemoglobin that takes part in ovary vascularization and LH secretion.

The functional analysis of the follicular population revealed 48 terms related to number of follicles and highlighted the importance of the following molecules: i) Neurepeptide receptor and kisspeptin associated with GnRH expression (Amstalden *et al*., 2014); ii) Cathepsin B that affects oocyte quality and control heat stress (Balboula *et al*., 2013); and iii) palmitic acid associated with apoptosis in follicular cells and with reproductive problems (Zeron *et al*., 2001).

The MeSH term enrichment of the GWAS results proved to be a reliable approach to get insight into the biology of the reproductive traits. These findings confirmed some of the candidate genes and molecular pathways identified in previous studies and could contribute to the research of the ARTs related traits.

**Conclusion and future perspectives**

The huge amount of data generated from high

throughput technologies has had a tremendous impact in the research for biomarkers for complex traits like those related to IVP procedures. Similarly, genomic analysis coupled with functional enrichment could expand the knowledge about the biology of conventional reproductive traits in important breeds such as Nellore cows. A better understanding of these traits such as number of follicles or pregnancy outcome is of great importance for the improvement of ARTs. In this context systems biology analysis aimed to identify the emergent properties from the "omic" frame is the most promising tool.

The next step is the validation of the candidate genes in bigger reference populations to test their real predictive power. The candidate genes could be validated with q-PCR, while secreted proteins encoded by the candidate genes could be tested directly *in vitro*. Moreover, further analysis (proteomics and cytofluorimetric studies) could be used to verify the correlation between atresia, gene expression and IVP performances. However, the integration of systems biology analysis across different biological layers proved to be a good methodology to get a complete picture of IVP traits that are controlled at different molecular levels and to improve the prediction of donor and recipient cow quality for *in vitro* embryo production.

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## References

**Amstalden M, Cardoso R, Alves B, Williams G**. 2014. Reproduction symposium: hypothalamic neuropeptides and the nutritional programming of puberty in heifers. *J Anim Sci*, 92:3211-3222.

**Aronson J**. 2005. Biomarkers and surrogate endpoints. *Br J Clin Pharmacol*, 59:491-494.

**Balboula AZ, Yamanaka K-I, Sakatani M, Kawahara M, Hegab A, Zaabel S, Takahashi M**. 2013. Cathepsin B activity has a crucial role in the developmental competence of bovine cumulus–oocyte complexes exposed to heat shock during in vitro maturation. *Reproduction*, 146:407-417.

**Bauersachs S, Ulbrich S, Gross K, Schmidt S, Meyer H, Einspanier R, Wenigerkind H, Vermehren M, Blum H, Sinowatz F**. 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. *J Mol Endocrinol*, 34:889-908.

**Bauersachs S, Ulbrich SE, Gross K, Schmidt SE, Meyer HH, Wenigerkind H, Vermehren M, Sinowatz F, Blum H, Wolf E**. 2006. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction*, 132:319-331.

**Bauersachs S, Ulbrich SE, Zakhartchenko V, Minten M, Reichenbach M, Reichenbach H-D, Blum H, Spencer T.E, Wolf E**. 2009. The endometrium responds differently to cloned versus fertilized embryos. *Proc Natl Acad Sci*, 106:5681-5686.

**Binelli M, Scolari SC, Pugliesi G, Van Hoeck V, Gonella-Diaza AM, Andrade SC, Gasparin GR, Coutinho LL**. 2015. The transcriptome signature of the receptive bovine uterus determined at early gestation. *PloS One*, 10:e0122874.

**Brazhnik P, de la Fuente A, Mendes P**. 2002. Gene networks: how to put the function in genomics. *Trends Biotechnol*, 20:467-472.

**Breiman L**. 2001. Random forests. *Mach Learn*, 45:5-32.

**Buchner DA, Nadeau JH**. 2015. Contrasting genetic architectures in different mouse reference populations used for studying complex traits. *Genome Res*, 25:775-791.

**Chuang H-Y, Hofree M, Ideker T**. 2010. A decade of systems biology. *Annu Rev **Cell** Dev Biol*, 26:721-744.

**Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M**. 2009. Mapping complex disease traits with global gene expression. *Nat Rev Genet*, 10:184-194.

**De Wit A, Wurth Y, Kruip T**. 2000. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. *J Anim Sci*, 78:1277-1283.

**Dixon AL, Liang L, Moffatt MF, Chen W, Heath S, Wong KC, Taylor J, Burnett E, Gut I, Farrall M**. 2007. A genome-wide association study of global gene expression. *Nat Genet*, 39:1202-1207.

**Feng W-G, Sui H-S, Han Z-B, Chang Z-L, Zhou P, Liu D-J, Bao S, Tan J-H**. 2007. Effects of follicular atresia and size on the developmental competence of bovine oocytes: a study using the well-in-drop culture system. *Theriogenology*, 67:1339-1350.

**Fernando R, Garrick D**. 2012. *GenSel: user manual for a portfolio of genomic selection related analyses*. Ames, IA: Iowa State University Animal Breeding &Genetics. 24 pp.

**Forde N, Lonergan P**. 2012. Transcriptomic analysis of the bovine endometrium: what is required to establish uterine receptivity to implantation in cattle? *J Reprod Dev*, 58:189-195.

**Friedman N, Linial M, Nachman I, Pe'er D**. 2000. Using Bayesian networks to analyze expression data. *J Comput Biol*, 7:601-620.

**Gilbert I, Robert C, Vigneault C, Blondin P, Sirard M-A**. 2012. Impact of the LH surge on granulosa cell transcript levels as markers of oocyte developmental competence in cattle. *Reproduction*, 143:735-747.

**Girard A, Dufort I, Douville G, Sirard M-A**. 2015. Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. *Reproductive Biology and Endocrinology*, 13:17. doi: 10.1186/s12958-015-0010-7.

**Hatzirodos N, Hummitzsch K, Irving-Rodgers HF, Harland ML, Morris SE, Rodgers RJ**. 2014a. Transcriptome profiling of granulosa cells from bovine ovarian follicles during atresia. *BMC Genomics*, 15:40. doi: 10.1186/1471-2164-15-40.

**Hatzirodos N, Irving-Rodgers HF, Hummitzsch K, Harland ML, Morris SE, Rodgers RJ**. 2014b. Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes. *BMC Genomics*, 15:24. doi: 10.1186/1471-2164-15-24.

**Heleil B, Kuzmina T, Alm H, Scotti O, Tuchscherer A, Torner H**. 2010. Involvement of granulosa cells in realization of prolactin effects on the developmental competence of bovine oocytes matured in vitro. *J Am Sci*, 6:796-805.

**Horvath S**, 2011. *Weighted Network Analysis*: *applications in genomics and systems biology*. New York, NY Springer Science & Business Media. 421 pp.

**Jiang J-Y, Xiong H, Cao M, Xia X, Sirard M-A, Tsang BK**. 2010. Mural granulosa cell gene expression associated with oocyte developmental competence. *J Ovarian Res*, 3:6. doi: 10.1186/1757-2215-3-6.

**Kadarmideen HN, Watson-Haigh NS, Andronicos NM**. 2011. Systems biology of ovine intestinal parasite resistance: disease gene modules and biomarkers. *Mol Biosyst*, 7:235-246.

**Kadarmideen HN**. 2014. Genomics to systems biology in animal and veterinary sciences: progress, lessons and opportunities. *Livest Sci*, 166:232-248.

**Kadarmideen H, Mazzoni G, Watanabe Y, Strøbech L, Baruselli P, Meirelles F, Callesen H, Hyttel P, Ferraz J, Nogueira M**. 2015. Genomic selection of in vitro produced and somatic cell nuclear transfer embryos for rapid genetic improvement in cattle production. *Anim Reprod*, 12, 389-396.

**Killeen AP, Morris DG, Kenny DA, Mullen MP, Diskin MG, Waters SM**. 2014. Global gene expression in endometrium of high and low fertility heifers during the mid-luteal phase of the estrous cycle. *BMC Genomics*, 15:234. doi: 10.1186/1471-2164-15-234.

**Kitano H**. 2002. Systems biology: a brief overview. *Science*, 295:1662-1664.

**Lu P, Vogel C, Wang R, Yao X, Marcotte EM**. 2007. Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nat Biotechnol*, 25:117-124.

**Mansouri-Attia N, Sandra O, Aubert J, Degrelle S, Everts RE, Giraud-Delville C, Heyman Y, Galio L, Hue I, Yang X**. 2009. Endometrium as an early sensor of in vitro embryo manipulation technologies. *Proc Natl Acad Sci*, 106:5687-5692.

**Matoba S, Bender K, Fahey AG, Mamo S, Brennan L, Lonergan P, Fair T**. 2014. Predictive value of bovine follicular components as markers of oocyte developmental potential. *Reprod Fertil Dev*, 26:337-345.

**Mazzoni G, Kogelman LJ, Suravajhala P, Kadarmideen HN**. 2015. Systems genetics of complex diseases using RNA-sequencing methods. *Int J Biosci Biochem Bioinforma*, 5:264.

**Mazzoni G, Salleh SM, Freude K, Pedersen HS, Stroebech L, Callesen H, Hyttel P, Kadarmideen HN**. 2017. Identification of potential biomarkers in donor cows for in vitro embryo production by granulosa cell transcriptomics. *PloS One*, 12:e0175464.

**McCulloch WS, Pitts W**. 1943. A logical calculus of the ideas immanent in nervous activity. *Bull Math Biophys*, 5:115-133.

**Minten MA, Bilby TR, Bruno RG, Allen CC, Madsen CA, Wang Z, Sawyer JE, Tibary A, Neibergs HL, Geary TW**. 2013. Effects of fertility on gene expression and function of the bovine endometrium. *PLoS One*, 8:e69444.

**Mitko K, Ulbrich SE, Wenigerkind H, Sinowatz F, Blum H, Wolf E, Bauersachs S**. 2008. Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. *Reproduction*, 135:225-240.

**Montojo J, Zuberi K, Rodriguez H, Kazi F, Wright G, Donaldson SL, Morris Q, Bader GD**. 2010. GeneMANIA Cytoscape plugin: fast gene function predictions on the desktop. *Bioinformatics*, 26:2927-2928.

**Moor R, Trounson A**, 1977. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. *J Reprod Fertil*, 49:101-109.

**Morota G, Peñagaricano F, Petersen J, Ciobanu DC, Tsuyuzaki K, Nikaido I**. 2015. An application of MeSH enrichment analysis in livestock. *Anim Genet*, 46:381-387.

**Nica AC, Dermitzakis ET**. 2013. Expression quantitative trait loci: present and future. *Phil Trans R Soc B*, 368:20120362.

**Nivet A-L, Vigneault C, Blondin P, Sirard M-**A. 2013. Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. *Reproduction*, 145:555-565.

**Orozco-Lucero E, Sirard M**. 2014. Molecular markers of fertility in cattle oocytes and embryos: progress and challenges. *Anim Reprod*, 11:183-194.

**Ponsuksili S, Murani E, Schwerin M, Schellander K, Wimmers K**. 2010. Identification of expression QTL (eQTL) of genes expressed in porcine M. longissimus dorsi and associated with meat quality traits. *BMC Genomics*, 11:572. doi: 10.1186/1471-2164-11-572.

**Ponsuksili S, Murani E, Schwerin M, Schellander K, Tesfaye D, Wimmers K**. 2012. Gene expression and DNA-methylation of bovine pretransfer endometrium depending on its receptivity after in vitro-produced embryo transfer. *PloS One*, 7:e42402.

**Saadi HAS, Vigneault C, Sargolzaei M, Gagné D, Fournier É, de Montera B, Chesnais J, Blondin P, Robert C**. 2014. Impact of whole-genome amplification on the reliability of pre-transfer cattle embryo breeding value estimates. *BMC Genomics*, 15:889. doi: 10.1186/1471-2164-15-889.

**Salilew-Wondim D, Hölker M, Rings F, Ghanem N, Ulas-Cinar M, Peippo J, Tholen E, Looft C, Schellander K, Tesfaye D**. 2010. Bovine pretransfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after embryo transfer. *Physiol Genomics*, 42:201-218.

**Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M**. 2011. Global quantification of mammalian gene expression control. *Nature*, 473:337-342.

**Simianer H**. 2016. Genomic and other revolutions: why

some technologies are quickly adopted and others are not. *Anim Front*, 6:53-58.

**Suravajhala P, Kogelman LJ, Kadarmideen HN**. 2016. Multi-omic data integration and analysis using systems genomics approaches: methods and applications in animal production, health and welfare. *Genet Sel Evol*, 48:38.

**Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, Simonovic M, Roth A, Santos A, Tsafou KP**. 2015. STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res*, 43, D447-D452.

**Tsuyuzaki K, Morota G, Ishii M, Nakazato T, Miyazaki S, Nikaido I**. 2015. MeSH ORA framework: R/Bioconductor packages to support MeSH over-representation analysis. *BMC Bioinformatics*, 16:45. doi: 10.1186/s12859-015-0453-z.

**Wang H, Misztal I, Aguilar I, Legarra A, Muir W**. 2012. Genome-wide association mapping including phenotypes from relatives without genotypes. *Genet Res*, 94:73-83.

**Wang L, Michoel T**. 2016. Detection of regulator genes and eQTLs in gene networks. *In*: *Systems Biology in Animal Production and Health*. Cham, Switzerland: Springer. vol. 1, pp. 1-23.

**Westra H-J, Franke L**. 2014. From genome to function by studying eQTLs. *Biochim Biophys Acta*, , 1842:1896-1902.

**Wurth Y, Kruip TA**. 1992. Bovine embryo production in vitro after selection of the follicles and oocytes. *In*: Proceedings of the 12th International Congress on Animal Reproduction, The Hague, The Netherlands. The Hague: ICAR. pp. 387-389.

**Zeron Y, Ocheretny A, Kedar O, Borochov A, Sklan D, Arav A**. 2001. Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction*, 121:447-454.

**Zhang ZH, Jhaveri DJ, Marshall VM, Bauer DC, Edson J, Narayanan RK, Robinson GJ, Lundberg AE, Bartlett PF, Wray NR**. 2014. A comparative study of techniques for differential expression analysis on RNA-Seq data. *PloS One*, 9:e103207.

**Zhao W, Langfelder P, Fuller T, Dong J, Li A, Hovarth S**. 2010. Weighted gene coexpression network analysis: state of the art. *J Biopharm Stat*, 20:281-300.

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# Antral follicle count in cattle: advantages, challenges, and controversy

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## Abstract

The antral follicle count (AFC) represents the number of follicles visualized by ultrasonography in the ovaries. Antral follicle count tends to be variable among cows but with high repeatability within the same individual. In the last decade, AFC has been considered a biological marker of fertility with many positive aspects of reproductive efficiency for those animals with high AFC. For instance, a larger number and better-quality embryos, better pregnancy rates, increased concentrations of circulating progesterone, in addition to other characteristics linked to fertility, are all responses observed in individuals with high compared with those with low AFC in *Bos taurus* cattle. However, the positive association between AFC and fertility did not follow the same pattern for *Bos indicus*. Recent articles showed no connection between fertility and number of antral follicles or better reproductive efficiency for high AFC group in Zebu cows. Thus, the aim of this review is to discuss the various data concerning AFC between *indicus* and *taurus* cattle. Additionally, we consider AFC to be a possible tool to improve cattle performance in reproductive biotechnology.

**Keywords**: antral follicle count, *Bos indicus*, *Bos taurus*, embryo production, fertility.

## Introduction

Assisted reproductive technologies, such as artificial insemination and embryo production, represent crucial tools to improve genetic merit in cattle production (Mapletoft and Hasler, 2005; Hansen, 2014; Bó *et al*., 2016). Recently, reports in the literature have documented improved reproductive parameters and response to reproductive biotechnologies in cattle with increased antral follicle count (Ireland *et al*., 2011; Rico *et al*., 2012; Silva-Santos *et al*., 2014a). These first reports described benefits of high AFC in *Bos taurus*, and the evaluation of antral follicles by ultrasound was considered the most practical strategy to classify a cow for reproductive purposes. Despite the considerable variability in AFC among cows, the number of antral follicles observed in the same animal is highly repeatable over several evaluations (Burns *et al.*, 2005; Morotti *et al*., 2017). This reproducibility of AFC in the same individual becomes a strategic resource for classifying an animal by the AFC with a single ultrasound examination. For *taurus* animals, AFC is directly correlated with the size of the ovarian follicular reserve (Ireland *et al*., 2011), which was not proven in *indicus* females when examining fetuses, heifers, and cows (Silva-Santos *et al*., 2011). However, other factors, such as genetics (Walsh *et al*., 2014), maternal environment, nutritional status, and healthiness (Ireland *et al*., 2011; Evans *et al*., 2012) also appear to influence the AFC. For example, nutritional status and general metabolism were mentioned as factors that affect follicular growth, oocyte quality, and secretion of reproductive hormones in cattle (Jimenez-Krassel *et al*., 2009; Mossa *et al*., 2010; Evans *et al*., 2012).

Considering *Bos taurus* cattle, the AFC has been directly linked to the female reproductive performance. Several reports suggest that high AFC is related to the total number of follicles that are morphologically healthy (Ireland *et al*., 2008, 2011). Additionally, the number of oocytes and blastocysts (Guerreiro *et al*., 2014), as well as the concentration of progesterone, were greater for cows with more antral follicles (Jimenez-Krassel *et al*., 2009). Moreover, females with high AFC showed a greater number of embryos produced by the donors in *Bos taurus* (Ireland *et al*., 2008), crossbred *indicus-taurus* (Silva-Santos *et al*., 2014a) and *Bos indicus* (Santos *et al*., 2016). Additionally, cows with high AFC presented greater pregnancy rates compared with those with low AFC (Evans *et al*., 2012; Mossa *et al*., 2012).

In contrast, other researchers reported controversial data regarding AFC and parameters of fertility (Santos *et al*., 2013). Recent studies with timed artificial insemination (TAI) in *indicus* and crosses of *indicus-taurus* cattle described no positive correlation between AFC and pregnancy rates (Mendonça *et al*., 2013; Santos *et al*., 2014). Interestingly, certain results suggest better pregnancy rates for low AFC cows (Santos *et al*., 2013).

Considering the application of AFC as a valuable tool to assist cattle performance and reproductive biotechnology, this review aims to discuss the following: i) the relationship between the number of antral follicles and physiological parameters; ii) the challenges to applying AFC in the field, and iii) the different data for AFC between several research teams.

## Repeatability of AFC and anti-müllerian hormone

Several studies performed in *taurus* (Burns *et al*., 2005; Ireland *et al*., 2008), *indicus* (Santos *et al*., 2012; Silva-Santos *et al*., 2014b) and *indicus-taurus* crosses (Silva-Santos *et al*., 2014a) reported AFC to be

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a parameter that was highly variable among cows but with a very consistent repeatability within the same animal. Based on this characteristic, the females can be classified as low, intermediate, or high AFC according to the number of antral follicles visualized by ultrasonography (Ireland *et a*l., 2008; Guerreiro *et al*., 2014; Silva-Santos *et al*., 2014a). An important aspect related to AFC is its high correlation with concentrations of the anti-müllerian hormone (AMH).

The AMH belongs to the β-growth factor superfamily and is synthesized from granulosa cells of antral and preantral follicles (Cate *et al*., 1986). The concentration of this hormone in blood is highly correlated with the AFC. Thus, AMH can be considered an endocrine marker of the AFC in *Bos indicus* and *Bos taurus* females (Batista *et al*., 2014; Morotti *et al*., 2015). The significant advantage of this property is the possibility of using AFC instead of AMH dosage, as a single ultrasound evaluation is less expensive and easier than determining the AMH concentration. In *taurus* animals, a high correlation ($r = 0.88$, $P < 0.001$) was observed between the AMH concentration and the ovarian AFC. The females classified as high AFC showed greater concentrations of AMH when compared to cows with low AFC (Ireland *et al*., 2008). The same pattern was observed in *Bos indicus,* with high AFC cows presenting greater AMH concentration than cows with low AFC (Batista *et al*., 2014). In this context, the AMH is recognized as a reliable indicator of ovarian activity and ability to respond to a super-stimulation protocol and ovum pick-up/*in vitro* production (IVP; Rico *et al*., 2009; Baruselli *et al*., 2016).

**Concentrations of progesterone and AFC**

The production of progesterone by the corpus luteum (CL) is necessary for maintaining the appropriate uterine environment, enabling embryonic development and maintaining pregnancy in domestic animals (Bazer *et al*., 2010; Pohler *et al*., 2012). Consequently, low progesterone concentration is associated with reproductive problems, such as embryonic mortality and slower development of the endometrium in cattle (Inskeep, 2004; Diskin and Morris, 2008).

Interestingly, the number of follicles in the ovary was associated with concentrations of progesterone, as described by Martinez *et al*. (2016), corroborating the data of previous studies (Ireland *et al*., 2011; Evans *et al*., 2012). All these authors reported greater concentrations of plasma progesterone in *taurus* cows with high AFC during diestrus and pregnancy compared with low-AFC females. The reduced concentration of progesterone in plasma of low-AFC females was associated with decreased capacity of luteal and granulosal cells to produce progesterone, and reduced abundance of STAR and mRNA for STAR and LH receptor in CL (Jimenez-Krassel *et al*., 2009).

**Antral follicle count and efficiency in embryo production**

Increased AFC has been related to a larger number of IVP embryos for both *Bos taurus* and *Bos indicus* cows (Taneja *et al*., 2000; Singh *et al*., 2004; Silva-Santos *et al*., 2014a). Considering *Bos taurus* cattle from Europe, the group classified with low AFC presented smaller number of embryo compared with cows classified in the high AFC group (Ireland *et al*., 2007). In the same way, *indicus* cows with high AFC also presented a greater number of embryos than those considered to have low AFC as shown in Table 1.

Table 1. Production of embryos and pregnancies according to the number of oocytes obtained by OPU/IVP (n = 656) from Nelore donors (n = 317). Values are presented per donor (mean ± SD).

| Donors according to oocyte production | N°. viable oocytes | N°. viable embryos | Blastocyst rate, % | N°. pregnancy 30 days | N°. pregnancy 90 days |
|---|---|---|---|---|---|
| Elevated (n = 78) | 47.06 ± 1.6[a] | 15.06 ± 0.86[a] | 32.00 | 5.62 ± 0.54[a] | 5.52 ± 0.81[a] |
| High (n = 80) | 24.95 ± 0.33[b] | 9.17 ± 0.63[b] | 36.75 | 3.63 ± 0.36[b] | 3.32 ± 0.33[b] |
| Intermediate (n = 79) | 15.57 ± 0.26[c] | 6.00 ± 0.39[c] | 38.54 | 2.10 ± 0.21[c] | 1.92 ± 0.20[b] |
| Low (n = 80) | 6.31 ± 0.38[d] | 2.42 ± 0.25[d] | 38.35 | 0.92 ± 0.13[d] | 0.85 ± 0.13[c] |

[a-d]Within a column, mean values with uncommon superscripts differ significantly ($P \leq 0.05$). Adapted from Pontes *et al*. (2011).

Considering the efficiency of embryonic production, and not only the total number of ova/embryos, the relationship between IVP and AFC in *indicus* donors is controversial. Several studies with *indicus* showed the same pattern of that obtained from *taurus* donors. For example, Nelore cows (n = 66) classified into high (>40 follicles), intermediate (18 to 25 follicles) or low AFC (<7 follicles) groups produced 42, 32 and 13% ($P < 0.05$) of total blastocyst rates, respectively (Santos et *al*., 2016). Considering *indicus-taurus* heifers classified in high (≥40 follicles) or low (≤10 follicles) AFC groups, the results showed a greater number of embryos produced in females with high AFC compared with those with low AFC (6.9 ± 5.3 *vs.* 1.9 ± 2.1; Silva-Santos *et al*., 2014a). However, Monteiro *et al*. (2017) did not find any advantage of a high AFC when considering IVP of embryos. Those authors classified *Bos indicus* females into high (>15) or low (<15) cumulus-oophorous complex (COC) counts, and data were collected through 12 consecutive OPU/IVP sessions. Differences in COC and number of blastocysts were observed, but production of blastocyst per COC did not differ according to category of COC (Tab. 1).

Similar results were obtained by another team. The authors studied a large number of Nelore donors (n = 356), which were classified into high (>92 follicles), intermediate (46 to 76 follicles), or low AFC groups (<31

follicles). *In vitro* embryo production did not differ among the three groups: 40, 36 and 38%, respectively (Rosa *et al*., 2015). Thus, although several studies involving Zebu animals demonstrate a positive correlation between embryo production and high AFC, this aspect is not entirely clear in *Bos indicus* cattle (Tab. 2). To date, only a quantitative advantage for high AFC is evident, but proportion of blastocyst obtained per COC is not supported by *indicus* donors with more antral follicles.

To date, it is not possible to clarify the controversial data regarding AFC and IVP. We have several considerations to comment regarding the contradicting results between *Bos taurus* and *Bos indicus*. First, we should consider the physiological differences involving reproductive patterns between *taurus* and *indicus* cattle. Several aspects have been reported on that (e.g., number of follicular waves, metabolism of hormones, and diameter of ovulatory follicle). The influence of AFC on IVP may be another physiological difference related to breeds of European origin compared with Zebu cattle. Another question involved in the different data of AFC and IVP is the classification of animals into high, intermediate, or low AFC groups. The literature in this area is heterogenous and there is no standard to establish the follicular groups. In summary, the full understanding of how AFC could influence IVP is not understood to date. We believe that further studies will certainly help to improve our knowledge on the subject.

Table 2. Cumulative production of blastocysts by Nelore donors (*Bos indicus*) according to the category (high, n = 18 or low, n = 18) of COC recovered (LSM ± SEM).

| Ovum pickup program | Cumulative blastocyst production per donor | | | P value |
|---|---|---|---|---|
| | Low COCs | High COCs | Overall | |
| After 3 months | 10.6 ± 1.6 | 25.6 ± 3.9 | 18.1 ± 2.4 | 0.001 |
| After 6 months | 22.2 ± 2.7 | 47.7 ± 6.8 | 34.9 ± 4.2 | 0.0004 |
| After 30 months | 43.6 ± 5.2 | 85.6 ± 11.4 | 64.6 ± 7.1 | 0.001 |

Adapted from Monteiro *et al*. (2017).

**Fertility parameters and AFC in cows**

The hypothesis that fertility is influenced by the number of follicles and oocytes in ovaries is an old idea (Hunter, 1787; Erickson, 1966). Over the years, several studies have described a high AFC as being positively correlated with female fertility. Many advantageous features that affect fertility have been related to the number of follicles in the ovary (Ireland *et al*., 2008), such as plasma progesterone concentration, (Jimenez-Krassel *et al*., 2009; Martinez *et al*., 2016), shorter interval between calvings (Mossa *et al*., 2012), and CL function and endometrial thickness (Jimenez-Krassel *et al*., 2009). However, it is important to emphasize that the positive correlation between AFC and cow fertility was mainly described in *Bos taurus* cattle (Mossa *et al*., 2012, 2013; Walsh *et al*., 2014; Jimenez-Krassel *et al*., 2015; McNeel and Cushman, 2015; Santos *et al*., 2016).

However, even for *taurus* cows, the association between AFC and fertility parameters may be controversial. When considering New Zealand *taurus* lactating dairy cows, it was not possible to verify any association between AFC and proportion of cows pregnant after artificial insemination, as shown in Table 3 (Martinez *et al*., 2016), but future studies may contribute to improving the accuracy of this information.

Table 3. Reproductive responses in *Bos taurus* New Zealand lactating dairy cows subjected to artificial insemination according to antral follicle count.

| Item[1] | High ≥ 30 follicles n = 104 | Intermediate 21-29 follicles n = 137 | Low ≤ 20 follicles n = 200 |
|---|---|---|---|
| Days to pregnancy (LSM ± SEM) | 82.4 ± 1.6[a] | 85.2 ±1.6[ab] | 87.3 ±1.2[b] |
| Number of AI per pregnancy (LSM ± SEM) | 1.2 ± 0.1 | 1.3 ± 0.1 | 1.3 ± 0.1 |
| Estrus-detected cows (%) | 50.9 | 48.9 | 40.9 |
| Cows with a CL at scanning (%) | 91.5[a] | 84.7[ab] | 82.4[b] |
| Pregnant to first AI (%) | 54.6 | 49.4 | 48.5 |
| Pregnant after 6 weeks of AI (%) | 70.0 | 70.4 | 61.2 |
| Pregnant overall (%) | 87.1 | 79.4 | 81.0 |

[a,b]Different superscripts within the same row indicate significant effect ($P < 0.05$). [1]AI = artificial insemination; CL = corpus luteum. Adapted from Martinez *et al*. (2016).

When considering studies with *indicus* (Nelore) and *indicus-taurus* (Braford), the connection between of AFC and fertility seems to be even more complex. The evaluation of follicular dynamics of Nelore cows with low AFC (<15 follicles) demonstrated greater follicular diameter at TAI (13.4 *vs*. 12.2 mm, $P \leq 0.05$) and increased pregnancy to insemination (62 *vs*. 50%) than cows with a high AFC (>45 follicles; Morotti *et al*.,

2017; unpublished data). An increased rate of follicular growth and larger follicular diameters were previously described in *indicus-taurus* (Santos *et al*., 2012) and *indicus* cows with low AFC (Morotti *et al*., 2014). A larger ovulatory follicle diameter has been clearly associated with better pregnancy per insemination (Sá Filho *et al*., 2010; Pfeifer *et al*., 2012), and this aspect reinforces the hypothesis that cows with low AFC may present increased pregnancy rates.

**Relationship between AFC and phenotypic and genotypic production characteristics**

A recent study was performed to verify the influence of the phenotypic and genotypic characteristics of the genetic improvement program on AFC *indicus-taurus* heifers. This study revealed only one parameter, the visual score for finishing precocity at weaning, that had a very small negative correlation with AFC, and all other parameters evaluated for genetic merit for beef cattle showed no correlation with AFC (Morotti *et al*., 2017).

Another recent study conducted in crossbred beef heifers (*Bos taurus,* n = 95) confirmed that AFC is associated with calving day. Heifers giving birth early in the calving season presented more antral follicles than heifers giving birth later in the calving season (McNeel and Cushman, 2015). Therefore, the AFC can be used as an ovarian phenotype for the reproductive tract in commercial production.

For dairy cattle, it was demonstrated that AFC is a reproductive characteristic with moderate heritability that is affected by age and lactation status but not correlated with milk production or other characteristics associated with the genetic merit (Walsh *et al*., 2014).

When considering beef and dairy cattle, the AFC seems to not be related to parameters of production, either for meat or milk. In this way, the number of antral follicles could be used as secondary criteria for considering the use of reproductive biotechnologies. The first aspect always must be the genetic merit of the dam. Next, an analysis of AFC may be considered depending on the technique to be used.

**Possible mechanisms involving AFC and fertility**

The physiological mechanisms regarding number of follicles and fertility parameters are not currently understood. To date, most of the published literature has described primarily applied results. In other words, most published articles focused on rates of pregnancy, embryo production, follicular growth, and similar responses; however, a thorough description of the molecular mechanisms that underlie the differences among individuals with distinct AFC remains to be elucidated.

The original articles regarding AFC pointed to several advantages for females with a larger number of antral follicles. However, a relationship with low AFC and better fertility, such as observed in recent studies with TAI in *Bos indicus* seems to be more easily explained. The first results about follicular dynamics and AFC have shown larger ovulatory follicles in low-AFC cows compared with those with a high AFC (Morotti *el al*., 2017; unpublished data). When considering fewer number of follicles to receive gonadotropin stimulation, it is possible to expect that each follicle would obtain a larger amount of FSH at the emergence of the follicular wave. Conversely, those females with a high AFC would have more follicles to share the same amount of gonadotropins. After comparing the diameter of the ovulatory follicle, many articles have described better pregnancy rates for cows with moderate to large pre-ovulatory follicle diameter compared with females with small follicle diameter at ovulation (Sá Filho *et al*., 2010; Pfeifer *et al*., 2012). In this way, the better pregnancy rates in cows with a low AFC compared with cows with a high AFC might be explained by those with low AFC having larger diameter of the ovulatory follicle.

Regarding the IVP, the majority of the studies in the literature did not present a clear advantage for any AFC category, or the studies' results are controversial. This might be explained by the artificial steps the oocytes undergo with *in vitro* embryo production. The nuclear and cytoplasmic maturation of oocytes are finished *in vitro* aside from the follicular environment. In this way, it is possible to believe that the influence of the follicle on the oocyte will be lower than in *in vivo* conditions, such as artificial insemination. We believe this possibility could at least partly explain the differences of AFC between artificial insemination and IVP programs.

**Challenges related to AFC**

The largest problem in AFC studies has been the significant variation in the evaluation criteria used to establish the categories of high, intermediate, or low counts, making it difficult to compare data (Morotti *et al*., 2015). For example, an intermediate category in one published paper may be considered a low or even a high category for another paper. This question becomes more critical when comparing AFC between *taurus* and *indicus* cattle. Zebu cattle usually show two, three, or even four times more follicles than female cattle of European breeds. Therefore, it is not surprising that differences in follicullogenesis between species or subspecies of domestic cattle might also affect how AFC is associated with reproduction, and extrapolation of data from *Bos taurus taurus* to *Bos taurus indicus* breeds might be incorrect and further work is warranted to identify the underlying mechanisms that explain such differences in responses.

Another critical question is the interval among AFC categories. Several articles have considered specific cut-points with only one follicle to distinguish one AFC category from another. For example, a low AFC as being fewer than 15 follicles and an intermediate AFC as being equal to or greater than 15 follicles. We believe that it is highly critical to keep the interval close, but at the same time it is easy to imagine that one follicle could be missed during an ultrasound evaluation. Therefore, we suggest a more conservative

approach with a safety interval of AFC categories, eliminating a portion of the animals with AFC in between categories to maintain a more accurate classification of individuals. In other words, low AFC as fewer than 15 follicles and the intermediate category of AFC could start at greater than 20 or even greater than 25 follicles. The total number of animals in the experiment would be less because many cows would be discarded. Conversely, it would be possible to introduce more consistency in the AFC comparisons, because individuals with borderline values for AFC would be excluded.

Another critical challenge regarding AFC is related to the technique used. For example, females with high AFC are advantageous when submitted to OPU and IVP. For *taurus* donors, there are benefits regarding total numbers of embryos, as well as better rates of embryonic development. For *indicus* cows, it is well-accepted that there is a better performance only on the number of embryos. However, for pregnancy rates after AI, the results between *indicus* and *taurus* cattle are notably conflicting. For *taurus* herds, it is well-established that high AFC will be associated with best pregnancy rates in AI programs. On the other hand, for Zebu females, the relationship between AFC and pregnancy per AI remains equivocal. In fact, several studies have shown improved proportion of pregnancy in cows with low or intermediate AFC (Jimenez-Krassel et al., 2017; Santos *et al*., 2013, 2016).

Conversely, there are many factors that warrant further study, such as the relation of AFC with follicular growth, quality of oocytes, nutritional and health status of the female. Additionally, the maternal environment during gestation seems to determine the size of the ovarian reserve in their offspring (Ireland *et al*., 2011; Evans *et al*., 2012), and this aspect of maternal imprinting merits better understanding.

## Final remarks

The relationship between AFC and reproductive performance represents a great challenge to the current reproductive scenario in cattle. Studies with *Bos taurus* herds have pointed to better reproductive performance in females with high AFC, regardless of biotechnology. However, studies conducted with *Bos indicus* animals have shown that AFC may be associated with performance differently according to the technique used (i.e., AI *vs*. IVP). Additionally, a weak correlation between genetic merit and number of antral follicle has been demonstrated. This finding may be used when considering AFC and the biotechnology to be used, as no interferences on genetic merit would be expected. We predict that AFC may be another tool for improving reproductive efficiency in cattle, perhaps something less determinant, as initially proposed years ago. However, AFC clearly remains a useful strategy for improving programs of reproductive performance in cattle.

## References

**Batista EOS, Macedo GG, Sala RV, Ortolan MDDV, Sá Filho MF, Del Valle TA, Jesus EF, Lopes RNVR, Renn FP, Baruselli PS**. 2014. Plasma antimullerian hormone as a predictor of ovarian antral follicular population in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. *Reprod Domest Anim*, 49:448-452.

**Baruselli PS, Batista EOS, Ferreira RM**. 2016. Plasma anti-müllerian hormone allows reproductive selection of donors with greater potential of embryo production. *Spermova*, 6:1-13.

**Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K**. 2010. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol Hum Reprod*, 16:135-152.

**Bó GA, de la Mata JJ, Baruselli PS, Menchaca,A**. 2016. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology*, 86:388-396.

**Burns DS, Jimenez-Krassel F, Ireland JLH, Knight PG, Ireland JJ**. 2005. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. *Biol Reprod*, 73:53-62.

**Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, Fisher RA, Bertonis JM, Torres G, Wallner BP, Ramachandran KL, Ragin RC, Manganaro TF, MacLaughlin DT, Donahoe PK**. 1986. Isolation of the bovine and human genes for mullerian inhibiting substance and expression of the human gene in animal cells. *Cell*, 45:685-698.

**Diskin MG, Morris DG**. 2008. Embryonic and early foetal losses in cattle and other ruminants. *Reprod Domest Anim*, 43:260-267.

**Erickson BH**. 1966. Development and senescence of the postnatal bovine ovary. *J Anim Sci*, 25:800-805.

**Evans ACO, Mossa F, Walsh SW, Scheetz D, Jimenez-Krassel F, Ireland JLH, Smith GW, Ireland JJ**. 2012. Effects of maternal environment during gestation on ovarian folliculogenesis and consequences for fertility in bovine offspring. *Reprod Domest Anim*, 47:31-37.

**Guerreiro BM, Batista EOS, Vieira LM, Sá Filho MF, Rodrigues CA, Castro Netto A, Silveira CRA, Bayeux BM, Dias EAR, Monteiro FM, Accorsi,M, Lopes RNVR, Baruselli PS**. 2014. Plasma anti-mullerian hormone: an endocrine marker for in vitro embryo production from *Bos taurus* and *Bos indicus* donors. *Domest Anim Endocrinol*, 49:96-104.

**Hansen P**. 2014. Current and future assisted reproductive technologies for mammalian farm animals. *Adv Exp Med Biol*, 752:1-22.

**Hunter J**. 1787. An experiment to determine the effect of extirpating one ovarium upon the number of young produced. *Philos Trans R Soc Lond*, 77:233-239.

**Inskeep EK**. 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. *J Anim. Sci,* 82:24-39.

**Ireland JJ, Ward F, Jimenez-Krassel F, Ireland JLH, Smith GW, Lonergan P, Evans ACO**. 2007.

Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum Reprod*, 22:1687-1695.

**Ireland JJ, Smith GW, Scheetz D, Jimenez-Krassel F, Folger JK, Ireland JLH, Mossa F, Lonergan P, Evans ACO**. 2011. Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-müllerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. *Reprod Fertil Dev*, 23:1-14.

**Ireland JLH, Scheetz D, Jimenez-Krasse F, Themmen APN, Ward F, Lonergan P, Smith GW, Perez GI, Evans ACO, Ireland JJ**. 2008. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biol Reprod*, 79:1219-1225.

**Jimenez-Krassel F, Folger JK, Ireland JLH, Smith GW, Hou X, Davis JS, Lonergan P, Evans ACO, Ireland JJ**. 2009. Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle. *Biol Reprod*, 80:1272-1281.

**Jimenez-Krassel F, Scheetz DM, Neuder LM, Ireland JLH, Pursley JR, Smith GW, Tempelman RJ, Ferris T, Roudebush WE, Mossa F, Lonergan P, Evans, ACO, Ireland JJ**. 2015. Concentration of anti-Müllerian hormone in dairy heifers is positively associated with productive herd life. *J Dairy Sci*, 98:3036-3045.

**Jimenez-Krassel F, Scheetz DM, Neuder LM, Pursley JR, Ireland JJ**. 2017. A single ultrasound determination of≥25 follicles ≥3 mm in diameter in dairy heifers is predictive of a reduced productive herd life. *J Dairy Sci*, 100:5019-5027.

**Mapletoft RJ, Hasler JF**. 2005. Assisted reproductive technologies in cattle: a review. *Rev Sci Tech*, 24:393-403.

**Martinez MF, Sanderson N, Quirke LD, Lawrence SB, Juengel JL**. 2016. Association between antral follicle count and reproductive measures in New Zealand lactating dairy cows maintained in a pasture-based production system. *Theriogenology*, 85:466-475.

**McNeel AK, Cushman RA**. 2015. Influence of puberty and antral follicle count on calving day in crossbred beef heifers. *Theriogenology*, 84:1061-1066.

**Mendonça HGR, Santos GMG, Silva-Santos KC, Barreiros TRR, Blaschi W, Morotti F, Moraes FLZ, Seneda MM**. 2013. Pregnancy rates following FTAI of Nelore heifers (*Bos indicus*) with high, intermediate and low numbers of antral follicles. *Anim Reprod*, 10:452. Abstract.

**Monteiro FM, Batista EOS, Vieira LM, Bayeux BM, Accorsi M, Campanholi SP, Dias EAR, Souza AH, Baruselli PS**. 2017. Beef donor cows with high number of retrieved COC produce more in vitro embryos compared with cows with low number of COC after repeated ovum pick-up sessions. *Theriogenology*, 90:54-58.

**Morotti F, Santos GMG, Silva-Santos KC, Moritz Y, Marcantonio TN, Seneda MM**. 2014. Follicular dynamics in Nelore females (*Bos indicus*) with high or low numbers of antral follicles. *Anim Reprod*, 11:352. Abstract.

**Morotti F, Barreiros TRR, Machado FZ, González SM, Marinho LSR, Seneda MM**. 2015. Is the number of antral follicles an interesting selection criterium for fertility in cattle? *Anim Reprod*, 12:479-486.

**Morotti F, Santos GMG, Koetz Júnior C, Silva-Santos KC, Roso VM, Seneda MM**. 2017. Correlation between phenotype, genotype and antral follicle population in beef heifers. *Theriogenology*, 91:21-26.

**Mossa F, Duffy P, Naitana S, Lonergan P, Evans AC**. 2007. Association between numbers of ovarian follicles in the first follicle wave and superovulatory response in ewes. *Anim Reprod Sci*, 100:391-396.

**Mossa F, Jimenez-Krassel F, Folger JK, Ireland JL, Smith GW, Lonergan P, Evans AC, Ireland JJ**. 2010. Evidence that high variation in antral follicle count during follicular waves is linked to alterations in ovarian androgen production in cattle. *Reproduction*, 140:713-720.

**Mossa F, Walsh SW, Butler ST, Berry DP, Carter F, Lonergan P, Smith GW, Ireland JJ, Evans ACO**. 2012. Low numbers of ovarian follicles ≥3 mm in diameter are associated with low fertility in dairy cows. *J Dairy Sci,* 95:2355-2361.

**Mossa F, Carter F, Walsh SW, Kenny DA, Smith GW, Ireland JLH, Hildebrandt TB, Lonergan P, Ireland JJ, Evans ACO**. 2013. Maternal undernutrition in cows impairs ovarian and cardiovascular systems in their offspring. *Biol Reprod*, 88:92. doi: 10.1095/biolreprod.112.107235.

**Pfeifer LFM, Leal SdCBdS, Schneider A, Schmitt E, Corrêa MN**. 2012. Effect of the ovulatory follicle diameter and progesterone concentration on the pregnancy rate of fixed-time inseminated lactating beef cows. *Rev Bras Zootec*, 41:1004-1008.

**Pohler KG, Geary TW, Atkins JA, Perry GA, Jinks EM, Smith MF**. 2012. Follicular determinants of pregnancy establishment and maintenance. *Cell Tissue Res*, 349:649-664.

**Pontes JHF, Melo-Sterza FA, Basso AC, Ferreira CR, Sanches BV, Rubin KCP, Seneda MM**. 2011. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (*Bos indicus*) donors. *Theriogenology*, 75:1640-1646.

**Rico C, Fabre S, Médigue C, Di Clemente N, Clément F, Bontoux M, Touzé JL, Dupont M, Briant E, Rémy B, Beckers JF, Monniaux D**. 2009. Antimullerian hormone is an endocrine marker of ovarian gonadotropin responsive follicles and can help to predict superovulatory responses in the cow. *Biol Reprod*, 80:50-59.

**Rico C, Drouilhet L, Salvetti P, Dalbiès-Tran R, Jarrier P, Touzé J-L, Pillet E, Ponsart C, Fabre S, Monniaux D**. 2012. Determination of anti-müllerian hormone concentrations in blood as a tool to select Holstein donor cows for embryo production: from the laboratory to the farm. *Reprod Fertil Dev*, 24:932-944.

**Rosa CO, Marinho LSR, Lunardelli PA, Machado**

**FZ, Gonzalez SM, Rosa PRA, Cesaro MP, Bueno JVR, Bordignon V, Seneda MM**. 2015. *In vitro* embryo development and gene expression in granulosa and cumulus cells from Bos indicus cows with different numbers of antral follicles. *Anim Reprod*, 12:762. Abstract.

**Sá Filho MF, Crespilho AM, Santos JE, Perry GA, Baruselli PS**. 2010. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled *Bos indicus* cows. *Anim Reprod Sci*, 120:23-30.

**Santos GMG, Silva-Santos KC, Siloto LS, Morotti F, Marcantonio TN, Marinho LSR, Thasmo RLO, Koetz JRC, Cintra DML, Seneda MM**. 2012. Dinâmica folicular em fêmeas bovinas de alta, média e baixa contagem de folículos antrais: resultados preliminares. *Acta Sci Vet*, 40:422. Abstract.

**Santos GMG, Silva-Santos KC, Barreiros TRR, Blaschi W, Morotti F, Silva CB, Moraes FLZ, Giudicissi DPL, Seneda MM**. 2013. Conception rates following FTAI of Nelore cows (*Bos indicus*) with high, intermediate and low numbers of antral follicles. *Anim Reprod*, 10:451. Abstract.

**Santos GMG, Silva-Santos KC, Barreiros TRR, Morotti F, Sanches, BV, Moraes FLZ, Blaschi W, Seneda MM**. 2014. High numbers of antral follicles influence the in vitro embryo production, but not the conception rate of fixed-time artificial insemination in nelore cattle. *Reprod Fertil Dev*, 27:206.

**Santos GMG, Silva-Santos KC, Barreiros TRR, Morotti F, Sanches BV, de Moraes FLZ, Blaschi W, Seneda MM**. 2016. High numbers of antral follicles are positively associated with *in vitro* embryo production but not the conception rate for FTAI in Nelore cattle. *Anim Reprod Sci*, 165:17-21.

**Silva-Santos KC, Santos GMG, Siloto LS, Hertel MF, Andrade ER, Rubinb MIB, Sturionc L, Melo-Sterza FA, Seneda MM**. 2011. Estimate of the population of preantral follicles in the ovaries of *Bos taurus indicus* and *Bos taurus taurus* cattle. *Theriogenology*, 76:1051-1057.

**Silva-Santos KC, Santos GMG, Koetz Júnior C, Morotti F, Siloto LS, Marcantonio TN, Urbano MR, Oliveira RL, Lima DCM, Seneda MM**. 2014a. Antral follicle populations and embryo production - in vitro and in vivo - of *Bos indicus-taurus* donors from weaning to yearling ages. *Reprod Domest Anim*, 49:228-232.

**Silva-Santos KC, Siloto LS, Santos GMC, Morotti F, Marcantonio TN, Seneda MM**. 2014b. Comparison of antral and pre-antral ovarian follicle populations between *Bos indicus* and *Bos indicus-taurus* cows with high or low antral follicles counts. *Reprod Domest Anim*, 49:48-51.

**Singh J, Dominguez M, Jaiswal R, Adams GP**. 2004. A simple ultrasound test to predict the superstimulatory response in cattle. *Theriogenology*, 62:227-243.

**Taneja M, Bols PE, Van De Velde A, Ju JC, Schreiber D, Tripp MW, Levine H, Echelard Y, Riesen J, Yang X**. 2000. Developmental competence of juvenile calf oocytes in vitro and in vivo: influence of donor animal variation and repeated gonadotrophin stimulation. *Biol Reprod*, 62:206-213.

**Walsh SW, Mossa F, Butler ST, Berry DP, Scheetz D, Jimenez-Krassel F, Tempelman RJ, Carter F, Lonergan P, Evans ACO, Ireland JJ**. 2014. Heritability and impact of environmental effects during pregnancy on antral follicle count in cattle. *J Dairy Sci*, 97:4503-4511.

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# Cryopreservation of *in vitro*-produced embryos: challenges for commercial implementation

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## Abstract

In the last several years, the high demand for embryo production has resulted in the need to study new methods to make the cryopreservation of bovine embryos produced *in vitro* more efficient. Despite the advantages offered by *in vitro* embryo production (IVEP), the major challenge to its greater dissemination is to improve embryonic survival after cryopreservation. Embryos that are produced *in vitro* are less resistant to cryopreservation compared to those produced *in vivo*, which is due to the higher accumulation of lipids in their cells, among other factors. In this context, changes in the culture conditions such as the addition of lipolytic chemical substances and the adjustment of fetal calf serum in the medium have been proposed to decrease the lipid amount within the embryos. Several years ago, vitrification allowed good results for *in vitro* produced (IVP) embryos because of its simplicity, speed and low cost. More recently, another technique applied to simplify the embryo post-thawing rehydration step *in vivo*, direct transfer (DT), is a strategy that has proven to be of interest in helping to overcome limitations to the cryopreservation of *in vitro* produced embryos. DT has been performed by commercial laboratories, ensuring good embryo viability after thawing. However, commercial and operational limitations prevent the large-scale use of these techniques. Thus, this review aims to discuss the use of strategies to improve the post-cryopreservation survival capacity and the aspects to be overcome so that the cryopreservation of IVP embryos becomes a well-established and commercially applicable technique in addition to presenting new guidelines for embryo transfer (ET) programs from a better selection of recipients.

**Keywords**: bovine, commercial limitation, cryopreservation, *in vitro*-produced embryos, recipient cow.

## Introduction

During the year 2015, almost 700.000 IVP embryos were produced, surpassing for the first time the number of bovine embryos produced *in vivo*. In this context, 269.353 bovine OPU IVP embryos were transferred in Brazil alone (Perry, 2016), which is considered the world's largest producer of bovine embryos. This situation is directly related to the predominance of *Bos indicus* cattle. Several studies have reported that Zebu females, when submitted to ovum pick-up (OPU) guided by transvaginal ultrasonography, had a higher number of oocytes aspirated than *Bos taurus* females (Segerson *et al*., 1984; Silva-Santos *et al*., 2011). This feature favors large-scale *in vitro* embryo production (IVEP) in both dairy and beef cattle (Pontes *et al*., 2011).

Furthermore, IVEP has advantageous conditions for its application in *Bos indicus* dairy cattle, since these animals, in addition to being good donors of oocytes, are adapted to a tropical climate and can produce milk even under high-temperature conditions (Marinho *et al*., 2015). Another advantage is the fact that embryos are more resistant than gametes when subjected to high body temperatures due to thermal stress (Chebel *et al*., 2008). Thus, the pregnancy rates are better in embryo transfer (ET) than artificial insemination (AI) throughout the year (Stewart *et al*., 2011; Ferreira, 2013).

Additionally, in the last decade, there has been a significant increase in the production of sexed embryos, especially due to the search for genetic improvement of dairy cattle (Pontes *et al*., 2010). Another advantage of IVEP compared with *in vivo* methods is the smaller number of viable sperm required for fertilization and, therefore, more efficient results in the use of sex-sorted semen (Pontes *et al*., 2010; Morotti *et al*., 2014).

In this context, the total embryo production is sometimes higher than the number of embryos transferred, so investment in research was increased to develop an efficient protocol for the cryopreservation of the remaining embryos in a program (Sanches *et al*., 2016). Despite the advantages provided by IVEP, the greatest challenge of this biotechnology is the lower resistance to the cryopreservation process that these embryos present (Sudano *et al*., 2011).

The high sensitivity to cooling of *in vitro* embryos is reported to be due to the greater accumulation of lipids in their cells (Abe *et al*., 2002), arranged in the form of cytoplasmic lipid droplets that are constituted predominantly of triglycerides (McKeegan and Sturmey, 2012). Additionally, there are indications that this high lipid content is because of the medium in which the embryos are cultured (Abe *et al*., 2002; Sanches *et al*., 2013). Thus, some strategies for improving post-cryopreservation survival capacity have been studied and tested to produce more cryotolerant embryos (Sudano *et al*., 2013).

Among cryopreservation techniques, vitrification

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has been used worldwide (Dode *et al*., 2013) because of its simplicity, speed, and low cost. However, this technique requires a high concentration of cryoprotectants in addition to a trained person to perform a morphological evaluation of embryo quality before the loading process (Vajta *et al*., 1998).

In contrast, Sanches *et al*. (2016) demonstrated that a technique used since the 1990s to simplify the post-thawing rehydration step of *in vivo* embryos — direct transfer (DT) — can also be used for frozen *in vitro* embryos. The DT strategy has been demonstrated to be helpful for overcoming limitations to *in vitro* embryo cryopreservation, since it has been recently performed by commercial laboratories, providing good embryo viability after thawing.

The choice of embryo recipients is another important step in the implementation of IVEP programs (Peixoto *et al*., 2004). Age, sanitary and nutritional conditions, such as synchrony between the recipients and embryonic stage, are important attributes to take into account in the choice of embryo recipients (Hasler *et al*., 1987; Sreenan and Diskin, 1987; Callesen *et al*., 1996; Jones and Lamb, 2008). Moreover, recent strategies for the synchronization of estrus/ovulation and the selection of recipients by fertility have been achieved (Marinho *et al*., 2012).

In contrast, there are still many commercial and operational limitations of bovine IVEP embryos and cryopreservation processes, which prevent its use on a large scale. Examples include the need for a qualified person to perform all stages of IVEP and the cryopreservation process, logistics between laboratory and recipients, as well as a trained technician in the field due the particularities of warming those cryopreserved embryos before transfer (Hasler, 2010; Saragusty and Arav, 2011).

Therefore, considering the importance of implementing an efficient IVEP and cryopreservation program, this review aims to discuss i) the use of strategies to improve embryo post-cryopreservation capacity; ii) the choice of recipients with good sanitary/nutritional conditions and reproductive characteristics to maintain a healthy pregnancy; and finally, iii) a team able to perform all stages of IVEP with rigorous quality control and the logistics necessary for making ET feasible in the field.

## Cryopreservation of bovine embryos

### *Methods and differences of cryotolerance in embryos produced* in vivo *and* in vitro

The process of embryo cryopreservation is the most challenging aspect of embryo biotechnology, and despite advances in recent years, the results are still inconsistent (Sudano *et al*., 2013). During embryo freezing, the cryopreservation method aims to avoid the formation of intracellular ice crystals and to decrease the toxic effects generated by the cryoprotectant agent, minimizing the osmotic stress to the cells (Pryor *et al*., 2009).

Cryopreservation protocols are based on two variables: type and concentration of cryoprotectant and cooling rates (Vajta and Kuwayama, 2006). Currently, slow freezing (classic) and vitrification (ultra-rapid) are the two main methods used commercially for IVEP embryo cryopreservation (Saragusty and Arav, 2011).

Vitrification is the predominant technique used for IVEP (Dode *et al*., 2013) due to being a simple, fast and low-cost method (Sanches *et al*., 2016). In this method, a high-osmolarity solution is used so that the embryonic intracellular water exits rapidly, dehydrating the embryonic cells and making them permeable to the cryoprotectant. Thus, the embryo is able to withstand direct immersion in liquid nitrogen (-196ºC) without the formation of ice crystals (Vajta *et al*., 1998).

On the other hand, high cryoprotectant concentrations have been described as promoting high cellular toxicity, even if exposed for a short period and a minimum volume of this solution (Vajta *et al*., 1998). Thus, different strategies have been developed for embryos to have rapid contact with liquid nitrogen and to reduce the volume of the cryoprotectant agent, such as the open pulled straw (OPS; Vajta *et al*., 1998), cryoloop (Lane *et al*., 1999), microdroplets (Papis *et al*., 2000) and cryotop techniques (Kuwayama *et al*., 2005).

In the classical slow-freezing protocol, the cooling rate is controlled to maintain a constant curve until the straws with embryos are immersed in the liquid nitrogen. The use of low concentrations of cryoprotectants is the main advantage of this technique since high concentrations are toxic to embryos. In addition, the process of thawing and the DT of embryos to cows make the slow freezing protocol more efficient for commercial use.

However, ice crystals can form and damage the structure of the embryo's membranes and organelles (Dode *et al*., 2013). In this way, the success of slow freezing and direct transfer of *in vitro* produced embryos invariably depends on the equilibrium between the rate of dehydration of the cell and the rate at which water is transformed into ice crystals (Visintin *et al*., 2002).

Despite the advances in cryopreservation methods, freezing and thawing processes impair the viability of the embryo. This impairment occurs due to the physical and chemical damages induced during the cryopreservation process (Overstrom, 1996; Baguisi *et al*., 2000). Sudano *et al*. (2012a) reported the effects of this damage by comparing the apoptosis rate caused by the stress of cryopreservation between fresh and vitrified blastocysts. In this study, there was a 2.4-fold increase ($P < 0.0001$) in the apoptosis rate of vitrified ($49.4 \pm 1.9$) in relation to fresh embryos ($20.8 \pm 1.1$). Similar apoptosis profiles were observed in other studies, which demonstrated increases of 3.7-fold (Park *et al*., 2006) and 1.7-fold (Márquez-Alvarado *et al*., 2004) in the apoptosis rate of cryopreserved embryos compared with fresh embryos.

Moreover, it has been definitively demonstrated that *in vitro* embryos are more sensitive to cryopreservation than *in vivo* embryos (Pollard and Leibo, 1994). This lower cryotolerance has been associated with the high lipid content present in the

cytoplasm of these embryos (Abe *et al*., 2002; Mucci *et al*., 2006) and the decrease in the density of mature mitochondria compared to embryos produced *in vivo* (Crosier *et al*., 2001; Farin *et al*., 2004). Additionally, the most abundant lipids in the plasma membranes of cells (phosphatidylcholine and sphingomyelin) also have different profiles (Sudano *et al*., 2012b).

Researchers suggest that lipid accumulation may be due to the uptake of the culture medium itself or to the inefficient and unregulated metabolism of the embryonic mitochondria (Farin *et al*., 2004; Barceló-Fimbres and Seidel, 2007a; Moore *et al*., 2007). Further, *in vitro* embryos have fewer transcripts levels for genes related to lipid metabolism compared to *in vivo*-produced embryos (Gad *et al*., 2012). Therefore, the addition of substances to the culture medium has been proposed in addition to adjusting the cryopreservation method to make the embryos more cryotolerant (Dode *et al*., 2013).

*Strategies to increase the cryotolerance of* in vitro *embryos*

Despite many advances in the last decades, the cryopreservation process of IVEP remains a major challenge in livestock, and the results are still inconsistent (Sudano *et al*., 2013). For example, the low cryotolerance of *in vitro* embryos is the main obstacle to the use of cryopreservation protocols (Sudano *et al*., 2011). The role of embryonic lipids in this regard is well described in the literature (Abe *et al*., 2002). Furthermore, strategies such as the use of serum-free culture media, the addition of chemical substances to promote changes in lipid metabolism, and the modulation of the membrane lipid composition can help improve the survival of *in vitro* embryos after cryopreservation (Sudano *et al*., 2013).

The cause of cytoplasmatic lipid deposition in *in vitro* embryos is not well established, but it has been suggested that the presence of serum in the culture medium may be directly involved in this process (Sanches *et al*., 2013). Studies have shown that the fetal calf serum (FCS) concentration affects the number of cytoplasmic lipid droplets of embryos (Leroy *et al*., 2005; Sudano *et al*., 2012a). Moreover, *in vitro* embryos cultured in a serum-free medium had decreased lipids and higher cryotolerance (Pereira and Marques, 2008).

An alternative to improving embryo freezeability is the use of lipolytic chemical agents, such as phenazine ethosulfate (PES), which reduces lipid accumulation and regulates energetic metabolism by NADPH to NADP oxidation (De La Torre-Sanchez *et al*., 2006; Sudano *et al*., 2011). Interestingly, it has been reported that PES, when used in the post-compaction period, promoted an increase in post-cryopreservation survival (Barceló- Fimbres and Seidel, 2007b).

In this sense, a study involving supplementation with FCS and PES showed an improvement in the blastocoele re-expansion rate after the embryo vitrification process when the serum concentration was reduced to 2.5% concomitant to the addition of PES to the culture medium on day 4 (Table 1; Sudano *et al*., 2011).

Table 1. Effects of fetal calf serum (FCS) and phenazine ethosulfate (PES) on blastocoele re-expansion (means ± SEM).

| Responses | Cryotolerance | |
|---|---|---|
| | Vitrified embryos (n) | Re-expansion rate (%) |
| FCS | | |
| 0% | 233 | $90.5 \pm 2.7^{a}$ |
| 2.5% | 346 | $81.6 \pm 2.5^{b}$ |
| 5% | 332 | $78.0 \pm 2.8^{bc}$ |
| 10% | 405 | $67.3 \pm 3.5^{c}$ |
| *In vivo* control | 15 | $93.3 \pm 6.7^{aA}$ |
| PES | | |
| Control | 474 | $72.0 \pm 3.0^{B}$ |
| PES day 2.5 | 362 | $79.9 \pm 2.8^{C}$ |
| PES day 4.0 | 480 | $86.2 \pm 2.4A^{C}$ |

$^{a-d}$Within a column, means without a common superscript differ ($P < 0.05$). $^{A-C}$Within a column, means without a common superscript differ ($P < 0.05$). Adapted from Sudano *et al*. (2011).

It is important to note that the addition of medium with 2.5% FCS did not decrease the embryonic cryotolerance (represented by the blastocoele re-expansion rate) compared to the group without FCS. However, independent of FCS concentration in the medium and the use of PES, the embryos in the *in vivo* group (control) had the highest survival after vitrification (Sudano *et al*., 2011).

Forskolin is another lipolytic chemical agent used to reduce the lipid content of *in vitro* embryos. This agent acts directly by activation of the adenylate cyclase, thus increasing the levels of cAMP and stimulating lipolysis to activate lipases (Men *et al*., 2006). Recently, Paschoal *et al*. (2017) demonstrated that forskolin was an effective lipolytic agent even at low concentrations, resulting in the formation of blastocysts with a larger number of cells than the untreated group. Additionally, this substance decreased embryo apoptosis caused by the cryopreservation method.

Therefore, it has been previously reported that treatment with forskolin before vitrification with the

cryotop method (a polypropylene rod in which the embryos are allocated next to minimum volumes of cryoprotectant solution) improved the cryotolerance and pregnancy rates of *Bos indicus in vitro* embryos after transfer to recipients (Sanches *et al*., 2013). The results are shown in Table 2.

Table 2. Pregnancy rates of *Bos indicus in vitro* embryos treated with or without the lipolytic agent forskolin for 48 hours in culture before the vitrification process.

| Treatment | Transferred embryos (n) | Pregnancy rate (%) |
|---|---|---|
| Control | 65 | 18.5[b] |
| Forskolin | 80 | 48.8[a] |

[a,b]Within a column, rates without a common superscript differed (P < 0.05). Adapted from Sanches *et al*. (2013).

According to these results, the use of forskolin and vitrification with the cryotop system as a strategic cryopreservation system could be a great alternative to facilitate the transport and export of embryos over long distances (Sanches *et al*., 2013).

In addition, the stage of development of blastocysts at the time they undergo cryopreservation is another factor that needs to be considered as a strategy to improve cryotolerance. For example, Kocyigit and Cevik (2016), showed a correlation between the diameter of embryos and their cryosurvival, in which early and expanded blastocysts were more sensitive to the damage promoted by vitrification and posterior warming compared to the blastocyst stage. In our experience, the ideal developmental stages are blastocyst and expanded blastocyst for both vitrification and direct transfer methods.

The slow freezing of embryos for later DT, despite having higher costs, eliminates the evaluation before transfer, which makes it more practical than vitrification. Moreover, smaller concentrations of cryoprotectants may also be used, thereby reducing toxicity to the embryos (Voelkel and Hu, 1992).

Briefly, in the DT method, the *in vitro* embryos are cryopreserved by the slow freezing method previously described for *in vivo* embryos (Vajta *et al*., 1998). The *in vitro* embryos are next exposed to a freezing solution consisting of 1.5 M ethylene glycol (EG), and at the end of the freezing curve, they are directly immersed in liquid nitrogen and stored until being transferred into the recipients.

Surprisingly, this strategy has been demonstrated to help overcome obstacles to *in vitro* embryo cryopreservation. On Table 3 pregnancy rates for fresh, vitrified, and frozen (direct transfer) *in vitro* embryos from dairy cows are presented.

Table 3. Pregnancy rates at 30 days after the transfer of fresh, vitrified or frozen (direct transfer) *in vitro*-produced embryos after ovum pick-up of Girolando cows.

| Group | Transferred embryos (n) | Pregnancy (%) |
|---|---|---|
| Fresh | 259 | 43.24 ± 1.23[a] |
| Vitrified | 234 | 31.19 ± 4.01[b] |
| Frozen | 311 | 34.72 ± 4.15[b] |

[a,b]Different letters in the same column indicate a significant difference (P < 0.05). Adapted from Sanches *et al.* (2016).

The results of this study revealed the possibility of using frozen embryos because the direct transfer optimized the logistics and can become a more practical approach for the transfer of cryopreserved *in vitro* embryos in the field (Sanches *et al*., 2016).

The direct transfer protocol has been used in large-scale operations, especially in the US and Brazil. In the near future, once other companies incorporate the direct transfer protocol in their operation, the majority of commercial IVEP embryos will probably be frozen, as currently occurs in the semen industry.

Despite the advances in cryopreservation methods, few players are using this technique, and some challenges remain in relation to the greater efficiency of the technique.

**Importance of recipient cow selection**

The choice of recipients is an important part of the success of bovine ET programs, since many of problems with this biotechnology application are related to the female conditions that will allow embryo implantation and maintenance of gestation until the fetus is born (Andrade *et al*., 2012).

Among the factors that directly interfere with the performance of fresh or cryopreserved transferred embryos, major highlighted aspects are the recipient's age, the sanitary and nutritional conditions of the recipients, and the degree of synchrony between the embryo stage and its recipient (Sreenan and Diskin, 1987; Hasler *et al*., 1987; Callesen *et al*., 1996; Peixoto *et al*., 2004, Jones and Lamb, 2008).

An interesting study evaluated the effects of synchrony between embryo stage and recipient on conceptus elongation and pregnancy rate. In this study, the authors showed that conceptus length was greater following transfer to an advanced uterus and that supplementation with progesterone resulted in short cycles in approximately 50% of recipients. Transfer of day 7 embryos to a synchronous uterus (day 7) resulted in a pregnancy rate of 47.3%. Transfer to an asynchronous uterus of day 5 (40.8%) or day 8

embryos (41.3%) moderately impacted the pregnancy rate ($P < 0.01$), but transfer to the uterus 2 days in advance (day 9, 24.4%) or 3 days behind (day 4, 27.0%) reduced ($P < 0.001$) the pregnancy rate compared with synchronous transfer (Randi *et al*., 2015). Interestingly, this study emphasized the importance of greater possible synchrony between the embryonic stage and the cycle day of the recipient.

Additionally, new technologies have been developed with the aim of helping the selection of recipients be more accurate by searching for genetic markers related to desirable characteristics. The sequencing of the bovine genome allowed genome-wide association studies (GWAS) to be conducted, which examine specific sites, such as single-nucleotide polymorphisms (SNPs), and associate them with certain phenotypes (Dairy Herd Management, 2017).

Recently, it has been possible to use commercial programs aimed at the identification of genomic loci associated with fertility in heifers and dairy cows. Some specific genes associated with fetal abnormalities that lead to abortion, embryonic death, or lower fertility, as well as genes associated with better reproductive efficiency, have been identified.

Therefore, this information associated with other methods and criteria for choosing the recipient can help the optimization and practical success of ET and consequently can improve the efficiency of IVEP in the field.

## Commercial and operational limitations to cryopreservation of *in vitro*-produced embryos

Cryopreservation of bovine embryos is a biotechnology that allows the storage of surplus embryos produced *in vitro* or through superovulation/embryo transfer programs, making feasible commercialization between countries and the transfer of embryos at a more convenient time (Sudano *et al*., 2012b).

However, the number of embryos cryopreserved in the past several years represented only 3 to 7% of the total embryo production in Brazil (Stroud, 2011, 2012; Viana, 2012). These data reflect the great challenges to the implementation of this technique.

As previously discussed, the low cryotolerance of *in vitro* embryos is a crucial obstacle to the use of cryopreservation processes in IVEP programs (Sudano *et al*., 2011). In this context, many efforts have been made by different research groups to improve the culture medium conditions during IVEP or to change cryopreservation protocols (Sudano *et al*., 2013). It is also important to emphasize that the survival capacity of the embryo after cryopreservation is a multifactorial event (Sudano *et al*., 2013).

Embryonic survival after freezing/thawing is influenced by important aspects, such as the culture medium composition (additives, supplementation with or without fetal calf serum, pH, and osmolarity), oocyte and semen quality, and the technician who produced the embryo in the laboratory (Gardner, 2008; Feugang *et al*., 2009; Hasler, 2010). Another feature to be considered is the atmosphere (lower or higher oxygen tension) in which the embryos are grown, which has been widely used to minimize oxidative stress; low oxygen tension improves metabolism and decreases the production of free radicals (Dode *et al*., 2013).

Finally, the qualification of the field veterinarian/technical responsible for performing the embryo transfer into the recipient uterus is another factor limiting the use of cryopreserved embryos in ET programs. In general, the professional must perform the process in a careful, rapid and accurate manner.

In our experience, this job position (embryo transfer) will be the next limitation to using IVEP cryopreservation on a large scale and globally. Once the technology is proven and well accepted, there will not be a sufficient number of field technicians able to perform embryo transfers.

Therefore, these factors, when considered together, will directly reflect pregnancy rates and may have positive impacts on the large-scale application of IVEP and embryo cryopreservation in cattle.

## Final comments

In the last decade, several technical advances have increased the efficiency of IVEP, making this reproductive strategy to have a greater impact on selection and genetic dissemination in cattle. On the other hand, the need for an efficient method to cryopreserve the surplus volume from embryo programs was generated. For *in vitro* embryos, vitrification has become the most frequently used technique for cryopreservation worldwide, which has contributed widely to the storage of embryos, as well as making IVEP programs more efficient.

Therefore, the implementation of a commercial program for IVEP and cryopreservation needs to overcome many challenges when using a vitrification protocol. There is no question that we must develop and improve the efficiency of direct transfer techniques to make the IVEP technology accessible to everyone everywhere. Along with the selection of the recipient according to good sanitary and nutritional status, adequate synchrony between embryo stage and recipient cycle, high maternal ability, and the choice of females with characteristics linked to fertility are aspects fundamental to the success of this biotechnology. Finally, the entire *in vitro* process for production or cryopreservation requires a highly qualified and trained team to perform each step of this journey.

## References

**Abe H, Yamashita S, Satoh T, Hoshi H**. 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol Reprod*, 61:57-66.

**Andrade GA, Fernandes MA, Knychala RM, Pereira Junior MV, Oliveira AJ, Nunes DP, Bonato G, Santos RM**. 2012. Fatores que afetam a taxa de prenhez de receptoras de embriões bovinos produzidos in vitro. *Rev Bras Reprod Anim*, 36:66-69.

**Baguisi A, Lonergan P, Overstrom E, Boland M**.

2000. Vitrification of bovine embryos: incidence of necrosis and apoptosis. *Theriogenology,* 55:162.Abstract.

**Barceló-Fimbres M, Seidel GEJ**. 2007a. Effects of either glucose or fructose and metabolic regulators on bovine embryo development and lipid accumulation *in vitro*. *Mol Reprod Dev*, 74:1406-1418.

**Barceló-Fimbres M, Seidel GEJ**. 2007b. Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during *in vitro* culture of bovine embryos on embryonic development after cryopreservation. *Mol Reprod Dev*, 74:1395-1405.

**Callesen H, Liboriussen T, Greve T**. 1996. Practical aspects of multiple ovulation-embryo transfer in cattle. *Anim Reprod Sci*, 42:205-214.

**Chebel RC, Demétrio DGB, Metzger J.** 2008. Factor affecting success of embryo collection and transfer in large dairy herds. *Theriogenology*, 69: 98-106.

**Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE**. 2001. Ultrastructural morphometry of bovine blastocysts produced in vivo or in vitro. *Biol Reprod*, 64:1375-1385.

**Dairy Herd Management** [homepage on the Internet]. 2017. The genomics of fertility and health traits. Available on: http://www.dairyherd.com/advice-and-tips/reproduction/genomics-fertility-and-health-traits. Accessed on: April 10, 2017.

**De La Torre-Sanchez JF, Gardner DK, Preis K, Gibbons J, Seidel GE**. 2006. Metabolic regulation of in vitro-produced bovine embryos. II. Effects of phenazine ethosulfate, sodium azide and 2,4-dinitrophenol during post-compaction development on glucose metabolism and lipid accumulation. *Reprod Fertil Dev*, 18:597-607.

**Dode MAN, Leme LO, Spricigo JFW**. 2013. Criopreservação de embriões bovinos produzidos in vitro. *Rev Bras Reprod Anim*, 37:145-150.

**Farin CE, Farin PW, Piedrahita JA**. 2004. Development of fetuses from *in vitro*-produced and cloned bovine embryos. *J Anim Sci*, 82(E-suppl):53-62.

**Ferreira G**. 2013. Reproductive performance of dairy farms in western Buenos Aires province, Argentina. *J Dairy Sci,* 96:8075-8080.

**Feugang JM, Camargo-Rodriguez O, Memili E**. 2009. Culture systems for bovine embryos. *Livest Sci*, 121:141-149.

**Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D**. 2012. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. *Biol Reprod*, 87:100. doi: 10.1095/biolreprod.112.099697.

**Gardner DK**. 2008. Dissection of culture media for embryos: the most important and less important components and characteristics. *Reprod Fertil Dev*, 20:9-18.

**Hasler JF, Mccauley AD, Lathrop WF, Foote RH**. 1987. Effect of donor-embryo-recipient interactions on pregnancy rate in a large-scale bovine embryo transfer program. *Theriogenology*, 27:139-168.

**Hasler JF**. 2010. Synthetic media for culture, freezing and vitrification of bovine embryos. *Reprod Fertil Dev*, 22:119-125.

**Jones AL, Lamb GC**. 2008. Nutrition, synchronization, and management of beef embryo transfer recipients. *Theriogenology*, 69:107-115.

**Kocyigit A, Cevik M**. 2016. Correlation between the cryosurvival, cell number and diameter in bovine in vitro produced embryos. *Cryobiology,* 73: 203-208.

**Kuwayama M, Vajta G, Kato O, Leibo SP**. 2005. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed,* 11:300-308.

**Lane M, Schoolcraft WB, Gardner DK**. 1999. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril*, 72:1073-1078.

**Leroy JL, Opsomer G, De Vliegher S, Vanholder T, Goossens L, Geldhof A, Bols PE, DE Kruif A, Van Soom A**. 2005. Comparison of embryo quality in high-yielding dairy cows, in dairy heifers and in beef cows. *Theriogenology*, 64:2022-2036.

**Marinho LSR, Untura RM, Morotti F, Moino LL, Rigo AG, Sanches BV, Pontes JHF, Seneda MM**. 2012. Large-scale programs for recipients of in vitro-produced embryos. *Anim Reprod*, 9:323-328.

**Marinho LSR, Sanches BV, Rosa CO, Tannura JH, Rigo AG, Basso AC, Pontes JHF, Seneda MM**. 2015. Pregnancy rates to fixed embryo transfer of vitrified IVP *Bos indicus*, *Bos Taurus* or *Bos indicus x Bos taurus* embryos. *Reprod Domest Anim*, 50:807-811.

**Márquez-Alvarado YC, Galina CS, Castilla B, León H, Moreno-Mendoza N**. 2004. Evidence of damage in cryopreserved and fresh bovine embryos using the TUNEL technique. *Reprod Domest Anim,* 39:141-145.

**McKeegan PJ, Sturmey RG**. 2012. The role of fatty acids in oocyte and early embryo development. *Reprod Fertil Dev,* 24:59-67.

**Men H, Agca Y, Riley LK, Critser JK**. 2006. Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. *Theriogenology*, 66:2008-2016.

**Moore K, Rodriguez-Sallaberry CJ, Kramer JM, Johnson S, Wroclawska,E, Goicoa S, Niasari-Naslaji A**. 2007. In vitro production of bovine embryos in medium supplemented with a serum replacer: effects on blastocyst development, cryotolerance and survival to term. *Theriogenology*, 68:1316-1325.

**Morotti F, Sanches BV, Pontes JHF, Basso AC, Siqueira ER, Lisboa LA, Seneda MM**. 2014. Pregnancy rate and birth rate of calves from a large-scale IVF program using reverse-sorted semen in *Bos indicus*, *Bos indicus-taurus*, and *Bos taurus cattle. Theriogenology*, 81:696-701.

**Mucci N, Aller J, Kaiser GG, Hozbor F, Cabodevila J, Alberio RH**. 2006. Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology*, 65:1551-1562.

**Overstrom EW**. 1996. In vitro assessment of embryo viability. *Theriogenology*, 45:3-16.

**Papis K, Shimizu M, Izaike Y**. 2000. Factors affecting the survivability of bovine oocytes vitrified in droplets. *Theriogenology*, 54:651-658.

**Park SY, Kim EY, Cui XS, Tae JC, Lee WD, Kim NH, Park SP, Lim JH**. 2006. Increase in DNA fragmentation and apoptosis-related gene expression in frozen–thawed bovine blastocysts. *Zygote*, 14:125-131.

**Paschoal DM, Sudano MJ, Schwarz KRL, Maziero RRD, Guastali MD, Crocomo LF, Magalhães LCO, Martins Jr, A, Leal ALV, Landim-Alvarenga FC**. 2017. Cell apoptosis and lipid content of in vitro–produced, vitrified bovine embryos treated with forskolin. *Theriogenology*, 87:108-114.

**Peixoto MGCD, Bergmann JAG, Alvim MTT, Penna VM**. 2004. Fatores que influenciaram a prenhez de embriões zebuínos em receptoras mestiças. *In*: Abstracts of the V Simpósio da Sociedade Brasileira de Melhoramento Animal, 2004, Pirassununga, SP. Pirassununga, SP: SBMA. 4 pp.

**Pereira RM, Marques CC**. 2008. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank*, 9:267-277.

**Perry G.** 2016. IETS 2016. 2015 Statistics of embryo collection and transfer in domestic farm animals. *Embryo Technology Newsletter*, 34: 1-75.

**Pollard JW, Leibo SP**. 1994. Chilling sensitivity of mammalian embryos. *Theriogenology*, 41:101-106.

**Pontes JHF, Silva KCF, Basso AC, Rigo AG, Ferreira CR, Santos GMG, Sanches, BV, Porcionato, JPF, Vieira, PHS, Faifer, FS, Sterza, FAM, Schenkd, JL, Seneda, MM**. 2010. Large-scale *in vitro* embryo production and pregnancy rates from *Bos taurus, Bos indicus*, and *indicus-taurus* dairy cows using sexed sperm. *Theriogenology*, 74:1349-1355.

**Pontes JHF, Melo Sterza FA, Basso AC, Ferreira CR, Sanches BV, Rubin KC, Seneda MM**. 2011. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (*Bos indicus*) donors. *Theriogenology*, 75:1640-1646.

**Pryor JH, Trant JA, Ponchirolli-Schneider CB, Looney CR, Long CR, Forrest DW**. 2009. The use of forskolin and its effect on *in vitro*-produced brahman-sired embryos submitted to slow cool freezing or vitrification. *Reprod Fertil Dev*, 22:214-214.

**Randi F, Fernandez-Fuertes B, McDonald M, Forde N, Kelly AK, Amorin HB, Lima EM, Morotti F, Seneda MM, Lonergan P**. 2015. Asynchronous embryo transfer as a tool to understand embryo–uterine interaction in cattle: is a large conceptus a good thing? *Reprod Fertil Dev*, 28:1999-2006.

**Sanches BV, Marinho LSR, Filho BDO, Pontes JHF, Basso AC, Meirinhos, MLG, Silva-Santos KC, Ferreira CR, Seneda MM**. 2013. Cryosurvival and pregnancy rates after exposure of IVF-derived *Bos indicus* embryos to forskolin before vitrification. *Theriogenology*, 80:372-377.

**Sanches BV, Lunardelli PA, Tannura JH, Cardoso BL, Pereira MHC, Gaitkoski G, Basso AC, Arnold DR, Seneda MM**. 2016. A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology*, 85:1147-1151.

**Saragusty J, Arav A.** 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction,* 141: 1-19.

**Segerson EC, Hansen TR, Libby DW, Randel RD, Getz WR**. 1984. Ovarian and uterine morphology and function in Angus and Brahman cows. *J Anim Sci*, 59:1026-1046.

**Silva-Santos KC, Santos GMG, Siloto LS, Hertel MF, Andrade ER, Rubin MIB, Sturion L, Melo-Sterza FA, Seneda MM**. 2011. Estimate of the population of preantral follicles in the ovaries of *Bos taurus indicus* and *Bos taurus taurus* cattle. *Theriogenology*, 76:1051-1057.

**Sreenan JM, Diskin MG**. 1987. Factors affecting pregnancy rate following embryo transfer in the cow. *Theriogenology*, 27:99-113.

**Stewart BM, Block J, Morelli P, Navarette AE, Amstalden M, Bonilla L, Hansen PJ, Bilby TR**. 2011. Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced in vitro with sex-sorted semen. *J Dairy Sci,* 94:3437-3445.

**Stroud B**. 2011. IETS 2011 Statistics and data retrieval committee report: the year 2010 worldwide statistics of embryo transfer in domestic farm animals. *Embryo Trans Newslett,* 29:14–23.

**Stroud B**. 2012. IETS 2012 statistics and data retrieval committee report: the year 2011 world wide statistics of embryo transfer in domestic farm animals. *Embryo Trans Newslett,* 30:16-26.

**Sudano MJ, Paschoal DM, Rascado TD, Magalhaes LCO, Crocomo LF, Lima-Neto JF, Landim-Alvarenga FD**. 2011. Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. *Theriogenology*, 75:1211-1220.

**Sudano MJ, Santos VG, Tata A, Ferreira CR, Paschoal DM, Machado R, Buratini J, Eberlin MN, Landim-Alvarenga FDC**. 2012b. Phosphatidylcholine and sphingomyelin profiles vary in *Bos taurus indicus* and *Bos taurus taurus* in vitro-and in vivo-produced blastocysts. *Biol Reprod*, 87:1-11. doi: 10.1095/biolreprod.112.102897.

**Sudano MJ, Paschoal DM, Maziero RRD, Rascado TS, Guastali MD, Crocomo LF, Magalhães LCO, Monteiro BA, Martins Jr A, Machado R, Landim-Alvarenga FDC**. 2013. Improving postcryopreservation survival capacity: an embryo-focused approach. *Anim. Reprod*, 10:160-167.

**Sudano MJ, Paschoal DM, Rascado TS, Crocomo LF, Magalhaes LC, Junior AM, Machado R, Landim-Alvarenga FC**. 2012a. Crucial surviving aspects for vitrified in vitro-produced bovine embryos. *Zygote*, 22:124-131.

**Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H**. 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev*, 51:53-58.

**Vajta G, Kuwayama M**. 2006. Improving cryopreservation systems. *Theriogenology*, 65:236-244.

**Viana JHM**. 2012. Levantamento estatístico da produção de embriões bovinos no Brasil em 2011: mudanças e tendências futuras. *O Embrião*, 51:6-10.

**Visintin JA, Martins JFP, Bevilacqua EM, Mello MRB, Nicácio AC, Assumpção MEOA**. 2002. Cryopreservation of *Bos taurus* vs. *Bos indicus* embryos: are they really different? *Theriogenology*, 57:345-359.

**Voelkel SA, Hu YX**. 1992. Direct transfer of frozen-thawed bovine embryos. *Theriogenology,* 37:23-37.

DOI: 10.21451/1984-3143-AR996

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# Does maternal size, nutrition and metabolic status affect offspring production traits in domestic species?

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## Abstract

The Developmental Origins of health and Disease state that environmental conditions during pregnancy affect long term outcomes in offspring. In the present paper, effects of maternal size and breed as well as maternal nutrition on offspring size, growth and production traits are described. Although birthweight is mostly not affected, metabolic perturbations are often observed in adult offspring. In animal production, however, the relation between developmental conditions and long-term offspring outcome may remain unnoticed. Nevertheless, improving dams' health and nutrition before and during pregnancy may help improving production traits in domestic animals.

**Keywords**: DOHaD, embryo transfer, fetal programming, nutrition, pregnancy.

## Introduction

In mammals, developmental conditions at the time of conception, during pregnancy and the neonatal period are known to affect long-term post-natal health, as known under the term "Developmental Origins of Health and Disease" (DOHaD). This phenomenon, is associated with modifications in gene expression due to environmentally induced epigenetic mechanisms. Maternal environment, such as maternal metabolism and nutrition, or the use of reproductive biotechnologies, may have an effect on feto-placental development, growth and subsequent adult health, thus affecting offspring performance and longevity.

This article aims to summarize existing knowledge on long term effects of maternal phenotype in domestic species and their potential impact on animal health, fertility and welfare. Future directions both in research and for improvement of field management are discussed.

## The other side of genetics: effect of maternal phenotype/genotype

Genetic selection for production traits is the basis of animal breeding. Taking into consideration maternal genetic value and production, the sire is selected based on his genetic indices and heritability, in order to improve desired production traits. Maternal genotype and phenotype are also seminal in determining the environment in which the embryo and fetus will develop, regardless of production traits. This can be studied by comparing cross-bred offspring born to dams of different genotypes, or by studying phenotypic variation in genetically identical animals (Fig. 1).

In pigs, the cross-breeding between Meishan sows (200 kg adult weight) and Yorkshire males (300 kg adult weight) yields lighter piglets than the opposite crossing (Meishan males and Yorkshire sows; Biensen *et al.*, 1999). Similarly, in cattle, calves born to South Devon cows (790 kg adult weight) and Dexter bulls (340 kg adult weight) were approximately 6 kg heavier than crossbred calves born to a Dexter cow (Joubert and Hamond, 1958). Moreover, Charolais breed embryos transferred into Brahman cows are lighter at birth (mean 29 kg) compared to Charolais embryos transferred into Charolais recipients (mean 63 kg). Inversely, Brahman embryos transferred into Charolais recipients result in calves with heavier birthweight (mean 41 kg) than those produced by the transfer of Brahman embryos into Brahman cows (mean 19 kg; Ferrell, 1991). These results indicate that maternal breed and consequently maternal size and environment will affect offspring weight and size at birth, regardless of genetic potential.

Further consequences on postnatal development have been explored in horses. In the first half of the 20th century, Walton and Hammond elegantly demonstrated, using cross-breeding between large Shire horses and small Shetland ponies, that crossbred offspring whose dam was a Shetland pony were smaller at birth and remained smaller as adults than those whose dam was a Shire mare (Walton and Hammond, 1938). Almost 50 years later, Tischner *et al.* showed that Polish pony embryos transferred into draft mares produced foals that were larger at birth and remained larger as adults, compared to those that had been transferred into mares of their own breed (Tischner *et al.*, 2000). More recently, the transfer of pony embryos into mares of larger breeds was shown to consistently increase fetal and postnatal growth until adulthood. Conversely, foals from a larger breed born to pony mares were small at birth and only partially caught-up to controls of the same breed (Allen *et al.*, 2004; Peugnet *et al.*, 2014). Moreover, both excess and reduced fetal growth were associated with osteoarticular lesions and metabolic perturbations, some of which still present at 2 years of age (Peugnet *et al.*, 2014, 2016).

## Effect of maternal nutrition

Procedures in terms of maternal nutrition in domestic animals vary greatly depending on breed, location, availability of feedstuff and season, amongst other factors. The choice of dietary treatments in

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experimental protocols is also very diverse, rendering it difficult to draw clear conclusions. In general, the effects of maternal nutrition on offspring phenotype are marginal, except when dietary treatments are severe and prolonged as reviewed recently (Funston *et al.*, 2012; Chavatte-Palmer *et al.*, 2015, 2016; Sinclair *et al.*, 2016; Opsomer *et al.*, 2017). The list of studies presented here does not claim to be exhaustive as the authors have selected key examples to illustrate each nutritional condition.

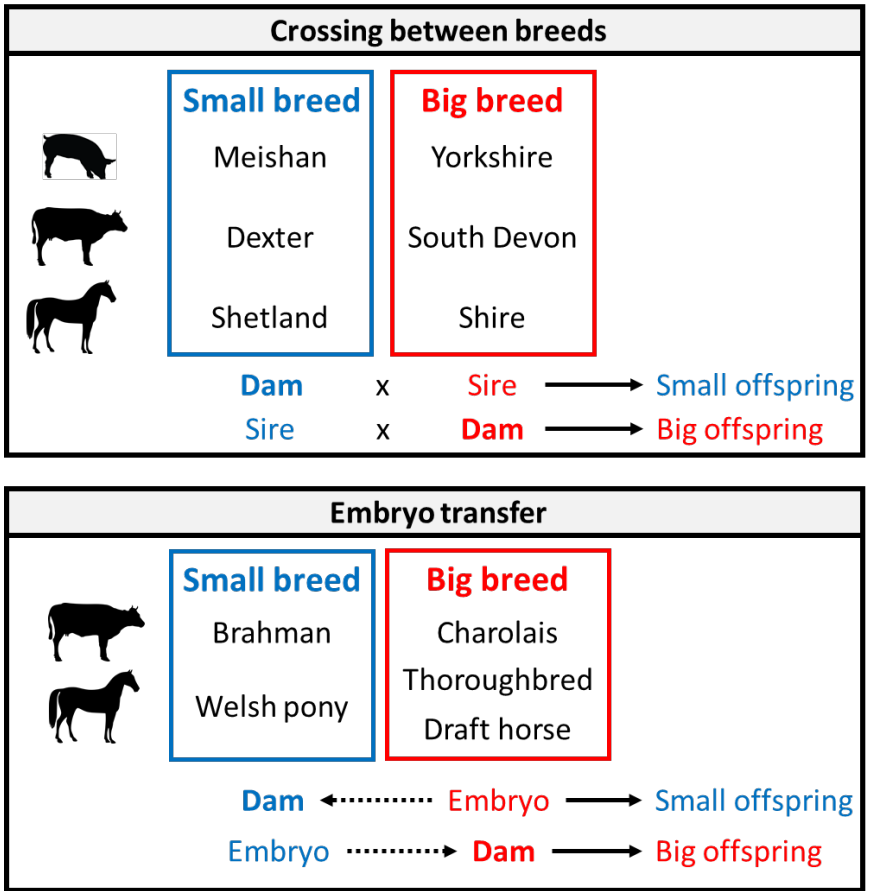


Figure 1. Effect of maternal and partenal size on offspring size after artificial insemination or embryo transfer. After Walton and Hamon (1938); Joubert and Hamon (1958); Ferrel. (1991); Biensen *et al.* (1999); Tischner *et al.* (2000); Allen *et al.* (2004); Peugnet *et al.* (2014).

*Excess nutrition and obesity*

In many studies, excess nutritional intake during pregnancy is confounded with maternal obesity. Obesity can be defined as excess adiposity above a certain level as defined by the authors depending on studies, before breeding and during pregnancy. Here we tried to discriminate studies with maternal gestational overfeeding from studies with maternal obesity prior to breeding.

Effects of excess maternal nutrition have been mainly studied in sheep (Table 1) with little observed effects on lamb birthweight and postnatal growth (Hoffman *et al.*, 2014; Khanal *et al.*, 2014; Kleemann *et al.*, 2015; Sen *et al.*, 2016). Nevertheless, expression of Insulin Growth Factor 1 (IGF1) is increased in the lamb liver (Hoffman *et al.*, 2014) resulting in increased plasma IGF1 concentrations (Hoffman *et al.*, 2016) and lipid accumulation is also observed in the lambs' muscle (Hoffman *et al.*, 2014; Reed *et al.*, 2014), together with increased insulin resistance (Hoffman *et al.*, 2016), increased adiposity (Khanal *et al.*, 2014), hyperglycemia and alteration of hepatic signaling pathways (Philp *et al.*, 2008). Finally, increased ovarian size and reduced ovarian follicular numbers have also been observed (Da Silva *et al.*, 2003; Kleemann *et al.*, 2015). *Ad libitum* access to feedstuff at adulthood (19-22 months) increased food intake, weight gain, visceral and subcutaneous fat, basal glycemia and insulinemia in all animals but offspring born to obese dams were less affected than offspring born to control dams (Long *et al.*, 2010, 2015).

When maternal obesity was induced by overfeeding dams starting 2 months before breeding and until lambing, offspring birthweight was not affected but glucose metabolism was consistently and durably altered. The number of pancreatic β-cells was reduced in fetal life, resulting in hyperglycemia, un hypoinsulinemia and reduced pancreatic weight at birth and increased insulin resistance and altered glycemic regulation in adults. Moreover, muscular fibrosis and hyperleptinemia were observed (Long *et al.*, 2010, 2015; Huang *et al.*, 2012; Zhang *et al.*, 2012).

Table 1. Summary of studies performed on the effects of overnutrition in pregnant ewes on the post-natal development of the offspring. The level of excess nutrition is expressed as a percentage of the energy content ingested by the control group.

| Level of overnutrition | Period of overnutrition | Function | Age | Phenotype | Source |
|---|---|---|---|---|---|
| Periconceptioal overnutrition | | | | | |
| 150% | From 17 days before insemination to 6 days after | Growth | Birth | = Live weight | Kleeman *et al*., 2015 |
| | | | 5 days | ↗ Ovary weight | |
| Overnutrition in the begining of gestation | | | | | |
| *Ad libitum* | The 100 first days of gestation (2 first third) | Endocrinology | 103 days of gestation | ↘ Progesterone concentration | Da Silva *et al*., 2003 |
| | | Ovary function | | ↘ Number of primordial follicles<br>↘ Number of total follicles | |
| 175% | From 30 to 80 days of gestation | Growth | From birth to 5 months | = Live weight | Sen *et al*., 2016 |
| | | Muscle function | 5 months | ↗ Fibres density | |
| Overnutrition in the end of gestation | | | | | |
| 126% | From 116 days of gestation | Growth | 1 day | = Live weight<br>↗ Heart weight | Hoffman *et al*., 2014 |
| | | | 3 months | ↘ Live weight | |
| | | Endocrinology | From 1 day to 3 months | ↘ IGFBP3 Concentration<br>↗ Leptin Concentration | |
| | | Gene expression | 1 day | ↗ Liver *IGF1* expression<br>↗ Muscle *β-catenin* expression (stimulate the differentiation of stem cells into muscle cells) | |
| 155% | From 115 days of gestation | Carbohydrate metabolism | From 1 to 30 days | ↗ Fasting glycaemia<br>↘ % of phosphorylated AMPK in the liver (can contribute to ↗ the production of glucose by the liver) | Philp *et al*., 2008 |
| 150% | From 105 days of gestation | Growth | From birth to 2 months | = Live weight | Khanal *et al*., 2014 |
| | | | From 2 to 6 months | ↘ Live weight | |
| | | Body condition | 6 months | ↘ % subcutaneous adipose tissue<br>↘ Subcutaneous adipose tissue / Visceral adipose tissue | |

| Level of overnutrition | Period of overnutrition | Function | Age | Phenotype | Source |
|---|---|---|---|---|---|
| Overnutrition during the most of gestation | | | | | |
| 140% | From 31 days of gestation | Growth | From 1 day to 3 months | ↗ Live weight, thoracic perimeter | Reed *et al*., 2014<br>Hoffman *et al*., 2016<br>Pillai *et al*., 2017 |
| | | | 1 day | ↗ Length | |
| | | Endocrinology | 3 months | ↗ IGF1 Concentration<br>↗ IGFBP2 Concentration | |
| | | Carbohydrate metabolism | 3 months | ↗ Leptin concentration<br>↗ Insulin/basal glucose ratio<br>↗ Insulin resistance | |
| | | Muscle function | 1 day | ↗ Fibres area (cross section)<br>↗ *Myostatin* expression | |
| | | | 3 months | ↘ Fibres area (cross section) | |
| | | | From 1 day to 3 months | ↗ Lipid accumulation in muscle | |
| Modelling of obesity – Overnutrition throughout gestation | | | | | |
| 150% | From 60 days before insemination | Gestation | Gestation length | ↘ Gestation length | Long *et al*., 2010<br>Huang *et al*., 2012<br>Zhang et al., 2012<br>Long et al., 2015 |
| | | Growth | From birth to 19 months | = Live weight | |
| | | Carbohydrate metabolism | 135 days of gestation | ↘ β pancreatic cells<br>↗ Production of insulin by remaining β cells | |
| | | | Birth | ↗ Basale glycaemia<br>↘ Basale insulinemia<br>↘Pancreas weight | |
| | | | 19 months | ↗ Insulin resistance<br>↘ Glucose disposition by insulin independent glucose transporters | |
| | | Muscle function | 2.5 years (males) | ↗ Collagen concentration (=↗ fibrosis)<br>↘ Metalloproteases expression<br>↗ Metalloproteases inhibitor expression | |
| | | Endocrinology | 2-3 years (males) | ↗ Leptin concentration | |

IGF1: Insulin Growth Factor 1; IGFBP3: Insulin growth Factor Binding Protein 3; AMPK: AMP-activiated protein kinase; IGFBP2: Insulin growth Factor Binding Protein 2.

In order to understand the importance of preconceptional obesity, embryos produced in adult obese or control ewes were transferred in adolescent control or obese ewes (Wallace *et al.*, 2017). Pregnancy length was shorter in obese recipients and resulted in reduced lamb birthweight compared to controls, regardless of donor group. The colostrum quality was also affected by obesity (Wallace *et al.*, 2017).

Finally, in cattle, feeding with 125% requirements from 3 months of gestation increases calf birthweight but weaning weight and carcass quality at 5 months of age were not different between groups (Wilson *et al.*, 2016). Thus, excess maternal nutrition and maternal obesity both affect lipid and glucose metabolism in offspring and may also alter body composition and muscle quality.

*Undernutrition*

As for excess nutrition, maternal undernutrition has been extensively studied in the ewe (Table 2). Only severe maternal undernutrition reduces birthweight whereas moderate undernutrition appears un-noticed in terms of offspring birthweight (Bispham *et al.*, 2003; Gardner *et al.*, 2005; Ford *et al.*, 2007; Hoffman *et al.*, 2014, 2016; Field *et al.*, 2015; Kleemann *et al.*, 2015; Sen *et al.*, 2016; Whorwood *et al.*, 2016). Nevertheless, lambs born to undernourished ewes have reduced plasma IGF1 and T3 (triiodothyronin) concentrations (Hoffman *et al.*, 2014; Field *et al.*, 2015) and physiological pathways involving corticosteroid hormones are disturbed (Whorwood *et al.*, 2016). Maternal undernutrition has also been associated with alterations in glucose metabolism, including hyperglycemia or hyperinsulinemia, increased insulin secretion by β-cells and reduced glucose tolerance (Gardner *et al.*, 2005; Ford *et al.*, 2007; Hoffman *et al.*, 2016). Intra-muscular lipid depositions are also increased in these lambs together with modifications in muscular fiber development (Ford *et al.*, 2007; Reed *et al.*, 2014) and increased perirenal fat mass was observed with or without reduction of subcutaneous fat (Gardner *et al.*, 2005; Ford *et al.*, 2007; Hoffman *et al.*, 2014, 2016).

In goats, a progressive maternal undernutrition (goats were fed 50 to 80% of the spontaneous intake of controls) in the last third of gestation reduced birthweight in male kids only although Non-esterified fatty Acid concentrations (NEFA) were increased in all kids (Laporte-Broux *et al.*, 2011). Subsequently at 1 and 2 years of age, restricted female offspring ate more than controls but no difference in energy metabolism was evidenced between groups (Laporte-Broux *et al.*, 2012).

In beef cattle, feeding cows at 80% requirements between 3 and 6 months of gestation reduced subcutaneous rib fat thickness and increased the intra- to inter-muscular fat ratio in 7 months old calves (Mohrhauser *et al.*, 2015). The birthweight of calves born to Angus cross-bred cows fed 60% of their requirements between 30 and 85 days or between 30 and 140 days of gestation was the same as offspring born to cows fed 100% of requirements but their liver was heavier (Prezotto *et al.*, 2016). Moreover, nutritional supplementation of restricted beef heifers during pregnancy did not increase offspring birthweight nor subsequent performance (Summers *et al.*, 2015) but increased feedlot efficiency and altered carcass characteristics with a tendency for high fat concentrations in the meat of animal born to restricted, non-supplemented heifers (Summers *et al.*, 2015).

In rabbits, a 50% maternal undernutrition from 7 to 19 days or from 20 to 27 days of gestation (31 days pregnancy) reduced pups' birthweight but post-natal growth, feeding behavior and body composition were not altered until 2,5 months of age (Lopez-Tello *et al.*, 2017).

Thus, whatever the species, although maternal undernutrition may not alter birthweight, offspring lipid and glucose metabolism are usually disturbed, affecting body composition and muscular development.

*Effect of maternal metabolism*

Independently from nutrition, maternal metabolism can be affected by many factors. Insulinoresistance is usually linked to obesity but can also be associated to production. Indeed, high yielding dairy cattle are prone to insulinoresistance because of their high energy requirements for milk production inducing a negative energy balance and this lactational insulinoresistance can persist for subsequent pregnancies (Bossaert *et al.*, 2008; De Koster and Opsomer, 2013; Zachut *et al.*, 2013; Opsomer *et al.*, 2017). Dairy cows insulinoresistant in late gestation produce lighter calves with reduced IGF1 plasma concentrations and increased insulinemia at birth (Kawashima *et al.*, 2016). Effects on subsequent offspring production have not been studied but epidemiological data indicate a slightly reduced milk yield if offspring from dams inseminated at peak lactation (González-Recio *et al.*, 2012).

*Effect of maternal parity*

The study of maternal age and parity on offspring development is difficult in production animals as age and parity are usually linked. Heifers are also non-lactating at breeding in contrast to cows.

In the horse, primiparous mares produce smaller and lighter foals at birth than multiparous mares (Fig. 2; Doreau *et al.*, 1991; Lawrence *et al.*, 1992; Pool-Anderson *et al.*, 1994; Cymbaluk and Laarveld, 1996; Wilsher and Allen, 2003; Elliott *et al.*, 2009; Klewitz *et al.*, 2015; Vazquez *et al.*, 2015; Meirelles *et al.*, 2017). Moreover, it has been shown that foals born to primiparous mares remain smaller until 1 year of age and lighter until 4 months of age compared with foals born to multiparous mares (Pool-Anderson *et al.*, 1994; Cymbaluk and Laarveld, 1996; Zoch *et al.*, 2016; Meirelles *et al.*, 2017). This difference of growth seems to be linked with decreased IGF-1 serum concentration in primiparous foals (Cymbaluk and Laarveld, 1996).

Table 2. Summary of studies performed on the effects of undernutrition in pregnant ewes on the post-natal development of the offspring. The level of undernutrition is expressed as a percentage of the energy content ingested by the control group.

| Undernutrition | Undernutrition period | Function | Age | Phenotype | Source |
|---|---|---|---|---|---|
| | | | Periconceptional undernutrition | | |
| 70% | From 17 days before insemination to 6 days after | Growth | Birth | = Live weight | Kleeman *et al*., 2015 |
| | | | 5 days | ↗ Liver weight | |
| | | | Undernutrition in early gestation | | |
| 50% | 28 to 79 days of gestation | Growth | 135 days of gestation | = Live weight | Field *et al*., 2015 |
| 50% | 28 to 78 days of gestation | Growth | Birth | ↘ Live weight | Ford *et al*., 2007 |
| | | | From 4 to 8 months | ↗ Live weight | |
| | | Body condition | 8 months | ↗ Perirenal and muscle adipose tissue | |
| | | Carbohydrate metabolism | 2 months | ↗ Basal glycaemia<br>↗ Pancreatic β cell response | |
| | | | 8 months | ↘ Pancreatic β cell response | |
| 50% | 28 to 77 days of gestation | Growth | Birth | = live weight, ↗ total length, ↗ kidneys weight | Whorwood *et al*., 2016 |
| | | Gene expression | | ↗ G*lucocorticoid receptor* expression (adrenal, liver, lung, perirenal adipose tissue, kidneys)<br>↘ *11- βHSD2 e*xpression (adrenals, kidneys) | |
| 40% | 28 to 80 days of gestation | Growth | Birth | ↗ Live weight | Bispham *et al*., 2003 |
| 50% | 30 to 80 days of gestation | Growth | Birth | = Live weight | Sen *et al*., 2016 |
| | | | 5 months | ↘ Live weight | |

| Undernutrition | Undernutrition period | Function | Age | Phenotype | Source |
|---|---|---|---|---|---|
| **Undernutrition in late gestation** | | | | | |
| 60% | From 116 days of gestation | Growth | 1 day | ↘ Live weight, ↘ thoracic perimeter | Hoffman *et al.,* 2014 |
| | | | 3 months | ↘ Back subcutaneous adipose tissue thickness | |
| | | Endocrinology | 1 day | ↘ T3 concentration | |
| | | | From 1 day to 3 months | ↘ IGF1 concentration<br>↘ IGFBP3 concentration | |
| 50% | From 110 days of gestation | Growth | From birth to 1 year | = Live weight | Gardner *et al*., 2005 |
| | | Carbohydrate metabolism | 1 year | ↘ Glucose tolerance<br>↘ GLUT4 expression in the perirenal adipose tissue | |
| | | Body condition | 1 year | ↗ Fat mass | |
| 50% | From 105 days of gestation | Growth | From birth to 6 months | ↘ Live weight | Khanal et al., 2014 |
| | | | 6 months | ↘ Subcutaneous adipose tissue / visceral adipose tissue ratio | |
| **Undernutrition throughout gestation** | | | | | |
| 50% | From 28 days of gestation | Growth | 135 days of gestation | = Live weight, ↗ liver weight | Field *et al*., 2015 |
| | | Carbohydrate metabolism | | ↗ Umbilical insulinemia | |
| | | Endocrinology | | ↗ Umbilical IGF1 concentration | |
| 60% | From 31 days of gestation | Growth | From 1 day to 3 months | = Live weight, total length | Reed *et al*., 2014<br>Hoffman *et al*., 2016 |
| | | | 3 months | ↗ Heart weight | |
| | | Body condition | 3 months | ↘ Back subcutaneous adipose tissue thickness | |
| | | Carbohydrate metabolism | 3 months | ↗ Basal insulinemia<br>↗ Basal insulin/glucose ratio | |
| | | Muscle function | 1 day | ↗ Fibres area (cross section) | |
| | | | 3 months | ↘ Fibres area (cross section) | |
| | | | From 1 day to 3 months | ↗ Lipid accumulation in muscle | |

11-βHSD: 11β-hydroxusteroid dehydrogenase : converts cortison into cortisone; T3: Triiodothyronine; IGF1: Insulin Growth Factor 1; IGFBP3: Insulin growth Factor Binding Protein 3; GLUT4: Glucose transporter 4.

In dairy cattle, heifers have a shorter gestation length and produce smaller (-9%) and lighter calves compared to cows (Kertz *et al.*, 1997; Kamal *et al.*, 2014, 2015) with a reduced body mass index (weight/ withers' heigth * crown-rump length) at birth (Kamal *et al.*, 2014, 2015). Nevertheless, alterations of the glucose metabolism were not observed at birth in these calves (Kamal *et al.*, 2015). Finally, heifers' colostrum contains less calcium, phosphorus and magnesium than multiparous cow colostrum, indicating that colostrum quality is altered, maybe for other components (Kume and Tanabe, 1993).

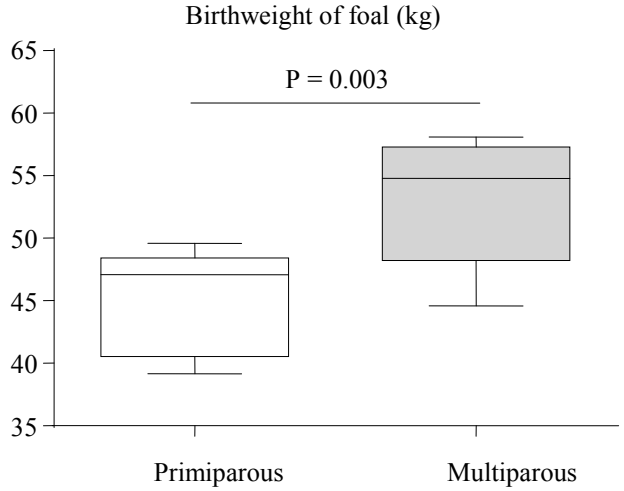


Figure 2. Birthweight of foals depending on the parity of the mare. Data derived from Doreau *et al*. (1991); Lawrence *et al*. (1992); Wilsher and Allen (2003); Elliott *et al*. (2009); Meirelles *et al*. (2017).

**Practical implications for embryo transfer**

The data presented above show clear evidence that maternal size and nutrition may influence offspring size but also metabolism and production. Other production and health traits, such as immunity, feeding behavior but also fertility may also be affected (Chadio and Kotsampasi, 2014; Chavatte-Palmer *et al.* 2014). This pleads for a very careful choice of embryo recipients in terms of breed and size but also underlines the importance of the management of these animals before and during pregnancy. The molecular basis for these effects is epigenetic mechanisms (Gonzalez-Recio *et al*., 2015; Triantaphyllopoulos *et al.*, 2016). Future research is needed to explore if epigenetic markers could be used as predictors of long term outcomes in offspring.

**References**

**Allen W, Wilsher S, Tiplady C, Butterfield R**. 2004. The influence of maternal size on pre- and postnatal growth in the horse: III postnatal growth. *Reproduction,* 127:67-77.

**Biensen NJ, Wilson ME, Ford SP**. 1999. The impacts of uterine environment and fetal genotype on conceptus size and placental vascularity during late gestation in pigs. *J Anim Sci,* 77:954-959.

**Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Pipkin FB, Stephenson T, Symonds ME**. 2003. Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: Consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. *Endocrinology,* 144:3575-3585.

**Bossaert P, Leroy J, Vliergher S, Opsomer G**. 2008. Interrelations between glucose-induced insulin response, metabolic indicators and time of first ovulation in high-yielding dairy cows. *J Dairy Sci,* 91:3363-3371.

**Chadio S, Kotsampasi B**. 2014. The role of early life nutrition in programming of reproductive function. *J Dev Orig Health Dis*, 5:2-15.

**Chavatte-Palmer P, Debus N, Dupont,C, Camous S**. 2014. Nutritional programming and the reproductive function of the offspring. *Anim Prod Sci*, 54:1166-1176.

**Chavatte-Palmer P, Richard C, Peugnet P, Robles M, Rousseau-Ralliard D, Tarrade A**. 2015. The developmental origins of health and disease: importance for animal production. *Anim Reprod Sci,* 12:505-520.

**Chavatte-Palmer P, Tarrade A, Rousseau-Ralliard D**. 2016. Diet before and during pregnancy and offspring health: The importance of animal models and what can be learned from them. *Int J Environ Res Public Health,* 13:E586. doi: 10.3390/ijerph13060586.

**Cymbaluk N, Laarveld B**. 1996. The ontogeny of serum insulin-like growth factor-I concentration in foals: effects of dam parity, diet, and age at weaning. *Domest Anim Endocrinol,* 13:197-209.

**Da Silva P, Aitken RP, Rhind SM, Racey PA, Wallace JM**. 2003. Effect of maternal overnutrition during pregnancy on pituitary gonadotrophin gene expression and gonadal morphology in female and male foetal sheep at day 103 of gestation. *Placenta*, 24:248-257.

**De Koster JD, Opsomer G**. 2013. Insulin resistance in dairy cows. *Vet Clin North Am Food Anim Pract,* 29:299-322.

**Doreau M, Boulot S, Martin-Rosset W**. 1991. Effect of parity and physiological state on intake, milk production and blood parameters in lactating mares differing in body size. *Anim Prod,* 53:111-118.

**Elliott C, Morton J, Chopin J**. 2009. Factors affecting foal birth weight in Thoroughbred horses. *Theriogenology,* 71:683-689.

**Evans AC, Mossa F, Walsh SW, Scheetz D, Jimenez-Krassel F, Ireland JL, Smith GW, Ireland JJ**. 2012. Effects of maternal environment during gestation on ovarian folliculogenesis and consequences for fertility in bovine offspring. *Reprod Domest Anim*, 47(suppl. 4):31-37.

**Ferrell CL**. 1991. Maternal and fetal influences on uterine and conceptus development in the cow: I. Growth of tissues of the gravid uterus. *J Anim Sci,* 69:1945-1953.

**Field ME, Anthony R V., Engle TE, Archibeque SL, Keisler DH, Han H**. 2015. Duration of maternal undernutrition differentially alters fetal growth and hormone concentrations. *Domest Anim Endocrinol,* 51:1-7.

**Ford SP, Hess BW, Schwope MM, Nijland MJ, Gilbert JS, Vonnahme KA, Means WJ, Han H, Nathanielsz PW**. 2007. Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *J Anim Sci*, 85:1285-1294.

**Funston RN, Summers AF, Roberts AJ**. 2012. Alpharma Beef Cattle Nutrition Symposium: implications of nutritional management for beef cow-calf systems. *J Anim Sci*, 90:2301-2307.

**Gardner D, Tingey K, Van Bon B, Ozanne S, Wilson V, Dandrea J, Keisler D, Stephenson T, Symonds M**. 2005. Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *Am J Physiol Regul Integr Comp Physiol,* 289:947-954.

**González-Recio O, Ugarte E, Bach A**. 2012. Trans-generational effect of maternal lactation during pregnancy: a Holstein cow model. *PLoS One,* 7:e51816.

**Gonzalez-Recio O, Toro MA, Bach A**. 2015. Past, present, and future of epigenetics applied to livestock breeding. *Front Genet*, 6:305. doi: 10.3389/fgene.2015.00305.

**Hoffman ML, Rokosa MA, Zinn SA, Hoagland TA, Govoni KE**. 2014. Poor maternal nutrition during gestation in sheep reduces circulating concentrations of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in offspring. *Domest Anim Endocrinol,* 49:39-48.

**Hoffman ML, Peck KN, Forella ME, Fox AR, Govoni KE, Zinn SA**. 2016. The effects of poor maternal nutrition on postnatal growth and development of lambs. *J Anim Sci*, 94:789-799.

**Huang Y, Zhao JX, Yan X, Zhu MJ, Long NM, McCormick RJ, Ford SP, Nathanielsz PW, Du M**. 2012. Maternal obesity enhances collagen accumulation and cross-linking in skeletal muscle of ovine offspring. *PLoS One,* 7:1-8.

**Joubert D, Hamond J**. 1958. A crossbreeding experiment with cattle, with special reference to maternal effect in South Devon-Dexter crosses. *J Agric Sci*, 51:325-341.

**Kamal MM, Van Eetvelde M, Depreester E, Hostens M, Vandaele L, Opsomer G**. 2014. Age at calving in heifers and level of milk production during gestation in cows are associated with the birth size of Holstein calves. *J Dairy Sci,* 97:5448-5458.

**Kamal MM, Van Eetvelde M, Bogaert H, Hostens M, Vandaele L, Shamsuddin M, Opsomer G**. 2015. Environmental factors and dam characteristics associated with insulin sensitivity and insulin secretion in newborn Holstein calves. *Animal,* 9:1490-1499.

**Kawashima C, Munakata M, Shimizu T, Miyamoto A, Kida K, Matsui M**. 2016. Relationship between the degree of insulin resistance during late gestation and postpartum performance in dairy cows and factors that affect growth and metabolic status of their calves. *J Vet Med Sci,* 78:739-745.

**Kertz AF, Reutzel LF, Barton BA, Ely RL**. 1997. Body weight, body condition score, and wither height of prepartum holstein cows and birth weight and sex of calves by parity: a database and summary. *J Dairy Sci,* 80:525-529.

**Khanal P, Husted SV, Axel AMD, Johnsen L, Pedersen KL, Mortensen MS, Kongsted AH, Nielsen MO**. 2014. Late gestation over- and undernutrition predispose for visceral adiposity in response to a post-natal obesogenic diet, but with differential impacts on glucose-insulin adaptations during fasting in lambs. *Acta Physiol,* 210:110-126.

**Kleemann DO, Kelly JM, Rudiger SR, McMillen IC, Morrison JL, Zhang S, MacLaughlin SM, Smith DH, Grimson RJ, Jaensch KS, Brien FD, Plush KJ, Hiendleder S, Walker SK**. 2015. Effect of periconceptional nutrition on the growth, behaviour and survival of the neonatal lamb. *Anim Reprod Sci,* 160:12-22.

**Klewitz J, Struebing C, Rohn K, Goergens A, Martinsson G, Orgies F, Probst J, Hollinshead F, Bollwein H, Sieme H**. 2015. Effects of age, parity, and pregnancy abnormalities on foal birth weight and uterine blood flow in the mare. *Theriogenology*, 83:721-729.

**Kume S, Tanabe S**. 1993. Effect of parity on colostral mineral concentrations of Holstein cows and value of colostrum as a mineral source for newborn calves. *Journal of dairy science*, 76:1654-1660.

**Laporte-Broux B, Roussel S, Ponter A, Perault J, Chavatte-Palmer P, Duvaux-Ponter C**. 2011. Short-term effects of maternal feed restriction during pregnancy on goat kid morphology, metabolism, and behavior. *J Anim Sci,* 89:2154-2163.

**Laporte-Broux B, Roussel S, Ponter A, Giger-Reverdin S, Camous S, Chavatte-Palmer P, Duvaux-Ponter C**. 2012. Long-term consequences of feed restriction during late pregnancy in goats on feeding behavior and emotional reactivity of female offspring. *Physiol Behav,* 106:178-184.

**Lawrence L, DiPietro J, Ewert K, Parrett D, Moser L, Powell D**. 1992. Changes in body weight and condition of gestating mares. *J Equine Vet Sci*, 12:355-358.

**Long NM, George LA, Uthlaut AB, Smith DT, Nijland MJ, Nathanielsz PW, Ford SP**. 2010. Maternal obesity and increased nutrient intake before and during gestation in the ewe results in altered growth, adiposity, and glucose tolerance in adult offspring. *J Anim Sci,* 88:3546-3553.

**Long NM, Rule D, Tuersunjiang N, Nathanielsz P,**

**Ford S**. 2015. Maternal obesity in sheep increases fatty acid synthesis, upregulates nutrient transporters, and increases adiposity in adult male offspring after a feeding challenge. *PLoS One*, 10:e0122152.

**Lopez-Tello J, Arias-Alvarez M, Jimenez-Martinez M-A, Garcia-Garcia R-M, Rodriguez M, Lorenzo Gonzalez P, Bermejo-Poza R, Gonzalez-Bulnes A and Garcia Rebollar P**. 2017. Competition for materno-fetal resource partitioning in a rabbit model of undernourished pregnancy. *PLoS One*, 12:e0169194.

**Meirelles MG, Veras MM, Alonso MA, de Fátima Guimarães C, Nichi M, Fernandes CB**. 2017. Influence of maternal age and parity on placental structure and foal characteristics from birth up to two years of age. *J Equine Vet Sci,* 56:68-79.

**Mohrhauser DA, Taylor AR, Underwood KR, Pritchard RH, Wertz-Lutz AE, Blair AD**. 2015. The influence of maternal energy status during midgestation on beef offspring carcass characteristics and meat quality. *J Anim Sci,* 93:786-793.

**Opsomer GA, Van Eetvelde M, Kamal MA, Van Soom A**. 2017. Epidemiological evidence for metabolic programming in dairy cattle. *Reprod Fertil Dev,* 29:52-57.

**Peugnet P, Wimel L, Duchamp G, Sandersen C, Camous S, Guillaume D, Dahirel M, Dubois C, Jouneau L, Reigner F, Berthelot V, Chaffaux S, Tarrade A, Serteyn D, Chavatte-Palmer P**. 2014. Enhanced or reduced fetal growth induced by embryo transfer into smaller or larger breeds alters post-natal growth and metabolism in pre-weaning horses. *PLoS One,* 9:e102044.

**Peugnet P, Mendoza L, Wimel L, Duchamp G, Dubois C, Reigner F, Caudron I, Deliège B, Toquet M-P, Richard E, Chaffaux S, Tarrade A, Lejeune J-F, SerteynD, Chavatte-Palmer P**. 2016. Longitudinal study of growth and osteoarticular status in foals born to between-breed embryo transfers. *J Equine Vet Sci,* 37:24-38.

**Philp LK, Muhlhausler BS, Janovska A, Wittert GA, Duffield JA, McMillen IC**. 2008. Maternal overnutrition suppresses the phosphorylation of 5'-AMP activated protein kinase (AMPK) in liver, but not skeletal muscle, in fetal and neonatal sheep. *Am J Physiol Regul Integr Comp Physiol*, 295:1982-1990.

**Pillai S, Jones A, Hoffman M, McFadden K, Reed S, Zinn S, Govoni, K. 2017**. Fetal and organ development at gestational days 45, 90, 135 and at birth of lambs exposed to under- or over-nutrition during gestation. *Translational Animal Science*, 1:16-25.

**Pool-Anderson L, Raub R, Warren J**. 1994. Maternal influences on growth and development of full-sibling foals. *J Anim Sci,* 72:1661-1666.

**Prezotto LD, Camacho LE, Lemley CO, Keomanivong FE, Caton JS, Vonnahme KA, Swanson KC**. 2016. Nutrient restriction and realimentation in beef cows during early and mid-gestation and maternal and fetal hepatic and small intestinal in vitro oxygen consumption. *Animal*, 10:1-9.

**Reed SA, Raja JS, Hoffman ML, Zinn SA, Govoni KE**. 2014. Poor maternal nutrition inhibits muscle development in ovine offspring. *J Anim Sci Biotechnol*, 5:43. doi: 10.1186/2049-1891-5-43.

**Sen U, Sirin E, Ensoy U, Aksoy Y, Ulutas Z, Kuran M**. 2016. The effect of maternal nutrition level during mid-gestation on postnatal muscle fibre composition and meat quality in lambs. *Anim Prod Sci,* 56:834-843.

**Sinclair KD, Rutherford KMD, Wallace JM, Brameld JM, Stöger R, Alberio R, Sweetman D, Gardner DS, Perry VEA, Adam CL,** Ashworth CJ**,** Robinson JE**,** Dwyer CM. 2016. Epigenetics and developmental programming of welfare and production traits in farm animals. *Reprod Fertil Dev*, 28:1443-1478.

**Summers AF, Meyer TL, Funston RN**. 2015. Impact of supplemental protein source offered to primiparous heifers during gestation on I. Average daily gain, feed intake, calf birth body weight, and rebreeding in pregnant beef heifers. *J Anim Sci,* 93:1865-1870.

**Tischner M**. 2000. Maternal influence on pre- and postnatal growth of foals born after embryo transfer. *J Reprod Fertil Suppl*, 56:705-708.

**Triantaphyllopoulos KA, Ikonomopoulos I and Bannister AJ**. 2016. Epigenetics and inheritance of phenotype variation in livestock. *Epigenetics Chromatin* 9:31. doi: 10.1186/s13072-016-0081-5.

**Vazquez J, Esteller-Vico A, Irizarri A, Liu I, Troedsson M**. 2015. Influence of stallion, donor and recipient mare's height and parity in foal height from birth to three years old in an embryo transfer program. A retrospective study. *In*: Proceedings of the 4th International Congress of the World Equine Veterinary Association (WEVA), 2015, Guadalajara, Mexico. Guadalajara: WEVA.

**Wallace J, Milne J, Adam C, Aitken R**. 2017. Impact of donor and recipient adiposity on feto-placental growth in adolescent sheep. *Reproduction*, 153:381-394.

**Walton A, Hammond J**. 1938. The maternal effects on growth and conformation in Shire horse-Shetland pony crosses. *Proc R Soc London*, 125:311-335.

**Whorwood CB, Firth KM, Budge H, Symonds ME**. 2016. Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11-hydroxysteroid receptor in neonatal sheep. *Endocrinology*, 142:2854-2864.

**Wilsher S, Allen W**. 2003. The effects of maternal age and parity on placental and fetal development in the mare. *Equine Vet J*, 35:476-483.

**Wilson TB, Faulkner DB and Shike DW**. 2016. Influence of prepartum dietary energy on beef cow performance and calf growth and carcass characteristics. *Livest Sci,* 184:21-27.

**Zachut M, Honig H, Striem S, Zick Y, Boura-Halfon S, Moallem U**. 2013. Periparturient dairy cows do not exhibit hepatic insulin resistance, yet adipose-specific insulin resistance occurs in cows prone to high weight loss. *J Dairy Sci,* 96:5656-5669.

**Zhang L, Long N, Hein S, Ma Y, Nathanielsz, Ford S**. 2012. Maternal obesity in the ewe results in reduced fetal pancreatic β-cell numbers in late gestation, and decreased circulating insulin concentration at term. *Domest Anim Endocrinol,* 40:30-39.

**Zoch ML, Clemens TL, Riddle RC**. 2016. New insights into the biology of osteocalcin. *Bone,* 82:42-49.

DOI: 10.21451/1984-3143-AR997

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# Understanding the uterine environment in early pregnancy in cattle: How have the *omics* enhanced our knowledge?

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## Abstract

Early pregnancy loss in cattle can be attributed to a myriad of sources. One key factor that can influence early pregnancy success or loss is the influence and interactions between the maternal environment and the developing embryo/conceptus. Recent advances in high-throughput 'omics' technologies coupled with improved bioinformatics capabilities represent a promising avenue for enhancing our understanding of fundamental developmental events – which would have direct agricultural, veterinary, and economic benefits. Thusly this review revolves around recent applications of advanced transcriptomic, proteomic, and metabolomic analyses within a bovine uterine secretomic and interactomic context, with an overriding aim to highlight the advantages of these emerging fields whilst identifying areas for improvement, consideration, and further research and development.

**Keywords**: phenomics, interactomics, proteomics, hormonomics, metabolomics, transcriptomics, epigenomics, genomics, uterine biology, reproductive physiology, maternal-embryo communication.

## Introduction

Successful pregnancy of all species is contingent on a carefully orchestrated series of events along the reproduction continuum such that the foetus is born alive. A significant proportion of reproductive wastage occurs in the first 2-3 weeks of pregnancy (Bazer *et al.,* 2011). The initial obstacle is the successful deposition of the male gametes into the female reproductive tract, at the optimal time, with the objective to fertilise a high-quality oocyte, released from the ovulatory ovarian follicle. This ovulatory follicle subsequently undergoes a luteinising hormone (LH)-driven remodelling process to form a corpus luteum (CL), which produces progesterone (P4). This result is a post-ovulatory rise in P4 that is ordinarily sufficient for establishing an appropriate uterine environment, conducive to embryo growth and receptive to subsequent conceptus (embryo proper and extra-embryonic membranes) implantation.

Successful early embryo and conceptus development, however, is a bilateral process, requiring reciprocal signalling from the conceptus to the mother. Initial maternal recognition of pregnancy occurs on day 16 post-oestrus in cattle. Such reciprocality between mother and offspring is a complex and still poorly understood process but known to be influenced by maternal metabolic parameters in addition the quality of embryo present (Reviewed by Lonergan *et al*., 2016; Spencer *et al*., 2016).

One way of enhancing our understanding of early pregnancy loss is to elucidate the physiological biological and biochemical processes underpinning and regulating such fundamental reproductive events. Large scale 'omics' technologies offer scope for addressing many specific questions remaining around early pregnancy, and are addressed in this review.

The 'omics' suffix loosely describes the study of big data sets within functional biological niches, or the holistic study of intra-domain interactions, and is therefore considered high dimensional biology (Horgen and Kenny, 2011). This has been facilitated by advances in *en masse* sequencing technologies (Mochida and Shinozaki, 2011) and accompanying enhanced bioinformatic analysis capabilities (Gandomi and Haider, 2015).

Whilst the 'omics' have traditionally revolved around the central dogma of biology – genomics, transcriptomics, and proteomics, increasing attention is being afforded to multi-level inter-domain interactions, in addition to the emergence of additional domains, including epigenomics, interactomics, hormonomics, metabolomics, and phenomics. Figure 1 schematically depicts the hierarchical and overlapping nature of our current understanding of these 'omics' domains. All of the aforementioned are pivotal to understanding several fundamental reproductive processes and Fig. 2 schematically depicts various inter-domain interactions within an 'omics' context which underpin the formation and regulation of the uterine secretome.

This review focuses on the bovine and aims to summarise how these different 'omics' technologies have, and can, enhance our understanding of how the uterine environment successful supports early pregnancy, how the conceptus itself affects this bilateral communication, in addition to how factors in the maternal environment modify such interactions.

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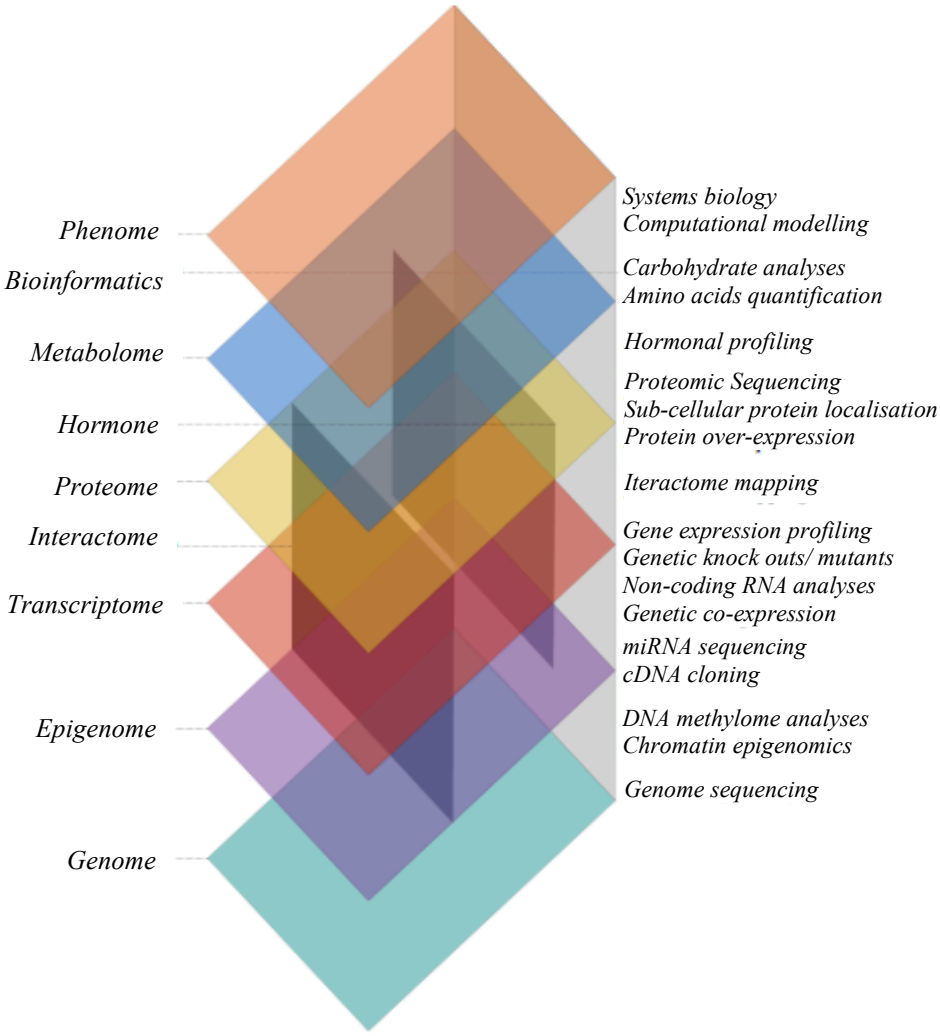


Figure 1. Multi-level linear relationship between 'omics' domains, with common corresponding available techniques for studying respective domains. Adapted from Mochida and Shinozaki (2011).

**Uterine epithelial cell transcriptomics**

*Detecting changes due to steroid hormones*

Initial reports of using transcriptomics to understand the physiology of the endometrium sought to determine the role that steroid hormones played in modifying gene expression patterns. Specifically, work by Bauersachs *et al.* (2005) compared endometria from day 0 of the oestrus cycle (low P4 and high oestradiol - E2) to that recovered from cycle animals on day 12 (dioestrus; high P4) to identify 133 differentially expressed genes. Additional studies determined that elevating (Forde *et al*., 2009) or decreasing (Forde *et al*., 2011a, 2012b) P4 in circulation advanced or delayed, respectively, the normal temporal changes that occur in the endometrium during the oestrus cycle. This altered the endometrial transcriptome and modified the elongation trajectory of the conceptus (Carter *et al*., 2008; Forde *et al*., 2011a) – a phenomenon previously demonstrated in cattle (Garrett *et al*., 1988). More recently, RNA sequencing has been used to identify how ovulation of different sized follicles (and the different endocrine environments that the endometrium is therefore exposed to), alters the endometrial transcriptome (Mesquita *et al*., 2015).

*Transcriptomic changes induced by the conceptus*

Dating back to the late 1970s and early 1980s it was understood that conceptus presence has an anti-leutolytic effect on the CL and this was required to occur by day 16 following oestrus *i.e*. day 16 was the day of pregnancy recognition (Betteridge *et al*., 1980; Northey and French, 1980). It was later identified that a secretory protein component of the conceptus could inhibit uterine prostaglandin F2 alpha (PGF2α) production as well as extend CL formation (Knickerbocker *et al*., 1986a, b) and was confirmed as a type 1 interferon, interferon tau (IFNT; Helmer *et al*., 1989). Prior to the advent of large-scale transcriptomics, most effects of the conceptus on the endometrium, targeted alterations to candidate genes involved in the prostaglandin pathway (both production and inhibition) as well as candidate classical interferon stimulated genes (ISGs) (Reviewed in Forde and Lonergan, 2012). The initial work in the bovine endometrium was performed using microarray technology using a combination of both 'in-house' (Bauersachs *et al*. , 2006; Klein *et al*., 2006; Mansouri-Attia *et al*., 2009a)

and commercially available arrays used (Forde *et al*., 2011b; Walker *et al*., 2010). As with the candidate gene approach, the majority of changes induced by the conceptus were classical ISGs.

Interestingly, Gene Set Enrichment Analysis (GSEA) performed on the lists of differentially expressed transcripts from these different studies using different platforms (in-house *vs*. affymetrix™), performed in different laboratories, on different samples (days, 12, 15, 16, 18, and 20 of confirmed pregnant compared to cyclic heifers) identified a consistent transcriptomic signature in the endometrium associated with the presence of the conceptus during the peri-implantation period of pregnancy in cattle (Bauersachs *et al*., 2012). More recent uses of RNA sequencing (a more sensitive technique) have identified that milder embryo/conceptus induced changes are detectable prior to pregnancy recognition on day 13 (Forde *et al*., 2012a) with some as early as day 6 (Binelli *et al*., 2015).

Intriguingly two separate studies also identified a transcriptomic signature as early as day 18 of pregnancy in the intercaruncular endometrium that is specific to the type of conceptus present. Even on day 18 of pregnancy, the endometrium responds differently to embryos with known different developmental outcomes and modifies its response to IVF-produced conceptus *vs*. cloned conceptus (Bauersachs *et al*., 2009). This altered endometrial transcriptomic response is magnified by day 20, particularly in the caruncular region of the endometrium (Mansourri-Attia *et al*., 2009a). More recently, attempts were made to access whether the endometrium alters its response to male compared to female conceptuses (Forde *et al*., 2016). However, despite there being a large component of sexual dimorphism both at the blastocyst stage (Bermejo-Alvarez *et al*., 2010) and during the peri-implantation period (Forde *et al*., 2016) no appreciable difference in the endometrial transcriptomic response was detected between male and female conceptuses. This may indicate that the endometrium does not 'favour' one over the other is simply due to a non-transcritomic response yet to be identified.

## The uterine secretome

The aforementioned work describing endometrial transcriptomics changes have enhanced our understanding of the temporal changes that occur in the endometrium as well as how the conceptus alters expression patterns. Such transcriptomic changes however are not always reflected further up the 'omics' pipeline, *i.e*. those mRNA changes that we observe in the endometrium are not always translated into changes in the protein content of the uterine luminal fluid (ULF) or the metabolic molecules that comprise the ULF. These collectively make up the uterine secretome – a spatiotemporally dynamic secreted, or actively transported, milieu in which the free-floating conceptus is bathed and which is required for growth of the conceptus past the hatched blastocyst stage of development (Gray *et al.,* 2001; Brandão *et al.,* 2004; Alexopoulos *et al.,* 2005).

As depicted by Fig. 2, the uterine secretome, also known as ULF or histotroph, composition is influenced by four parameters. Whilst the vasculature (*i*) contributes a plethora of factors including ovarian sex hormones (Einer-Jensen and Hunter, 2005) and immune cells (Singh *et al*., 2008; Healy *et al*., 2014; Sheldon *et al*., 2014), uterine secretions are predominantly produced by the (*ii*) uterine glandular and (*iii*) epithelial cells. The physiological conceptus (*iv*), if present, also secretes factors which alter the microenvironmental consistency. The nature of such uterine secretions is largely transcriptomic (miRNAs), metabolic (carbohydrates and amino acids), and proteomic (enzymes and signalling molecules), and are discussed below.

### *Secretome proteomics*

Previous studies looking at the composition of bovine ULF have been limited, not only by access to the sample matrix aforementioned, but also by antibody availability. Recent advances, particularly in mass spectrometry (and in label free quantitative analysis of protein composition such at iTRAQ analysis) have advanced 'omics' technologies to enable the evolution from analysing whole ULF composition for basic constituents, including proteins and metabolites (discussed below), towards wholesale temporal screening of complex uterine and embryo derived exosomes, or nanovesicles (Saadeldin *et al*., 2015; Campoy *et al*., 2016).

Similarly to the temporal changes observed in the endometrial transcriptome, iTRAQ analysis of the ULF from different stages of pregnancy identified a temporal change not just in the composition of proteins in the ULF, but also in the quantity of specific proteins (Forde *et al*., 2014a). However, there is an order of magnitude in the difference in the number of transcripts detected compared to the numbers of proteins – which may reflect technological limitations as opposed to a true biological phenomenon. In addition, there are considerably more proteins present, or being produced, by the conceptus on day 16 of pregnancy (Forde *et al*., 2015) than IFNT alone. Transcriptomic analysis of genes differentially expressed due to the presence of the conceptus are similar but not solely attributable to IFNT production (Bauersachs *et al*., 2012) with previous studies in both cattle (Bartol *et al*., 1985) and sheep (Brooks *et al*., 2014) determining that additional molecules modify the endometrium during the time of pregnancy recognition including cortisol and prostaglandins. Therefore, it is likely the bovine conceptus also produces proteins in addition to IFNT that modify endometrial function, and may not always be secretory in nature (Forde *et al*., 2015).

Recently, extracellular vesicles (EVs) have emerged as a non-traditional form of cell-to-cell communication (paracrine signalling). EVs are membrane-bound vesicles (classified by size) and contain microRNAs (miRNA), proteins, as well as other RNA molecules capable of being incorporated into target tissues (Raposo and Storvel, 2013). One of the first reports of this phenomenon in the uterus was

through the incorporation of endogenous retroviral envelope proteins that were shed from the endometrial epithelium and incorporated into the trophectoderm cells of sheep conceptuses, with more recent data confirming that the mode of transfer of these retroviral particles was via exosomes (Black *et al.,* 2010; Burns *et al*., 2014, 2016). This phenomenon has been reported in other species *e.g*. sheep and cattle at different points on the reproductive axis (Machtinger *et al*., 2012). It is likely that EVs pay a role in bovine conceptus-maternal communication via RNA species, including miRNA, transport. Detailed analyses of the composition of EVs is now possible with advances in mRNA, miRNA and protein sequencing.

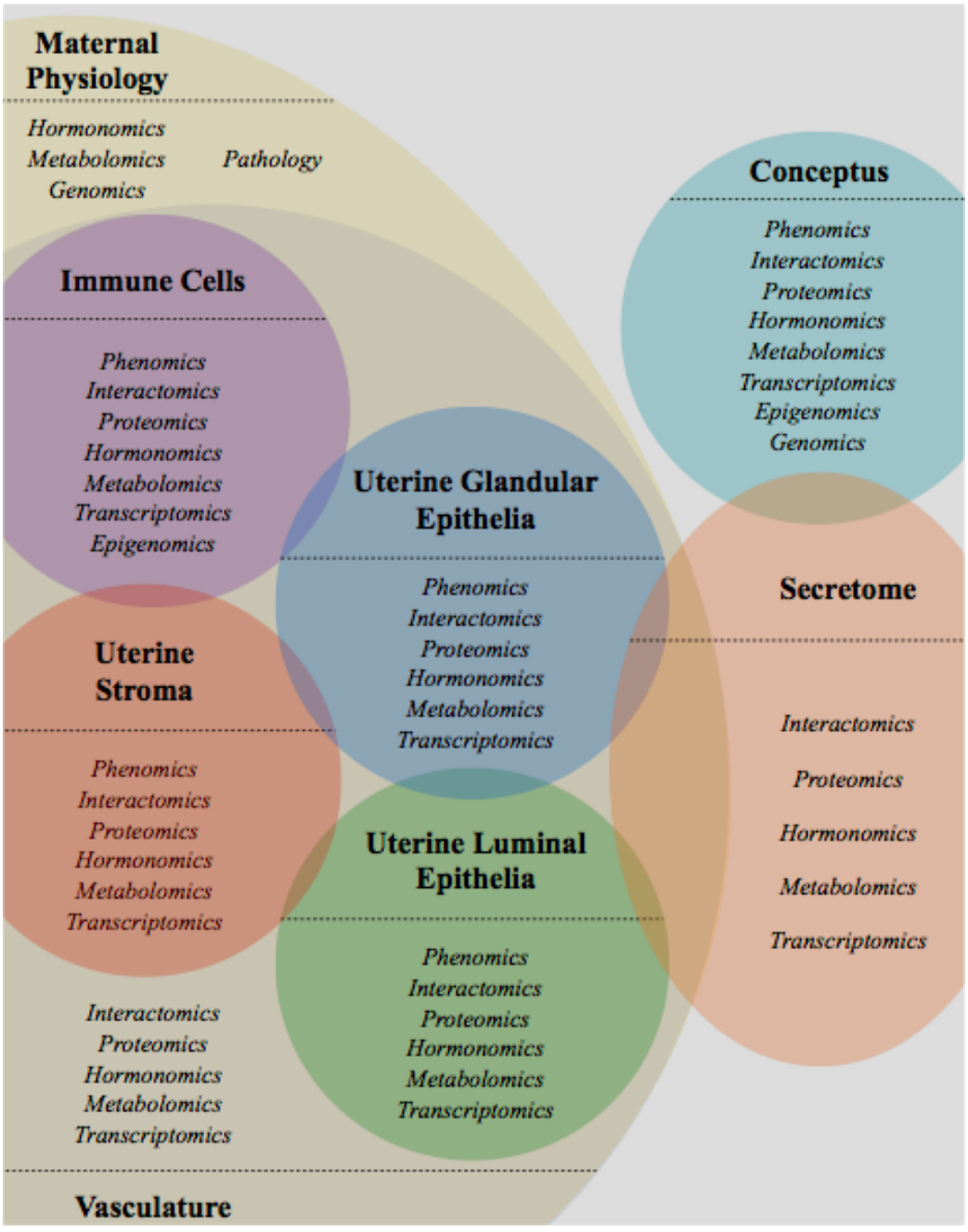


Figure 2. Schematic representation of the constituents which contribute to the bovine uterine secretome. Overlapping depicts the influence each constituent has on one another — the secretome is primarily influenced by uterine glandular and luminal epithelia in addition to conceptus presence, and to a lesser extent, leak-through from the maternal vasculature, whose composition is in turn influenced by maternal physiology. For a comprehensive review on embryo 'omics' please refer to Krisher *et al*. (2015).

*Secretome metabolomics*

Aside from passive fluid seepage into the uterine lumen from the oviducts, cervix, vagina, and the ejaculate post-intercourse, histotroph composition is largely attributable to, and regulated by, the uterine luminal and glandular epithelia, thus considered the uterine lumen gatekeepers (Leese *et al*., 2008). Combined with the vast transcriptomic data from these gatekeepers described, providing a clearer picture of the

spatiotemporally dynamic behaviour of these cells, the evolution in metabolic profiling analyses (cheaper, quicker, more robust, and more sensitive) has enabled a shift away from analysing whole uterine luminal fluid for basic constituents – such as pH (Hugentobler *et al*., 2004), ions (Hugentobler *et al*., 2007b), amino acids (Hugentobler *et al*., 2007a), carbohydrates (Hugentobler *et al*., 2008), and single enzymes, such as N-acetyl-beta-D-glucosaminidase (Hussain *et al*., 1989) – on a few days and in a few cows, towards comprehensively profiling the uterine fluid of a plethora of animals and on numerous days, whilst simultaneously comparing and contrasting this data between animals with varying physiologies and metabolic statuses.

Within the context of maternal-conceptus interactions, for example, whilst the importance of amino acids for embryo development *in vivo* and *in vitro* have been well established (Morris *et al.,* 2002; Wirtu *et al*., 2003; Sturmey *et al.,* 2010; Wale and Gardner 2010; Leese 2012), and has been comprehensively studied in the sheep (Gao *et al*., 2009), little was known about the basic requirements for conceptus elongation and successful pregnancy recognition in cattle. For this reason Forde *et al*. (2014b), in brief, analysed *en masse* the amino acid composition of beef heifer uteri which had been inseminated (n = 59) *vs*. a non-inseminated cyclic control group (n = 24) on days 7, 10, 13, 16 or 19 of pregnancy and days 7, 10, 13, or 16 of cyclic animals. Whilst several differences between the two groups were observed, when both pregnancy and day effects were considered, only differences in threonine, glutamate, and valine flux in ULF were observed, thus offering scope for targeted experimentation into the role of these amino acids in day 7-19 embryo development. Moreover, the total amino acid content of ULF increased as the blastocyst progressed to an elongated filamentous conceptus, most likely to accommodate for the greater metabolic demands of the developing offspring (Souza *et al.,* 2015). Interestingly, this data loosely corroborates earlier findings that total ULF amino acid concentrations are lower in sub-fertile animals (Meier *et al.,* 2014) and in cattle carrying developmentally compromised (cloned) embryos (Groebner *et al.,* 2011).

As aforementioned, transcriptomic advances – such as endometrial transcriptomic profiling at day 20 being predictive of the type of conceptus present (*in vivo vs. in vitro* derived *vs.* cloned; Bauersachs *et al.,* 2009; Mansouri-Attia *et al.,* 2009b) – led to the question of whether the same was the case for male *vs.* female conceptuses, and if such transcriptomic changes were reflected in the amino acid composition of the respective ULF. Thus Forde *et al*. (2016) inseminated 30 heifers prior to uterine and conceptus recovery on day 19 following oestrus. Their data showed that 9 amino acids (asparagine, histidine, glutamine, arginine, tryptophan, methionine, phenylalanine, isoleucine, and lysine) were present in higher levels in the ULF of XY containing uteri *vs.* XX positive counterparts however, there was no altered endometrial transcription of amino acid transporters observed. This interactomics inconsistency suggests that the amino acid consumption of XX *vs.* XY conceptuses is different whilst ULF secretions are constant. In support of this, Sturmey *et al.* (2010) demonstrated that male bovine blastocysts consumed less amino acids and exhibited a lower amino acid turnover compared to female blastocysts.

A significant challenge to the dairy industry is cattle sub-fertility arising from a systemic negative energy balance (NEB). Data show that a NEB perturbs endometrial transcriptomics (Cerri *et al.,* 2012), however unclear was whether lactation status influences ULF composition. To this end Bauersachs *et al*., 2017 (Forde 2017; Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, UK; unpublished data) analysed the amino acid and carbohydrate composition of ULF from lactating (n = 6) *vs.* non-lactating (dry) cows (n = 7) *vs.* maiden heifers (n = 4). Glutamate was the only differentially transported amino acid, lower in lactating cows relative to heifers but not dry cows. Conversely, lactate was higher in the lactating cohort compared against heifers but not relative to dry cows. Glucose and pyruvate remained unchanged. A potential aetiology for this inconsistency between transcriptomic and secretomic data, however, may lie in the fact that the heifer group was artificially inseminated (AI) whereas lactating and dry animals had embryos transferred. In other words, conceptus origin (AI *vs.* transfer) may have a greater impact on ULF composition than maternal lactating physiology.

An interesting supplementary observation arising from the *en masse* data analyses aforementioned was that the ULF from ipsilateral uterine horns comprised more glycine than those contralateral on day 19 of pregnancy Forde *et al*., 2017 (Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, UK; unpublished data). Groebner *et al.* (2011) too reported an amino acid abundance increase in the ipsilateral horn on day 18, suggestive that conceptus presence stimulates differential amino acid flux into the lumen. A second hypothesis worthy of further investigation is that the ipsilateral endometrium is exposed to more P4 released from the large luteal cells of the corpus luteum therein signalling greater glycine secretion into the ipsilateral uterine horn.

*Additional considerations*

It is worth noting that, unlike in the human (Cheong *et al*., 2013), obtaining bovine ULF aspirates is unfeasible owing to minuscule volume, and viscous consistency, of physiological ULF. Thus the field has been reliant on studying the composition of ULF by flushing tracts. Advantages of flushes include the recovery of ample volumes for subsequent 'omics' analyses (Velazquez *et al*., 2010). On the other hand, the greatest disadvantage is perhaps the inability to calculate dilution coefficients precisely as less fluid is recovered than injected into the uterine horn. Additional robust sampling methodologies are available such as the direct ULF sampling via uterine exteriorisation and catheterisation of anaesthetised heifers (Hugentobler *et*

*al*., 2007a), which also have associated advantages and disadvantages. For a comprehensive review pertinent to the relative merits of various complementary reproductive tract sampling technologies please refer to Leese *et al*. (2008) and Velazquez *et al*. (2010).

Another potentially fruitful area for future work resides in telocytes – a recently discovered cell type (Popescu and Faussone-Pellegrini, 2010) identified and characterised in the human myometrium (Ciontea *et al*., 2005), endometrium (Cretoiu *et al*., 2013), rat endometrium (Hatta *et al*., 2012), and recently the bovine oviduct (Abd-Elhafeez and Soliman, 2017), though not in the bovine uterine environment yet. Telocytes are reported to secrete exosomes (45 ± 8 nm), ectosomes (128 ± 28 nm), and multi vesicular cargos (MVCs; 1 ± 0.4 μm; Roatesi *et al*., 2015) and thus may – indirectly, through paracrine interactions with the stroma and epithelium, and directly, via contributing to the uterine secretome – play a role in maternal-embryo dialogue (Saadeldin *et al*., 2015; Campoy *et al*., 2016).

In the wider context of reproductive 'omics' an interesting recent development is the identification that miRNA molecules in circulation in bovine plasma can be used to identify pregnancy as early as day 8 (Ioannidis and Donadeu, 2017). Utilising such RNA sequencing capabilities for elucidating biomarkers for identifying potentially subfertile pathophysiologies in cattle, as in the human (Sathyapalan *et al*., 2015), will be an important development for both selectively mating, inseminating, or embryo transferring into heifers, in addition to enhancing our holistic understanding of fundamental reproductive events (Fig. 2).

There is a "clear need for a new means of interrogating the intrauterine environment" (Cheong *et al.,* 2013). Owing to the technical, and often ethical, limitations surrounding *in vivo* sampling, one direction towards addressing this includes *in vitro* modelling. Advances have been made regarding the production of bovine *in vitro* derived oviduct fluid (*iv*DOF) which resemble *in vivo* derived fluid (Simintiras and Sturmey 2017; Simintiras *et al.,* 2017) and moreover supports early embryo development (Chen *et al*., 2017), however, this can be considered currently under optimisation and has yet to be recapitulated to the uterus. The delay in an *in vitro* uterine luminal fluid (*iv*ULF) is partly attributable to the increased complexity of the endometrium, notably the presence of glands and a greater number of different cell types. Thus, for the time being ULF must be obtained using *in vivo* and *ex situ* techniques.

## Conclusions

What is clear from the application of these large-scale transcriptomic analyses of concept-maternal interactions is that significant advances in the field can be made. Nonetheless there are obstacles to utilising these technologies. Firstly, International standards for best practice, in addition to the free sharing of such big data, is critical to the sound interpretation of resulting omics analysis. Secondly, the integration of the data generated from these various 'omics' platforms, although advancing, is still not fully integrative from an interactomics perspective. Last but not least, sound interpretation of such data relies on a cognisant understanding of the biological question being asked – *i.e*. whether one is using these technologies as a hypothesis generating tool, or to further understand the non-canonical roles and factors central to successful early pregnancy.

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## References

**Abd-Elhafeez HH, Soliman SA**. 2017. New description of telocyte sheaths in the bovine uterine tube: an immunohistochemical and scanning microscopic study. *Cells Tissues Organs*, 203:295-315.

**Alexopoulos NI, Vajta G, Maddox-Hyttel P, French AJ, Trounson AO**. 2005. Stereomicroscopic and histological examination of bovine embryos following extended in vitro culture. *Reprod Fertil Dev*, 17:799-808.

**Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW**. 1985. Characterization of proteins produced in vitro by periattachment bovine conceptuses. *Biol Reprod,* 32:681-693.

**Bauersachs S, Ulbrich SE, Gross K, Schmidt SE, Meyer HH, Einspanier R, Wenigerkind H, Vermehren M, Blum H, Sinowatz F, Wolf E**. 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. *J Mol Endocrinol*, 34:889-908.

**Bauersachs S, Ulbirch SE, Gross K, Schmidt SEM, Meyer HH, Wenigerkind H, Vermehren M, Blum H, Wolf E**. 2006. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction*, 132:319-331.

**Bauersachs S, Ulbrich SE, Zakhartchenko V, Minten M, Reichenbach M, Reichenbach HD, Blum H, Spencer TE, Wolf E**. 2009. The endometrium responds differently to cloned versus fertilized embryos. *Proc Natl Acad Sci*, 106:5681-5686.

**Bauersachs S, Ulbrich SE, Reichenbach HD, Reichenbach M, Buttner M, Meyer HH, Spencer TE, Minten M, Sax G, Winter G, Wolf E**. 2012. Comparison of the effects of early pregnancy with human interferon, alpha 2 (IFNA2), on gene expression in bovine endometrium. *Biol Reprod*, 86:46. http://doi.org/10.1095/biolreprod.111.094771.

**Bazer FW, Spencer TE, Johnson GA, Burghardt RC**. 2011. Uterine receptivity to implantation of blastocysts in mammals. *Front Biosci (Schol Ed)*, 3:745-767.

**Berjemo-Alvarez P, Rizos D, Rath D, Lonergan P,**

**Gutierrez-Adan A.** 2010. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc Natl Acad Sci*, 107(8):3394-3399.

**Betteridge KJ, Eaglesome MD, Randall GCB, Mitchell D**. 1980. Collection, description and transfer of embryos from cattle 10-16 days after oestrus. *J Reprod. Fertil,* 59:205-216.

**Binelli M, Scolari SC, Pugliesi G, Van Hoeck V, Gonella-Diaza AM, Andrade SCS, Gasparin GR, Coutinho LL**. 2015. The transcriptome signature of the receptive bovine uterus determined at early gestation. *PLoS One*, 10:e0122874. doi:10.1371/journal. pone.0122874.

**Black SG, Arnaud F, Burghardt RC, Satterfield MC, Fleming JA, Long CR, Hanna C, Murphy L, Biek R, Palmarini M, Spencer TE**. 2010. Viral particles of endogenous betaretroviruses are released in the sheep uterus and infect the conceptus trophectoderm in a transspecies embryo transfer model. *J Virol*, 84:9078-9085.

**Brandão DO, Maddox-Hyttel P, Lovendahl P, Rumpf R, Stringfellow D, Callesen D**. 2004. Post hatching development: a novel system for extended in vitro culture of bovine embryos. *Biol Reprod*, 71:2048-2055.

**Brooks K, Burns G, Spencer TE**. 2014. Conceptus elongation in ruminants: roles of progesterone, prostaglandin, interferon tau and cortisol. *J Anim Sci Biotechnol*, 5:53. https://doi.org/10.1186/2049-1891-5-53.

**Burns G, Brooks K, Wildung M, Navakanitworakul R, Christenson LK, Spencer TE**. 2014. Extracellular vesicles in luminal fluid of the ovine uterus. *PLoS One*, 9:e90913.
https://doi.org/10.1371/journal.pone.0090913.

**Burns GW, Brooks KE, Spencer TE**. 2016. Extracellular vesicles originate from the conceptus and uterus during early pregnancy in sheep. *Biol Reprod*, 94:1-11. https://doi.org/10.1095/biolreprod.115.134973.

**Campoy I, Lanau L, Altadill T, Sequeiros T, Cabrera S, Cubo-Abert M, Pérez-Benavente A, Garcia 4, Borrós S, Santamaria A, Ponce J, Matias-Guiu X, Reventós J, Gil-Moreno A, Rigau M, Colas E**. 2016. Exosome-like vesicles in uterine aspirates: a comparison of ultracentrifugation-based isolation protocols. *J Transl Med*, 14:180. http://doi.org/10.1186/s12967-016-0935-4.

**Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF, Lonergan P**. 2008. Effect of increasing progesterone concentration from day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reprod Fertil Dev*, 20:368-375

**Cerri RL, Thompson IM, Kim IH, Ealy AD, Hansen PJ, Staples CR, Li JL, Santos JE, Thatcher WW**. 2012. Effects of lactation and pregnancy on gene expression of endometrium of Holstein cows at day 17 of the estrous cycle or pregnancy. *J Dairy Sci*, 95:5657-5675.

**Chen S, Palma-Vera SE, Langhammer M, Galuska SP, Braun BC, Krause E, Lucas-Hahn A, Schoen J**. 2017. An air-liquid interphase approach for modeling the early embryo-maternal contact zone. *Sci Rep*, 7:42298. http://doi.org/10.1038/srep42298.

**Cheong Y, Boomsma C, Heijnen C, Macklon N**. 2013. Uterine secretomics: a window on the maternal-embryo interface. *Fertil Steril*, 99:1093-1099.

**Ciontea SM, Radu E, Regalia T, Ceafalan L, Cretoiu D, Gherghiceanu M, Braga RI, Malincenco M, Zagrean L, Hinescu ME, Popescu LM**. 2005. C-kit immunopositive interstitial cells (Cajal-type) in human myometrium. *J Cell Mol Med*, 9:407420. **Cretoiu SM, Cretoiu D, Marin A, Radu BM, Popescu LM**. 2013. Telocytes: ultrastructural, immunohistochemical and electrophysiological characteristics in human myometrium. *Reproduction*, 145:357-370.

**Einer-Jensen N, Hunter RHF**. 2005. Counter-current transfer in reproductive biology. *Reproduction*, 129:9-18.

**Forde N, Spencer TE, Bazer FW, Song G, Roche JF, Lonergan P**. 2009. Effect of pregnancy and progesterone concentration on expression of genes encoding for transporters or secreted proteins in the bovine endometrium. *Phys Genomics*, 41:53-62.

**Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, O'Gaora P, Roche JF, Lonergan P, Crowe MA**. 2011a. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. *Biol Reprod*, 84:266-278.

**Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, Mehta JP, McBride JP, O'Gaora P, Roche JF, Lonergan P**. 2011b. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? *Biol Reprod*, 85:144-156.

**Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, Mansouri-Attia N, Sandra O, Loftus BJ, Crowe MA, Fair T, Roche JF, Lonergan P, Evans ACO**. 2012a. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. *Physiol Genomics*, 44:799-810.

**Forde N, Lonergan P**. 2012. Transcriptomic analysis of the bovine endometrium: What is required to establish uterine receptivity to implantation in cattle? *J Reprod Dev*, 58:189-195.

**Forde N, Mehta, JP, Minten M, Crowe MA, Roche JF, Spencer TE, Lonergan P**. 2012b. Effects of low progesterone on the endometrial transcriptome in cattle. *Biol Reprod*, 87:1–11. http://doi.org/10.1095/biolreprod.112.103424.

**Forde N, McGettigan PA, Mehta JP, O'Hara L, Mamo S, Bazer FW, Spencer TE, Lonergan P**. 2014a. Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle. *Reproduction*, 147:575-587.

**Forde N, Simintiras CA, Sturmey RG, Mamo S, Spencer TE, Bazer FW, Lonergan P**. 2014b. Amino acids in the uterine luminal fluid reflect the temporal changes in transporter expression in the endometrium and conceptus during early pregnancy in cattle. *Plos One*,9(6):e100010. doi: 10.1371/journal.pone.0100010.

**Forde N, Bazer FW, Spencer TE, Lonergan P**. 2015. Conceptualizing the endometrium: identification of conceptus-derived proteins during early pregnancy in cattle. *Biol Reprod*, 92:1-13. http://doi.org/10.1095/biolreprod.115.129296.

**Forde N, Maillo V, O'Agora P, Simintiras CA, Sturmey RG, Ealy AD, Spencer TE, Gutierrez-Adan A, Rizos D, Lonergan P**. 2016. Sexually dimorphic gene expression in male and female bovine conceptuses at the initiation of implantation. *Biol Reprod*, 95:92. doi: biolreprod.116.139857.

**Gandomi A, Haider M**. 2015. Beyond the hype: big data concepts, methods, and analytics. Int J Info Manag, 35:137-144.

**Gao H, Wu G, Spencer TE, Johnson GA, Li X, Bazer FW**. 2009. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. *Biol Reprod*, 93:86-93.

**Garrett JE, Geisert RD, Zavy MT, Morgan** GL. 1988. Evidence for maternal regulation of early conceptus growth and development in beef cattle. *J Reprod Fertil*, 84:437-446.

**Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, Bazer FW, Spencer TE**. 2001. Developmental biology of uterine glands. *Biol Reprod*, 63:1311-1323.

**Groebner AE, Rubio-Aliaga I, Schulke K, Reichenbach HD, Daniel H, Wolf E, Meyer HHD, Ulbrich SE**. 2011. Increase of essential amino acids in the bovine uterine lumen during preimplantation development. *Reproduction*, 141:685-695.

**Hatta K, Huang M, Weisel RD**. 2012. Culture of rat endometrial telocytes. *J Cell Mol Med*, 16:1392-1396.

**Healy LL, Cronin JG, Sheldon IM**. 2014. Endometrial cells sense and react to tissue damage during infection of the bovine endometrium via interleukin 1. *Sci Rep*, 4:7060. http://doi.org/10.1038/srep07060.

**Helmer SD, Hansen PJ, Thatcher WW, Johnson JW. Bazer FW**. 1989. Intrauterine infusion of highly enriched bovine trophoblast protein- complex exerts an antiluteolytic effect to extend corpus luteum lifespan in cyclic cattle. *J Reprod Fertil*, 87:89-101.

**Horgen RP, Kenny LC**. 2011. Omic technologies: genomics, transcriptomics, proteomics and metabolomics. *Obstetr Gynaecol***,** 13:189-195.

**Hugentobler SA, Morris DG, Kane MT, Sreenan JM**. 2004. In situ oviduct and uterine pH in cattle. *Theriogenology*.2004 61:1419-1427.

**Hugentobler SA, Diskin MG, Leese HJ, Humpherson, PG, Watson T, Sreenan JM, Morris DG**. 2007a. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Mol Reprod Dev*, 74:445-454.

**Hugentobler SA, Morris DG, Sreenan JM, Diskin MG**. 2007b. Ion concentrations in oviduct and uterine fluid and blood serum during the estrous cycle in the bovine. *Theriogenology*, 68:538-548

**Hugentobler SA, Humpherson PG, Leese HJ, Sreenan JM, Morris DG**. 2008. Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. *Mol Reprod Dev*, 75:496-503.

**Hussain AM, Daniel RCW, O'Boyle D**. 1989. N-acetyl-β-D-glucosaminidase activity in bovine uterine fluid and blood serum during the postpartum period. *Theriogenology*, 32:507-514.

**Ioannidis J, Donadeu FX**. 2017. Changes in circulating microRNA levels can be identified as early as day 8 of pregnancy in cattle. *PLoS One*, 12:e0174892. https://doi.org/10.1371/journal.pone.0174892.

**Klein C, Bauersachs S, Ulbrich SE, Einspanier R, Meyer HH, Schmidt SE, Reichenbach HD, Vermehren M, Sinowatz F, Blum H, Wolf E**. 2006. Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. *Biol Reprod*, 74:253-264.

**Knickenbocker JJ, Thatcher WW, Bazer FW, Drost M, Barron DH, Fincher KB, Roberts RM**. 1986a. Proteins secreted by day 16 to 18 bovine conceptuses extend corpus luteum function in cows. *J Reprod Fertil*, 77:381-391.

**Knickerbocker JJ, Thatcher WW, Foster DB, Wolfenson D, Bartol FF, Caton D**. 1986b. Uterine prostaglandin and blood flow responses to estradiol-17 beta in cyclic cattle. *Prostaglandins*, 31:757-776.

**Krisher RL, Schoolcraft WB, Katz-Jaffe MG**. 2015. Omics as a window to view embryo viability. *Fertil Steril*, 103:333-341.

**Leese HJ. Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear SL, Sreenan JM**. 2008. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reprod Fertil Dev*, 20:1-8.

**Leese HJ**. 2012. Metabolism of the preimplantation embryo: 40 Years on. *Reproduction*, 143:417-427.

**Lonergan P, Forde N, Spencer TE**. 2016. Progesterone and conceptus-derived factors important for conceptus survival and growth. *Anim Reprod*, 13:143-152.

**Machtinger R, Combelles CMH, Missmer SA, Correia KF, Fox JH, Racowsky C**. 2012. The association between severe obesity and characteristics of failed fertilized oocytes, *Hum Reprod*, 27:3198-3207.

**Mansouri-Attia N, Aubert J, Reinaud P, Giraud-Delville C, Taghouti G, Galio L, Everts RE, Degrelle S, Richard C, Hue I, Yang X, Tian XC, Lewin HA, Renard JP, Sandra O**. 2009a. Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. *Physiol Genomics,* 39:14-27.

**Mansouri-Attia N, Sandra O, Aubert J, Degrelle S, Everts RE, Giraud-Delville C, Heyman Y, Galio, L, Hue I, Yang X, Tian XC, Lewin HA, Renard JP**. 2009b Endometrium as an early sensor of *in vitro* embryo manipulation technologies. *Proc Natl Acad Sci USA*, 106:5687-5692.

**Meier S, Mitchell MD, Walker CG, Roche JR, Verkerk GA**. 2014. Amino acid concentrations in uterine fluid during early pregnancy differ in fertile and subfertile dairy cow strains. *J Dairy Sci*, 97:1364-1376.

**Mesquita FS, Ramos RS, Pugliesi G, Andrade SCS, Van Hoeck V, Langbeen A, Oliveira ML, Gonella-Diaza AM, Gasparin G, Fukumasu H, Pulz LH, Membrive CM, Coutinho LL, Binelli M**. 2015. The

receptive endometrial transcriptomic signature indicates an earlier shift from proliferation to metabolism at early diestrus in the cow. *Biol Reprod*, 93:52. doi: 10.1095/biolreprod.115.129031.

**Mochida K, Shinozaki K**. 2017. Advances in omics and bioinformatics tools for systems analyses of plant functions. *Plant Cell Physiol*, 52:2017-2038.

**Morris DG, Humpherson PG, Leese HJ, Sreenan JM**. 2002. Amino acid turnover by elongating cattle blastocysts recovered on days 14-16 after insemination. *Reproduction*, 124:667-673.

**Northey DL, French LR**. 1980. Effect of embryo removal and intrauterine infusion of embryonic homogenates on the lifespan of the bovine corpus luteum. *J Anim Sci*, 50:298-302.

**Popescu LM, Faussone-Pellegrini MS**. 2010. Telocytes: a case of serendipity: the winding way from interstitial cells of cajal (ICC), via interstitial cajal-like cells (ICLC) to telocytes. *J Cell Mol Med*, 14:729-740.

**Raposo G. Stoorvogel W**. 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*, 200:373-383.

**Roatesi I, Radu BM, Cretiou D, Cretiou SM**. 2015. Uterine telocytes: a review of current knowledge. *Biol Reprod*, 93:10. doi:10.1095/biolreprod.114.125906.

**Saadelin IM, Oh HJ, Lee BC**. 2015. Embryonic-maternal cross-talk via exosomes: potential implications. *Stem Cells and Cloning*, 8:103-107.

**Sathyapalan T, David R, Gooderham NJ, Atkin SL**. 2015. Increased expression of circulating miRNA-93 in women with polycystic ovary syndrome may represent a novel, non-invasive biomarker for diagnosis. *Sci Rep,* 5:16890. http://doi.org/10.1038/srep16890.

**Sheldon IM, Cronin JG, Healey GD, Gabler C, Heuwieser W, Streyl D, Bromfield JJ, Miyamoto A, Fergani C, Dobson H**. 2014. Innate immunity and inflammation of the bovine female reproductive tract in health and disease. *Reproduction*, 48:41-51.

**Simintiras CA, Fröhlich T, Sathyapalan T, Arnold G, Ulbrich S, Leese HJ, Sturmey RG**. 2017. Modelling aspects of oviduct fluid formation in vitro. *Reproduction*, 153:23-33.

**Simintiras CA, Sturmey RG**. 2017. Genistein crosses the bioartificial oviduct and alters secretion composition. *Reprod Toxicol,* 71:63-70.

**Singh J. Murray RD, Mshelia G, Woldehiwet Z**. 2008. The immune status of the bovine uterus during the peripartum period. *Vet J*, 175:301-309.

**Souza DK, Salles LP, Rosa e Silva AAM**. 2015. Aspects of energetic substrate metabolism of in vitro and in vivo bovine embryos. *Braz J Med Biol Res*, 48:191-197.

**Spencer TE, Forde N, Lonergan P**. 2016. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *J Dairy Sci*,2016 99:5941-5950.

**Sturmey RG, Rizos D, Leese HJ**. 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Mol Reprod Dev*, 77:285-296.

**Velazquez MA, Parrilla I, Soom AV, Verberckmoes S**. 2010. Sampling techniques for oviductal and uterine luminal fluid in cattle. *Theriogenology*,; 73:758-767.

**Wale PL, Gardner DK**. 2010. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod BioMed Online*, 21:402-410.

**Walker CG, Meier S, Littlejohn MD, Lehnert K, Roche JR, Mitchell MD**. 2010. Modulation of the maternal immune system by the pre-implantation embryo. *BMC Genomics*, 11:1-13. https://doi.org/ 10.1186/1471-2164-11-474.

**Wirtu G, Pope CE, Damiani P, Miller F, Dresser BL, Short CR, Godke RA, Bavister BD**. 2003. Development of in-vitro-derived bovine embryos in protein-free media: effects of amino acids, glucose, pyruvate, lactate, phosphate and osmotic pressure. *Reprod Fertil Dev*, 15:439-449.

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# Reproductive programs for beef cattle: incorporating management and reproductive techniques for better fertility

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## Abstract

This review summarizes strategies to increase overall pregnancy rates to TAI protocols, and potential areas for improvement in reproductive management of Nelore cows. Low BCS at calving and postpartum BCS loss negatively impacted percentage of pregnant females to TAI, with primiparous cows being the most sensitive to the effects of low BCS during the postpartum period. The current reproductive management recommended for replacement heifers (with TAI on day 0) consist of intravaginal progesterone device insertion from day -35 to -23, 0.6 mg i.m. injection of estradiol cypionate on day -23, insertion of another intravaginal progesterone device and 2.0 mg i.m. injection of estradiol benzoate on day -11, 12.5 mg i.m. injection of dinoprost tromethamine on day -4, intravaginal progesterone device removal and 0.6 mg i.m. injection of estradiol cypionate on day -2, and TAI on day 0. Supplementing corn for 41 days after the first TAI of the breeding season increased pregnancy to a second AI in primiparous cows, increased final proportion of pregnancy in primiparous cows, but decreased the final proportion of pregnancy in mature cows. In turn, supplementing melengestrol acetate or Ca salts of PUFA during the expected time of luteolysis was beneficial to pregnancy rates. Vaccinating cows against BoHV-1, BVDV, and *Leptospira* spp., particularly when both doses were administered before TAI, improved cow reproductive performance. Cow temperament has direct implications not only on reproductive efficiency of *B. indicus* females, but also on overall production efficiency in cow-calf system based on *B. indicus* cattle. Lastly, concentration of pregnancy-associated glycoproteins was driven by the ability of pregnancy maintenance and by sire used at TAI, whereas exploring this relationship might be interesting to improve sire fertility regarding late embryonic loss in Nelore females.

**Keywords**: beef females, puberty, timed artificial insemination, Nelore, management strategies, reproduction.

## Introduction

Hormonal treatments have been developed to synchronize the time of ovulation allowing fixed time artificial insemination (TAI) in anestrous or cycling cows, without the need for detection of estrus. The Group Specialized in Applied Herd Reproduction (Grupo Especializado em Reprodução Aplicada de Rebanho - GERAR (https://www.grupogerar.agr.br) was created in 2006 and composed primarily of veterinarians from all regions in Brazil that use the TAI protocol described by Meneghetti *et al*. (2009). The overall results of the GERAR are described in Fig. 1. This review summarizes some strategies that might increase overall pregnancy rates to TAI protocols, and also potential areas for improvement.

## Body condition score (BCS)

The present study evaluated the effects of BCS at calving and postpartum BCS change on reproductive performance of suckled Nelore cows submitted to TAI ( Carvalho *et al*., 2017; UNESP, Department of Animal Production, Botucatu, SP, Brazil; unpublished data). A total of 1,909 cows were allocated based on age and days postpartum into 17 groups. All cows were submitted to a TAI protocol (Meneghetti *et al*., 2009) at approximately 38 ± 2.4 days postpartum (day 0 of the protocol). Pregnancy status were evaluated 30 days post-TAI. Cows were individually scored for body condition at calving, on the day of TAI, and 30 days post-TAI using a 1 to 5 scale with 0.25 increments (1 = thin to 5 = fat). Cows were divided into three groups according to BCS at calving: high (H; BCS $\geq$ 3.5), moderate (M; BCS = 3.00 to 3.25) and low (L; BCS $\leq$ 2.75). Thereafter, cows were categorized into 1 of 7 groups based on BCS at calving and BCS change from calving to pregnancy diagnosis: high-maintaining (HM), high-losing (HL), moderate-gaining (MG), moderate-maintaining (MM), moderate-losing (ML), low-gaining (LG) and low-maintaining (LM). Low BCS at calving and postpartum BCS loss negatively impacted percentage of pregnant females to TAI, with primiparous cows being the most sensitive to the effects of low BCS during the postpartum period (Tables 1, 2 and 3).

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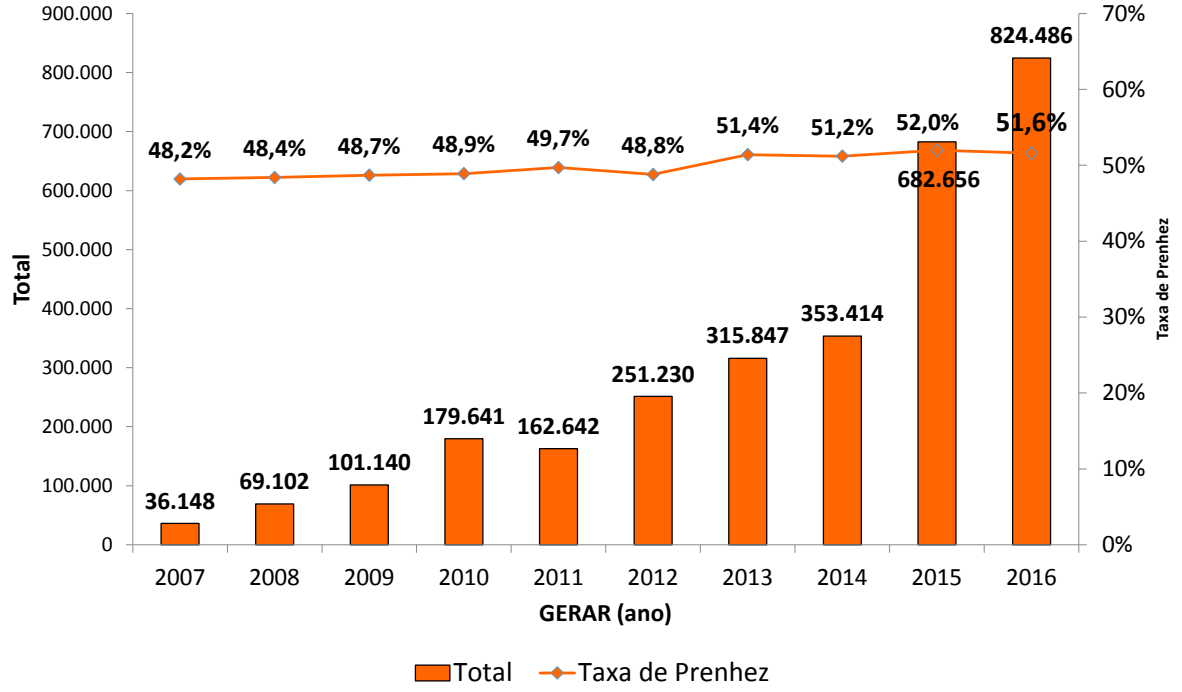


Figure 1. Overall pregnancy to timed artificial insemination by year. Adapted from GERAR group.

Table 1. Effect of BCS at calving and BCS change from parturition to 80 days postpartum on pregnancies at first TAI (%P/1st AI) for primiparous suckled Nelore cows (total cows = 593).

| BCS at calving | Cows | %cows[1] | %P/1st AI | BCS Change | %cows[1] | %P/1st AI |
|---|---|---|---|---|---|---|
| High (H) BCS ≥ 3.5 | 142 | 23.9 | 47.4[a] | Maintaining (HM) | 31.0 | 54.9[a] |
| | | | | Losing (HL) | 69.0 | 43.0[ab] |
| Moderate (M) BCS = 3.00 - 3.25 | 358 | 60.4 | 44.3[a] | Gaining (MG) | 25.7 | 54.5[a] |
| | | | | Maintaining (MM) | 19.8 | 53.9[a] |
| | | | | Losing (ML) | 54.5 | 35.7[b] |
| Low (L) BCS ≤ 2.75 | 93 | 15.7 | 25.9[b] | Gaining (LG) | 50.5 | 33.2[bc] |
| | | | | Maintaining (LM) | 49.5 | 19.4[c] |
| SEM | | | 4.4 | | | 6.0 |
| P-value | | | 0.0023 | | | 0.0001 |

[a,b,c]Values within a column with different superscript letters differ at P < 0.05. [1]% of cows according to BCS group at calving. From Carvalho *et al*., 2017; UNESP, Department of Animal Production, Botucatu, SP, Brazil; unpublished data.

Table 2. Effect of BCS at calving and BCS change from parturition to 80 days postpartum on pregnancies at first TAI (%P/1st AI) for second parity suckled Nelore cows (total cows = 423).

| BCS at calving | cows | %cows | %P/1st AI | BCS Change | %cows[1] | %P/1st AI |
|---|---|---|---|---|---|---|
| Moderate (M) BCS 3.00 – 3.25 | 138 | 32.6 | 63.2[a] | Gaining (MG) | 29,0 | 69.6[a] |
| | | | | Maintaining (MM) | 33,3 | 68.0[a] |
| | | | | Losing (ML) | 37,7 | 55.6[ab] |
| Low BCS (L) ≤ 2.75 | 285 | 67.4 | 41.4[b] | Gaining (LG) | 53,3 | 45.6[bc] |
| | | | | Maintaining (LM) | 46,7 | 35.7[c] |
| SEM | | | 6.8 | | | 8.3 |
| P-value | | | < 0.0001 | | | 0.0002 |

[a, b,c] Values within a column with different superscript letters differ at P < 0.05. [1]% of cows according to BCS group at calving. From Carvalho *et al*., 2017; UNESP, Department of Animal Production, Botucatu, SP, Brazil; unpublished data.

Table 3. Effect of BCS at calving and BCS change from parturition to 80 days postpartum on pregnancies at first TAI (%P/1st AI) for multiparous suckled Nelore cows (total cows = 893).

| BCS at calving | cows | %cows[1] | %P/1st AI | BCS Change | %cows[1] | %P/1st AI |
|---|---|---|---|---|---|---|
| High (H) BCS ≥ 3.5 | 347 | 38.9 | 57.5[a] | Maintaining (HM) | 40.6 | 59.9[x] |
| | | | | Losing (HL) | 59.4 | 56.0[xy] |
| Moderate (M) BCS = 3.00 - 3.25 | 314 | 35.1 | 51.4[ab] | Gaining (MG) | 20.1 | 62.6[x] |
| | | | | Maintaining (MM) | 23.6 | 49.6[xyz] |
| | | | | Losing (ML) | 56.4 | 48.1[yz] |
| Low (L) BCS ≤ 2.75 | 232 | 26.0 | 45.9[b] | Gaining (LG) | 43.5 | 47.3[yz] |
| | | | | Maintaining (LM) | 56.5 | 44.7[z] |
| SEM | | | 3.2 | | | 4.9 |
| P-value | | | 0.0303 | | | 0.0718 |

[a,b,c]Values within a column with different superscript letters differ at $P < 0.05$. [x, y,z]Values within a column with different superscript letters differ at $P < 0.1$. [1] % of cows according to BCS group at calving. From Carvalho *et al*., 2017; UNESP, Department of Animal Production, Botucatu, SP, Brazil; unpublished data.

Meneghetti and Vasconcelos (2008) evaluated the association between BCS change during the pre and postpartum periods in beef heifers (Nelore and crossbred Nelore/Angus) bred to AI and calving from September to December in Brazil. The BCS were evaluated monthly during pre and post-partum periods (June to February). Heifers that calved earlier had greater ($P < 0.001$) reduction on BCS (Fig. 2). In the Brazilian beef system, it is not beneficial to pregnancy to TAI for heifers to calve earlier, if the plan is to utilize TAI at the start of the next breeding season as primiparous cows. Heifers that calve earlier will become primiparous cows that lose more BCS during the postpartum period, and thus, experience decreased pregnancy rates to TAI. However, if these heifers calve later or closer to the actual time of AI, there is an increased chance of them becoming pregnant as a primiparous cow.

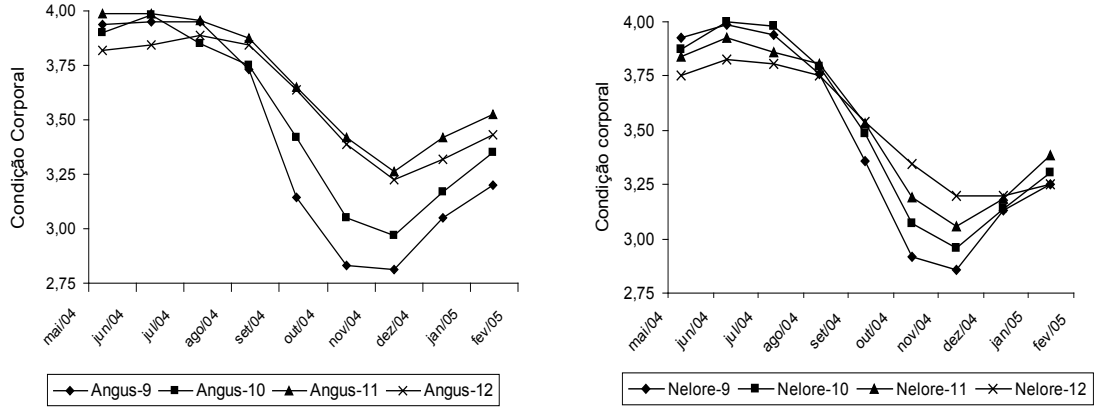


Figure 2. Body condition score change pre and post-partum in heifers (Nelore and ½ Nelore + ½ Red Angus) inseminated to calve from September to December.

**Puberty attainment**

Strategies to increase the number of pregnant beef heifers at the correct moment during the breeding season might affect the maintenance of these animals in the herd. The major factor that leads to failure in pregnancy in beef heifers during their first breeding season is the failure to attain puberty. Claro Junior *et al*. (2010) evaluated the use of intravaginal progesterone devices to induce puberty in Nelore prepubertal heifers. Heifers that received the intravaginal progesterone device had greater percentage of estrus detected, conception, and pregnancy rates compared to heifers that not treated with intravaginal progesterone device. Rodrigues *et al*. (2013) evaluated additional hormonal strategies to further increase puberty attainment in Nelore heifers assigned to the protocol proposed by Claro Junior *et al*. (2010). Rodrigues *et al*. (2013) concluded that the addition of 0.5 mg of estradiol cypionate increased the percentage of Nelore heifers detected in estrus and that became pregnant. In another study, Rodrigues *et al*. (2014) evaluated the optimal interval to start a timed-AI protocol after the puberty induction protocol. Using a 12-day interval in relation to a 10-day interval resulted in greater pregnancy rates in Nelore heifers. Furthermore, the induction of puberty

followed by TAI program proposed by Rodrigues *et al*. (2014) were evaluated in Nelore heifers regardless of their puberty status before the start of the study (Rodrigues *et al*., 2016). Pregnancy rates of heifers submitted to puberty induction protocol before the TAI program did not differ compared with pubertal heifers submitted only to a standard TAI protocol (Fig. 3). Therefore, it is not necessary to evaluate pre-breeding puberty status of Nelore heifers, and all heifers could be simultaneously assigned to the same estrus and ovulation synchronization protocol. The reproductive program indicated for heifers consisted of an intravaginal progesterone device insertion for 12 days (day -35 to day -23), 0.6 mg i.m. injection of estradiol cypionate on day -23, insertion of another intravaginal progesterone device and 2.0 mg i.m. injection of estradiol benzoate on day -11 (beginning of the timed-AI protocol), 12.5 mg i.m. injection of dinoprost tromethamine on day -4, intravaginal progesterone device removal and 0.6 mg i.m. injection of estradiol cypionate on day -2, and TAI on day 0 (48 h after device removal). With the inclusion of a puberty induction protocol before the TAI protocol (Rodrigues *et al*., 2014), heifers will be artificially inseminated about 30 days later than mature cows. Therefore, heifers will calve closer to the start of their subsequent breeding season and experience improved reproductive performance compared with heifers calving at earlier months. According to Meneghetti and Vasconcelos (2008), primiparous cows inseminated with a few days postpartum have greater BCS at TAI and more likely to become pregnant to TAI compared with primiparous cows with longer postpartum interval and smaller BCS at TAI.

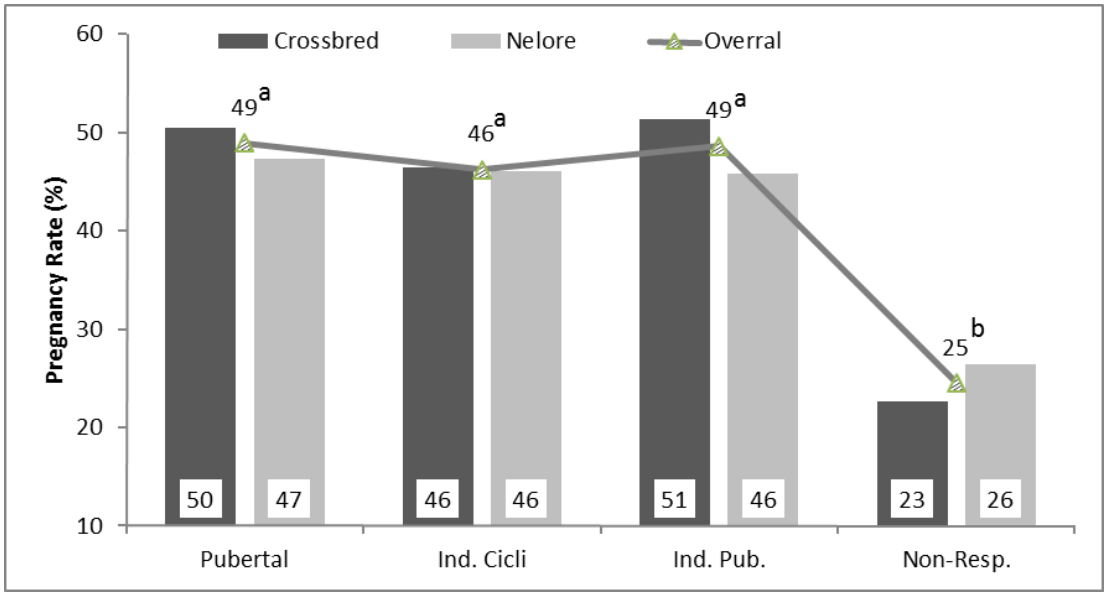


Figure 3. Overall pregnancy rates for the experimental groups: Pubertal (n = 967), induced cyclic heifers (n = 841), induced pre-pubertal heifers (n = 2,052) and non-responded pre-pubertal heifers (n = 1,424). Bars represent the experimental groups in each breed: Crossbred (n = 380, 102, 957 and 816) and Nelore (n = 587, 739, 1,095 and 608) for pubertal, induced cyclic heifers, induced pre-pubertal heifers and non-responded pre-pubertal heifers, respectively

**Energy supplementation**

As suggested by Carvalho (2017; UNESP, Department of Animal Production, Botucatu, SP, Brazil; unpublished data), BCS loss could negatively affect the postpartum fertility of Nelore cows. A recent study by Peres *et al*. (2016) evaluated the effects of corn supplementation beginning at the start of a TAI protocol (day 0) until pregnancy diagnosis, 42 d later, on reproductive performance of Nelore females. In experiment 1, 1, 681 primiparous cows (BCS = 2.84 ± 0.36 and body weight = 407 ± 42.6 kg) were used. In experiment 2, Nelore females (n = 2,395) were divided into 20 groups that included heifers (n = 648; 4 groups), primiparous (n = 635; 6 groups) and multiparous (n = 1,112; 10 groups), with an overall BCS = 2.96 ± 0.36 and body weight = 401 ± 50.1 kg. All primiparous and multiparous cows were assigned to a resynchronization protocol and second TAI if they were diagnosed as nonpregnant on day 41. Calf weaning weight was evaluated in experiment 2. After calving, each group of cows was randomly assigned into two treatments, no corn supplementation (NS) or daily corn supplementation at 1.0 kg/cow (as-fed basis) from day 0 to 11 (adaptation to supplementation), and 2.2 kg/cow from day 11 to 41 (CS). Both treatments reameind on pastures with free choice access to water and mineral supplementation. The TAI protocols started approximately 35 days after calving. In experiment, there was no difference ($P \geq 0.17$) on pregnancy to TAI (44.0 *vs*. 50.0%), pregnancy to second TAI (36.0 *vs*. 40.0 %), and final proportion of pregnant cows (67.5%) between NS and CS treatments, respectively. In experiment, there was no difference ($P = 0.50$) in pregnancy per TAI between NS and CS cows (51.0 *vs*. 52.4%, respectively), but mature cows had greater ($P < 0.01$) pregnancy per TAI (56.6%) compared with nulliparous (48.0%) and primiparous cows (50.5%).

Cows with BCS below 2.75 on day 0 of the TAI protocol had less (P < 0.01) pregnancy per TAI compared with cows with BCS 2.75 or more (44.1 *vs*. 55.5%). Pregnancy to the second TAI tended (P = 0.10) to increase for CS *vs*. NS cows (44.3 *vs*. 38.5%, respectively). Mature cows had greater (P = 0.01) pregnancy to the second AI compared with primiparous cows (46.2 *vs*. 36.6%). There was an interaction (P < 0.01) between treatment and cow category: CS increased final proportion of pregnant cows in primiparous cows (77.8 *vs*. 65.7%; P < 0.05), but reduced that in mature cows (86.9 *vs*. 91.9%; P < 0.05), but had no effect on final proportion of pregnancy in nulliparous cows (89.3%; P = 0.37). Calves from primiparous cows that received corn supplementation were heavier (P < 0.01) at weaning compared with calves from NS primiparous cows (208 ± 1.54 *vs*. 195 ± 1.73 kg). Mature and primiparous cows that received CS (425 ± 2.36 and 464 ± 1.81 kg) were heavier (P = 0.01) at the end of the breeding season compared with NS mature and primiparous cows (415 ± 2.47 and 458 ± 1.84 kg). In conclusion, corn supplementation for 41 days increased pregnancy to a second AI in primiparous and multiparous cows, increased final proportion of pregnancy in primiparous cows, but decreased the final proportion of pregnancy in mature cows despite the increased body weight of primiparous and multiparous cows at the end of the breeding season.

**Polyunsaturated fatty acids (PUFA) supplementation**

Another nutritional strategy that can improve pregnancy rates of beef cows is the supplementation of Ca salts of PUFA. Five experiments evaluated the effects of supplemental Ca salts of saturated fatty acids compared with Ca salts of unsatured fatty acids (Ca salts of PUFA) on reproductive function of Nelore cows (Lopes *et al*., 2009, 2011 ). In experiment 1, 51 nonlactating and multiparous grazing cows were assigned to receive (as-fed basis) 0.1 kg/day of a protein-mineral mix + 0.1 kg/day of ground corn, and 0.1 kg/day of: 1) Ca salts of PUFA (PF; Megalac-E, Quimica Geral do Nordeste, Rio de Janeiro, Brazil), 2) Ca salts of saturated fatty acids (SF; Megalac, Church and Dwight, Princeton, NJ), or 3) kaolin, a rumen-inert compound to equalize DM intake (control). Treatments were offered from day 0 to 20 of the estrous cycle. No treatment effects were detected on serum progesterone concentrations (P = 0.83), day of luteolysis (P = 0.86), or incidence of short cycles (P = 0.84). In experiment 2, 43 non-lactating, multiparous Nelore cows were assigned to receive PF, SF, or control from day 0 to 8 of the estrous cycle. On day 6, all cows received 25 mg i.m. injection of PGF2α. No treatment effects were detected on serum progesterone concentrations on day 6 (P = 0.37), and incidence (P = 0.67) or estimated time of luteolysis (P = 0.44). In experiment 3, 27 lactating, multiparous Nelore cows were assigned to receive daily supplementation of PF or Control for 10 days, beginning at the first postpartum ovulation (30 to 40 days postpartum). No treatment effects were detected (P = 0.85) for incidence of short cycles. In experiment 4, 1,454 lactating, multiparous Nelore cows were assigned to receive 1 of the 7 treatments for 28 days after TAI (day 0; 40 to 60 days postpartum): 1) control from day 0 to 28; 2) SF from day 0 to 14 and control from day 14 to 28; 3) PF from day 0 to 14 and then control from day 14 to 28; 4) SF from day 0 to 21 and then control from day 21 to 28; 5) PF from day 0 to 21 and then control from day 21 to 28; 6) SF from day 0 to 28; and 7) PF from day 0 to 28. Cows receiving daily PF supplementation for more than 21 days after TAI had greater (P < 0.01) pregnancy to TAI compared with all other treatments combined (50.4 *vs*. 42.4 %, respectively). In experiment 5, 501 lactating, multiparous Nelore cows were assigned to receive 1 of the 4 treatments for 21 days after TAI (day 0; 40 to 60 d postpartum): 1) PF from day 0 to 14 and control from day 14 to 21, 2) control from day 0 to 6 and PF from day 6 to 21; 3) control from day 0 to 13 and PF from day 14 to 21; and 4) PF from day 0 to 21. Cows receiving PF after day 14 had greater (P = 0.02) pregnancy rate to TAI compared to cows not receiving PF during the same period (46.8 *vs*. 33.1 %, respectively). In summary, supplemental Ca salts of PUFA during the expected time of luteolysis increased pregnancy to TAI in Nelore cows.

*Vaccination against reproductive diseases*

Decrease pregnancy losses could increase the proportion of cows calving at the beginning of the calving season. The objectives of the experiments described below were to assess the incidence of pregnancy loss, associate this outcome with immunization programs against reproductive diseases, and evaluate the effects of vaccination against bovine herpesvirus-1 (BoHV-1), bovine viral diarrhea virus (BVDV), and *Leptospira* spp., on reproductive efficiency of commercial cow-calf operations (Aono *et al*., 2013). In study 1, 7614 lactating Nelore cows from 18 cow-calf operations were assigned to the same estrous synchronization + TAI protocol (Meneghetti *et al*., 2009). Pregnancy status was determined with transrectal ultrasonography on days 30 and 120 after TAI. Pregnancy loss was defined as cows that were diagnosed as pregnant on day 30, but non-pregnant on day 120. Incidence of pregnancy loss across all ranches was 4.1%; pregnancy losses were detected in 14 operations, but not detected in four operations. Pregnancy loss was less (P ≤ 0.02) in farms using vaccination against BoHV-1, BVDV, and *Leptospira* spp. compared with farms that did not use vaccination, or only vaccinated against *Leptospira* spp. In experiments 2 and 3, lactating Nelore cows (n = 950 and 793, respectively) from operations that did not have a history of vaccinating against reproductive diseases (experiment 2), or only vaccinated against *Leptospira* spp. (experiment 3), were assigned to the same TAI protocol used in study 1. Within each operation, cows received vaccination against BoHV-1, BVDV, and *Leptospira* spp. at the beginning of the TAI protocol (day -11) and 30 days after TAI (day 41; VAC) or remained as unvaccinated controls (control). In experiment 2, VAC cows had greater (P ≤ 0.05)

pregnancy rates compared with control cows on days 30 and 120 (Table 4). In experiments 2 and 3, pregnancy loss was reduced ($P \leq 0.03$) in primiparous VAC compared with control cohorts. In experiment 4, 367 primiparous, lactating Nelore cows previously vaccinated against *Leptospira* spp. were assigned to received the vaccine at the beginning (VAC) of the TAI protocol (day -11) and 30 days after TAI (day 41) or at 30 days prior to (day -41) and at the beginning (day -11) of the TAI protocol (PREVAC). Pregnancy rates on days 30 and 120 were greater ($P \leq 0.05$) in PREVAC cows compared with VAC cows. In conclusion, pregnancy losses impacted reproductive performance of beef cows and may be associated with BoHV-1, BVDV, and *Leptospira* spp. infections. Hence, vaccinating cows against these pathogens, particularly when both doses are administered before TAI, improved reproductive performance.

Table 4. Pregnancy rates 30 and 120 days after fixed-time AI in cows from experiments 2, 3, and 4[1].

| Experiment[2] | Pregnancy status[3] | |
|---|---|---|
| | 30 days | 120 days |
| Experiment 2 | | |
| VAC | 55.1 (546/935) | 53.5 (532/935) |
| COM | 49.8 (548/1015) | 45.9 (523/1015) |
| SEM | 2.8 | 2.8 |
| P-Value | 0.05 | 0.01 |
| | | |
| Experiment 3 | | |
| VAC | 47.3 (599/1292) | 46.8 (579/1292) |
| COM | 46.7 (726/1501) | 44.7 (692/1501) |
| SEM | 4.8 | 4.9 |
| P-Value | 0.84 | 0.45 |
| | | |
| Experiment 4 | | |
| PREVAC | 55.6 (129/232) | 54.7 (127/232) |
| VAC | 45.2 (61/135) | 42.9 (58/135) |
| SEM | 3.8 | 3.8 |
| P-Value | 0.05 | 0.03 |

[1]Pregnancy rates to fixed-time AI are reported as least square means. Values in parentheses represent number of pregnant cows/total inseminated cows. [2]In experiment 2 and 3, cows received (VAC) or not (CON) vaccination against IBR, BVD, and leptospirosis on day -11 and day 30 relative to fixed-time AI (day 0). In experiment 4, cows received vaccination against IBR, BVD, and leptospirosis (at two different schedules relative to fixed-time AI (day 0): 1) day -41 and day -11 (PREVAC), or 2) day -11 and day 30 (VAC = 135). In experiment 3 and 4, cows already received biannual vaccination against leptospirosis. [3]Pregnancy status was verified by detecting a fetus with transrectal ultrasonography at 30 and 120 days after fixed-time AI.

*Melengerol acetate (MGA) supplementation*

Another strategy to increase the proportion of cows calving at the beginning of the calving season is MGA supplementation after TAI. This experiment compared pregnancy rates in *Bos indicus* cows assigned to temporary calf weaning (TCW) or eCG administration during estrus synchronization, and receiving or not MGA supplementation after AI (Costa *et al*., 2015). A total of 3,042 lactating, multiparous, nonpregnant Nelore cows were managed in 48 groups, and assigned to an estrous synchronization + TAI protocol (day -11 to 0). On day -11, groups were randomly assigned, in a 2 x 3 factorial arrangement of treatments, to receive 1 of 2 gonadotropic stimulus: 48-h TCW from day -2 to AI on day 0 (n = 9 groups, 604 cows) or 300 IU i.m. injection of eCG on day -2 (n = 39 groups, 2438 cows). Then, on day 1, groups were assigned to receive 1 of 3 MGA treatments: 1) 0.5 mg of MGA/cow from day 5 to 18 (M5-18; n = 16 groups, 1074 cows) or from day 13 to 18 (M13-18; n = 16 groups, 971 cows), or no MGA supplementation (CON; n = 16 groups, 997 cows). Estrus expression was evaluated by painting the tailhead of each cow on day -2, and verifying if paint completely disappeared at TAI. Body condition score (1 to 9 scale) was evaluated at TAI, and cows were classified as having adequate (≥4.5) or inadequate (<4.5) BCS. Pregnancy rates on days 30 and 80 were greater ($P \leq 0.05$) in M5-18 and M13-18 compared to CON cows, and were similar ($P \geq 0.79$) among M5-18 and M13-18 (Table 5). The MGA supplementation × gonadotropic stimulus interaction was not detected ($P \geq 0.41$), whereas no interactions of main effects with cow BCS and estrus expression were detected ($P \geq 0.21$) for pregnancy outcomes. Supplementing Nelore beef cows with MGA post-AI increased pregnancy rates compared with non-supplemented cows, and this outcome was independent of period and length of MGA supplementation, gonadotropic stimulus, cow BCS status, and estrus expression during the synchronization protocol.

Table 5. Pregnancy outcomes in *Bos indicus* beef cows assigned to different gonadotropic stimulus (eCG administration or temporary calf weaning (TCW)] and receiving or not melengerol acetate (MGA) supplementation after fixed-time AI.

| | MGA supplementation[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Item | TCW | eCG | P = | COM | M5to18 | M13to18 | SEM | P = |
| Pregnant,[3] % | | | | | | | | |
| day 30 | 62.6 (384/604) | 58.1 (1432/2438) | 0.17 | 55.3[a] (554/997) | 62.9[b] (648/1074) | 62.9[b] (614/971) | 2.8 | 0.05 |
| day 80 | 57.6 (348/604) | 54.3 (1349/2438) | 0.40 | 50.5[a] (513/997) | 58.1[b] (604/1074) | 59.2[b] (580/971) | 3.4 | 0.10 |
| Pregnancy loss,[4] % | 9.4 (36/384) | 6.8 (83/1432) | 0.29 | 9.6 (41/554) | 8.3 (44/648) | 6.4 (34/614) | 2.0 | 0.54 |

[1]Cows were assigned to the same estrus synchronization + timed-AI protocol (Meneghetti *et al*., 2009; day -11 to 0). On day -11, cows were assigned to receive 48-h TCW from day -2 until after fixed-time AI on day 0 (n = 9 groups, 604 cows total), 2) 300 IU injection (i.m.) of eCG (Novormon, Zoetis, São Paulo, SP, Brazil) administered on day -2 (n = 39 groups, with 2438 cows total). [2]After fixed-time AI, cows were randomly assigned to receive: 1) 0.5 mg of MGA per cow from day 5 to 18 after fixed-time AI (MGA5to18; n = 16 groups, with 1074 cows total), 2) 0.5 mg of MGA (Zoetis) per cow from d 13 to 18 after fixed-time AI (MGA13to18; n = 16 groups, with 971 cows total), or 3) no MGA supplementation (CON; n = 16 groups, with 997 cows total). [3]Pregnancy status to AI was verified on day 30 and 80 after timed-AI by detecting, respectively, a viable conceptus and fetus via transrectal ultrasonography (Mindray-2200VET DP; Mindray Bio-Medical Electronics Co., Shenzhen, China). Values within parenthesis represent number of pregnant cows divided by number of total cows assigned to fixed-time AI. [4]Pregnancy loss was considered in cows that were pregnant on 30 days after fixed-time AI, but non-pregnant 80 days after fixed-time AI. Values within parenthesis represent number of cows that lost AI pregnancy divided by number of diagnosed as pregnant 30 days after AI.

**Cattle temperament**

Research has shown that behavioral and physiological responses associated with excitable temperament are detrimental to reproductive efficiency of *B. taurus* beef cows (Cooke, 2014), independently if cows are assigned to insemination by natural service or TAI protocols. Excitable temperament is detected more frequently in *B. indicus* cattle compared with *B. taurus* and *B. taurus*-crosses (Cooke, 2014). Therefore, cattle temperament might be of even greater importance for reproductive efficiency of cow-calf operations based on *B. indicus* cows, such as the cow-calf industry in Brazil. To address this subject, Cooke *et al*. (2011) assessed the effects of temperament on reproductive performance of Nelore females by associating temperament characteristics and pregnancy rates to TAI in Brazilian cow-calf operations. Were evaluated 761 multiparous cows from 4 different commercial cow-calf ranches for temperament when cows were processed for TAI (protocol described by Meneghetti *et al*., 2009). Temperament was assessed by chute score and exit velocity. Chute score was assessed by a single technician, immediately before TAI, based on a 5-point scale where: 1 = calm with no movement, 2 = restless movements, 3 = frequent movement with vocalization, 4 = constant movement, vocalization, shaking of the chute, and 5 = violent and continuous struggling. Exit velocity was assessed immediately after TAI by determining the speed of the cow exiting the squeeze chute by measuring rate of travel over a 1.9-m distance. Further, within each ranch group, cows were divided in quintiles according to their exit velocity, and assigned a score from 1 to 5 (exit score; 1 = cows within the slowest quintile; 5 = cows within the fastest quintile). Individual temperament scores were calculated by averaging cow chute score and exit score. Cows were also classified according to the final temperament score (temperament type) as adequate temperament (temperament score ≤3) or excitable temperament (temperament score >3).

Cooke *et al*. (2011) reported that pregnancy to TAI tended (P = 0.08) to be negatively affected by temperament score (Fig. 4). The probability of cows becoming pregnant to TAI was negatively associated with temperament score (linear effect). Accordingly, pregnancy rates were reduced (P = 0.05) in cows with excitable temperament compared to cows with adequate temperament (35.3 *vs*. 42.8% of pregnant cows/total cows, respectively). These results demonstrated that excitable temperament was detrimental to pregnancy to TAI in *B. indicus* beef cows, likely by stimulating, during handling for TAI, neuroendocrine stress responses that directly impair physiological mechanisms required for fertility in females. In fact, pregnancy rates were reduced by 17% when comparing cows with excitable temperament and cows with adequate temperament (35.3 % divided by 42.8), or by 43% when comparing cows with the highest temperament score with those with the lowest temperament score (29.3% divided by 51.4% for temperament score 4.5 and 1, respectively). Based on these outcomes, we concluded that temperament has a significant impact on reproductive performance of *B. indicus* cows exposed to TAI protocols, whereas strategies to ameliorate temperament or the cowherd (such as selecting cattle for adequate temperament or encouraging interaction between young cattle and humans) are necessary to optimize reproductive and overall efficiency of Brazilian cow-calf operations. A follow-up experiment was conducted to further comprehend the impacts of temperament on productive and reproductive outcomes in *B. indicus* cowherds, including reproductive and overall productivity in females following a typical TAI + bull breeding season, calving season, and at offspring weaning (Cooke *et al*., 2017). In this experiment, temperament was evaluated in 953 multiparous Nelore cows at the time of TAI as in Cooke *et al*. (2011). Cows not pregnant to first TAI were assigned to a second TAI protocol or to bulls for 60 days. Cows with excitable temperament had greater (P < 0.01) serum cortisol concentrations at TAI, reduced (P = 0.09) pregnancy rate to first TAI, greater (P = 0.05) pregnancy loss, reduced (P = 0.04) calving rate, as well as reduced (P = 0.09) weaning rate compared with cows with adequate temperament (Table 6). Moreover, calves born from cows with excitable temperament were lighter and younger at weaning (P ≤ 0.05) compared with calves from cows with adequate temperament (Table 6); hence, kg of calf weaned/cow exposed to breeding was reduced (P = 0.04) in cows with excitable temperament (Table 6). Based on these outcomes, this experiment demonstrated that cattle temperament has direct implications not only on reproductive efficiency of *B. indicus* females, but also on overall production efficiency in cow-calf system based on *B. indicus* cattle.

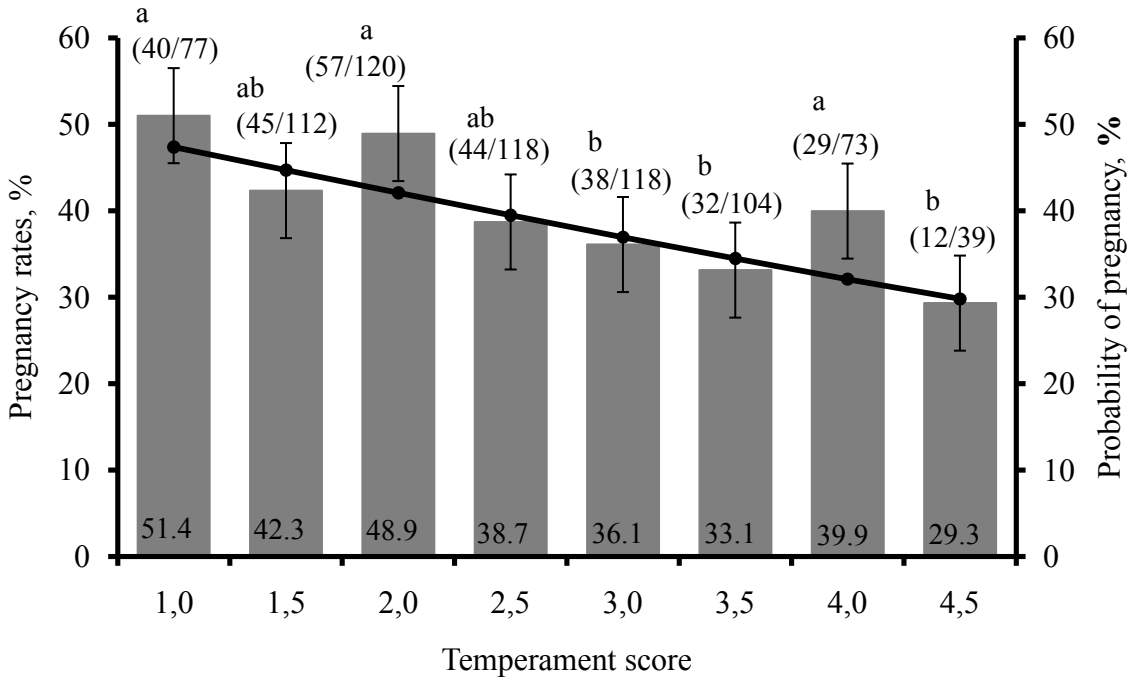


Figure 4. Pregnancy rates (bars) and probability of pregnancy (line) to TAI in Nelore (*B. indicus*) beef cows according to temperament score. Pregnancy rates tended (P = 0.08) to be negatively affected whereas probability of pregnancy was negatively associated (linear effect, P < 0.01) with temperament score. Values within bars correspond to means. Values in parenthesis correspond to pregnant cows divided by total cows assigned to the AI protocol. Means with different superscripts (a *vs*. b) differ at P < 0.05. Adapted from Cooke *et al*. (2011).

Table 6. Serum cortisol concentrations and overall productive responses of Nelore (*Bos indicus*) beef cows according to temperament evaluated at fixed-time AI. [1]

| Item | Temperament type[1] | | SEM | P = |
|---|---|---|---|---|
| | Adequate | Excitable | | |
| Physiological responses | | | | |
| Serum cortisol, ng/ml | 39.1 | 49.1 | 1.0 | < 0.01 |
| Reproductive responses | | | | |
| Pregnancy rates, % | | | | |
| First TAI | 47.3 | 41.0 | 3.6 | 0.09 |
| Second TAI | 43.1 | 39.2 | 5.1 | 0.56 |
| Natural breeding | 58.4 (157/269) | 54.4 (49/90) | 4.1 | 0.52 |
| Final (TAI + natural) | 79.5 (577/726) | 75.8 (172/227) | 2.1 | 0.23 |
| Calving rate, % | 74.8 (543/726) | 68.3 (155/227) | 2.2 | 0.04 |
| Pregnancy loss, % | 5.9 (34/577) | 9.9 (17/172) | 1.4 | 0.05 |
| Calf parameters | | | | |
| Calf weaning age, d | 212 | 209 | 1 | 0.05 |
| Calf weaning BW, kg | 210 | 204 | 2 | 0.04 |
| Cow-calf production parameters | | | | |
| Calf loss from birth to weaning, % | 7.2 (39/543) | 6.4 (10/155) | 1.5 | 0.75 |
| Weaning rate, % | 69.4 (504/726) | 63.9 (145/227) | 2.4 | 0.09 |
| Kg of calf weaned per cow exposed, kg | 146 | 130 | 5 | 0.04 |

[1]Values within parenthesis represent positive responses (pregnant cows, cows that gave birth to a calf, cows that lost pregnancy, number of dead calves, or number of calves weaned) divided by cows exposed to breeding (or total calves born for calf loss results only). Adapted from Cooke *et al*. (2016).

**Sire fertility**

The variation in pregnancy to TAI caused by sire fertility is substantial (Table 7). Understanding the causes that affect conception and maintenance of pregnancy is fundamental to developing management

strategies to increase fertility. Placental insufficiency is a cause of embryonic mortality (evaluated from days 30 to 100 of gestation) and bovine pregnancy-associated glycoproteins (PAG) have been used as a marker of placental function. Although the functional role of PAG is unclear, it has been shown that many factors affect PAG concentrations including pregnancy stage, breed, parity, sire and fetal sex. Limited data have been reported on sire effects on PAG concentration, however, based on the influence sire has on placental development, we were interested in this potential relationship. A previous study from our group pointed toward a relationship between bull fertility/pregnancy loss and circulating concentrations of PAG in early gestation in postpartum Nelore cows (Pohler *et al*., 2016). Thus the objectives of this study were to determine how sire used for TAI influences embryonic loss rate and PAG concentration at day 30. Postpartum Nelore beef cows (n = 736) were artificially inseminated using 6 Angus sires at a fixed time (day 0) after synchronization of ovulation (Franco *et al*., 2017; unpublished data). Pregnancy diagnosis by ultrasound was performed and serum samples were collected on day 30. Serum concentrations of PAG were quantified using an in house PAG ELISA with antibodies raised against PAGs expressed early in gestation. Overall pregnancy rate at day 30 was 53.75 % and late embryonic loss was 6.21%. Mean concentration of PAGs of pregnant cows at day 30 was 8.81 ± 0.24 ng/ml, and cows that maintained a pregnancy from days 30 to 100 of gestations had significantly (P = 0.004) greater serum concentrations of PAG on day 30 compared with cows that did not maintain a pregnancy until day 100 (8.98 ± 0.25 *vs*. 5.95 ± 1.02 ng/ml). Although there was variation in sire conception rate to TAI, there was no linear relationship between sire pregnancy rate and circulating concentrations of PAGs (Sire 1 = 51.56%, 7.72 ng/ml; Sire 2 = 49.17%, 8.96 ng/ml; Sire 3 = 55.28%, 8.81 ng/ml; Sire 4 = 55.28%, 10.14 ng/ml; Sire 5 = 55.28%, 8.42 ng/ml, and Sire 6 = 35.29%, 9.52 ng/ml). Then, sires were classified according to percentage on total embryonic mortality between days 30 and 100 as high embryonic loss (Sire 1 = 20%; Sire 2 = 28%, and Sire 3 = 24%) or low embryonic loss (Sire 4 = 16%; Sire 5 = 4%, and Sire 6 = 8%). After removing all cows that lost pregnancy after day 30, pregnancies by sires classified as high embryonic loss had lower PAG compared to pregnancies by low embryonic loss sires (8.5 ± 0.35 *vs*. 9.48 ± 0.36 ng/ml; P =0.05). In summary, PAG concentration was driven by the ability of pregnancy maintenance and by sire used at TAI. Exploring this relationship might be interesting to improve sire fertility regarding late embryonic loss.

The overall conclusion is that pregnancy rates to timed artificial insemination is influenced by various factors. In this review, we proposed multiple strategies that could increase the fertility of Nelore cows.

Table 7. Overall pregnancy to timed artificial insemination (P/TAI) by AI center and variation by sire. Data used included multiparous cows with BCS at AI between 2.75 and 3.75 and sires with more than 100 AI.

| AI center | Number of AI | P/TAI | P/TAI Range |
|---|---|---|---|
| A | 45,231 | 54.8% | 38.3 to 79.1% |
| B | 128,443 | 55.4% | 30.9 to 70.2% |
| C | 9,434 | 50.5% | 38.1 to 57.9% |
| D | 19,311 | 56.7% | 42.8 to 76.9% |
| E | 25,522 | 54.8% | 28.2 to 72.4% |
| F | 32,397 | 52.5% | 32.1 to 62.7% |
| G | 7,042 | 54.9% | 22.8 to 81.3% |

Adapted from GERAR group.

## References


**Aono FH, Cooke RF, Alfieri AA, Vasconcelos JLM**. 2013. Effects of vaccination against reproductive diseases on reproductive performance of beef cows submitted to fixed-timed AI in Brazilian cow-calf operations. *Theriogenology*, 79:242-248.

**Claro Junior I, Sa Filho OG, Peres RFG, Aono FHS, Day ML, Vasconcelos JLM**. 2010. Reproductive performance of prepubertal *Bos indicus* heifers after progesterone-based treatments. *Theriogenology*, 74:903-911.

**Cooke RF, Bohnert DW, Meneghetti M, Losi TC, Vasconcelos JLM**. 2011. Effects of temperament on pregnancy rates to fixed-timed AI in *Bos indicus* beef cows. *Livest Sci* 142:108-113.

**Cooke RF**. 2014. Temperament and acclimation to human handling influence growth, health, and reproductive responses in *Bos taurus* and *B. indicus* cattle. *J Anim Sci*, 92:5325:5333.

**Cooke RF, Schubach KM, Marques RS, Peres RG, Silva LGT, Carvalho R, Cipriano RS, Bohnert DW, Pires AV, Vasconcelos JLM**. 2017. Effects of temperament on physiological, productive, and reproductive responses in *Bos indicus* beef cows. *J Anim Sci*, 95:1-8. doi: 10.2527/jas.2016.1098.

**Costa WM, Carvalho ER, Pereira MHC, Peres RFG, Vasconcelos JLM**. 2015. Supplementation with melengestrol acetate post TAI improves fertility in suckled Nelore cows. *Anim Reprod*, 12:660. (abstract).

**Lopes CN, Scarpa AB, Cappellozza BI, Cooke RF, Vasconcelos JLM**. 2009. Effects of rumen-protected polyunsaturated fatty acid supplementation on reproductive performance of *Bos indicus* beef cows. *J Anim Sci*, 87:3935-3943.

**Lopes CN, Cooke RF, Reis MM, Peres RFG, Vasconcelos JLM**. 2011. Strategic supplementation of calcium salts of polyunsaturated fatty acids to enhance

reproductive performance of *Bos indicus* beef cows. *J Anim Sci*, 89:3116-3124.

**Meneghetti M, Vasconcelos JLM** . 2008. Mês de parição, condição corporal e resposta ao protocolo de inseminação artificial em tempo fixo em vacas de corte primíparas. *Arq Bras Med Vet Zootec,* 60:786-793.

**Meneghetti M, Sá Filho OG, Peres RFG, Lamb GC, Vasconcelos JLM**. 2009. Fixed-time artificial insemination with estradiol and progesterone for *B. indicus* cows I: Basis for development of protocols. *Theriogenology*, 72:179-189, 2009.

**Peres RFG, Carvalho R, Graff H, Furlan Junior JH, Rodrigues ADP, Franco GA, Keisler DH, Smith MF, Pohler KG, Vasconcelos JLM**. 2016. Corn supplementation during 41 days after the begginning of TAI protocol and its effects on metabolic hormones and reproductive performance of Nelore females, *Anim Reprod*, 13:382. (abstract).

**Pohler KG, Peres RFG, Greeen JA, Graff H, Martins T, Vasconcelos JLM, Smith MF**. 2016. Use of bovine pregnancy-associated glycoproteins to predict late embryonic mortality in postpartum Nelore beef cows. *Theriogenology*, 85:1652-1659.

**Rodrigues ADP, Peres RFG ; Lemes AP, Martins T, Pereira MHC; Day ML, Vasconcelos JLM**. 2013 Progesterone-based strategies to induce ovulation in prepubertal Nellore heifers.*Theriogenology*, 79:135-141.

**Rodrigues ADP, Peres RFG, Lemes AP, Martins T, Pereira MHC, Carvalho ER, Day ML, Vasconcelos JLM**. 2014. Effect of interval from induction of puberty to initiation of a timed AI protocol on pregnancy rate in Nellore heifers. *Theriogenology*. v.82:760-766.

**Rodrigues ADP, Peres RFG, Day ML, Vasconcelos JLM**. 2016. Effect of eCG and P4 level in timed AI programs in *bos indicus* and *bos indicus* x *bos taurus* heifers. *J Dairy Sci*, 99(E-suppl. 1):502. (abstract).

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# Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil

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## Abstract

Beef and dairy productivity depends directly on the reproductive efficiency and genetic gain of the herd, which can be related to the appropriate use of biotechnologies, such as timed artificial insemination (TAI). When considering variations in synchronizations protocols, longer or shorter periods of progesterone (P4) device treatment could provide benefits to fertility. However, our studies evidenced that protocols with six (J-synch), seven, eight and nine days of P4 device treatment had similar pregnancy per AI (P/AI). In cyclic cows, the early prostaglandin (PGF) administration, moving from the day of P4 device removal to two days earlier, which results in four handlings of cows, or the administration of one extra dose of PGF at the onset of the protocol and a single PGF on the day of P4 device removal (three handlings) are both efficient to induce early luteolysis, reducing serum P4 concentrations and, therefore, stimulating LH pulsatility, which improves growth of the dominant follicle and results greater P/AI when compared with protocols with the administration of PGF only on the day of P4 device removal. Resynchronization is another valuable tool to reduce the interval between AI. Traditional Resynch is initiated at pregnancy diagnosis (28 to 32 days after TAI) and the interval between AI is around 40 days; Resynch 22 and Resynch 14 respectively initiates 22 and 14 days after the previous AI in all cows (unknown status of pregnancy) and reduces the interval between AI to 32 and 24 days. The novelty about Resynch 14 is the need to use of Doppler ultrasonography for pregnancy diagnosis [evaluation of corpus luteum (CL) vascularization]. Similar P/AI after Resynch 22 and 14 were found in Nelore cows. In dairy cattle, reproductive management is carried out throughout the year, thus, it is important to adapt the reproductive management to few established days of the week. Therefore, traditional Resynch and Resynch 25 were set to start 32 and 25 days after previous TAI, respectively. The hastening of reproductive age of Nelore heifers aims to reduce age at first calving and increase productivity. Factors such as age, weight, body condition score (BCS), uterine score (USC), average daily weight gain (ADG), withers height/depth of rib relationship (dRIB) and subcutaneous fat thickness (SCFT) were associated with an increase in the success of gestational establishment at TAI and can be used to select the heifers that are more suitable for reproduction. These technologies can contribute to improve the national production of kilograms of meat and liters of milk per hectare, and consequently improve livestock profitability.

**Keywords**: hastening of reproduction age, reproductive management, resynchronization programs, reproductive efficiency.

## Introduction

Brazil has a distinguished position in the global beef industry. In addition to being one of the leaders in the beef export market, it is prominent in the scientific development and commercial application of applied reproductive biotechnologies. The correct use of biotechnologies in farms plays a critical role on productivity. Among the most used reproductive biotechnologies, timed artificial insemination (TAI) - which eliminates the need for estrous detection - deserves to be highlighted for facilitating management and by improving reproductive efficiency and genetic gain of the herds. Data comprised in our laboratory in 2016 showed that in 2015, TAI moved approximately R$567 million (~U$175 million) in Brazil, with an estimate of 3,500 veterinarians directly involved with this activity. Timed AI is currently implemented on 8.2 million beef cows, therefore generating an increase of 8% on calves' production, which represents approximately 656 thousand more calves per year or an additional income of R$820 million/year (~U$253 million) compared with natural service breeding. Time AI also hastens parturition and adds genetic gain to the herds, generating an average gain of 20 kg on the weaning weight of calves, which represents 3.3 million weaned calves with extra 20 kg or, extra R$400 million (~U$123 million). Also, from weaning to slaughtering, TAI calves gain an additional 15 kg of carcass, generating extra R$482.2 million (~U$149 million). Thus, TAI aggregates to the bovine beef chain around R$1.7 billion (more than half billion US $) per year (Baruselli, 2016).

As for dairy herds, TAI also has impact and adds around extra R$900 million (~U$278 million) per year by reducing the calving interval and using genetically superior bulls. It is estimated that TAI reduces one month of calving interval, increasing by 10% the annual milk production, what in Brazil represents extra 690 million liters of milk or R$759 million (~U$234 million) of additional income per year. Additionally, the use of bulls with superior genetics

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adds around 300 liters of milk per lactation of the daughter, resulting in an extra income of R$113.9 million (~U$35 million; Baruselli, 2016). Thus, it is estimated that the impact of TAI on dairy and beef chain together achieves around R$2.6 billion (~U$800 million) per year of extra income.

In 2016, TAI reached the mark of 11,034,119 procedures, which represents a growth of 5.1% in relation to the previous year (Fig. 1; non-published data from VRA-USP-Brazil; 2017), according to a carried out survey based on the number of protocols sold for TAI (information provided by companies in the sector) and the number of commercialized semen straws (Associação Brasileira de Inseminação Artificial - ASBIA, 2017). Currently, TAI procedures correspond to 85% of the insemination performed in Brazil (Fig. 1). Thus, it is evident that TAI holds a relevant place in the AI market. The strong progress made in recent years is indicative that the technology has been consolidated in the market, as it has positive results for livestock and qualified professionals for its execution. In spite of the proved benefits of TAI for reproductive efficiency, only 10 to 12% of females in reproductive age are in fact inseminated in Brazil (Baruselli, 2016). Thus, new strategies for expanding the AI application and optimizing the results should be incorporated to maximize its use and improve even more its economic impact.

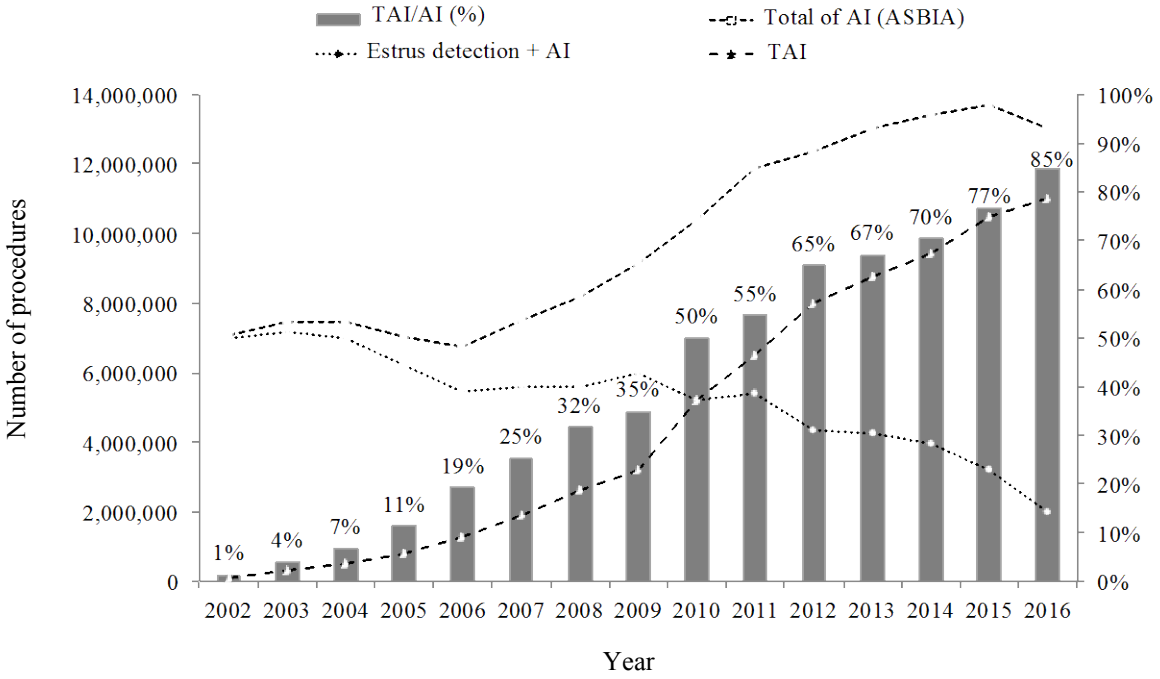


Figure 1. Time evolution of artificial insemination (AI) in Brazil. The numbers of AI done after estrus detection and following treatments for timed AI (TAI) are estimated based on the number of protocols sold for TAI (information provided by companies in the sector) and the number of commercialized semen straws (ASBIA, 2017). Data was organized by Departamento de Reprodução Animal-FMVZ-USP, São Paulo, Brazil, 2017.

## Recent conquests for improving the TAI efficiency in the field

In this topic, the evolution and main perspectives of TAI programs used in beef and dairy cattle in Brazil will be discussed. Variations of current synchronization protocols, such as duration of the progesterone (P4) device and early prostaglandin F2α (PGF) treatment, were investigated to optimize animal handling and the efficiency of TAI protocols. Additionally, the evolution of programs to resynchronize ovulation, allowing subsequent management of TAI during the breeding season may reduce the calving interval and accelerate the genetic gain. Finally, the reduction of the heifers' age as they enter TAI programs is another great tool to maximize the calve production of genetic superior females earlier, hastening the age at parturition and accelerating the genetic advance.

### *The evolution and flexibility of TAI protocols*

Over the past years, TAI protocols for beef and dairy cattle have gone through several modifications aiming to improve pregnancy outcomes. The length of treatment with P4 or progestin devices (summarized in Table 1) and early administration of PGF (from the day of device removal to two days before device removal; summarized in Table 2) were the main alterations evaluated. Briefly, in most experiments, the basic protocol used as control was the insertion of a P4 or progestin-releasing device plus the administration IM of estradiol benzoate (EB) at random days of the estrous cycle defined as day 0, device removal and intramuscular administration of PGF, estradiol cypionate (EC) and equine chorionic gonadotropin (eCG) on day 8 and TAI 48 h later.

The modifications of the duration of P4 or progestin device treatment in cows are based in the following evidences. The increased duration of device treatment, from 8 to 9 days, could provide more growth time to the dominant follicle, guaranteeing the ovulation of follicles with larger diameter with greater ovulation rates (Mantovani *et al*., 2005, 2010; Sá Filho *et al*., 2010) and, consequently generating larger corpus luteum (CL) with greater capacity of P4 synthesis (Mantovani *et al*., 2005). On the other hand, excessively long periods of follicular dominance may disrupt oocyte quality, resulting in reduced fertility (Cerri et al., 2009; Mihm *et al*., 1999; Roche *et al*., 1999; Lonergan, 2011). Another hypothesis is that the reduction of the period of P4 exposition (to six or seven days) may avoid adverse effects of follicular growth at the end of the protocol, extending proestrus and increasing estradiol concentration during this period (Bó *et al*., 2016). Briefly, none of these modifications tested and summarized in Table 1 improved pregnancy per artificial insemination (P/AI) following TAI. Protocols with six (J-synch), 7, 8, or 9 days of P4 device treatment were similarly efficient (Table 1). Similarly, previous studies have shown that the age of the ovulatory follicle did not influence P/AI in heifers and suckled beef cows (Abreu *et al*., 2014a, b). The absence of differences between protocols using P4/E2/eCG with various periods of P4 device treatment (6 to 9 days) may indicate that the new dominant follicle has a window of size and proestrus duration to ovulate an oocyte that is competent and suitable for fertilization. This would allow a flexible management for TAI protocols.

In countries that use GnRH and PGF based protocols, the beneficial effect of changing the duration of traditional Ovsynch protocol has been associated with greater circulating estradiol concentrations by prolonging the proestrus prior to ovulation and greater progesterone concentrations in the ensuing luteal phase, especially in the cows that do not ovulate after the first GnRH (Bridges *et al*., 2014). Furthermore, high estradiol concentrations in the proestrus period have been associated to a more appropriate uterine environment and smaller incidence of embryo loss (Jinks *et al*., 2013).

On the other hand, in the P4/E2/eCG-based protocol routinely used in Brazil, the lack of differences when using different periods for P4 device treatment may be due to diverse reasons: 1) the high efficiency in the synchronization of a new follicular wave at the beginning of the protocol; 2) the treatment with eCG to stimulate dominant follicle growth and to increase circulating P4 concentrations in the subsequent luteal phase; and 3) the treatment with estrogen that increase circulating estradiol concentrations prior to a synchronized ovulation.

The other proposal of protocol modification was related to earliness administration of PGF, from the day of device removal to two days earlier, aiming to induce early luteolysis and reduce serum P4 in cyclic cows. This would stimulate luteinizing hormone (LH) pulsatility and, consequently, improve the growth of the dominant follicle (Mantovani *et al*., 2005, 2010). In this context, greater P/AI was achieved when cycling Nelore cows (detection of CL at the onset of the protocol) received an early administration of PGF [(two days previous to device removal; day 7; 50.3% (86/171)]) compared with those receiving PGF on the day of device removal [day 9; 36.1% (56/155); $P < 0.05$]. Such difference was not observed in cows without a CL at the beginning of the treatment for TAI (Meneghetti *et al*., 2009). Increased P/AI was also observed with early PGF administration (from day 9 to day 7) for non-lactating Nelore cows (Peres *et al*., 2009). In dairy cows, the early administration of PGF from day 8 to day 7 also improved fertility to TAI and embryo transfer (Pereira *et al*., 2013).

Despite improving P/AI, the early administration of PGF demands an extra day of animal handling, which is undesirable, especially in beef farms. An alternative strategy to reduce P4 serum concentration during TAI protocol of cows with CL avoiding the extra handling is the inclusion of an extra dose of PGF at the onset of the protocol, keeping the second dose on the day of device removal (Carvalho *et al.*, 2008). In this context, several studies showed similar P/AI when cows were treated with a single PGF administration on day 7 (four animal handlings) or PGF on day 0 and again on the day of device removal (three animal handlings) for Nelore cows (Carvalhaes *et al*., 2016; Mingoti *et al*., 2016), Girolando heifers (Mendanha *et al*., 2012) and crossbred Nelore-Aberdeen Angus heifers (Colli *et al*., 2016), as summarized in Table 2. Thus, the inclusion of an extra dose of PGF at the onset of the protocol allows improving fertility by reducing P4 serum concentration during TAI protocols, without the need of an extra day of handling.

Table 1. Efficiency of TAI protocols with different periods of progesterone (P4) or progestin source in different categories of cattle.

| Reference | P4/progestin source | N | Category | Device, days | Pregnancy per AI | P value |
|---|---|---|---|---|---|---|
| Barbuio *et al*. (2016) | New FertilCare 1200® | 313 | Lactating Nelore Cow | 8 *vs.* 9 | 55.8% (87/156) *vs.* 56.1% (88/157) | 0.96 |
| Barbuio *et al*. (2016) | New Crestar® | 297 | Lactating Nelore Cow | 8 *vs.* 9 | 59.6% (87/146) *vs.* 62.9% (95/151) | 0.59 |
| Barbuio *et al*. (2016) | 1x Used Crestar® | 214 | Lactating Nelore Cow | 8 *vs.* 9 | 46.7% (50/107) *vs.* 43.0% (46/107) | >0.05 |
| Mingoti *et al*. (2016) | New Sincrogest® | 288 | Lactating Holstein Cow | *8 vs. 9* | 28.3% (41/145) *vs.* 23.8% (34/143) | 0.28 |
| Santos (2016) | New CIDR® | 655 | Lactating Nelore Cow | 7 *vs.* 9 | 56.2% (195/347) *vs.* 54.2% (167/308) | 0.49 |
| Motta *et al*. (2016) | 2x Used FertilCare 1200® | 211 | Nelore Heifer | 6 *vs.* 8 | 47.1 (48/102) *vs.* 48.6% (53/109) | >0.05 |
| Motta *et al*. (2016) | 2x Used FertilCare 1200® | 574 | Nelore *vs.*Angus Heifer | 6 *vs.* 8 | 55.0% (159/289) *vs.* 55.4% (158/285) | >0.05 |
| Elliff *et al*.(2017)* | Primer® | 505 | Lactating Holstein Cow | 7 *vs.* 8 | 27% (68/255) *vs.* 25% (64/250) | 0.72 |

*sent for publication - SBTE 2017: similar (P = 0.554) pregnancy per AI (day 40) was observed between groups treated with intravaginal device containing 1g P4 kept for 7 days (28%; 35/129) or 8 days (24%; 31/130) or containing 0.5g P4 kept for 7 days (26%; 33/126) or 8 days (27%; 33/120).

Table 2. Efficiency of TAI protocols with three (administration of PGF on day 0 and on the days of device removal) or four animal handlings (administration of PGF two days prior to device removal) in different cattle categories.

| Reference | Duration and source of P4 device | N | Category | Handling number | Pregnancy per AI | P Value |
|---|---|---|---|---|---|---|
| Mendanha *et al*. (2012) | CIDR® (9 days) | 451 | Girolando heifer | 3 *vs.* 4 | 40.3% (92/228) *vs.* 42.1% (94/223) | >0.05 |
| Colli *et al*. (2016) | 2x Used CIDR® (8 or 9 days) | 367 | Angus *vs.* Nelore heifer | 3 *vs.* 4 | 57.3% (110/192) *vs.* 57.1% (100/175) | 0.93 |
| Mingoti *et al*. (2016) | Sincrogest®† & CIDR®†(8* or 9 days) | 1.941 | Lactating Nelore Cow | 3 *vs.* 4 | 53.4% (518/971) *vs.* 53.9% (523/970) | 0.71 |
| Carvalhaes *et al*. (2016) | New CIDR® † (9 days) | 289 | Lactating Nelore Cow | 3 *vs.* 4 | 57.1% (84/147) *vs.* 64.8% (92/142) | 0.18 |

*3 cattle handlings with PGF treatment only on the day of P4 device removal. †New, 1x used and 2 used P4 device.

*Resynchronization of ovulation (Resynch programs)*

In order to achieve maximum reproductive efficiency, aggressive strategies to concentrate pregnancies early in the breeding season should be taken. For that, three steps should be considered: AI of all cows at the beginning of the breeding season, early identification of nonpregnant cows, and reinsemination of nonpregnant cows as early as possible. The most commonly management adopted to get nonpregnant cows pregnant soon after the first AI is the introduction of clean-up bulls for the remainder of the breeding season. However, modern alternatives known as Resynchronization Programs are a potential tool to reduce time for subsequent inseminations.

The term "resynchronization" or "Resynch" refers to the treatment for synchronization of follicular wave emergence and ovulation of a female that was previously subjected to TAI. The aim of the technique is to allow the subsequent AI of cows that have already gone through one or more AI without establishing pregnancy, eliminating the need for estrus detection, maximizing the use of selected bulls and improving reproductive efficiency associated with genetic gain. Consequently, it allows the reduction of the interval between AI and hastens postpartum conception, reducing the calving interval and accelerating herds' genetic evolution.

At first, the Resynch program (so called traditional Resynch) was initiated at pregnancy diagnosis around 28 to 32 days after TAI ( Stevenson *et al*., 2003; Marques *et al*., 2012, 2015). Cows diagnosed as nonpregnant immediately initiated a new protocol for TAI. This Resynch management is flexible (starts at date chosen for pregnancy diagnosis) and treatments are only performed in nonpregnant cows. However, the interval between AI is around 40 days and is still considered too long by some technicians compared with bull exposure, when mating occurs around 21 days after TAI. Although bull mating allows the reduction of the interval between two consecutive services, the service rate depends on the estrus return (around 50%; Sá Filho *et al*., 2013). The evolution of Resynch programs allowed the reduction of the interval between AI to compact the period of breeding season and the interval between two subsequent parturitions, with the benefit of guaranteeing 100% service rate. To reach this goal, the treatments should start earlier than pregnancy diagnosis and therefore should be done in all cows. The Resynch 22 was developed to initiate 22 days after the previous AI, eight days before pregnancy diagnosis (Sá Filho *et al*., 2014). At that time, cows diagnosed as pregnant are excluded from the following treatments and nonpregnant cows continue the synchronization treatment. The advantage of Resynch 22 is to reduce the interval between AI to 32 days, however, the first treatment (P4 device and estradiol) should be done in all cows and pregnancy diagnosis must be performed in a fixed schedule, differently from the traditional Resynch. Although most of the farms that use the traditional Resynch have a prescheduled date for pregnancy diagnosis in order to start the resynch protocol as soon as possible, this date is not mandatory (anytime the pregnancy diagnosis is done, it is possible to start a new protocol in open cows). On the other hand, for Resynch 22 or 14, the date of pregnancy diagnosis is mandatory because the protocol was already started eight days before.

More recently, a new approach of early Resynch was proposed using the technology of color Doppler ultrasonography to perform an earlier non-pregnancy diagnosis by analyzing the vascular patterns of the CL (and not the presence of an embryonic vesicle in the uterus as usually done; Siqueira *et al*., 2013; Pugliesi *et al*., 2017). This management is called Resynch 14, because the treatment starts 14 days after the previous TAI, followed by pregnancy diagnosis eight days later (22 days after TAI) using Doppler ultrasonography (Vieira *et al*., 2014). Again, this Resynch protocol demands the initial treatment in all animals (unknown pregnancy status) and a mandatory fixed schedule for pregnancy diagnosis. Additionally, it demands specific ultrasound equipment and a well-trained technician to perform pregnancy diagnosis by the evaluation of CL vascularization grade. However, it allows an impacting reduction in the interval between AI to 24 days, which it close to what is achieved with an ideal 21 days service rate (Fig. 2).

Regarding the early diagnosis of nonpregnancy in cows using the color Doppler ultrasonography, high accuracy and close to 100% sensitivity were observed using only the CL vascularization patters as an indicative of luteolysis to identify nonpregnant dairy cows (Siqueira *et al*., 2013). As for beef cattle, 100% sensibility and 91% accuracy was reported when the association of CL vascularization and size were evaluated to diagnose early pregnancy (Pugliesi *et al*., 2014). Thus, color Doppler ultrasonography is considered an accurate tool to diagnose early pregnancy, because there is a low possibility of a wrong diagnostic of a pregnant cow as nonpregnant (close to 0% of false negatives). The occurrence of pregnancies diagnosed in a subsequent B mode ultrasonography exam as non-pregnancies may be related to long estrous cycles, pregnancy loss, or a lack of synchronization to the first TAI protocol, and not necessarily is due to a wrong pregnancy diagnosis. In these cases, the nonpregnant cows may start a subsequent synchronization of follicular wave emergence and TAI.

Some peculiarities are inherent to each Resynch protocol, specially related to the initial treatment. For the traditional Resynch and Resynch 22 the treatment starts 30 or 22 days after TAI, respectively, and it consists on the insertion of a P4 intravaginal device plus the administration of 2 mg estradiol benzoate (Pessoa *et al*., 2015). The dose of EB used in Resynch 22 was determined based on a study using 1,426 cows (768 *B. taurus* and 728 *B. indicus*; Pessoa *et al*., 2015). Pregnancy to the first TAI and pregnancy loss between 30 and 62 days following AI was similar between cows receiving 1 mg (44.0 and 3.8%) or 2 mg EB (44.0 and 5.5%) on D22. However, pregnancy to Resynch and cumulative pregnancy were greater ($P < 0.01$) in cows treated with 2 mg EB (47.3 and 68.2%) than with 1 mg EB (36.1 and 62.8%). This difference may be related to more effective follicle wave synchronization with 2 mg EB than 1 mg EB in cows (Caccia and Bó, 1998).

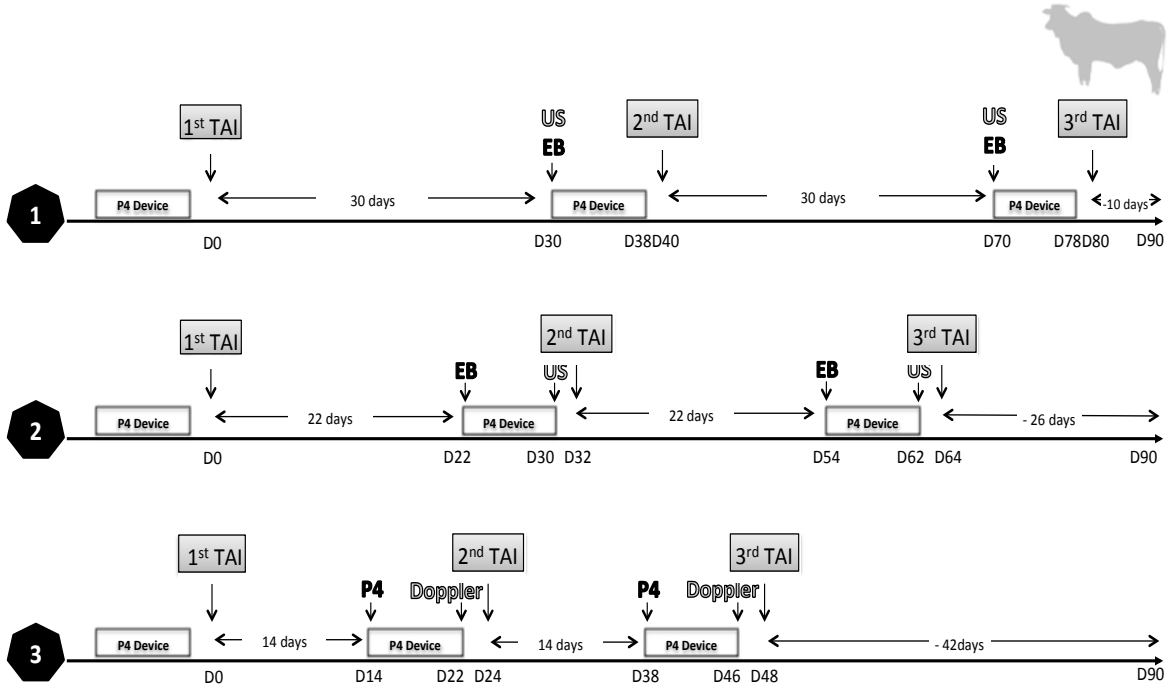


Figure 2. Scheme of three Resynch programs for timed artificial insemination (TAI) of beef females (based in a 90 day breeding season): 1) Traditional Resynch = treatment starts at pregnancy diagnosis (28-32 days after previous AI) in nonpregnant cows; 2) Resynch 22 = treatment starts in all cows (unknown pregnancy status) 22 days after the previous TAI and continue only in nonpregnant cows diagnosed eight days later by conventional ultrasonography (embryonic vesicle); 3) Resynch 14 = treatment stars in all cows (unknown pregnancy status) 14 days after the previous TAI and continue only in nonpregnant cows diagnosed eight days later by color Doppler ultrasonography (CL vascularization). TAI = timed artificial insemination; US = pregnancy diagnosis by conventional ultrasonography; P4 = progesterone; EB = estradiol benzoate.

However, for Resynch 14 the initial treatment starts 14 days after TAI and the administration of estradiol is replaced with the administration of 100 mg P4 IM. This is recommended based on previous studies showing that the use of EB 13 to 14 days after the previous AI induces luteolysis in some of the cows, reducing the previous AI conception rates (Cutaia *et al*., 2002; Vieira *et al*., 2014). This fact is not observed when estradiol is administered 22 days following AI for the Resynch 22 program, as reported previously ( Sá Filho *et al*., 2014; Pessoa *et al*., 2015).

These results are in agreement with previous data showing a reduced ability of the CL to produce P4 when females are treated with EB during mid-diestrus (Munro and Moore, 1985; El-Zarkouny and Stevenson, 2004). This might be related to the induction of PGF release driven by the increase on estradiol concentration (Thatcher *et al*., 1986; Araújo *et al*., 2009). Alternatively to estradiol, P4 can be employed to promote the atresia of the dominant follicle and the emergence of a new wave. In this context, Rezende *et al*. (2016) demonstrated the growth of a new follicular wave 3.0 ± 0.7 after treatment with 100 mg P4 IM in Nelore cows. Thus, synchronization of follicular wave emergence in Resynch 14 programs must be performed replacing EB with P4 on the first day of treatment.

P/AI after Resynch 22 and Resynch 14 were recently compared in 244 postpartum Nelore cows (Penteado *et al*., 2016). For that, cows subjected to the first TAI were allocated into one of the two Resynch programs, Resynch 22 (ECC = 3.0; n = 126) or Resynch 14 (ECC = 3.0; n = 118). Resynch 22 cows were treated with a P4 device and 2 mg EB IM 22 days after the previous AI (day 22). On day 30, the device was removed and pregnancy was diagnosed based on the presence or absence of an embryonic vesicle in the uterus (conventional ultrasonography). Nonpregnant cows had the P4 device removed and received 0.530 mg sodium cloprostenol (PGF), 1 mg estradiol cypionate and 300 IU of eCG IM, followed by TAI 48 h later on day 32. Resynch 14 cows were treated with a P4 device plus IM administration of 100 mg P4 (Afisterone®, HertapeCalier) 14 days after the previous AI (day 14). On day 22, pregnancy diagnosis was done by the assessment of CL vascularization using Collor Doppler ultrasonography. Cows with absence or low CL vascularization were considered open and proceeded the treatment (device removal, PGF, estradiol cypionate and eCG IM), and were TAI 48 h later on day 24. Cows with moderate or strong CL vascularity were considered pregnant and had the device removed without further treatments. Similar P/AI were observed for Resynch 22 and Resynch 14 cows following the first AI (48 *vs.* 53%; P = 0.57) and resynchronization (56 *vs.* 51%; P = 0.37), respectively. The cumulative pregnancy after 32 and 24 days of breeding season did not differ (P = 0.77) for Resynch 22 (77%; 97/126) and Resynch 14 cows (75%; 89/118), respectively. Besides keeping similar P/AI after subsequent TAI and reducing the interval between AI to 24 days, Resynch 14 considerably improved 21 days service rate from 66 to 87.5% in relation to Resynch 22 (Fig. 3).

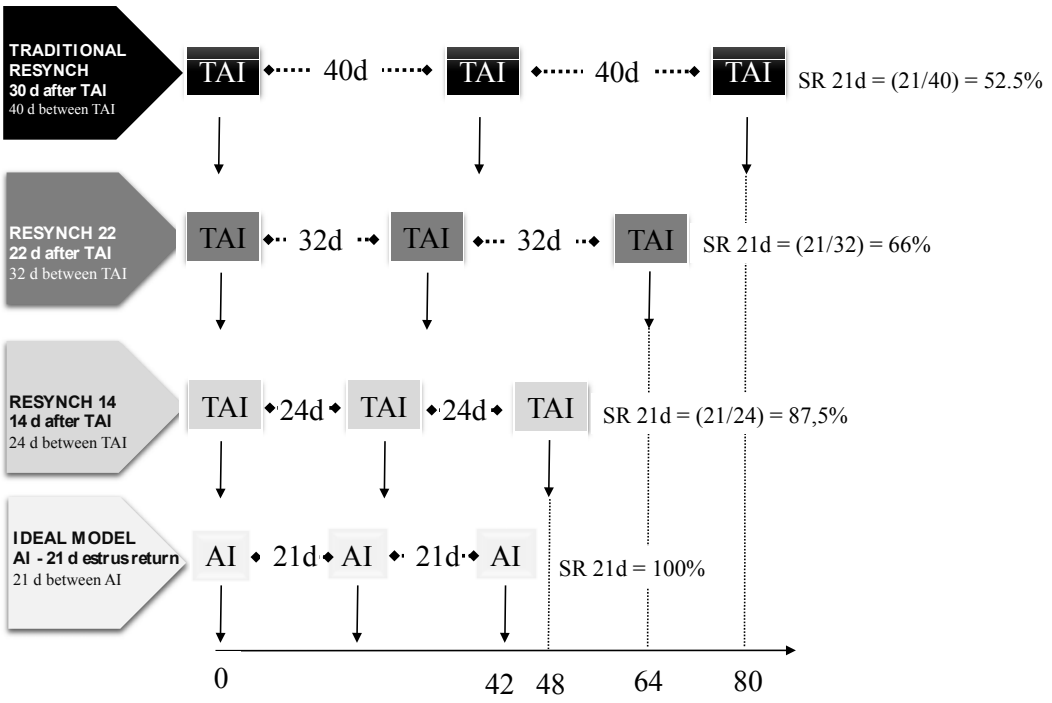


Figure 3. Service rate (SR) for different Resynch programs: Resynch 14 (starts 14 days after previous timed artificial insemination (TAI), with 24 days interval between AI and SR = 87.5%), Resynch 22 (starts 22 days after previous TAI, with 32 days interval between AI and SR = 66%), and traditional Resynch (starts after pregnancy diagnosis 30 days after previous TAI, with 40 days interval between AI and SR = 52.5%). Ideal model refers to a 21 day-interval between AI and SR = 100%.

The benefits of using Resynch programs are leading to the adoption of management exclusively with TAI, eliminating the need for clean-up bull in several farms. The use of three consecutive TAI using Resynch 22 (3 TAI) had similar pregnancy rates than those achieved with bull exposure after two TAI using Resynch 22 (2 TAI + bull) and greater pregnancy rate than one TAI followed by bull exposure (1 TAI + bull; Crepaldi *et al*., 2017). In this study, it was possible to achieve 87.4% of cumulative pregnancy rate at the end of a 64-day breeding season after three TAI using Resynch 22 (Fig. 4).

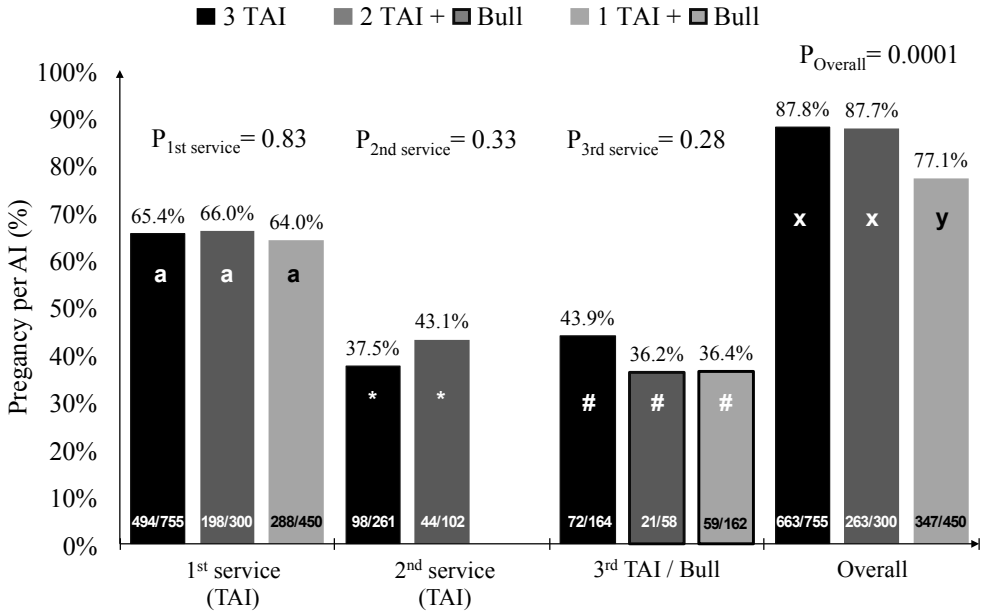


Figure 4. Pregnancy rate in *Bos indicus* beef cows after timed artificial insemination (TAI) with subsequent resynchronization for sequential TAI or bull exposure. Resynchronization was done using Resynch 22 program starting 22 days after the previous TAI. Groups were: 3 TAI (TAI + Resynch 22 and TAI + Resynch 22 and TAI; n = 450); 2 TAI (TAI + Resynch 22 and TAI + clean-up bulls; n = 300); 1 TAI (TAI+ clean-up bulls; n= 755). Pregnancy diagnosis of the 3rd TAI and bull mating were done at the end of the breeding season. Adapted from Crepaldi *et al*., 2017.

More recently, young Nelore heifers (12-15 months old) were subjected to three subsequent TAI using Resynch 14 program and achieved P/AI of 42.8% (270/631) at 1st AI, 34.1% (107/314) at 2nd TAI, 34.3% (59/172) at 3rd TAI and 72.1% (455/631) of cumulative pregnancy rate at the end of a 48-day breeding season (Colli *et al*., 2017; Fig. 5).

Pregnancy diagnosis was based on CL area and vascularization. Heifers with an area of CL ≥2 cm² and/or ≥25% CL blood flow were diagnosed as pregnant. False positive heifers were those diagnosed as pregnant at Doppler evaluation 22 days after AI and then as nonpregnant at pregnancy confirmation 30 days after AI. The false positive rate was 14.8% (47/317) and the P/AI of these heifers for the second time inseminated in D48 was 40.4% (19/47), increasing 2% in P/AI of day 48 (3rd TAI + false positive, 35.6%, 78/219).

These studies demonstrate that modern Resynch programs seems to be feasible and efficient on reducing the period of breeding season with similar cumulative pregnancy outcomes as obtained with bull exposure in traditional 90-day breeding season. It also brings the advantage of improving the number of pregnant animals by AI, accelerating the farms' genetic gain. Additionally, it concentrates calving births in the favorable calving season, consequently improving the weaning weight and accelerating the use of young females in the subsequent breeding season.

For dairy cattle, the Resynch protocols follow the same basis as for beef cattle. However, differently from beef, dairy herds reproductive management is accomplished all over the year, usually lacking a delimitated breeding season, and animals are managed several times a day. Thus, it becomes crucial to adapt the reproductive management so they do not interfere with the other daily management employed in a dairy farm. A good strategy is to concentrate the reproductive-related activities into established days of the week. In this basis, the traditional Resynch and Resynch 25 were set to start 32 and 25 days after previous AI, respectively (Fig. 6). This standardization of reproductive activities in specific weekdays enables the establishment of a well-planned routine in dairy farms, as shown in Figure 6.

In order to calculate reproductive efficiency of dairy cows in a Resynch 25 program, we considered that the first service occurs 58 days after parturition (average), 30% P/AI until third service, 20% P/AI between the fourth and sixth services (sixth service was established as the animals' last service) and 15% of pregnancy loss between 30 and 60 days of pregnancy. In this simulation, the herd achieves 82% of pregnant cows at 246 days in milk, 110.4 days interval between parturition and conception and 12.9 months of calving interval, which can be considered good reproductive efficiency. Therefore, studies were conducted in order to verify the applicability and viability of Resynch 25 in dairy properties in Brazil.

In one study, the efficiency of the association between gonadotropin-releasing hormone (GnRH) or EB and P4 at the onset of Resynch 25 to synchronize the new wave of follicular growth in Holstein cows without previous pregnancy diagnosis was evaluated (Vasconcellos *et al*., 2014). The authors verified that both associations (GnRH+P4 or EB+P4) were effective to synchronize the new wave of follicular growth and ovulation. Given this information, a second study was conducted to verify the possible influence of the administration of 2 mg EB at the onset of Resynch 25 on the establishment and maintenance of the previous pregnancy in Holstein cows (Vieira *et al*., 2015). Similar P/AI following first AI at 33 [EB: 33.0% (66/200), GnRH: 35.0% (69/197), EB+GnRH: 34.3% (70/204); P = 0.61] and 65 days [EB: 26.0% (52/200), GnRH: 28.9% (57/197), EB+GnRH: 26.5% (54/204); P = 0.26], as well as similar pregnancy loss between 33 and 65 days after first TAI [EB: 21.2% (14/66), GnRH: 17.4% (12/69), EB+GnRH: 22.9% (16/70); P = 0.47], were observed in cows resynchronized with EB, GnRH or a combination of both. The CL vascularization was also accessed by Doppler ultrasonography every 48 h, between days 25 and 33 in 42 lactating cows used in the previous experiment [EB: n = 15; GnRH: n = 12; EB+GnRH: n = 15]. It was found that the CL vascularization rate remained similar between the three groups during the experimental period (day 25: 76.1%, day 27: 79.1%, day 29: 77.5%, day 31: 76.3% and day 33: 77.1%). Thus, the resynch treatment using EB, GnRH or a combination of both after TAI results in similar P/AI after 1st TAI and after resynchronization. Also the administration of EB in pregnant lactating cows 25 days after AI does not induce pregnancy loss and does not compromise the CL vascularization.

Therefore, Resynch 25 can be an alternative for reproductive management of dairy properties. This program allows systematization and planning of the annual reproduction calendar of the farm, concentrating conceptions at the beginning of lactation. However, it is important to mention that other factors, such as nutrition and sanitary management, as well as organization and availability of qualified personal, may significantly influence the results of TAI programs with resynchronization in dairy herds.

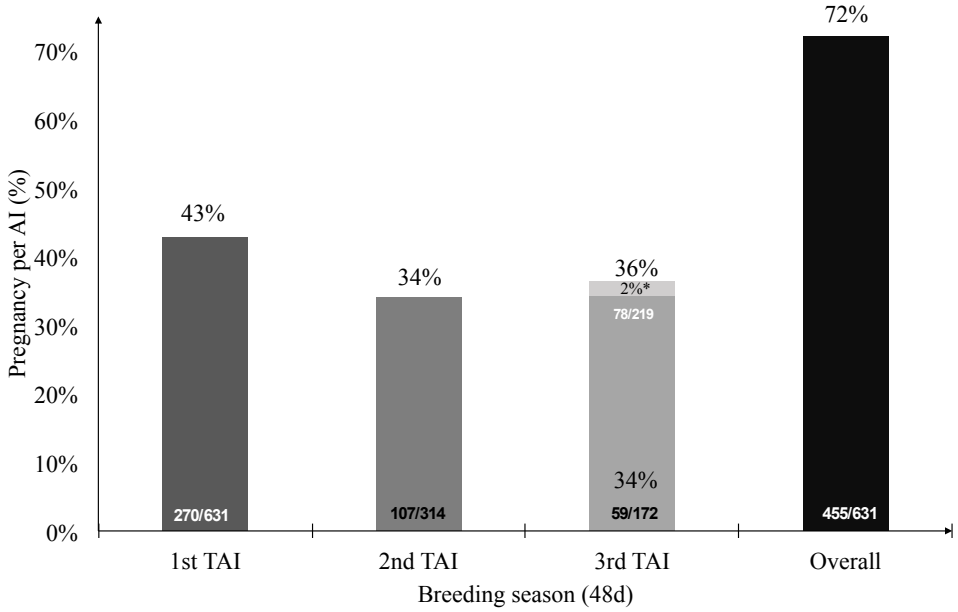


Figure 5. Pregnancy rates in young *Bos indicus* beef heifers (12-15 months old) after timed artificial insemination (TAI) with subsequent resynchronization using Resynch 14 (program starting 14 days after the previous TAI in all heifers, with unknown status of pregnancy). *Refers to heifers diagnosed as pregnant at first evaluation and them diagnosed as nonpregnant and reinsemination for the second time with the 3rd service heifers. Adapted from Colli *et al*., 2017.

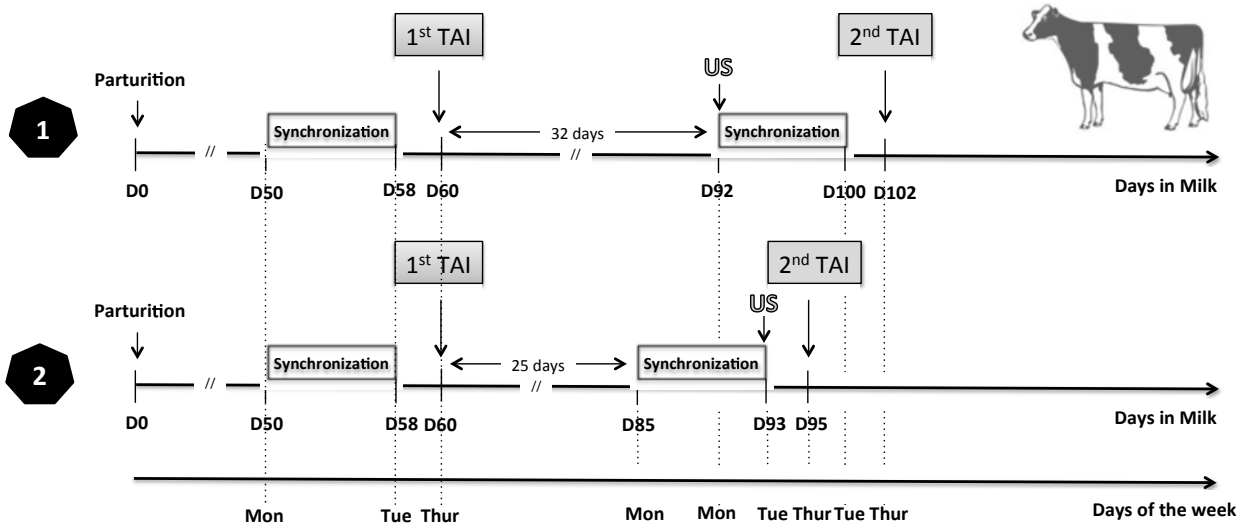


Figure 6. Scheme of two Resynch programs for timed artificial insemination (TAI) of dairy females: 1) Traditional Resynch = treatment starts at pregnancy diagnosis (32 days after previous AI) in nonpregnant cows, with 42 days interval between AI; 2) Resynch 25 = treatment starts in all cows (unknown pregnancy status) 25 days after the previous TAI and continue only in nonpregnant cows diagnosed eight days later by conventional ultrasonography (embryonic vesicle), with 35 days interval between AI. US = pregnancy diagnosis by conventional ultrasonography.

*Hasten of reproductive age of Nelore heifers*

Many factors may influence hasten or delay in the sexual maturity of heifers, such as age, weight after weaning and development of the reproductive tract. Knowing the real impact of these factors on the reproductive efficiency would be determinant for the development of future strategies that would accelerate even more the onset of reproduction of Nelore heifers in Brazil. Thus, the need to establish indicators of body development that determine improvements on the reproductive efficiency of yearling zebu heifers is evident. The main objective is to reduce age at first calving and increase productivity in Brazils' beef herd.

Recently, Freitas (2015) evaluated the body development and functioning of the reproductive tract in relation to gestational success in 650 14-month-old (13.9 ± 0.03 months) Nelore heifers that underwent TAI protocols. The heifers were kept on a pasture-based system. They were evaluated during 10 days before the onset of TAI protocol (day -10 to day 0). The evaluated characteristics were age (months), weight (kg, weight scale for squeeze chute), body condition score (BCS, 1 to 5 scale), withers height (hWIT, cm) and withers

height/depth of rib relationship (dRIB, %), reproductive tract score (RTS, 1 to 5 scale), cyclicity (presence of a CL) and subcutaneous fat thickness (SCFT, mm). All heifers were synchronized for TAI (Norgestomet year implant + 2 mg EB – 8d – PGF + 300 IU eCG + 0,6 mg estradiol cypionate - AI 48 hs). The cyclicity (presence of CL at day -10 and/or day 0) and P/AI 30 days after TAI were determined by ultrasonography. Briefly, hWIT and RTS were not associated with an increase in the success of gestational establishment. However, heifers that were older, heavier, had greater BCS, dRIB and SCFT had greater P/AI (Table 3).

Table 3. Cut-off points of the evaluated parameters and calculated by the receiver operation characteristics (ROC) curve, based on the pregnancy rate.

| Parameter | N | Area under curve | 95% confidence interval | P value |
|---|---|---|---|---|
| Age | | | 0.540 a 0.630 | 0.0003 |
| > 13.8 months | 351 | 58.5 | | |
| < 13.8 months | 298 | | | |
| Weight | | | | |
| > 248.00 kg | 429 | 56.8 | 0.529 a 0.607 | 0.003 |
| < 248.00 kg | 311 | | | |
| BCS | | | | |
| > 3.0 | 271 | 62.9 | 0.590 a 0.666 | < 0.0001 |
| < 3.0 | 469 | | | |
| hWIT | | | | |
| > 119.0 cm | 544 | 50.2 | 0.462 a 0.541 | 0.94 |
| < 119.0 cm | 196 | | | |
| dRIB | | | | |
| > 44.0 % | 393 | 61.0 | 0.571 a 0.648 | < 0.0001 |
| < 44.0 % | 347 | | | |
| SCFT | | | | |
| > 2.5 mm | 411 | 59.6 | 0.552 a 0.640 | < 0.0001 |
| < 2.5 mm | 314 | | | |

BCS = body condition score; hWIT = withers height; dRIB = rib depth; SCFT =subcutaneous fat BCS = body condition score. Adapted from Freitas, 2015.

It was found that older females showed greater success in pregnancy (> 13.8 months = 43.0 *vs*. ≤ 13.8 months = 27.2%; P = 0.04). No association was observed between hWIT and P/AI (> 119.0 cm = 34.1 *vs*. ≤ 119.0 cm = 40.0%; P = 0.46). In contrast to hWIT, heifers with greater dRIB had greater P/AI compared with heifers with smaller dRIB (> 44.0% = 41.9 *vs*. ≤ 44.0% = 27.0%; P = 0.02). In addition to dRIB, another characteristic that influenced P/AI was SCFT (> 2.5mm = 44.4 *vs*. ≤ 2.5 mm = 23.4%; P = 0.0003), heifers with greater SCFT showed greater probability of P/AI (Fig. 7; Freitas, 2015).

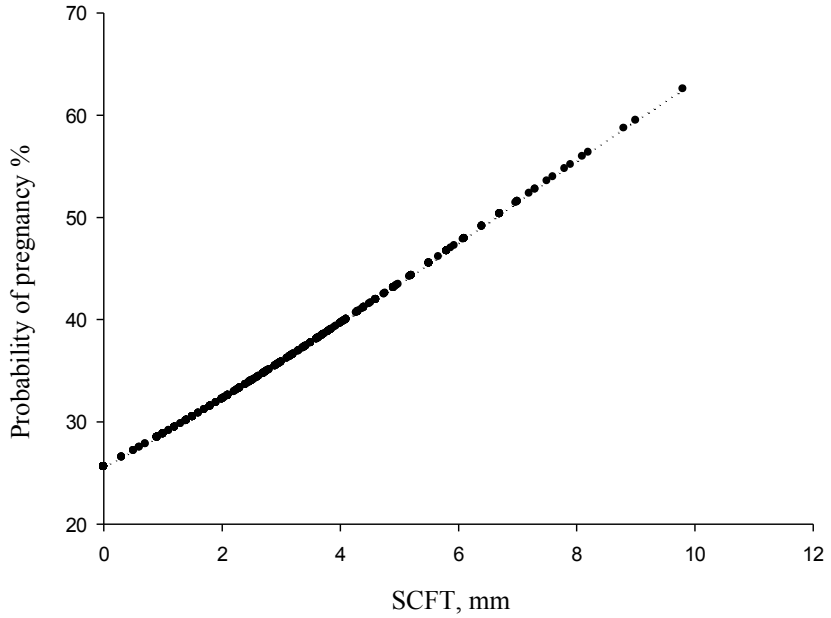


Figure 7. Probability of pregnancy to timed artificial insemination (TAI) as a function of the subcutaneous fat thickness (SCFT) of Nelore heifers (n = 650) with a mean of 13.9 months of age [Logit (SCFT) = 1.0662 + 0.1612 * SCFT; P = 0.0003]. Adapted from Freitas, 2015.

The zootechnics characteristics related to body development have different influence on P/AI. In the aforementioned study, hWIT and RTS isolated have no effect on pregnancy to TAI. However, age, weight, BCS, dRIB and SCFT are associated with an increase in the success of gestational establishment at TAI. When associated, the variables dRIB, age and SCFT are the characteristic that exert the most influence on P/AI (Freitas, 2015).

Garcia *et al*. (2002) showed that body weight accounted for most of the variation associated with the onset of puberty followed by the contribution of circulating leptin concentrations, which increased during a period of 16 weeks before first ovulation. Others studies found that the onset of puberty is related to growth rate and the amount of body fat (Nogueira, 2004). In a recent study, heifers were weaned at approximately 4 month of age and fed diets to promote relatively low (0.5 kg/day) or high (1.0 kg/day) rates of body weight gain until 8.5 month of age (Alves *et al*., 2015). Heifers that gained body weight at a greater rate exhibited greater circulating concentrations of leptin and reduced overall NPY expression in the arcuate nucleus. The authors suggest that such changes may mediate the nutritional programming of the reproductive neuroendocrine axis and facilitate an early onset of puberty in heifers (Alves *et al*., 2015).

More recently, two experiments were conducted with the objective of studying the factors that affect the pregnancy rate of 14 months old Nelore heifers submitted to TAI (n = 404) and natural breeding (n = 893; bull to heifer ratio was 1 to 30; Martins *et al*., 2017). On both studies, presence of CL, uterine score (USC; A = uterine horns diameter > 2 cm; B = uterine horns with diameter between 1.5 and 2 cm; and C = uterine horns with diameter < 1.5 cm), diameter of the largest follicle, daily average weight gain (DAWG), loin eye area (LEA), SCFT and hWIT were analyzed at the beginning of the breeding season. On TAI experiment, none of the heifers was cycling or had an A USC on the beginning of the breeding season. The P/AI was greater on heifers with greater USC [B = 41.1% (122/297) *vs*. C = 17.8% (19/107); P = 0.0005]. Pregnancy probability was greater for animals with greater SCFT ($r^2$ = 0.208; P = 0.005) and DAWG ($r^2$ = 0.168; P = 0.0007). The LEA ($r^2$ = 0.115; P = 0.13) and hWIT ($r^2$ = 0.309; P = 0.28) characteristics did not affect P/AI. This findings are in agreement with previous (Freitas, 2015). On natural breeding experiment, the cyclicity rate was 5.3% (47/893) at the beginning of the breeding season. Pregnancy diagnosed on day 50 of the breeding season was greater (P > 0.0001) for cycling (53.2%; 25/47) than anestrous (13.4%; 113/846) heifers and for heifers with greater uterine development [USC: A = 64.0% (16/25); B = 17.9% (116/647) and C = 3.2% (7/221); P < 0.0001]. The pregnancy probability was not influenced by SCFT ($r^2$ = 0.096; P = 0.42) and LEA ($r^2$ = 0.061; P = 0.61). However, DAWG positively influenced the probability of cyclicity ($r^2$ = 0.263; P < 0.0001) and pregnancy ($r^2$ = 0.093; P = 0.005); and hWIT negatively influenced pregnancy probability ($r^2$ = -0.082; P = 0.03). Pregnancy probability was also increased according to diameter of the largest follicle ($r^2$ = 0.117; *P* = 0.0004). Thus, it was possible to verify that heifers with greater SCFT and DAWG had greater pregnancy probability to TAI. Heifers submitted to natural breeding with greater DAWG and diameter of the largest follicle, and smaller hWIT had greater probability to become pregnant. Further, pregnancy rates to TAI and natural breeding were greater on heifers with greater USC (Martins *et al*., 2017).

Finally, the risk factors influencing P/AI of young Nelore heifers (aging 14.4 ± 0.92 months old; n = 631) subjected to three consecutive TAI using Resynch 14 and Doppler ultrasonography was evaluated (Colli *et al*., 2017). Weight, BCS, age and diameter of the largest follicle at the end of the synchronization program were evaluated. A positive correlation was found between P/AI 30 days after TAI and weight ($r^2$ = 0.09; P = 0.03), age ($r^2$ = 0.07; P = 0.06), BCS ($r^2$ = 0.07; P = 0.09), diameter of the largest follicle on D0 ($r^2$ = 0.20; *P* <0.0001) and diameter of the largest follicle at P4 device removal ($r^2$ = 0.11; P = 0.007). Also, a negative correlation was observed between the incidence of false positives (heifers diagnosed as pregnant at Doppler evaluation 22 days after AI and then as nonpregnant at pregnancy confirmation 30 days after AI) and weight ($r^2$ = -0.15; P = 0.009), age ($r^2$ = -0.10; P = 0.07), largest follicle at device removal ($r^2$ = -0.10; P = 0.10) and largest follicle at AI ($r^2$ = -0.15; P = 0.01).

Thus, it is possible to obtain pregnancy rates > 70% in Nelore heifers with 14 months old after three TAI (Colli *et al*., 2017; Fig. 5) and that there is a positive correlation between weight, age, BCS, SCFT, and diameter of the largest follicle at the end of the synchronization protocol and P/AI 30 days after TAI.

## Conclusions and future directions

The reduced reproductive efficiency of Brazilian bovine herd is still a limiting factor for the sustained growth of beef and dairy chains. We are well below the capacity of production of calves per cow per year (~68.5% weaning rate) and the quality of produced calves is far short of the ideal (~12% of females are inseminated). Besides, our heifers start reproductive life still very belatedly (around three to four years old), and reproductive efficiency is still impaired. In dairy, Brazil is not self-sufficient to supply the national market and the low productivity per cow (less than 2,000 L of milk per lactation) evidences the low genetic selection. Thus, our production system requires the development of complementary strategies to hasten and maximize the use of AI in dairy and beef herds, with easy and direct application and high reproductive efficiency to improve productivity.

In this context, several synchronization protocols (i.e. with different duration of P4 source treatment and acceleration of luteolysis) for dairy and beef cows and heifers were well studied and are shown herein. Also, the use of Resynch programs can reduce the interval between services and allow the massive use of AI in farms, resulting in the production of greater quantity and quality of calves. Finally, heifers'

reproductive age can be hasten by correctly managing their mothers so they give birth in the most adequate season of the year (greater weaning weight), associated with calves' and heifers' correct nutrition and the use of adequate characteristics of evaluation as age, weight, BCS, USC, DAWG, dRIB and SCFT to select the heifers that are more suitable for early reproduction.

The dissemination of the use of these strategies isolated or in combination enables the production of greater quantity (reduction of the calving interval and hastening of reproductive age of heifers) and quality (maximization of the use of AI) of beef calves, and the production of dairy females with greater milk production per lactation (reduction of the calving interval associated to genetic gain). These technologies can contribute to improve the national production of kilograms of meat and liters of milk per hectare (what is a reflection of production efficiency), and consequently improve livestock profitability.

## References


**Alves BRC, Cardoso RC, Prezotto LD, Thorson JF, Bedenbaugh M, Sharpton SM, Caraty A, Keisler DH, Tedeschi LO, Williams GL**. 2015. Elevated body weight gain during the juvenile period alters neuropeptide y-gonadotropin-releasing hormone circuitry in prepubertal heifers. *Biol Reprod*, 92:46, 1-10. doi: 10.1095/biolreprod.114.124636.

**Abreu FM, Cruppe LH, Maquivar M, Utt MD, Madsen CA, Vasconcelos JLM, Mussard ML, Day ML, Geary TW**. 2014a. Effect of follicle age on conception rate in beef heifers. *J Anim Sci*, 92:1022-1028.

**Abreu FM, Geary TW, Cruppe LH, Madsen CA, Jinks EM, Pohler KG, Vasconcelos JLM, Day ML**. 2014b. The effect of follicle age on pregnancy rate in beef cows. *J Anim Sci*, 92:1015-1021.

**Araújo RR, Ginther OJ, Ferreira JC, Palha MM, Beg MA, Wiltbank MC**. 2009. Role of follicular estradiol-17beta in timing of luteolysis in heifers. *Biol Reprod*, 81:426-437.

**Associação Brasileira de Inseminação Artificial (ASBIA)**. 2017. [WWW Document]. Available on: http://www.asbia.org.br/novo/relatorios/.

**Barbuio JP, Girotto RW, Marques MO, Penteado L, Rezende RG, Mingoti RD, Vieira LM, Colli MHA, Baruselli PS.** 2016. Pregnancy rate of lactating Nelore cows synchronized with progesterone devices or new progestogen implants or used for eight or nine days. *Anim Reprod*, 13:419. (abstract).

**Baruselli PS**. 2016. IATF supera dez milhões de procedimentos e amplia o mercado de trabalho. *Rev CFMV*, 69:57-60.

**Bó GA, de la Mata JJ, Baruselli PS, Menchaca A**. 2016. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology*, 86:388-396.

**Bridges GA, Mussard ML, Hesler LA, Day ML**. 2014. Comparison of follicular dynamics and hormone concentrations between the 7-day and 5-day CO-Synch + CIDR program in primiparous beef cows. *Theriogenology*, 81:632-638.

**Caccia M, Bó GA**. 1998. Follicle wave emergence following treatment of CIDR-B implanted beef cows with estradiol benzoate and progesterone. *Theriogenology*, 49:341 (abstract).

**Carvalhaes RPA, Colli MHA, Almeida TT, Mingoti RD, Vieira LM, Santos MH, Sales JNS, Baruselli PS**. 2016. Pregnancy rate following FTA of nelore (*Bos indicus*) cows submitted to protocols of 3 or 4 cattle handling with administration of PGF in different moments. *Anim Reprod*, 13:432. (abstract).

**Carvalho JBP, Carvalho NAT, Reis EL, Nichi M, Souza AH, Baruselli PS**. 2008. Effect of early luteolysis in progesterone-based timed AI protocols in *Bos indicus*, *Bos indicus* x *Bos taurus*, and *Bos taurus* heifers. *Theriogenology*, 69:167–175.

**Cerri RL, Rutigliano HM, Chebel RC, Santos JEP**. 2009. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. *Reproduction*, 137:813-823.

**Colli MHA, Carvalhaes RPA, Mingoti RD, Vieira LM, Rezende RG, Santos MH, Sales JNS, Baruselli PS**. 2016. Pregnancy rate to FTAI of Crossbred heifers (B. indicus x B. taurus) submitted to protocols of 3 or 4 handling cattle. *Anim Reprod*, 13:425. (abstract).

**Colli MHA, Rezende RG, Elliff FM, Zanatta GM, Mingoti RD, Gonçales Junior WA, Ferreira RM, Baruselli P.S**. 2017. Factors influencing the pregnancy rate of 14 month old Nelore heifers submitted to 3 consecutive TAI. *Anim Reprod*, 14. (abstract).

**Crepaldi GA, Freitas BG, MIngoti RD, Colli MHA, Gonçales Junior WA, Ferreira RM, Baruselli PS**. 2017. Reproductive efficiency of Nelore cows submitted to three different reproductive strategies in a 64 days breeding season. *Anim Reprod*, 14. (abstract).

**Cutaia L, Tribulo R, Tegli J, Moreno D, Bó GA**. 2002. The use of estradiol and progesterone inserts during mid-diestrus to synchronize return to estrus in beef cows and heifers. *Theriogenology*, 58:373. (abstract).

**Elliff FM, Rezende RG, Carneiro TO, Barreto AENP, Reis EL, Consentini, CEC, Mingoti RD, Belli RS, Baruselli PS**. 2017. Effect of time of permanence (7 vs. 8) of different intravaginal progesterone devices (PRIMER® Multidose or Monodose) on conception rate of dairy cows. *Anim Reprod*, 14. (abstract).

**El-Zarkouny SZ, Stevenson JS**. 2004. Resynchronizing estrus with progesterone or progesterone plus estrogen in cows of unknown pregnancy status. *J Dairy Sci*, 87:3306-3321.

**Freitas BG**. 2015. Influência do desenvolvimento corporal na resposta aos programas de sincronização para inseminação artificial em tempo fixo em novilhas Nelore de 14 meses de idade. São Paulo, SP: Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo. Dissertation.

**Garcia MR, Amstalden M, Williams SW, Stanko RL, Morrison CD, Keisler DH, Nizielski SE, Williams GL**. 2002. Serum leptin and its adipose gene expression during pubertal development, the estrous cycle, and different seasons in cattle. *J Anim Sci*, 80;2158-2167.

**Jinks EM, Smith MF, Atkins JA, Pohler KG, Perry GA, MacNeil MD, Roberts AJ, Waterman RC, Alexander LJ, Geary TW**. 2013. Preovulatory estradiol and the establishment and maintenance of pregnancy in suckled beef cows. *J Anim Sci*, 91:1176-1185.

**Lonergan P**. 2011. Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology*, 76:1594-1601.

**Mantovani AP, Reis EL, Gacek F, Bó GA, Binelli M, Baruselli PS**. 2005. Prolonged use of a progesterone-releasing intravaginal device (CIDR®) for induction of persistent follicles in bovine embryo recipients. *Anim Reprod*, 2:272-277.

**Mantovani AP, Nichi M, Sá Filho MF, Ayres H, Vettorato LF, Bó GA, Baruselli PS**. 2010. Follicular growth and plasma progesterone patterns in *Bos indicus* x *Bos taurus* heifers submitted to different PGF2 α/progesterone-based synchronization protocols. *Anim Reprod*, 7:91-96.

**Marques MO, Ribeiro Júnior M, Silva RCP, Sá Filho MF, Vieira LM, Baruselli PS**. 2012. Ressincronização em bovinos de corte. *In*: 5º Simpósio Internacional de Reprodução Animal Aplicada (SIRAA), 2012, Londrina, PR, Brazil. Londrina: UEL. pp. 82-92

**Marques MDO, Morotti F, Silva CB, Ribeiro Júnior M, Silva RCP, Baruselli PS, Seneda MM**. 2015. Influence of category - heifers, primiparous and multiparous lactating cows − in a large-scale resynchronization fixed-time artificial insemination program. *J Vet Sci*, 16:367-371.

**Martins CM, Elliff FM, Ferreira RM, Mingoti RD, Reis PO, Baruselli PS**. 2017. Factors that affect pregnancy rate to TAI and to natural breeding of 14 months old Nelore heifers . *Anim Reprod*, 14. (abstract).

**Mendanha MF, Sala RV, Reis TANPS, Carvalho FJ, Guimarães LHC, Macedo GG, Sá Filho MF, Baruselli PS**. 2012. Momento da administração de prostaglandina e número de utilizações do dispositivo de progesterona na eficiência da IATF em novilhas girolando. *In*: Anais da XXVI Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE), Fóz do Iguaçu, PR, Brazil.Foz do Iguaçu: SBTE. pp. 370. (abstract).

**Meneghetti M, Sá Filho OG, Peres RFG, Lamb GC, Vasconcelos JLM**. 2009. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows I: basis for development of protocols. *Theriogenology*, 72:179-189.

**Mihm M, Curran N, Hyttel P, Knight PG, Boland MP, Roche JF**. 1999. Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. *J Reprod Fertil*, 116:293-304.

**Mingoti RD, Freitas BG, Ramos RS, Bastos MR, Teixeira AA, Rezende MLG, Castro MW, Faquim A, Sá Filho MF, Baruselli PS**. 2016. Pregnancy rate to TAI Nelore (*Bos indicus*) protocol submitted to 3 or 4 managements using Sincrogest or CIDR New and Reused. *Anim Reprod*, 13:415. (abstract).

**Motta JCL, Colli MHA, Penteado L, Bayeux BM, Mingoti RD, Bó GA, Lugo LC, Rezende RG, Baruselli PS.** 2016. Pregnancy rate to FTAI in Nelore and crossbreed heifers submitted to J-Synch protocol (6 days)**.** *Anim Reprod*, 13:401. (abstract).

**Munro RK, Moore NW**. 1985. Effects of progesterone, oestradiol benzoate and cloprostenol on luteal function in the heifer. *J Reprod Fertil*, 73:353–359.

**Nogueira GP**. 2004. Puberty in South American *Bos indicus* (Zebu) cattle. *Anim Reprod Sci*, 82/83:361-372.

**Penteado L, Rezende RG, Mingoti RD, Colli MHA, Sá Filho MF, Santos FB, Lemos Motta JC, Bayeux BM, Vieira LM, Baruselli PS**. 2016. Pregnancy rate of Nelore cows submitted to resynchronization starting 14 or 22 days after prior FTAI. *Anim Reprod*, 13:450. (abstract).

**Pereira MH, Sanches CP, Guida TG, Rodrigues AD, Aragon FL, Veras MB, Borges PT, Wiltbank MC, Vasconcelos JL**. 2013. Timing of prostaglandin F2α treatment in an estrogen-based protocol for timed artificial insemination or timed embryo transfer in lactating dairy cows. *J Dairy Sci*, 96:2837-2846.

**Peres RFG, Claro Junior I, Sá Filho OG, Nogueira GP, Vasconcelos JLM**. 2009. Strategies to improve fertility in *Bos indicus* postpubertal heifers and nonlactating cows submitted to fixed-time artificial insemination. *Theriogenology*, 72:681-689.

**Pessoa GA, Martini AP, Chaiben MFC, Vieira LM, Girotto RW, Pugliesi G, Santin T, Rubin MIB, Baruselli PS, Sá Filho MF**. 2015. Adjustment of the estradiol benzoate dose in the resynchronization protocol with unknown pregnancy status in suckled beef cows. *Anim Reprod*, 12:, 610. (abstract).

**Pugliesi G, Miagawa BT, Paiva YN, França MR, Silva LA, Binelli M**. 2014. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: how early can we detect pregnancy? *Biol Reprod*, 91:95, 1-12.

**Pugliesi G, Rezende RG, Silva JCB, Lopes E, Nishimura TK, Baruselli PS, Madureira EH, Binelli M**. 2017. Uso da ultrassonografia Doppler em programas de IATF e TETF em bovinos. *Rev Bras Reprod Anim*, 41:140-150.

**Rezende RG, Freitas BG, Mingoti RD, Colli MHA, Carvalho JBP, Sá Filho MF, Motta JCL, Macedo GG, Baruselli PS**. 2016. Follicular dynamics of Nelore cows submitted to resynchronization 14 days after TAI using injectable P4 for synchronization of follicular wave. *Anim Reprod*, 13:375. (abstract).

**Roche JF, Austin EJ, Ryan M, O'Rourke M, Mihm M, Diskin MG**. 1999. Regulation of follicle waves to maximize fertility in cattle. *J Reprod Fertil Suppl*, 54:61-71.

**Sá Filho MF, Ayres H, Ferreira RM, Marques MO, Reis EL, Silva RCP, Rodrigues CA, Madureira EH, Bó GA, Baruselli PS**. 2010. Equine chorionic gonadotropin and gonadotropin-releasing hormone enhance fertility in a norgestomet-based, timed artificial insemination protocol in suckled Nelore (*Bos indicus*) cows. *Theriogenology*, 73, 651-658.

**Sá Filho MF, Penteado L, Reis EL, Reis TANPS, Galvão KN, Baruselli PS**. 2013. Timed artificial

insemination early in the breeding season improves the reproductive performance of suckled beef cows. *Theriogenology*, 79:625-632.

**Sá Filho MF, Marques MO, Girotto R, Santos FA, Sala RV, Barbuio JP, Baruselli PS**. 2014. Resynchronization with unknown pregnancy status using progestin-based timed artificial insemination protocol in beef cattle. *Theriogenology*, 81:284-290.

**Santos, MH.** 2016. Desenvolvimento de protocolos para IATF com 7 dias de permanência do CIDR® em fêmeas Nelore. 80f. Dissertation (Masters in Science) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2016.

**Siqueira LG, Areas VS, Ghetti AM, Fonseca JF, Palhao MP, Fernandes CA, Viana JH**. 2013. Color Doppler flow imaging for the early detection of nonpregnant cattle at 20 days after timed artificial insemination. *J Dairy Sci*, 96:6461-6472.

**Stevenson JS, Cartmill JA, Hensley BA, El-Zarkouny SZ**. 2003. Conception rates of dairy cows following early not-pregnant diagnosis by ultrasonography and subsequent treatments with shortened Ovsynch protocol. *Theriogenology*, 60:475-483.

**Thatcher WW, Bazer FW, Sharp DC, Roberts RM**. 1986. Interrelationships between uterus and conceptus to maintain corpus luteum function in early pregnancy: sheep, cattle, pigs and horses. *J Anim Sci*, 62:25-46.

**Vasconcellos GSFM, Vieira LM, Guerreiro BM, Freitas BG, Kametani MK, Miranda MS, Moreira RC, Vianna FP, Baruselli PS**. 2014. Evaluation of the resynchronization efficiency using GnRH or estradiol benzoate at the beginning of the protocol in lactating Holstein cows. *Anim Reprod*, 11:361. (abstract).

**Vieira LM, Sá Filho MF, Pugliesi G, Guerreiro BM, Cristaldo MA, Batista EOS, Freitas BG, CarvalhoFJ, Guimaraes LHC, Baruselli PS**. 2014. Resynchronization in dairy cows 13 days after TAI followed by pregnancy diagnosis based on corpus luteum vascularization by color doppler. *Anim Reprod*, 11:378. (abstract).

**Vieira LM, Guerreiro BM, Batista EOS, Freitas BG, Sá Filho MF, Vasconcellos GSFM, Soares JG, Bragança LGM, Plá SS, Baruselli PS**. 2015. Estradiol, GnRH or their association at the beginning of the resynchronization protocol results in similar reproductive efficiency in dairy cows. *Anim Reprod*, 12:635. (abstract).

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# Practical implications of sperm selection techniques for improving reproduction

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## Abstract

Sperm selection techniques are needed to separate spermatozoa from seminal plasma and extender for *in vitro* fertilization (IVF) and to improve sperm quality for a range of assisted reproduction techniques. Apart from sperm washing, which removes some but not all of the seminal plasma, the selection techniques that are currently used are mainly swim-up and colloid centrifugation; filtration through Sephadex columns or glass wool is seldom used in the field. Although swim-up can be used to prepare sperm samples for IVF, the low recovery rate and lack of selection for sperm quality other than motility make this technique ineffective for routine use. Colloid centrifugation is used to prepare semen for all types of assisted reproduction. The method has been scaled-up for voluminous ejaculates e.g. from stallion and boar, and scaled-down to accommodate small volumes of thawed semen (e.g. from bull). Sperm quality and fertility are improved, as shown in laboratory assays and in various fertility trials. Some normal spermatozoa are lost during the selection process but overall the advantages of improved longevity and fertility in the selected spermatozoa outweigh the disadvantages. Since spermatozoa are separated from bacteria in the ejaculate, it may be possible to reduce antibiotic usage in semen extenders. New applications of colloid centrifugation include extracting camelid spermatozoa from viscous seminal plasma, selecting spermatozoa with condensed chromatin (i.e. with fewer free thiols), and using the number of spermatozoa passing through the colloid as a diagnostic tool to indicate male fertility.

**Keywords**: chromatin integrity, colloid centrifugation, extended longevity, fertility, single layer centrifugation.

## Introduction

Fertility in some species e.g. dairy cattle, has been declining in recent decades (Rodriguez-Martinez *et al*., 2007; López-Gatius, 2013). This decline may be partly due to negative energy balance in high yielding dairy cows (Diskin and Morris, 2008), but other factors may also be involved. For example, the number of bull spermatozoa included in an insemination dose has decreased, especially for sexed sperm samples (Seidel, 2014); two million spermatozoa may not be sufficient to give good pregnancy rates for all bulls (Andersson *et al*., 2004). In addition, changes have been made to freezing protocols, such as the avoidance of extenders containing egg yolk (Leite *et al*., 2010; Röpke *et al*., 2011) and increasing pre-freezing equilibration times (Leite *et al*., 2010; Shahverdi *et al*., 2014). In horse breeding, there has been a decrease in the foaling rate after artificial insemination (AI) to approximately 65% (Rota *et al*., 2004) but this could be due to the more widespread use of cooled shipped semen nowadays, or to a real decrease in fertility. The porcine AI industry still uses liquid (fresh) semen for AI in preference to cryopreserved semen (unless for export), because pregnancy rates and litter sizes are perceived to be better with fresh semen, despite considerable progress in developing cryopreservation protocols that result in more consistent sperm survival (Roca *et al*., 2006.)

One point is certain, however: good quality gametes are needed to produce good quality blastocysts (Vandaele and Van Soom, 2011; Morrell *et al*., 2016). Embryo quality, assessed using the guidelines established by the International Embryo Transfer Society (Stringfellow and Seidel, 1998), was linked with oocyte quality following embryo production *in vitro* in pigs (Chen *et al.,* 2012), cattle (O´Hara *et al*., 2014; Saini *et al*., 2015), stallions (Colleoni *et al*., 2011 and human patients (Kurosawa *et al*., 2016).

Many studies have attempted to define the factors contributing to "good quality" spermatozoa: *in vivo*, bull sperm morphology was correlated with pregnancy rate (Attia *et al*., 2016; boar sperm fertility was linked independently with morphology, active mitochondria, beat cross frequency and oscillatory frequency (Schultze *et al*., 2013). The DNA integrity of stallion spermatozoa was correlated with pregnancy rate (Cuervo-Arango *et al*., 2009; Morrell *et al*., 2014c; Barrier Battut *et al*., 2016), whereas Underwood *et al*. (2010) linked the stresses of sex-sorting and re-freezing bull spermatozoa with low pregnancy rate. Ohlweiler *et al*. (2013) showed that there was no difference in fertilization rate when semen from bulls of low vigor was used to fertilize good and poor quality oocytes, whereas for semen of high vigor, the blastocyst rate was higher for good quality oocytes than for poor quality oocytes.

Sperm selection can be used to improve sperm quality (Morrell and Rodriguez-Martinez, 2009), which is the subject of this review, although oocyte quality and sperm/uterine interaction are vital components in fertilization and embryo production. In this review, the impact of sperm quality on fertility will be described briefly, followed by a discussion of sperm selection

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techniques, especially colloid centrifugation. After describing the practical uses of colloid centrifugation, the implications of sperm selection by this method for animal breeding will be presented.

**Relationship between sperm quality and fertility**

Sperm quality is a term used to define the predicted functionality of a sperm population. It incorporates a number of components that affect the ability of the spermatozoa to move through the reproductive tract to locate and interact with the oocyte. These factors include sperm motility, morphology, membrane integrity, degree of apoptosis, acrosome integrity, capacitation, mitochondrial membrane potential, chromatin integrity, chromatin maturity, production of reactive oxygen species, and ability to bind to the zona pellucida. Relationships have been found between a number of these parameters and fertility, either *in vitro* or following AI; examples have been described in detail in another review (Morrell and Rodriguez-Martinez, 2009) and are summarized in Table 1.

Table 1. Examples of the relationship between parameters of sperm quality and fertility in various species.

| Parameter | Reference |
|---|---|
| Normal morphology (cattle) | Wiltbank and Parrish, 1986; Menon *et al*., 2011 |
| Normal morphology (pigs) | Tsakmakidis *et al*., 2010 |
| Normal morphology (horses) | Parlevliet *et al*., 1999; Kavak *et al*., 2004 |
| Chromatin integrity (boars) | Lopez Fernandez *et al*., 2008 |
| Chromatin integrity (horses) | Love and Kenny, 1998 |
| Chroamtin integrity (bulls) | Karoui *et al*., 2012 |
| Acrosome integrity (buffalo) | Ahmed *et al*., 2003 |
| Acrosome integrity (bulls) | Al-Mahkzoomi *et al*., 2008 |
| Plasma membrane integrity (bulls) | Christiansen *et al*., 2011 |
| Mitochondrial membrane potential | Pena *et al*., 2009 |
| Reactive oxygen species | Aitken *et al*., 2012 |

**Sperm selection techniques**

Since sperm quality appears to affect fertility and therefore reproduction, selecting sperm sub-populations with certain characteristics known to be associated with fertility should lead to increased reproductive efficiency. Several sperm selection techniques are available, as reviewed previously (Morrell *et al*., 2016). The purpose of sperm selection techniques is to separate specific sperm sub-populations from the ejaculate and to separate them from seminal plasma. In this respect, laboratory procedures mimic events occurring in the female reproductive tract, whereby normal spermatozoa interact with parts of the female reproductive tract whereas abnormal spermatozoa do not (Suarez, 2007). The hypothesis is that this interaction with the female reproductive tract enables unsuitable spermatozoa to be removed, thereby prevented them from reaching the oocyte. Since laboratory selection techniques mimic a biological activity, they are known as "biomimetic".

Selection can be done by migration e.g. swim-up, by filtration e.g. through Sephadex or glass wool, or by colloid centrifugation. Cell sorting can be done in a flow cytometer or magnetic cell sorter, but these techniques are mostly too time-consuming to be used routinely, except on the larger semen collection stations. Swim-up is used mostly to prepare spermatozoa for *in vitro* fertilization or ICSI; colloid centrifugation is used for these applications and also to prepare semen for AI, especially the scaled-up versions (Morrell *et al*., 2009b), and for cryopreservation (Hoogewijs *et al*., 2011).

In the swim-up technique, collection medium is pipetted on top of extended semen in a centrifuge tube, which is then placed in an incubator. Motile spermatozoa move away from the rest of the sample and can be harvested from the collection medium. However, selection is only for sperm motility; there is no selection for normal morphology or chromatin integrity (Somfai *et al*., 2002). The procedure takes 45-60 min and results in the recovery of approximately 10% of the spermatozoa. Whilst such small sperm numbers may be adequate for IVF, they are clearly impractical when preparing spermatozoa for AI.

In filtration, spermatozoa pass through a column of Sephadex or glass wool; spermatozoa with damaged membranes or defective acrosomes bind to the Sephadex and are retained on the column (Bussalleu *et al*., 2008). Although a higher recovery rate is achieved with this technique than with swim-up, sperm quality may only be improved if there is a high proportion of damaged spermatozoa in the original sperm sample. This technique is used mainly in the laboratory or for research: there are no reports of its routine use under field conditions.

Colloid centrifugation involves layering extended semen over colloid e.g. silane-coated silica, in a centrifuge tube, followed by centrifugation for 20 min at a low g force, typically 300 g. After centrifugation, the sperm pellet contains mostly highly motile, morphologically normal spermatozoa with intact membranes and good chromatin integrity (Johannisson *et al*., 2009) that are not producing hydrogen peroxide (Morrell *et al*., 2017). Immotile or damaged spermatozoa are retained at the semen:colloid interface while seminal plasma remains above the colloid (boar semen; Kruse *et al*., 2011). This technique has been used to prepare stallion spermatozoa in the field for AI (Morrell *et al*., 2011a, 2014b) as well as bull and boar spermatozoa for IVF (Thys *et al*., 2009; Sjunnesson *et al*., 2013) or stallion spermatozoa for ICSI (Colleoni *et al*., 2011. Single Layer centrifugation (SLC) is a

modification of density gradient centrifugation (DGC) in which only one layer of colloid is used i.e. no density gradient is present. The advantage of SLC is that it is easier to use than DGC, requiring fewer steps in the preparation, and it can be scaled-up to process large volumes of semen e.g. 15-20 ml per 50 ml tube for stallion semen (Morrell *et al*., 2009b) or up to 150 ml per 500 ml tube for boar semen (Morrell *et al*., 2011b). It can also be scaled-down to process only 250 µl thawed bull semen on 1 ml colloid in a 15 ml tube (Abraham *et al*., 2016); a higher sperm yield is obtained if a 15 ml centrifuge tube is used rather than a small (1.5 ml) tube.

## Practical uses of colloid centrifugation

*For* in vitro *fertilization or intracytoplasmic sperm injection*

When preparing spermatozoa for IVF or ICSI it is important to remove seminal plasma, which contains decapacitating factors, and to replace the cryopreservation medium with capacitating medium or fertilization medium. The simplest way to do this is by centrifugation to pellet the spermatozoa, followed by resuspension of the sperm pellet in the new medium. However, all the spermatozoa, including the dead and dying or abnormal spermatozoa, will appear in the pellet since there is no selection for normal spermatozoa with this method. Such centrifugation can also cause damage to intact spermatozoa and may result in the release of reactive oxygen species that attack sperm membranes and DNA.

An important consideration for IVF or ICSI is that the selection of normal spermatozoa that occurs in the female reproductive tract is not present (Suarez, 2007) potentially allowing abnormal spermatozoa or those with damaged chromatin to fertilize the oocyte (Rath *et al*., 2008). Although the oocyte is fertilized and activated, there may be problems due to the damaged chromatin during embryo development or even after implantation (Katari *et al*., 2009; Evenson, 2016). It can be speculated that this may be one of the reasons why so many conceptuses or early pregnancies are lost in cattle, since approximately 90% of dairy cow oocytes are fertilized but less than half of these result in the birth of a calf (Diskin and Morris, 2008; Lopez-Gatius, 2013).

Karoui *et al*. (2012 observed that sperm DNA fragmentation (chromatin dispersion test) could be used to identify the least fertile bulls in a cohort study. Evenson (2016) reviewed many studies on the association between %DFI (evaluated by the Sperm Chromatin Structure Assay) and fertility; he reported threshold values for %DFI in various species above which there was likely to be an impact on fertility as: pigs 6%, bulls 10-20%, horses 28%, humans 25-30%.

*For artificial insemination*

The ejaculate contains a heterogeneous population of spermatozoa at different stages of maturity, some of which may be abnormal. These abnormal spermatozoa are included in the insemination dose and thus may reduce the number of normal spermatozoa reaching the oviducts to below the threshold required in order to ensure fertilization. This is particularly important for cattle where the number of spermatozoa in the insemination dose has been reduced to a small fraction of those in the original ejaculate. Alternatively, spermatozoa with damaged chromatin may compete with those with intact chromatin to fertilize the oocyte. Selecting normal spermatozoa with good chromatin integrity would be an obvious way of circumventing this problem.

Does colloid centrifugation really overcome this problem? This question can be answered using stallion spermatozoa as an example. Usually stallion semen is inseminated within 24-36 h after semen collection, being stored in the meantime at approximately 6°C (Varner *et al*., 1987). There are occasional reports of pregnancies being achieved after longer periods of cooled storage but this is not usual within the equine breeding industry and not feasible for some stallions. For some stallion semen, it is not possible to cool it at all; it must be inseminated within a short time of collection in order to obtain pregnancies. Preparing stallion semen by SLC allows even spermatozoa from these so-called “poor coolers” to be cooled and transported to other stud farms for insemination after 24 h (Morrell *et al*., 2011a). Chromatin integrity is higher in SLC-selected samples, and this integrity is maintained during storage (Table 2). Semen from normal stallions, processed by SLC, survive for at least 96 h after semen collection (Richter *et al*., 2016) and have good fertilizing ability (Lindahl *et al*., 2012. In a controlled trial inseminating mares with cooled sperm samples 24 h after semen, a higher number of mares were pregnant with the SLC-selected spermatozoa (54/78) than with control (unselected; 37/82) sperm samples (Morrell *et al*., 2014b). The SLC-selected samples showed an increased proportion of morphologically normal, membrane-intact spermatozoa with good chromatin integrity than control samples (Morrell *et al*., 2009a; 2010). Interestingly, production of hydrogen peroxide is markedly decreased in these SLC-selected samples (Morrell *et al*., 2017), which may contribute to the longer survival and retention of fertilizing capacity compared to control sperm samples. *In vitro* fertilizing capacity is also enhanced in SLC-selected sperm samples; the number of SLC-selected boar spermatozoa used for IVF had to be drastically reduced to avoid polyspermy (Sjunnesson *et al*., 2013). The SLC preparation technique has also been used to prepare stallion spermatozoa for ICSI (Table 3).

Table 2. DNA fragmentation index (%DFI) in stallion sperm samples prepared by Single Layer Centrifugation compared to uncentrifuged controls immediately after preparation and again after 24 h storage at 6ºC.

| Time after preparation (number) | Uncentrifuged | SLC |
|---|---|---|
| 0 h (n = 144) | 15.1 ± 9.3 | 10.2 ± 8.9 |
| 24 h (n = 77) | 26.0 ± 14.0 | 13.9 ± 14.0 |

Modified from Morrell *et al*. (2010).

Table 3. Blastocyst development after intracytoplasmic sperm injection of equine oocytes, using spermatozoa prepared by colloid centrifugation.

| Treatment | No. injected oocytes | No. blastocysts | Development to blastocyst (%) |
|---|---|---|---|
| DGC fertile (control) | 17 | 4 | 21 |
| SLC fertile (control) | 21 | 4 | 19 |
| DGC infertile | 104 | 15 | 21 |
| SLC infertile | 107 | 21 | 30 |

Note: DGC = density gradient centrifugation, SLC = Single Layer Centrifugation; fertile and infertile refer to the fertility status of the two stallions used as a source of spermatozoa. From Colleoni *et al*. (2011).

*In cryopreservation*

Stallion spermatozoa selected by SLC show better cryosurvival than non-selected sperm samples (Hoogewijs *et al*., 2011) and may have longer post-thaw survival (Hoogewijs *et al*., 2012). This latter attribute may also be a result of decreased hydrogen peroxide production in the thawed sperm samples, although reactive oxygen species production was not measured in their study. However, in studies with boar spermatozoa, SLC-selected spermatozoa were found to produce less hydrogen peroxide than non-selected spermatozoa (Martinez-Alborcia *et al*., 2012); and showed increased cryosurvival (Martinez-Alborcia *et al*., 2012, 2013). A higher mitochondrial membrane potential was observed in frozen-thawed SLC-selected bull spermatozoa compared to controls (Nongbua *et al*., 2017). In a previous study with fresh bull spermatozoa, chromatin integrity was significantly better in SLC-selected samples compared to controls (Goodla *et al*., 2014).

*Removal of bacteria*

When considering semen quality, one factor that is often overlooked is the bacterial content of the semen (Morrell and Wallgren, 2014). Almost all ejaculates become contaminated with bacteria during semen collection and subsequent handling (Maes *et al*., 2008); a negative correlation has been found between bacterial contamination above a certain threshold and litter size in pigs (Maroto Martin *et al*., 2010). Therefore, antibiotics are added to semen extenders to control microbial growth. Nowadays, there is increasing awareness of antibiotic resistance arising from the widespread use of antibiotics, both in human and veterinary medicine (Catry *et al*., 2010). Genes for resistance spread between bacteria in different host species (Johansson *et al*., 2004). There are anecdotal accounts that many bacteria found in semen are resistant to the antibiotics that are added to semen extenders; therefore the widespread practice of adding antibiotics to semen extenders may contribute to the problem of antibiotic resistance. An alternative to antibiotics would be to physically separate spermatozoa from bacteria in semen. Colloid centrifugation removes, or substantially reduces, bacterial contamination in boar (Morrell and Wallgren 2011) and stallion semen (Morrell *et al*., 2014a). Such an alternative would seem to be an excellent solution to avoid the further development of antimicrobial resistance, particularly when one considers the scale of usage of antibiotic-containing extenders in the semen production industry.

**Recent developments with colloid centrifugation**

*Camelid semen*

Processing camelid semen is known to be a problem due to the high viscosity of the seminal plasma, which also prevents penetration of cryoprotectants into spermatozoa during freezing. One solution to this problem has been to use enzymes to break down the viscous seminal plasma, although there is concern that the enzymes might damage the spermatozoa. Llama semen processed SLC after treatment with enzymes resulted in the production of hatched blastocysts in IVF (Trassoras *et al*., 2012). Obviously it would be preferable to avoid enzymes completely, if at all possible, but separating the spermatozoa from the enzyme-containing medium after a short exposure appears to be an option. A further development is that dromedary camel semen, processed by SLC after mechanical breakdown of the viscous seminal plasma by gentle pipetting, survived freezing and thawing (Malo *et al*., 2017), and pregnancies have been obtained.

*Indicators of fertility*

It was reported previously that the number of spermatozoa passing through the colloid could be used as an indicator of potential fertility of the original ejaculate. This was found to be the case for stallions where the yield of spermatozoa after SLC was found to be related to the pregnancy rate of mares inseminated with cooled semen from the same ejaculates (Morrell *et al*., 2014b). In a similar study with boar semen, the number of spermatozoa passing through the colloid was

related to the pregnancy rate in inseminated sows (Martinez-Alborcia *et al*., 2017). Thus, SLC could be used to provide a rapid means of identifying less highly fertile boars without the necessity of waiting for the outcome of AI trials, to allow their removal from the breeding pool at an early stage.

*Additional marker of chromatin integrity*

Chromatin integrity can be evaluated either by means of the Sperm Chromatin Structure Assay or by the Chromatin Dispersion Assay. However, there are other methods of evaluating chromatin structure, e.g. by measuring free thiols (as an indicator of lack of disulfide bond formation). Preliminary studies with bull spermatozoa indicate that SLC-selected sperm samples have fewer free thiols than control samples (Fig. 1). If this result is confirmed in a larger sample size, it could indicate that SLC selects for mature spermatozoa with highly condensed chromatin.

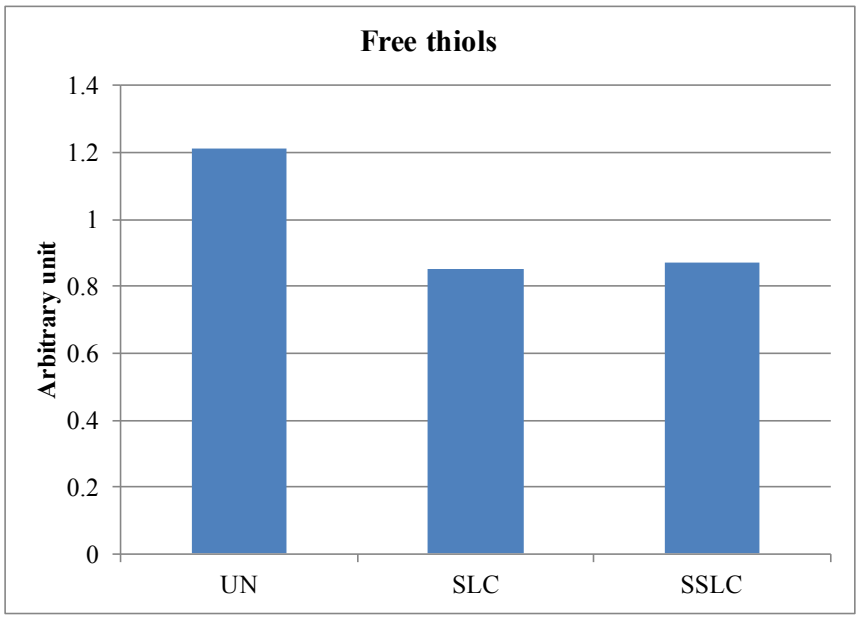


Figure 1. free thiols in SLC and control bull sperm samples. Note: un = control (uncentrifuged); SLC = single layer centrifugation; SSLC = stored for 24 h befor SLC.

**Implications of colloid centrifugation to animal breeding**

As described in the preceding section, colloid centrifugation has a number of practical uses; there are many advantages and some disadvantages to the technique, as summarized in Table 4. The main advantages are the improved sperm quality and fertilizing ability, which are retained for longer in the selected sperm samples than in unselected ones. However, since only good quality spermatozoa are selected, there can be a considerable reduction in sperm numbers during processing, depending on the quality of the original ejaculate (Hoogewijs *et al*., 2011; Martinez-Alborcia *et al*., 2013), and some good quality spermatozoa may be lost. The advantages outweigh the disadvantages: the vast majority of the spermatozoa recovered are functional and retain this functionality because they are not in the presence of damaged spermatozoa and hydrogen peroxide. Since the spermatozoa are highly fertile, it may be possible to reduce the number in the insemination dose. The small volume of colloid needed for the scaled-down version is economically more attractive than the larger volume when preparing spermatozoa for IVF or ICSI.

The ability to separate spermatozoa from seminal plasma without causing damage opens up the possibility of manipulating semen samples to obtain desirable attributes (de Graaf *et al*., 2008). This could involve substituting seminal plasma from an animal of low fertility (or poor freezability) with that from a high fertility (or good freezability) male (Morrell *et al*., 2014d; Nongbua *et al*., 2016a).

The possibility of separating spermatozoa from bacteria without the use of antibiotics has the potential to be of considerable importance, to slow the development of antimicrobial resistance. The animal breeding industry uses very large amounts of antibiotics at present, and there is a need to reduce this non-therapeutic use, which should be possible with SLC. The industry would also like to have more reliable indicators of a male´s potential fertility; by extrapolating from the results with stallion semen, SLC appears to offer such an indicator

Table 4. Advantages and disadvantages of colloid centrifugation for sperm preparation.

| Advantages | Disadvantages |
|---|---|
| Species-specific colloid formulations are available commercially | Colloid is expensive |
| Technique is not complicated; little training is required | Centrifuge required; 20 minute centrifugation time |
| Highly fertile spermatozoa recovered | Some good quality spermatozoa are lost |
| Motile, morphologically normal spermatozoa with intact membranes are selected | |
| Spermatozoa retain fertilizing ability for longer than unselected spermatozoa | |
| Spermatozoa with damaged DNA are removed → embryo development not impeded | |
| Fewer spermatozoa may be needed in insemination dose | |
| Reduction in bacterial contamination can obviate the need for antibiotics | |
| May be used as an indicator of fertility | |
| Can be used to extract camel spermatozoa from viscous seminal plasma | |
| May select spermatozoa with highly condensed chromatin (fewer free thiols) | |

## Conclusions

Sperm selection techniques are used when preparing sperm samples for assisted reproduction. Single layer centrifugation is especially beneficial since it selects the best quality spermatozoa from the rest of the ejaculate and also separates them from contaminating bacteria. The technique is simple to use, even in the field, although some good quality spermatozoa are also lost during processing. The technique shows promise for handling semen from unusual species such as camelids, and may be used in other contexts such as to differentiate between elite males and less highly fertile ones. It has advantages over migration or filtration techniques in selecting for sperm chromatin integrity.

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## References

**Abraham MC, Johannisson A, Morrell JM**. 2016. Effect of sperm preparation on development of bovine blastocyst in vitro. *Zygote,* 24:825-830.

**Ahmed Z, Anzar M, Shahab M, Ahmad N, Andrabi SMH**. 2003. Sephadex and sephadex ion-exchange filtration improves the quality and freezability of low-grade buffalo semen ejaculates. *Theriogenology,* 59:1189-1202.

**Aitken RJ, De Iuliis GN, GibbZ, Baker MA**. 2012. New horizons on an old landscape - oxidative stress, DNA damage and apoptosis in the male germ line. *Reprod Domest Anim,* 47(suppl. 4):7-14.

**Al-Makhzoomi A, Lundeheim N, Håård M, Rodriguez-Martinez H**. 2008. Sperm morphology and fertility in progeny-tested AI dairy bulls in Sweden. *Theriogenology*, 70:682-691.

**Andersson M, Taponen J, Koskinen E, Dahlbom M**. 2004. Effect of insemination with doses of 2 or 15 million frozen-thawed spermatozoa and semen deposition site on pregnancy rate in dairy cows. *Theriogenology*, 61:1583-1588.

**Attia S, T Katila T, Andersson M**. 2016 The effect of sperm morphology and sire fertility on calving rate of Finnish Ayrshire AI bulls. *Reprod Domest Anim,* 51:54-58.

**Barrier Battut I, Kempfer A, Becker J, Lebailly L, Camugli S, Chevrier L**. 2016. Development of a new fertility prediction model for stallion semen, including flow cytometry *Theriogenology* 86:1111-1131.

**Bussallou E, Pinart E, Rivera MM, Arlas X, Briz M, Sancho S, Garcia-Gil N, Bassols J, Prunedo A, Yeste M, Casas I, Rigau, T, Rodriguez-Gil JE, Bonet S**. 2008. Effects of filtration of semen doses from subfertile boars through neuter Sephadex columns. *Reprod Domest Anim* 43:48-52.

**Catry B, van Duijkere E, Pomba MC, Greko C, Moreno MA, Pyörälä, S, Ruzauskas M, Sanders P, Threlfall EJ, Ungemach F, Törnele K, Munoz-Madero C, Torren-Edo J**, **Scientific Advisory Group on Antimicrobials (SAGAM)**. 2010. Reflection paper on MRSA in food-producing and companion animals: epidemiology and control options for human and animal health. *Epidemiol Infect*, 138:626-644.

**Chen TY, Stott Pathorn RZ, Bouwman EG, Langendijk P.** 2012. Undernutrition during early follicle development has irreversible effects on ovulation rate and embryos *Reprod Fertil Dev*, 24:886-892

**Christiansen P, Labouriau R, Birck A, Boe-Hansen GB, Pedersen J, Borchersen S**. 2011. Relationship among seminal quality measures and field fertility of young dairy bulls using low-dose inseminations. *J Dairy Sci,* 94:1744-1754.

**Colleoni S, Lagutina I, Rodriguez-Martinez H, Lazzari G, Galli C, Morrell JM**. 2011. New techniques for selecting spermatozoa for equine assisted reproduction. *J Equine Vet Sci,* 31:536-541.

**Cuervo-Arango J, Aguilar J, Newcombe JR**. 2009. Effect of type of semen, time of insemination relative to ovulation and embryo transfer on early equine embryonic vesicle growth as determined by ultrasound. *Theriogenology*, 71:1267-1275.

**de Graaf SP, Leahy T, Marti J, Evans G, Maxwell, WMC**. 2008. Application of seminal plasma in sex-sorting and sperm cryopreservation. *Theriogenology,* 70:1360-1363.

**Diskin MG, Morris DG**. 2008. Embryonic and early foetal losses in cattle and other ruminants. *Reprod Domest Anim,* 43(suppl. 2):260-267.

**Evenson DP**. 2016. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Anim Reprod Sci,* 169:56-75.

**Goodla L, Morrell JM, Yusnizar Y, Stålhammar H, Johannisson A**. 2014. Quality of bull spermatozoa after preparation by single layer centrifugation. *J Dairy Sci,* 97:2204-2212.

**Hoogewijs M, Morrell JM, Van Soom A, Govaere J, Johannisson A, Piepers P, De Schauwer C, de Kruif A, De Vliegher S**. 2011. Sperm selection using single layer centrifugation prior to cryopreservation can increase post thaw sperm quality in stallions. *Equine Vet J*, 43(suppl. 40):35-41.

**Hoogewijs M, Piepers S, Govaere J, De Schauwer C, de Kruif, A Morrell JM**. 2012. Sperm longevity following pre-freeze sperm selection. *J Equine Vet Sci*, 32:489. doi: http://dx.doi.org/10.1016/j.jevs.2012.06.048

**Johannisson A, Morrell JM, Thorén J, Jonsson M, Dalin A-M, Rodriguez-Martinez H**. 2009. Colloidal centrifugation with Androcoll-E™ prolongs stallion sperm motility, viability and chromatin integrity. *Anim Reprod Sci*, 116:119-128.

**Johansson A, Greko C, Engström BE, Karlsson M**. 2004. Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of Clostridium perfringens from poultry, and distribution of tetracycline resistance genes. *Vet Microbiol,* 99:251-257.

**Karoui S, Díaz C, González-Marín C, Amenabar ME, Serrano M, Ugarte E, Gosálvez J, Roy R, López-Fernández C, Carabaño MJ**. 2012. Is sperm DNA fragmentation a good marker for field AI bull fertility? *J Anim Sci,* 90:2437-2449.

**Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, Gaughan JP, Coutifaris C, Sapienza C.** 2009. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Hum Mol Genet,* 18:3769-3778.

**Kavak A, Lundeheim N, Aidnik M, Einarsson S. 2004.** Sperm morphology in Estonian and Tori breed stallions. *Acta Vet Scand.* 45:11-8.

**Kruse R, Dutta PC, Morrell JM**. 2011. Colloid centrifugation removes seminal plasma and cholesterol from boar spermatozoa. *Reprod Fertil Dev*, 23:858-865.

**Kurosawa H, Utsunomiya H, Shiga N, Takahashi A, Ihara M, Ishibashi M, Nishimoto M, Watanabe Z, Abe H, Kumagai J, Terada Y, Igarashi H, Takahashi T, Fukui A, Suganuma R, Tachibana M, Yaegashi N.** 2016. Development of a new clinically applicable device for embryo evaluation which measures embryo oxygen consumption. *Hum Reprod*, 31:2321-2330.

**Leite TG, Vale-Filho VR, Arruda RP, Andrade AF, Emerick LL, Zaffalon FG, Martins JA,Andrade VJ.** 2010. Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. *Anim Reprod Sci,* 120:31-38.

**Lindahl J, Dalin A-M, Stuhtmann G, Morrell JM.** 2012. Stallion spermatozoa selected by Single Layer Centrifugation are capable of fertilization after storage for up to 96h at 6˚C prior to artificial insemination. *Acta Vet Scand*, 54:40-45.

**Lopez-Fernandez C, Perez-Llano B, Garcia-Casado P, Sala R, Gosalbez A, Arroyo F, Fernandez JL, Gosalvez J.** 2008. Sperm DNA fragmentation in a random sample of the Spanish boar livestock. *Anim Reprod Sci,* 103:87-98.

**López-Gatius F**. 2013. Approaches to increase reproductive efficiency in artificially inseminated dairy cows. *Anim Reprod*, 10:143-147.

**Love CC, Kenney RM**. 1998. The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. *Theriogenology*, 50:955-972.

**Maes D, Nauwynck H, Rijsselaere T, Mateusen B, Vyt P, de Kruif A, Van Soom A**. 2008. Diseases in swine transmitted by artificial insemination: an overview. *Theriogenology* 70:1337-1345.

**Malo C, Crichton E, Morrell JM, Pukazhenthi B, Skidmore J.** 2017. Single layer centrifugation of fresh dromedary camel semen improves sperm quality and in vitro fertilization capacity compared with simple sperm washing. *Reproduction in Domestic Animals In Press*

**Maroto Martin LO., Cruz Munoz E, de Cupere F, van Driessche E, Echemendia-Blanco D, Machado Rodriguez J, Beeckmans S**. 2010. Bacterial contamination of boar semen affects the litter size. *Anim Reprod Sci*, 120:95-104.

**Martinez-Alborcia MJ, Morrell JM, Parrilla I, Barranco I, Vázquez JM, Martinez EA, Roca J**. 2012. Improvement of boar sperm cryosurvival by using single-layer colloid centrifugation prior to freezing. *Theriogenology*, 78:1117-1125.

**Martinez-Alborcia MJ, Morrell JM, Barranco I, Maside C, Gil MA, Parrilla I, Vazquez JM, Martinez EA, Roca J**. 2013. Suitability and effectiveness of single layer centrifugation using Androcoll-P in the cryopreservation protocol for boar spermatozoa. *Anim Reprod Sci*, 140:173-179.

**Menon AG, Barkema HW, Wilde R, Kastelic JP, Thundathil JC**. 2011. Associations between sperm abnormalities, breed, age, and scrotal circumference in beef bulls. *Can J Vet Res,* 75:241-247.

**Morrell JM, Johannisson A, Dalin A-M, Rodriguez-Martinez H**. 2009a. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. *Reprod Domest Anim*, 44:512-517.

**Morrell JM, Johannisson A, Dalin A-M, Rodriguez-Martinez H**. 2009b. Single Layer Centrifugation with Androcoll™-E can be scaled-up to allow large volumes of stallion ejaculate to be processed easily. *Theriogenology,* 72:879-884.

**Morrell JM, Rodriguez-Martinez H**. 2009. Biomimetic techniques for improving sperm quality in animal breeding: a review. *Open Androl J*, 1:1-9. doi**:** 10.2174/1876827X00901010001.

**Morrell JM, Rodriguez-Martinez H, Johannisson A.** 2010. Single Layer Centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size: data from 3 breeding seasons. *Equine Veterinary Journal* 42: 579-585.

**Morrell JM, Mari G, Kutvölgyi G, Meurling S, Iacono E, Mislei B, Rodriguez-Martinez H**. 2011a. Spermatozoa from stallion ejaculates processed by Single Layer Centrifugation with Androcoll™-E are capable of fertilization after artificial insemination. *Reprod Domest Anim*, 46:642-645.

**Morrell JM, van Wienen M, Wallgren M**. 2011b. Single Layer Centrifugation can be scaled-up further to process up to ml150semen . *ISRN Vet Sci*, 2011:art.183412. doi:10.5402/2011/183412.

**Morrell JM, Wallgren M**. 2011. Removal of bacteria from boar ejaculates by Single Layer Centrifugation can reduce the use of antibiotics in semen extenders. *Anim Reprod Sci,* 123:64-69.

**Morrell JM, Klein C, Lundeheim N, Erol E, Troedsson MHT.** 2014a. Removal of bacteria from stallion semen by colloid centrifugation. *Anim Reprod Sci*, 145:47-53.

**Morrell JM, Richter J, Martinsson G, Stuhtmann G, Hoogewijs M, Roels K, Dalin A-M.** 2014b. Pregnancy rates are higher after artificial insemination with cooled stallion spermatozoa selected by Single Layer Centrifugation than with control semen doses. *Theriogenology*, 82:1102-1105.

**Morrell JM, Stuhtmann G, Meurling S, Lundgren A, Winblad C, Macias Garcia B, Johannisson A**. 2014c. Sperm yield after Single Layer Centrifugation with Androcoll-E is related to the potential fertility of the original ejaculate *Theriogenology,* 81:1005-1011.

**Morrell JM, Georgakas A, Lundeheim N, Nash D, Davis-Morel MCG, Johannisson A.** 2014d. Effect of heterologous and homologous seminal plasma on stallion sperm quality. *Theriogenology* 82:176-183.

**Morrell JM, Wallgren M**. 2014. Alternatives to antibiotics in semen extenders: a review. *Pathogens*, 3:934-946.

**Morrell JM, Sabes Alsina M., Abraham MC, Sjunnesson Y**. 2016. Practical applications of sperm selection techniques for improving reproductive efficiency. *Anim Reprod,* 13:340-345.

**Morrell JM, Lagerquist A, Humblot P, Johannisson A.** 2017. Effect of single layer centrifugation on reactive oxygen species and sperm mitochondrial membrane potential in cooled stallion semen. *Reprod Fertil Dev*, 29:1039-1045.

**Nongbua T, Johannisson A, Edman A, Morrell JM**. 2016a. Effect of bovine seminal plasma on sperm motility and chromatin integrity. *Anim Reprod Sci*, 169:113-114.

**Nongbua T, Yongzhi G, Edman A, Humblot P, Morrell JM**. 2016b. Effect of bovine seminal plasma on bovine endometrial epithelial cells proliferation in culture. *Theriogenology*, 86:348. (Abstract).

**Nongbua T, Johannisson A, Edman A, Morrell JM**. 2017. Effects of Single Layer Centrifugation (SLC) on bull spermatozoa prior to freezing on post-thaw semen characteristics. *Reprod Domest Anim*, 52:596-602.

**O'Hara L, Forde N, Kelly AK, Lonergan P**. 2014. Effect of bovine blastocyst size at embryo transfer on day 7 on conceptus length on day 14: can supplementary progesterone rescue small embryos? *Theriogenology*, 81:1123-1128.

**Ohlweiler LU, Brum DS, Leivas FG, Moyses AB, Ramos RS, Klein N., Mezzalira JC, Parlevliet JM, Colenbrander BC**. 1999. Prediction of first season stallion fertility of 3-year-old Dutch Warmbloods with prebreeding assessment of percentage of morphologically normal live sperm. *Equine Vet J*, 31:248-251.

**Parlevliet JM, Colenbrander B.** 1999. Prediction of first season stallion fertility of 3-year-old Dutch Warmbloods with prebreeding assessment of percentage of morphologically normal live sperm. *Equine Vet J.* 31:248-51.

**Pena FJ, Rodrıguez Martınez H, Tapia JA, Ortega Ferrusola C, Gonzalez Fernandez L, Macıas Garcıa B**. 2009. Mitochondria in mammalian sperm physiology and pathology:a review. *Reprod Domest Anim*, 44:345-349.

**Rath D, Schuberth HJ, Taylor U**. 2008. Sperm interaction from insemination to fertilization. *Reprod Domest Anim*, 43(suppl. 5):2-11.

**Richter J, Stuhtmann G, Martinsson G, Sieme H, Morrell JM**. 2016. Stallion sperm survival is increased after colloid centrifugation compared to sperm washing, or extension only, in INRA82 or INRA96. *J Equine Vet Sci*, 43:S78-S79.

**Roca J, Vazquez JM, Gil MA, Cuello C, Parrilla I, Martínez EA.** 2006. Challenges in pig artificial insemination. *Reprod Domest Anim*, 41(suppl. 2):43-53.

**Rodríguez-Martínez H, Hultgren J, Båge R, Bergqvist A-S, Svensson C, Bergsten C**, **Lidfors L, Gunnarsson S, Algers B, Emanuelson U, Berglund B, Andersson G, Håård M, Lindhé B, Stålhamm H, Gustafsson H**. 2007. Reproductive performance in high-producing dairy cows: can we sustain it under current practice? *In*: IVIS Reviews in Veterinary Medicine Ithaca NY: International Veterinary Information Service (www.ivis.org). 23 pp. Available on: https://pub.epsilon.slu.se/3502/1/RodriguezM_etal_090122.pdf..

**Röpke T, Oldenhof H, Leiding C, Sieme H, Bollwein H, Wolkers WF**. 2011. Liposomes for cryopreservation of bovine sperm. *Theriogenology,* 76:1465-1472

**Rota A, Furzi C, Panzani D, Camillo F**. 2004. Studies on motility and fertility of cooled stallion spermatozoa. *Reprod Domest Anim,* 39:103-109.

**Saini N, Singh MK, Shah SM, Singh KP, Kaushik R,**

**Manik RS, Singla SK, Palta P, Chauhan MS**. 2015 Developmental competence of different quality bovine oocytes retrieved through ovum pick-up following in vitro maturation and fertilization. *Animal*, 9:1979-1985.

**Schulze M, Ruediger K, Mueller K, Jung M, Wella C, Reissmann M**. 2013. Development of an in vitro index to characterize fertilizing capacity of boar ejaculates *Anim Reprod Sci*, 140:70-76

**Seidel GE Jr**. 2014. Update on sexed semen technology in cattle. *Animal*, 8(suppl. 1):160-164.

**Shahverdi A, Rastegarnia A, Topraggaleh TR**. 2014. Effect of extender and equilibration time on post thaw motility and chromatin structure of buffalo bull (*Bubalus bubalis*) spermatozoa. *Cell J*, 16:279-288.

**Sjunnesson YC, Morrell JM, González R**. 2013. Single Layer Centrifugaction-selected boar spermatozoa are capable of fertilization in vitro**.** *Acta Vet Scand,* 55:20-26

**Somfai T, Bodo S, Nagy S, Papp AB, Ivancsics J, Baranyai B, Gocza E, Kovacs K**. 2002. Effect of swim-up and Percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. *Reprod Domest Anim,* 37:285-290.

**Stringfellow D, Seidel SM**. 1998. *Manual of the International Embryo Transfer Society*. 3rd ed. Savoy, IL, USA: International Embryo Transfer Society.

**Suarez S**. 2007. Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. *Reprod Fertil Dev*, 19:103-110.

**Thys M, Vanadele L, Morrell JM, Mestach J, Van Soom A, Hoogewijs M, Rodriguez-Martinez H**. 2009. In vitro fertilising capacity of frozen-thawed bull spermatozoa separated by colloidal centrifugation through single-layer or gradients. *Reprod Domest Anim*, 44:390-394.

**Trasorras V, Giuliano S, Chaves G, Neild D, Agüero A, Carretero M, Pinto M, Baca Castex C, Alonso A, Rodríguez D, Morrell J, Miragaya M**. 2012. In vitro embryo production in Llamas (*Lama glama*) from in vivo matured oocytes with raw semen processed with Androcoll-e using defined embryo culture media. *Reprod Domest Anim*, 47:562-567.

**Tsakmakidis IA, Lymberopoulos AG, Khalifa TA**. 2010. Relationship between sperm quality traits and field-fertility of porcine semen. *J Vet Sci,* 11:151-4

**Underwood SL, Bathgate R, Ebsworth M, Maxwell W.M.C., Evans G**. 2010. Pregnancy loss in heifers after artificial insemination with frozen-thawed, sex-sorted, re-frozen-thawed dairy bull sperm. Anim Reprod Sci, 118:7-12

**Vandael L,Van Soom A**. 2011. Intrinsic factors affecting apoptosis in bovine in vitro produced embryos. *Verh K Acad Geneeskd Belg,* 73:79-104.

**Varner DD, Blanchard TL, Love CC, Garcia MC, Kenney RM**. 1987. Effects of semen fractionation and dilution ratio on equine spermatozoa motility parameters. *Theriogenology*, 28:709-723.

**Wiltbank JN, Parish NR**. 1986. Pregnancy rate in cows and heifers bred to bulls selected for semen quality. *Theriogenology,* 25:779-783.

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# Basic and practical aspects of pregnancy establishment in cattle

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## Abstract

Bovine embryos are increasingly produced using reproductive technologies, e.g. ovum pick-up (OPU), *in vitro* embryo production (IVP) and embryo transfer (ET). Such *in vitro* manipulated embryos are known to deviate in several aspects compared to *in vivo* derived embryos. Pregnancy establishment in cattle involves timed biological events including fine-tuned communication, initiated and carried out by both the embryo and the endometrium. This stimulates research to increase the understanding of events and interactions taking place in the uterus after embryo transfer, both from a biological and systems biology point of view. This review will focus on the biological events taking place during early embryonic development, implantation and beginning of placentation, with focus on transfer of *in vitro* produced embryos, including a systems biology approach for selection of superior embryo recipients.

**Keywords**: embryo recipient quality, *in vitro* embryo production, pregnancy establishment, reproductive technologies, systems biology.

## Introduction

For food producing animals such as cattle, reproductive health plays an important role in relation to farm economy and is essential for improving breeding progress. Several biological and management-related factors have an impact on successful fertilization, establishment and maintenance of pregnancy, such as oocyte competence, semen quality, hormone levels, nutrition, milk production and parity. In cattle, the fertilization rate after insemination or natural mating has been estimated to 90%, with an average calving rate of 55% (Sartori *et al*., 2002; Diskin *et al*., 2006; Wiltbank *et al*., 2016). Most pregnancy losses occur during the early embryonic period, and the biological reasons include both the embryo and the mother in terms of oocyte and embryo quality, impaired function of the endometrium and sub-optimal embryo-maternal communication.

The last decades have shown an increase in both development and use of new technologies for improved reproductive efficiency and for improving the genetic merit of a herd. Among these are ovum pick-up (OPU) followed by oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) also using sex-sorted semen, *in vitro* embryo culture (IVC) and embryo transfer (ET). In 2015, more than 520,000 *in vivo* derived and 427,000 *in vitro* produced bovine embryos were transferred worldwide (Perry, 2016). *In vitro* produced embryos are still showing impaired results compared to *in vivo* derived, both in tolerance to cryopreservation, pregnancy rates and early embryo loss (Alberto *et al*., 2013). To improve the output of OPU-IVP-ET, the identification and selection of high-quality oocyte donors and embryo recipients as well as improved culture systems, resulting in improved embryo development and pregnancy rates, would make these technologies even more attractive.

One important aspect of the increasing use of artificially produced and *in vitro* manipulated embryos is a growing knowledge about the delicate interactions existing between the embryo and the endometrium. These interactions are dependent on the quality of both parts, the embryo and the endometrium.

This review will focus on the biological events taking place during early embryonic development, implantation and beginning of placentation, with focus on transfer of *in vitro* produced embryos, including a systems biology approach for selection of superior embryo recipients.

## Establishment of the pregnancy

### *Early embryonic development*

In the few hours after ovulation of the mature oocyte, gamete interaction occurs in the ampulla of the oviduct. At that time greater portions of the oocyte's zona pellucida is devoid of cumulus cells and the fertilizing spermatozoon has easy access to the zona surface (Hyttel *et al*., 1988). At 2-3 h after ovulation, the spermatozoon has undergone the acrosome reaction, penetrated zona pellucida, and both the sperm head and tail are found in the ooplasm. Consequently, the oocyte is activated resulting in resumption of meiosis and release of the cortical granules that elicits zona hardening and the resulting block to polyspermic penetration. Over the coming hours, the second polar body is abstracted, and smooth endoplasmic reticulum is attracted to the sperm head as well as to the retained maternal chromatin in order to build nuclear envelopes of the two pronuclei. Around 5-7 h after ovulation, the pronuclei have developed to spherical structures that

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migrate to close apposition around 15-19 h after ovulation. During this developmental period the S-phase of the first mitotic cell cycle takes place (Laurincik *et al*., 1994). At around 20 h, the nuclear envelopes are dissolved into the smooth endoplasmic reticulum, and the maternal and paternal chromosomes align during formation of the prophase and metaphase of the first mitotic division.

Around 24 h after ovulation, the cleavage to the 2-cell stage occurs followed by two rather short cell cycles bringing the embryo to the 8-cell stage. This fourth cell cycle is considerably longer and includes major activation of the embryonic genome during which process the blastomere's nucleoli are activated to initiate transcription and ribosome production (King *et al*., 1988; Laurincik *et al*., 2000). Around the morula stage (16-32 cells), the embryo passes from the oviduct to the uterus.

With the activation of the embryonic genome, the embryo achieves the ability to form different cell lineages, and at the 16-32-cell stage compaction of the morula occurs. The pluripotent inner cell mass (ICM) is formed, and the outer trophectoderm (TE) is sealed by tight junctions and desmosomes and develops water transporting capacity leading to the formation of the fluid filled blastocyst. Around day 8 after ovulation, the blastocyst hatches from the zona pellucida.

Around the time of hatching, the ICM develops into an upper pluripotent epiblast and a lower epithelium, the hypoblast (Maddox-Hyttel *et al*., 2003). The latter epithelium develops on the inside of the blastocyst to form an inner lining of both the epiblast and the TE. Around day 12, the TE covering of the epiblast (Rauber's layer) becomes extremely thin, and finally the epiblast penetrates the TE and becomes incorporated into the outer layer of the conceptus, which at this time of development is two-layered. The epiblast establishes the embryonic disc of pluripotent cells that will give rise to the embryo proper.

Around day 14 after ovulation, the embryonic disc initiates gastrulation by formation of the primitive streak through which cells start to ingress to form endoderm and mesoderm. The endoderm becomes integrated in the hypoblast whereas the mesoderm forms a loose mesenchyme between the epiblast and the hypoblast as well as the longitudinal rod, the notochord (Maddox-Hyttel *et al*., 2003). The epiblast located longitudinally over the notochord will develop into the neural ectoderm, whereas the more peripheral parts of the overlying epiblast will develop into the surface ectoderm. Along with the development of the embryonic disc and gastrulation, the conceptus elongates to a length of several centimeters on day 15 after ovulation at the time of embryonic-maternal signaling.

The implantation process begins day 16-18, i.e. after embryo elongation, with placentation starting around day 22. Implantation and placentation occur at the caruncular areas of the endometrium. Reduction or loss of an anti-adhesive molecule from the uterine endometrium is necessary to prepare for implantation. Also, an appearance of cell-adhesion molecules (e.g. Integrins) on the surface of the endometrium is important to attachment and invasion (Mansouri-Attia *et al*., 2009). Fusions of placental cotyledons with the caruncles form placentomes that are involved in fetal-maternal gas exchange and provision of nutrients.

*Maternal-embryonic communication*

From the mid-1970ies, the view on maternal-embryonic communication during early pregnancy has changed. At that time, the function of the oviduct and the uterine horn was considered to simply keep and transport the gametes under proper conditions, but with no specialized signaling and interaction. In some ways, the success with *in vitro* embryo production (IVP) during the same period only supported such a view, also because these processes could be performed in a well-defined medium at the right temperature and atmosphere. However, the reports on Large Offspring Syndrome (LOS) from the early 1990ies challenged this view (e.g. Lazarri *et al*., 2002), even though the solution seemed to be a simple reduction in serum concentration in IVP media.

Parallel to the technological omics-revolution, a quite new insight into the mechanisms has gradually appeared, illustrating that the events are complex, interactive and fine-tuned involving both the embryo and the endometrium. The complex biological events have been expressed as "Thus, a receptive endometrium, an implantation-competent blastocyst and a synchronized dialogue between maternal and embryonic tissues is a pre-requisite for successful implantation" (Salilew-Wondim *et al*., 2012).

Today, it is well described that there is "cross-talking" going on at many points of the reproductive process in different species (e.g. Oestrup *et al*., 2011; Alminana *et al*., 2012; Forde *et al*., 2012a; Salilew-Wondim *et al*., 2012; Ulbrich *et al*., 2013; Fazelli and Holt, 2016; Klein, 2016). This cross-talking reflects quality at several points with some species differences, but the overall pattern is the same. In the oviduct, it involves timing of ovulation, tuba collection of the ovulated cumulus-oocyte-complex, the oocyte-sperm interactions and oviductal cilia movements to transport the zygote and early embryo (Avilés *et al*., 2015; Maillo *et al*., 2016a). *In vivo*, it has been demonstrated in mice that unfertilized vs fertilized oocytes trigger a different gene response in the oviduct (Lee *et al*., 2002), and in the horse are unfertilized oocytes not allowed to pass the utero-tubal junction, probably influenced by missing embryo secretion of prostaglandin E2 (Klein, 2016). Today, the active role of the oviduct is becoming more and more clear with a growing list of activities both as preparation for an embryo to arrive as well as reactions to its actual presence (Artemenko *et al*., 2015; Gonella-Diaza *et al*., 2015, 2017; Maillo *et al*., 2016b). One challenge for this kind of work seems to be able to detect the changes when they are only caused by a single embryo (Maillo *et al*., 2015).

Some of the cross-talking involves presence of semen in the reproductive tract. The first reports were *in vitro* studies with oviduct epithelial cells responding to

the presence of spermatozoa in cattle (Ellington *et al*., 1993) and in horse (Thomas *et al*., 1995). Since then, a number of studies have demonstrated various reactions in the oviduct from the arrival of sperm (Maillo *et al*., 2016b), also showing that the oviduct seems to be able to differentiate between X- and Y-bearing spermatozoa (Alminana *et al*., 2014). Seminal plasma is described to have a positive influence on embryo development, implantation and pregnancy in different species, although there are mixed results with respect to bovine (Maillo *et al*., 2016b). In several species has an ovulation-inducing-factor been demonstrated in seminal fluid that induces ovulation and possibly influences the properties of the progesterone-producing capacity of the corpus luteum (Ratto *et al*., 2012).

In the uterus, the interaction and communication continues through hatching and implantation. The hatching is a mutual process, where the embryo is active with collapses and re-expansions, the zona is thinned by secretion of trypsin from the endometrium, and small projections of specialized cellular TE through the zona induce the focal opening(s) through one of which the embryo escapes, aided by TE proteinase secretion (Kirkegaard *et al*., 2013). In human, an active embryo secretion seems to be essential for establishment of the pregnancy (Brosens et *al*., 2014).

Mechanisms of communication between embryo and recipient are also under investigation. It has been known for a longer time that an immunological reaction is initiated by the alien sperm/embryo that alert the maternal immune system, however without rejection of the gametes (Fazelli and Holt, 2016). Therefore, it must be well under control, and interferon (IFN-t) secreted by the elongating conceptus (around day 15 in cattle to block luteal regression) is considered to be one of the most important candidates in regulating the immune response (Oestrup *et al*., 2011). During the last ten years a messenger system including small vesicles has appeared (miRNA, exosomes; Saadeldin *et al*., 2015) that is so far speculated to be one way of communication between the maternal tract and the gametes (Maillo *et al*., 2016b).

Taken together, it is getting more and more clear that there is a testing of process and product quality going on at several points during the early phase of the reproductive process – and that this has regulatory consequences, sometimes resulting in embryonic/fetal loss, sometimes leading to long-term effects observed in the offspring (e.g. Fleming *et al*., 2015).

*Preparations in the embryo recipient*

During the estrous cycle, the cow prepares herself for a potential pregnancy by establishing an environment supporting embryonic development (Pohler *et al*., 2012; Atkins *et al*., 2013). High levels of estradiol produced by ovarian follicles during proestrous and estrous result in increased uterine blood flow, promote uterine contractions assisting sperm transport, and affect the uterine environment increasing the chance of embryo survival, possibly by sustaining embryonic growth and the development of placenta (Madsen *et al*., 2015) The ovulatory follicle's production of estradiol and the subsequent corpus luteum's progesterone production are now also shown to be related to changes in tissue, cells and secretions in both the oviduct (Gonella-Diaza *et al*., 2015, 2017) and the uterus (Binelli *et al*., 2015). These changes are also stimulated by the conceptus (INF-t, prostaglandins, cortisol), and the endometrium (prostaglandins, cortisol), and altogether are affecting uterine physiology and receptivity (Forde *et al*., 2009, 2011). The uterine preparation for pregnancy includes thickening of the endometrium and development of uterine muscles and glands, including production of uterine histotroph required for embryo survival and growth. The histotroph consists of different substances (e.g. amino acids, carbohydrates, proteins, lipids) transported into the uterine lumen by endometrial epithelial cells from the blood and as specific secretory products encoded by genes expressed in the endometrial epithelium (Bazer, 1975; Gray *et al*., 2001; Forde *et al*., 2014). Embryo development in uterine-gland knock-out sheep has shown to be retarded from day 9-14, indicating the importance of the endometrial epithelial secretions (Gray *et al*., 2002).

On the molecular level, the progesterone-induced changes in gene expression in the endometrium result in up- or down-regulation of genes involved in processes such as cellular transports, cell cycle, cell growth and differentiation, lipogenesis, metabolism, cell adhesion, signal transduction, biosynthesis and immune response (Bauersachs *et al*., 2006; Forde *et al*., 2009, 2011, 2012b; Simmons *et al*., 2009; Binelli *et al*., 2015). The progesterone-induced changes in the endometrial transcriptome seem to be independent of pregnancy status up to the time of conceptus elongation and maternal recognition of pregnancy on day 15 (Forde *et al*., 2011), but an embryo-dependent programming of endometrial function has recently been demonstrated already from day 7 in the estrous cycle (Sponchiado *et al*., 2017). Also on day 7, Binelli *et al*. (2015) showed endometrial gene expressions in the uterine horn contralateral to an AI that illustrated the readiness and preparedness of the endometrium to receive an incoming embryo. In addition, pretransfer endometrial biopsies from heifers on day 7 of the estrous cycle revealed differences in gene expression according to pregnancy diagnosis in the following cycle after transfer of *in vivo* derived embryos (Salilew-Wondim *et al*., 2010) and *in vitro* produced embryos (Ponsuksili *et al*., 2012). Differences in endometrial gene expression have shown to be related to the chance of pregnancy in fertility-classified heifers on day 14 (Minten *et al*., 2013). For pregnant animals, a difference in endometrial gene expression was demonstrated on day 17 between fertile and subfertile dairy cow strains (Walker *et al*., 2012). This information on endometrial transcriptomic profiles express the status of uterine receptivity at least at a given time, but it is still not known how well it can characterize that animal (or strain).

As stated above, progesterone stimulates and

maintains endometrial functions necessary for a pregnancy establishment. Insufficient plasma progesterone concentrations have been linked to poor embryo development and maternal-embryonic signaling in terms of decreased INF-t production by the embryo (Mann and Lemming, 2001), and high levels of progesterone have shown to advance conceptus elongation (Carter *et al*., 2008). Several studies have investigated the effect of exogenous post-insemination progesterone treatment in heifers and cows, but results differ with regard to a potential beneficial effect on embryo development and pregnancy outcome (Sreenan and Diskin, 1983; Mann and Lamming, 1999; Yan *et al*., 2016).

### *In vitro* produced embryos

*In vitro* produced embryos are in general less robust in establishing pregnancies compared to their *in vivo* counterparts. In terms of cryopreservation, *in vitro* produced embryos have decreased survival rates post-thawing after conventional freezing and post-warming after vitrification (Papadopoulos *et al*., 2002). Furthermore, there are morphological differences such as an overall lower cell number of both TE and ICM compared to *in vivo* derived embryos (Farin *et al*., 1995). The increasing use of *in vitro* produced embryos adds a challenge to the successful outcome of ET, and prediction of embryo quality prior to transfer could result in a major improvement of pregnancy rate.

The impact of culture conditions during IVP on bovine embryos is still not sufficiently investigated, and therefore the knowledge of how this parameter is reflected in the pregnancy establishment can be improved. It is, however, well known that culture conditions and IVP media have a high impact on embryonic gene expression and hence on embryo quality. Addition of serum to the embryo culture medium was earlier considered to cause abnormalities during pregnancy and at calving (LOS; Lazzari *et al*., 2002; Chen *et al*., 2013). This problem was to a high degree reduced considerably after use of serum-reduced or serum-free media, but the incidence of LOS still creates concern in commercial IVP also today. Other aspects of using reproductive technologies have been identified, such as an increase in the frequency of epigenetic abnormalities that may lead to congenital malformation syndromes including higher birth weight (DeBaun *et al*., 2003). Therefore, thorough control of conditions in the IVP laboratory as well as the protocol for embryo production could improve embryo quality and thus the overall IVP result. One example is the conditions during shipping of oocytes, a procedure that has increased enormously the last decade, that has been demonstrated to have a large impact on embryo development (Hashem *et al*., 2017). Furthermore, freezing and vitrification protocols also influence epigenetics and should be taken into consideration in attempting to further reduce the LOS incidences.

It is therefore still a major objective to increase the knowledge of embryo quality assessment to improve establishment of pregnancies and healthy live born offspring in both human assisted reproductive technologies as well as the cattle industry. Presently, few predictors are available for embryo quality evaluation. The subjective characterization based mainly on embryo morphology and kinetics is an insufficient predictor for IVP embryo survival and pregnancy outcome; however, it is still the most commonly used method. Other available *in vitro* techniques to assess embryo quality are hatching rates, degree of apoptosis (Antunes *et al*., 2010), chromosome analyses and to a lesser degree gene expression techniques (Jakobsen *et al*., 2006). More recent technologies are focusing more on developing new non-invasive methods, such as infrared spectroscopy to predict embryo quality and sex after analysis of spent culture medium (Gomez *et al*., 2008; Munoz *et al*., 2014). To monitor kinetics during early embryonic development assessing cleavage rate, synchronicity and even-sized blastomeres through time-lapse systems is widely used within the human IVF industry and has increased in the recent years (e.g. Kovacs, 2014). Metabolomics and proteomics profiling technologies may allow determination of the metabolites associated with embryo viability and thereby predicting pregnancy outcome (Gardner *et al*., 2001; Sturmey *et al*., 2010). Metabolomics, the newest emerging technology, includes analysis of spent culture media for the small non-coding RNA, including microRNA (Rødgård *et al*., 2015), demonstrated to be important to embryogenesis and development (Goossens *et al*., 2013). Therefore, new screening tools based on embryo quality and viability assessment could have a huge impact on prediction of pregnancy rates and the efficiency of ET programs with IVP embryos.

### Application of quantitative genetics for selection of embryo recipients

While the heritability of fertility traits is low (0.05), OPU-IVP related traits (number of cumulus-oocyte complexes, quality of cumulus-oocyte complexes, number and proportion of cleaved embryos at day 4, and number and proportion of total and transferable embryos at day 7 of culture) have shown a heritability from 0.10 to 0.25 (Merton *et al*., 2009). Thus, genetic improvement could be faster for ART traits such as OPU-IVP-ET than for conventional fertility traits in dairy cattle (Kadarmideen *et al*., 2000). Alternative approaches have to select successfully for this type of traits, and a possibility is to use molecular genomic information in animal breeding including genomic selection (GS; Kadarmideen, 2014).

Genomic selection is based on computing genomic estimated breeding values (GEBVs) by estimating SNP effects from prediction equations (Meuwissen *et al*., 2001). Two major advantages of genomic selection compared with traditional selection based on pedigree and phenotype alone are: (i) it can select animals accurately early in life using their GEBVs from genomic prediction and (ii) increased accuracy of GEBVs for phenotypes that are very difficult or expensive to measure including fertility

(Hayes *et al*., 2013). Genomic selection has made a substantial economic impact (Kadarmideen, 2014; Suravajhala *et al*., 2016) increasing the genetic gain or income with 60-120% compared to traditional methods of progeny testing and performance tests in livestock (Schaeffer, 2006; Pryce and Daetwyler, 2012). Numerous genomic prediction models have been developed, which vary according to several assumptions regarding the variance of traits of interest and the distribution of the SNP effect.

The principles behind genomic selection of recipient cows is the same as for any traditional phenotype in cattle breeding. Genomic prediction accuracy gets better with increasing trait heritability and reference population used for calculating GEBVs. Before applying any quantitative genetics or breeding method, the high quality reproductive data recording traits will be an essential step. Thus, it is important to set up a reference population where a large number of recipient cattle are recorded for pregnancy rates after OPU-IVP-ET. Once a good reference population is collected, the Best Linear Unbiased Prediction (BLUP) methods (e.g. GBLUP and single-step BLUP) (Henderson, 1975; Meuwissen *et al*., 2001; Aguilar *et al*., 2010; Goddard *et al*., 2011) can produce GEBVs for all these animals. Based on the ranking of GEBVs, breeding animals can then be selected and used in OPU-IVP-ET, increasing the recipient cattle reproductive performance, i.e. pregnancy success.

In this context, integrative systems biology could provide useful information for GS. IVP and ET performances are complex traits, so more holistic approaches are needed to identify biological mechanisms and biomarkers associated with these traits. Systems biology approaches identify the emerging properties of a biological system (Kitano, 2002; Breitling, 2010). Therefore, systems biology represents a promising tool for OPU-IVP-ET related traits. The function of the endometrium is important to the chance of embryo implantation in recipient cows. Therefore, transcriptomic of endometrial tissue can be used to perform systems biology analysis of recipient cow performances (Orozco-Lucero and Sirard, 2014). The biological mechanisms and the molecular markers identified through the systems biology analysis of transcriptomic data could be integrated in multi-omics analysis, for example eQTL studies. The eQTL studies integrate transcriptomic with genomic data to identify genomic regions controlling the expression of a certain gene (Westra and Franke, 2014). If the expression of the genes is associated to the trait of interest, the eQTL identified can be indirectly associated with the traits (Ponsuksili *et al*., 2010). The information provided by integrative systems biology studies, for example eQTLs, could be included in GS methods utilizing functional information.

The understanding of the biological basis of the molecular regulation of the complex reproductive events is improving significantly these years. One main reason is the fruitful interaction between the biological and molecular sciences that form a very strong platform, and the combined action "can provide a strong continuation to the understanding of traits related to ARTS" (Mazzoni *et al*., 2017).

## Conclusions and perspectives

The establishment of a pregnancy in cattle includes interactions between the embryo and the mother at all stages of the pregnancy. Timed biological events and communication take place to maintain and accomplish the pregnancy and to reach the final goal, i.e. the birth of a healthy live offspring. The embryo and the endometrium handle and adapt to different challenges and conditions, based on signaling from both sides and influenced by e.g. the use of reproductive technologies and the origin of the embryo. Also, many factors affect uterine receptivity and finally, the synchrony between the embryo and recipient is important. Therefore, to improve *in vitro* embryo production conditions and to increase the output from OPU-IVP-ET, it is highly relevant to continue the research into the complex biological mechanisms, but also to further investigate and develop methods based on a systems biology approach. One ultimate goal for this combined action will be to obtain a tool to improve selection of recipients for transfer of *in vitro* produced embryos.

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## References

**Aguilar I, Misztal I, Johnson D, Legarra A, Tsuruta S, Lawlor TJ**. 2010. Hot topic: a unified approach to utilize phenotypic, full pedigree and genomic information for genetic evaluation of Holstein final Score. *J Dairy Sci*, 93:743-752.

**Alberto ML, Meirelles FV, Perecin F, Ambrósio CE, Favaron PO, Franciolli ALR, Mess AM, Dos Santos JM, Rici REG, Bertolini M, Miglino MA**. 2013. Development of bovine embryos derived from reproductive techniques. *Reprod Fertil Dev*, 25:907-917.

**Alminana C, Heath PR, Wilkinson S, Sanchez-Osorio J, Cuello C, Parrilla I, Gil MA, Vazuez JL, Vazquez JM, Roca J, Martinez EA, Fazeli A**. 2012. Early developing pig embryo mediate their own environment in the maternal tract. *PLoS One*, 7:e33625. doi.org/10.1371/journal.pone.0033625.

**Alminana C, Caballero, Heath PR, Maleki-Dizaji S, Parrilla I, Cuello C, Gil MA, Vazquez JL, Vazquez JM, Roca J, Martinez EA, Holt WV, Fazeli A**.. 2014. The battle of sexes starts in the oviduct: modulation of oviductal transcriptome by X and Y-bearing spermatozoa. *BMC Genomics*, 15:293. doi**:** 10.1186/1471-2164-15-293

**Antunes G, Chaveiro A, Santos P, Marques A, Jin HS, Moreira da Silva F**. 2010. Influence of apoptosis in bovine embryo's development. *Reprod Domest Anim*,

45:26-32.

**Artemenko K, Horáková J, Steinberger B, Besenfelder U, Brem G, Bergquist J, Mayrhofer C**. 2015. A proteomic approach to monitor the dynamic response of the female oviductal epithelial cell surface to male gametes. *J Proteomics*, 113:1-14. doi: 10.1016/j.jprot.2014.09.016.

**Atkins JA, Smith MF, MacNeil MD, Jinks EM, Abreu FM, Alexander LJ, Geary TW**. 2013. Pregnancy establishment and maintenance in cattle. *J Anim Sci*, 91:722-733.

**Avilés M, Coy P, Rizos D**. 2015. The oviduct: a key organ for the success of early reproductive events. *Anim Front*, 5:25-31.

**Bauersachs S, Ulbrich SE, Gross K, Schmidt SEM, Meyer HHD, Wenigerkind H, Vermehren M, Sinowatz F, Blum H, Wolf E**. 2006. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction*, 132:319-331.

**Bazer FW**. 1975. Uterine protein secretions: relationship to development of the conceptus. *J Anim Sci*, 41:1376-1382.

**Binelli M, Scolari SC, Pugliesi G, Van Hoeck V, Gonella-Diaza AM, Andrade SCS, Gasparin GR, Coutinho LL**. 2015. The transcriptome signature of the receptive bovine uterus determined at early gestation. *PLoS On2*: 10:e0122874. doi: 10.1371/journal.pone.0122874.

**Breitling R**. 2010. What is systems biology? *Front Physiol*, 1:9. doi: 10.3389/fphys.2010.00009.

**Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, Steel JH, Christian M, Chan YW, Boomsma CM, Moore JD, Hartshorne GM, Sućurović S, Mulac-Jericevic B, Heijnen CJ, Quenby S, Koerkamp MJ, Holstege FC, Shmygol A, Macklon NS**. 2014. Uterine selection of human embryos at implantation. *Sci Rep,* 4:3894. doi: 10.1038/srep03894.

**Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF, Lonergan P**. 2008. Effect of increasing progesterone concentration from day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reprod Fertil Dev*, 20:368-375.

**Chen Z, Hagen DE, Elsik CG, Ji T, Morris CJ, Moon LE, Riviera RM**. 2013. Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction. *Proc Natl Acad Sci USA*, 112:4618-4623.

**DeBaun MR., Niemitz EL, Feinberg AP**. 2003. Association of in vitro fertilization with beckwith-wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet*, 72:156-160.

**Diskin MG, Murphy JJ, Sreenan JM**. 2006. Embryo survival in dairy cows managed under pastoral conditions. *Anim Reprod Sci*, 96:297-311.

**Ellington JE, Ignotz GG, Ball BA, Meyers-Wallen VN, Currie WB**. 1993. De novo protein synthesis by bovine uterine tube (oviduct) epithelial cells changes during co-culture with bull spermatozoa. *Biol Reprod*, 48:851-856.

**Farin PW, Britt JH, Shaw DW, Slenning BD**. 1995. Agreement among evaluators of bovine embryos produced in vivo or in vitro. *Theriogenology*, 44:339-349.

**Fazelli A, Holt WV**. 2016. Cross talk during the periconception period. *Theriogenology*, 86:438-442.

**Fleming TP, Watkins AJ, Sun C, Velazquez MA, Smyth NR, Eckert JJ**. 2015. Do little embryos make big decisions? How maternal dietary protein restriction can permanently change and embryo's potential, affecting adult health. *Reprod Fertil Dev*, 27:684-692.

**Forde N, Carter F, Fair T, Crowe MA, Evans AC, Spencer TE, Bazer FW, McBride R, Boland MP, O'Gaora P, Lonergan P, Roche JF**. 2009. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biol Reprod*, 81:784-794.

**Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, Ó'Gaora P, Roche JF, Lonergan P, Crowe MA**. 2011. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. *Biol Reprod*, 84:266-278.

**Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, Mansouri-Attia N, Sandra O, Loftus BJ, Crowe MA, Fair T, Roche JF, Lonergan P, Evans AC**. 2012a. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. *Physiol Genomics*, 44:799-810.

**Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE, Lonergan P**. 2012b. Effects of low progesterone on the endometrial transcriptome in cattle. *Biol Reprod*, 87:124, 1-11. doi: 10.1095/biolreprod.112.103424.

**Forde N, McGettigan PA, Mehta JP, O'Hara L, Mamo S, Bazer FW, Spencer TE, Lonergan P**. 2014. Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle. *Reproduction*, 147:575-587.

**Gardner DK, Lane M, Stevens J, Schoolcraft WB**. 2001. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril*, 76:1175-1180.

**Goddard ME, Hayes BJ, Meuwissen THE**. 2011. Using the genomic relationship matrix to predict the accuracy of genomic selection. *J Anim Breed Genet*, 128:409-421.

**Gómez E, Rodríguez A, Muñoz M, Caamaño JN, Hidalgo CO, Morán E, Facal N, Díez C**. 2008. Serum free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification. *Theriogenology*, 69:1013-1021.

**Gonella-Diaza AM, da Silva Andrade SC, Sponchiado M, Pugliesi G, Mesquita FS, Van Hoeck V, de Francisco Strefezzi R, Gasparin GR, Coutinho LL, Binelli M**. 2015. Size of the ovulatory follicle dictates spatial differences in the oviduct transcriptome in cattle. *PLoS One*, 10:e0145321. doi: 10.1371/journal.pone.0145321.

**Gonella-Diaza AM, da Silva Andrade SC, Sponchiado M, Pugliesi G, Mesquita FS, Van Hoeck V, de Francisco Strefezzi R, Gasparin GR, Coutinho LL, Binelli M**. 2017. Oviductal transcriptional profiling of a bovine fertility mdel by next-generation

sequencing. *Genomics Data*, 13:27-29.

**Goossens K, Mestdagh P, Lefever S, Van Poucke M, Van Zeveren A, Van Soom A, Vandesompele J, Peelman L**. 2013. Regulatory microRNA network identification in bovine blastocyst development. *Stem Cells Dev*, 22:1907-1920.

**Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, BAzer FW, Spencer TE**. 2001. Developmental biology of uterine glands. *Biol Reprod*, 65:1311-1323.

**Gray CA, Burghardt RC, Johnson GA, BAzer FW, Spencer TE**. 2002. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction*, 124:289-300.

**Hashem N, Secher, J. Pryor JH, Long CR, Looney CR, Avery B, Hyttel P, Stroebech L**. 2017. Bovine embryo development rates are affected when oocytes are matured in different vials containing HEPES/Bicarbonate buffered medium. *Reprod Fertil Dev*, 29:179. doi: 10.1071/RDv29n1Ab141.

**Hayes BJ, Lewin HA, Goddard ME**. 2013. The future of livestock breeding: genomic selection for efficiency, reduced emissions intensity, and adaptation. *Trends Genet,* 29:206-214.

**Henderson CR**. 1975. Best linear unbiased estimation and prediction under a selection model. *Biometrics*, 31:423-447.

**Hyttel P, Greve T, Callesen H**. 1988. Ultrastructure of in vivo fertilization in superovulated cattle. J Reprod Fertil, 82:1-13.

**Jakobsen AS, Thomsen PD, Avery B**. 2006. Few polyploid blastomeres in morphologically superior bovine embryos produced in vitro. *Theriogenology*, 65:870-881.

**Kadarmideen HN, Thompson R, Simm G**. 2000. Linear and threshold model genetic parameters for disease, fertility and milk production in dairy cattle. *Anim Sci*, 71:411-419.

**Kadarmideen HN**. 2014. Genomics to systems biology in animal and veterinary sciences: progress, lessons and opportunities. *Livest Sci,* 166:232-248.

**King WA, Niar A, Chartrain I, Betteridge KJ, Guay P**. 1988. Nucleolus organizer regions and nucleoli in preattachment bovine embryos. *J Reprod Fertil*, 82:87-95.

**Kirkegaard K, Hindkjaer JJ, Ingerslev HJ**. 2013. Hatching of in vitro fertilized human embryos is influenced by fertilization method. *Fertil Steril*, 100:1277-1282.

**Kitano H**. 2002. Systems biology: a brief overview. *Science*, 295:1662-1664.

**Klein C**. 2016. Maternal recognition of pregnancy in the context of equine embryo transfer. *J Equine Vet Sci,* 41:22-28.

**Kovacs P**. 2014. Embryo selection: the role of time-lapse monitoring. *Reprod Biol Endocrinol*, 12:124. doi: 10.1186/1477-7827-12-124.

**Laurincik J, Kopecny V, Hyttel P**.1994 Pronucleus development and DNA synthesis in bovine zygotes in vivo. *Theriogenology*, 42:1285-1293.

**Laurincik J, Thomsen PD, Hay-Schmidt A, Avery B, Greve T, Ochs RL, Hyttel P**. 2000. Nucleolar proteins and nuclear ultrastructure in preimplantation bovine embryos produced in vitro. *Biol Reprod*, 62:1024-1032.

**Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C**. 2002. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the Large Offspring Syndrome. *Biol Reprod*, 67:767-775.

**Lee KF, Yao YQ, Kwok KL, Xu JS, Yeung WS**. 2002. Early developing embryos affect the gene expression patterns in the mouse oviduct. *Biophys Res Commun*, 292:564-570.

**Maddox-Hyttel P, Alexopoulus NI, Vajta G, Lewis I, Rogers P, Cann L, Callesen H, Tveden-Nyborg P, Trounson A**. 2003. Immunohistochemical and ultrastructural characterization of the initial post-hatching development of bovine embryos. *Reproduction*, 125:607-623.

**Madsen CA, Perry GA, Mogck CL, Daily RF, MacNeil MD, Geary TW**. 2015. Effects of preovulatory estradiol on embryo survival and pregnancy establishment in beef cows. *Anim Reprod Sci*, 158:96-103.

**Maillo V, Gaora PÓ, Forde N, Besenfelder U, Havlicek V, Burns GW, Spencer TE, Gutierrez-Adan A, Lonergan P, Rizos D**. 2015. Oviduct-embryo inteactions in cattle: two-way traffic or a one-way street? *Biol Reprod*, 92:144, 1-8. doi: 10.1095/biolreprod.115.127969.

**Maillo V, Lopera-Vasquez R, Hamdi M, Gutierrez-Adan A, Lonergan P, Rizos D**. 2016a. Maternal-embryo interaction in the bovine oviduct: Evidence from in.-vivo and in vitro studies. *Theriogenology*, 86:443-450

**Maillo V, Sáchez-Calabuig MJ, Lopera-Vasquez R, Hamdi M, Gutierrez-Adan A, Lonergan P, Rizos D**. 2016b. Oviductal response to gametes and early embryos in mammals. *Reproduction*, 152:R127-R141.

**Mann GE, Lamming GE**. 1999. The influence of progesterone during early pregnancy in cattle. *Reprod Domest Anim*, 34:269-274.

**Mann GE, Lamming GE**. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction*, 121:175-180.

**Mansouri-Attia N, Sandra O, Aubert J, Degrelle S, Everts RE, Giraud-Delville C, Heyman Y, Galio L, Hue I, Yang X, Cindy Tian X, HA, Renard J-P**. 2009. Endometrium as an early sensor of in vitro embryo manipulation technologies. *Proc Natl Acad Sci USA*, 106:5687-5692.

**Mazzoni G, Pedersen HS, Oliveira Junior GA, Alexandre P, Razza EM, Callesen H, Hyttel P, Nogueira MFG, Ferraz JBS, Kadarmideen HN**. 2017. Application of integrative genomics and systems biology to conventional and *in vitro* reproductive traits in cattle. *Anim Reprod*, 14:507-513.

**Merton JS, Ask B, Onkundi DC, Mullaart E, Colenbrander B, Nielen M**. 2009. Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-in vitro production embryo-production program. *Theriogenology*, 72:885-893.

**Meuwissen THE, Hayes BJ, Goddard ME**. 2001.

Prediction of total genetic value using genome wide dense marker maps. Genetics, 157:1819-1829.

**Minten MA, Bilby TR, Bruno RGS, Allen CC, Madsen CA, Wang Z, Sawyer JE, Tibary A, Neibergs HL, Geary TW, Bauersachs S, Spencer TE**. 2013. Effects of fertility on gene expression and function of the bovine endometrium. *PLoS One*, 8:e69444. doi: 10.1371/journal.pone.0069444.

**Muñoz M, Uyar A, Correia E, Díez C, Fernandez-Gonzalez A, Caamaño JN, Martínez Bello D, Trigal B, Humblot P, Ponsart C, Guyader-Joly C, Carrocera S, Martin D, Marquant Le Guienne B, Seli E, Gomez E**. 2014. Prediction of pregnancy viability in bovine in vitro-produced embryos and recipient plasma with Fourier transform infrared spectroscopy. *J Dairy Sci*, 97:5497-5507.

**Oestrup E, Hyttel P, Oestrup O**. 2011. Embryo-maternal communication: signalling before and during placentation in cattle and pig. *Reprod Fertil Dev*, 23:964-975.

**Orozco-Lucero E, Sirard MA**. 2014. Molecular markers of fertility in cattle oocytes and embryos: progress and challenges. *Anim Reprod*, 11:183-194.

**Papadopoulos S, Rizos D, Duffy P, Wade M, Quinn K, Boland MP, Lonergan P**. 2002. Embryo survival and recipient pregnancy rates after transfer of fresh or vitrified, in vivo or in vitro produced ovine blastocysts. *Anim Reprod Sci*, 74:35-44.

**Perry G**. 2016. International Embryo Technology Society (IETS). *Embryo Technol Newslett*, 34:10-25.

**Pohler KG, Geary TW, Atkins JA, Perry GA, Jinks EM, Smith MF**. 2012. Follicular determinants of pregnancy establishment and maintenance. *Cell Tissue Res*, 349:649-664.

**Ponsuksili S, Murani E, Schwerin M, Schellander K, Wimmers K**. 2010. Identification of expression QTL (eQTL) of genes expressed in porcine M. longissimus dorsi and associated with meat quality traits. *BMC Genomics*, 11:572. doi: 10.1186/1471-2164-11-572.

**Ponsuksili S, Murani E, Schwerin M, Schellander K, Tesfaye D, Wimmers K**. 2012. Gene expression and DNA-methylation of bovine pretransfer endometrium depending on its receptivity after in vitro produced embryo transfer. *PLoS One*, 7:e42402. doi: 10.1371/journal.pone.0042402.

**Pryce J, Daetwyler H**. 2012. Designing dairy cattle breeding schemes under genomic selection: a review of international research. *Anim Prod Sci*, 52:107-114.

**Ratto MH, Leduc YA, Valderrama XP, Van Straaten KE, Delbaere LTJ, Pierson RA, Adams GP**. 2012. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci USA*, 109:15042-15047.

**Rødgård, TH, Heegaard P, Callesen H**. 2015. Non-invasive assessment of in vitro embryo quality to improve transfer success. *Reprod Biomed Online*, 31:585-592.

**Saadeldin IM, Oh HJ, Lee BC**. 2015. Embryonic-maternal cross-talk via exosomes: potential implications. *Stem Cells Cloning*, 8:103-107.

**Salilew-Wondim D, Hölker M, Rings F, Ghanem N, Ulas-Cinar M, Peippo J, Tholen E, Looft C, Schellander K, Tesfaye D**. 2010. Bovine pretransfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after embryo transfer. *Physiol Genomics*, 42:201-218.

**Salilew-Wondim D, Schellander K, Hoelker M, Tesfaye D**. 2012. Oviductal, endometrial and embryonic gene expression patterns as molecular clues for pregnancy establishment. *Anim Rep Sci*, 134:9-18.

**Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish JJ, Wiltbank MC**. 2002. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci*, 85:2803-2812.

**Schaeffer L**. 2006. Strategy for applying genome wide selection in dairy cattle. *J Anim Breed Genet*, 123:218-223.

**Simmons RM , Erikson DW, Kim J, Burghardt RC, Bazer FW, Johnson GA, Spencer TE**. 2009. Insulin-like growth factor binding protein one in the ruminant uterus: potential endometrial marker and regulator of conceptus elongation. *Endocrinology*, 150:4295-4305.

**Sponchiado M, Gomes NS, Fontes PK, Martins T, Del Collado M, Pastore AA, Puglies G, Nogueira MFG, Binelli M**. 2017. Pre-hatching embryo-dependent and - independent programming of endometrial function in cattle. *PLoS One*, 12:e0175954. doi: 10.1371/journal.pone.0175954.

**Sreenan JM, Diskin MG**. 1983. Early embryonic mortality in the cow: its relationship with progesterone concentration. *Vet Rec*, 112:517-521.

**Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P**. 2010. Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. *Mol Reprod Dev*, 77:285-296.

**Suravajhala P, Kogelman LJ, Kadarmideen HN**. 2016. Multi-omic data integration and analysis using systems genomics approaches: methods and applications in animal production, health and welfare. *Genet Sel Evol*, 48:38. doi: 10.1186/s12711-016-0217-x.

**Thomas PGA, Ball BA, Brinsko SP**. 1995. Changes associated with induced capacitation influence the interaction between equine spermatozoa and oviduct epithelial cell monolayers. *Biol Reprod Mono*, 1:697-705.

**Ulbrich SE, Wolf E, Bauersachs S**. 2013. Hosting the preimplantation embryo: potentials and limitations of diffferent approaches for analysing embryo-endometrium interactions in cattle. *Reprod Fertil Dev*, 25:62-70.

**Walker CG, Littlejohn MD, Mitchell MD, Roche JR, Meier S**. 2012. Endometrial gene expression during early pregnancy differs between fertile and subfertile cow strains. *Physiol Genomics*, 44:47-58.

**Westra HJ, Franke L**. 2014. From genome to function by studying eQTLs. *Biochim Biophys Acta*, 1842:1896-1902.

**Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PL, Melo LF, Ochoa JC, Santos JE, Sartori R**. 2016. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology*, 86:239-253.

**Yan L, Robinson R, Shi Z, Mann G**. 2016. Efficacy of progesterone supplementation during early pregnancy in cows: a meta-analysis. *Theriogenology*, 85:1390-1398.

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# Impact and mechanisms of inflammatory diseases on embryonic development and fertility in cattle

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## Abstract

Inflammatory diseases are prevalent in cattle and impair fertility. Cows affected by inflammatory disease from parturition to the day before breeding have reduced fertilization of oocytes, reduced survival of zygotes to the morula stage, impaired development to early stages of conceptus elongation, reduced secretion of interferon during the period of pregnancy recognition, altered transcriptome of preimplantation conceptus cells, and increased pregnancy loss. Consequently, these cows have reduced pregnancy and calving per breeding. Reduced oocyte competence is a likely reason for the carryover effects of diseases on developmental biology, but impaired uterine environment is also involved. Effects on pregnancy survival are observed up to 5 months after the diagnosis and treatment of disease, and effects on developmental biology seem to be extended into postnatal life in pregnancies that survive until term. Although the biological mechanism mediating the effects of inflammatory diseases are still not completely understood, control of inflammation during the clinical presentation of diseases seems to alleviate the negative effects on reproductive biology. It is increasingly evident that animal health, not only at the time of breeding or pregnancy development but also in the period preceding breeding, is imperative for optimal reproduction in cattle and should always be considered in herd evaluations and managerial decisions.

**Keywords**: dairy cattle, embryo, fertility, inflammation.

## Introduction

Optimization of reproductive efficiency is a necessity to maintain farms economically viable and sustainable (Ribeiro *et al.,* 2012; Rodgers *et al.,* 2012). Pregnancy loss in cattle, however, is substantial and impairs reproductive efficiency (Santos *et al.*, 2004). Although fertilization in cattle is estimated to be above 80, up to 50% of the potential zygotes fail to survive by the end of the fourth week of development (Ribeiro *et al.*, 2016a). Although less frequent, fetal mortality, after gestational day 42, is costly and reduce profitability significantly (De Vries, 2006).

Suboptimal uterine conditions and less competent embryos are ultimately the main reasons for reproductive failures, and these conditions are affected by many genetic and non-genetic factors in a complex series of interactions. Inflammatory diseases, however, have been identified as a major cause of reproductive failures in cattle. It is increasingly evident that animal health is imperative for optimal reproduction in cows. This review summarizes the current information evaluating the impact of inflammatory diseases on fertility in cattle, potential biological mechanisms involved and associated implications for health and reproductive management.

## Incidence of clinical diseases

Clinical diseases caused by microbial infection and tissue injury are prevalent in postpartum dairy cows (Santos *et al.*, 2010; Ribeiro *et al.*, 2013, 2016a). Approximately one-third of dairy cows have at least one clinical disease in the first 3-weeks of lactation, and they represent 60 to 80% all clinical cases occurring in lactating cows. The most common clinical diseases observed in dairy herds are metritis, mastitis, digestive problems, lameness, and respiratory problems. The incidence of these diseases in the first 2-months of lactation of 8,268 cows in eight large dairy herds in USA was 21.3, 13.8, 6.4, 5.5, and 2.4%, respectively (Ribeiro, 2015). Combined, these diseases affected 40% of all cows.

The increased susceptibility to diseases in the early postpartum is mostly explained by reduced immunocompetence of dairy cows during this period. The nutritional status and associated metabolic scenario observed postpartum impair function of immune cells and increase the susceptibility to opportunistic microbial infections (Sordillo, 2016). In addition, the enlarged uterus postpartum contains placenta remnants and lochia that favor proliferation of microbes and development of uterine infections (Sheldon *et al.*, 2009). All diseases described above are also seen in beef cows. However, the epidemiology of diseases in postpartum beef cows is not well documented. In general, diseases are less prevalent in beef cattle compared with dairy cattle but could become a significant problem in cases of nutritional deficiencies or environmental stress.

## Impact of clinical diseases on reproduction

Cows with clinical diseases have delayed resumption of estrous cyclicity postpartum (Santos *et al.*, 2010; Ribeiro *et al.,* 2013), which prolongs the interval between calving and first artificial insemination (AI) postpartum. In general, delayed first breeding causes reproductive inefficiency and economic losses (Ribeiro *et al.,* 2012). Timed AI programs can be used to assure

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proper time of first AI postpartum. However, the odds of being diagnosed pregnant 45 days after a timed AI is 30% smaller for cows that had postpartum disease compared with cows that did not have postpartum disease (Fig. 1). Further, the odds of pregnancy losses after day 45 of gestation are 2-times greater, and the odds of calving from first breeding postpartum are 42% smaller for cows that had postpartum diseases compared with cows that did not have disease (Fig. 1). Therefore, the impact of diseases is significant even when cows are subjected to timed AI programs. No differences in ovulation after synchronization of the estrous cycle or expression of estrus at timed AI were observed between cows that had or did not have postpartum diseases (Fig. 2). Therefore, the observed difference in pregnancy per breeding would be a result of reduced fertilization of oocytes and/or greater embryonic losses occurring before pregnancy diagnosis.

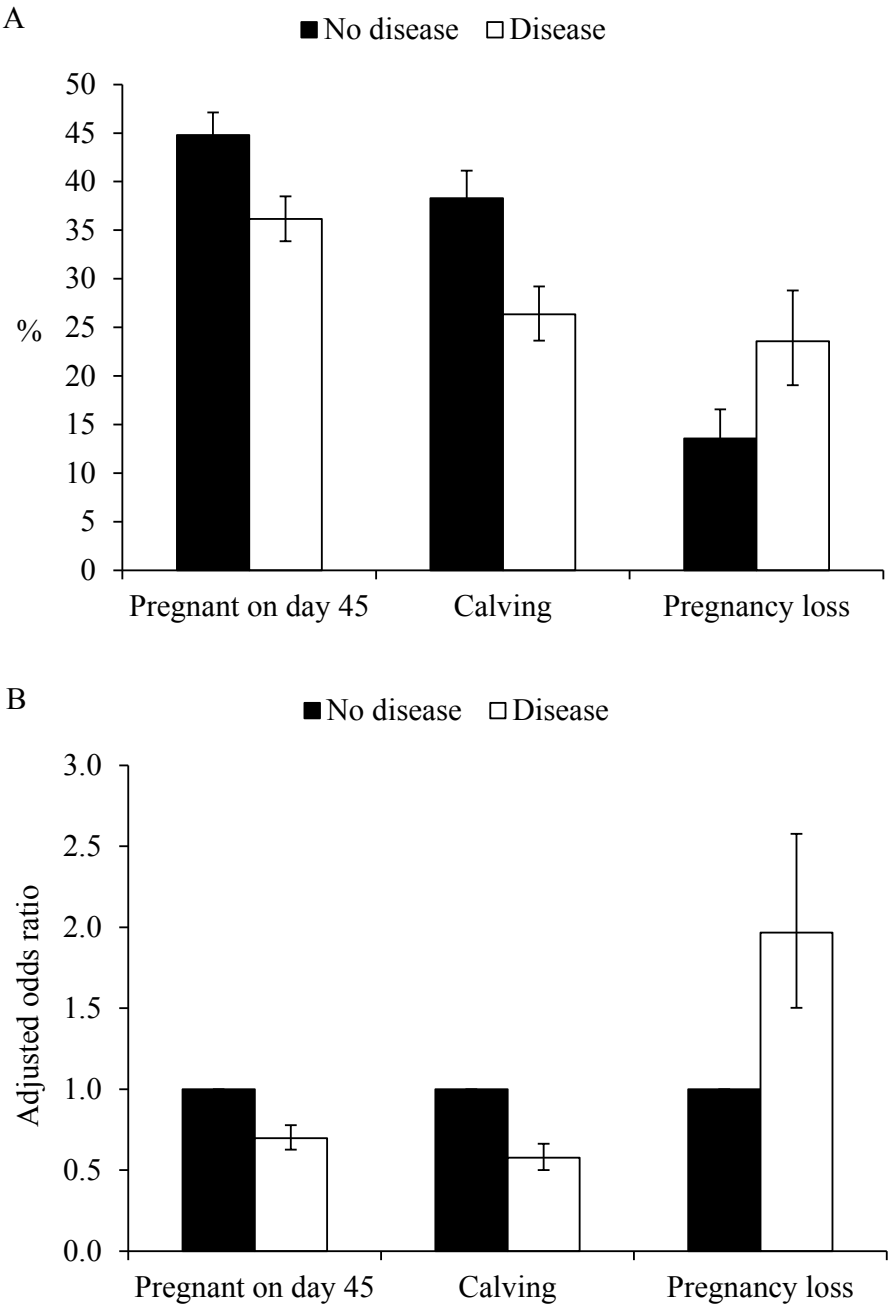


Figure 1. Adjusted means (Panel A) and adjusted odds ratio (Panel B) of the proportion of cows pregnant on day 45 after breeding, calving per breeding, and pregnancy loss after day 45 of gestation according incidence of clinical diseases before breeding. All outcomes were significantly ($P < 0.01$) affected by disease. Cows that did not have disease before breeding were used as reference for comparison (adjusted odds ratio = 1). Error bars represent the 95% confidence limits. Pregnancy on day 45 refers to data of 6,525 cows receiving their first breeding postpartum (Ribeiro, 2015). Calving per breeding and pregnancy loss after day 45 refer to data of 4,476 cows that were followed from first breeding postpartum until end of gestation (Ribeiro, 2015).

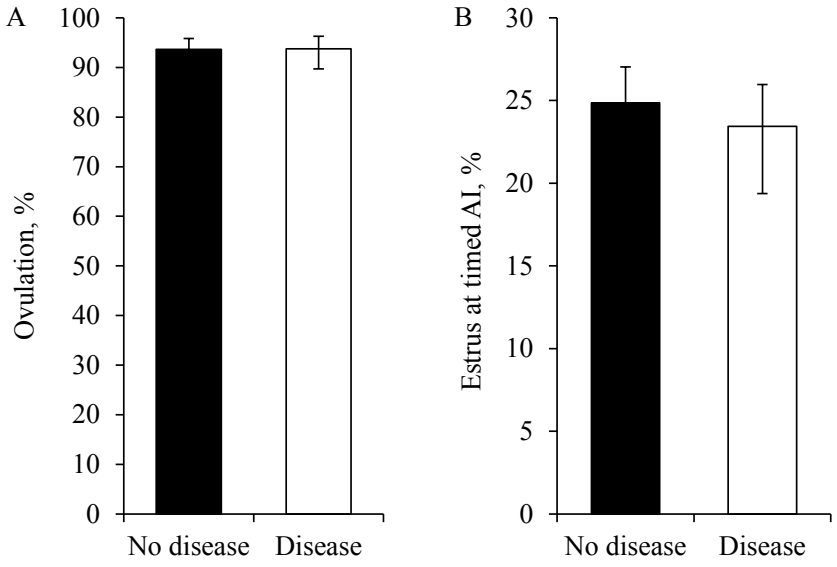


Figure 2. Adjusted means for the proportion of cows ovulating after synchronization of the estrous cycle (Panel A) and proportion of cows detected in estrus on the day of timed AI (Panel B) according to incidence of clinical diseases before AI. Disease before AI did not affect ovulation (P = 0.97) or estrous detection (P = 0.61). Error bars represent the 95% confidence limits. Data in Panel A refer to 746 cows receiving their first breeding postpartum whose ovulation was determined by ultrasonography examination of the ovaries 48 h after AI (Ribeiro, 2015). Data in Panel B refer to 1,265 cows receiving their first breeding postpartum whose detection of estrus was characterized by removal of tail chalk by the time of AI (Ribeiro, 2015).

To evaluate the impact of diseases on fertilization of oocytes, early embryo development and survival to morula stage, health information of 597 lactating cows was collected from parturition until first AI postpartum, and uterine flushing for recovery of ova-embryos was performed 5 or 6 days after AI. A total of 419 ova-embryos were recovered and evaluated for stage of development and quality. Cows with diseases before AI had reduced proportion of cleaved, live, and high-quality embryos relative to ova-embryos recovered (Ribeiro *et al.*, 2016a). Within cows with a recovered cleaved embryo, the odds of recovering a live embryo were reduced by 53.6% in cows with disease (Fig. 3). The reduction in cleaved embryos is likely caused by reduced fertilization of oocytes. Thus, the results indicate that postpartum disease reduces fertilization of oocytes and survival of zygotes in the first week of development.

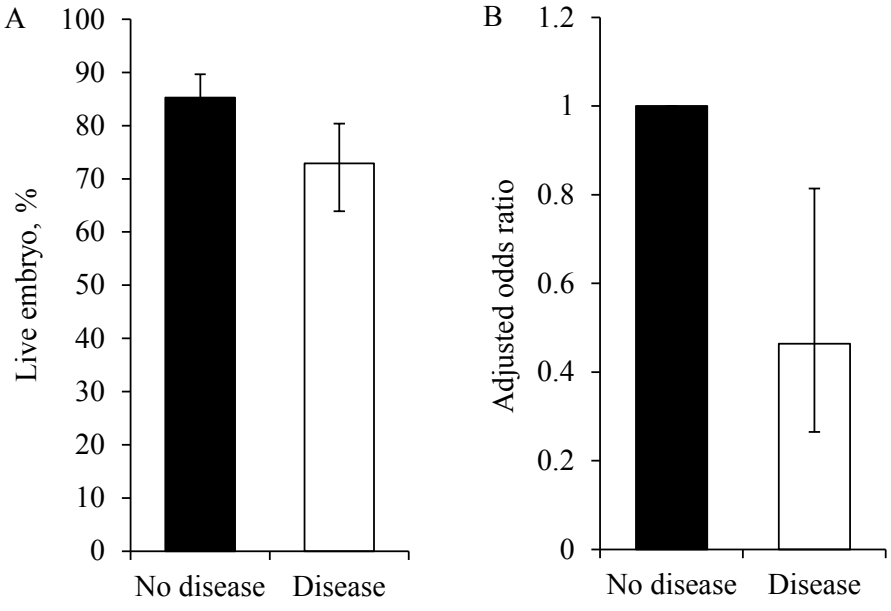


Figure 3. Adjusted means (Panel A) and adjusted odds ratio (Panel B) of the proportion of cows with a live embryo recovered from uterine flushes performed 5 or 6 days after AI according to incidence of diseases before AI. Cows that did not have disease before breeding were used as reference for comparison (adjusted odds ratio = 1). Error bars represent the 95% confidence limits. Data refer to 347 fertilized oocytes (cleaved embryos) recovered from 597 uterine flushes (Ribeiro *et al.*, 2016a).

To evaluate the impact of diseases on preimplantation conceptus elongation, health information of 148 lactating cows was collected from parturition until first AI postpartum, and uterine flushing for recovery of conceptuses was performed 15 or 16 days after AI. Cows with diseases had shorter conceptuses and reduced concentration of interferon (IFN)-τ in the uterine flush (Ribeiro *et al.*, 2016a). These results were supported by a second experiment that evaluated the transcript expression of IFN stimulated genes (ISGs) in peripheral blood leukocytes (PBL) on day 19 after AI (Ribeiro *et al.*, 2016a). Interferon-τ produced by the elongating conceptus in utero reaches maternal circulation and induces changes in gene expression in peripheral tissues including PBL (Oliveira *et al.*, 2008). Within cows that did not have disease before breeding, the expression of two ISGs (ISG15 and RTP4) was increased in cows later diagnosed as pregnant compared with those diagnosed not pregnant. However, this difference in gene expression of ISGs according to pregnancy status was not significant in cows that had diseases before AI, suggesting that production of IFN-τ by the elongating conceptuses in utero of cows that had postpartum diseases was reduced (Ribeiro *et al.*, 2016a).

**Site of infection or tissue injury**

In order to characterize the impact of diseases on reproductive biology of cattle, it is also important to understand how the impact is mediated, so that strategies to mitigate this negative association between diseases and reproduction might be developed. The site of infection or tissue injury is an important factor because the impact on reproduction and the mediator mechanism might change accordingly. Uterine diseases cause endometrial lesions that have detrimental effects on tissue integrity and physiology, hence suboptimal embryonic development and survival. Diseases that occur outside the uterus (i.e. mastitis, lameness, acidosis) have effects on reproductive biology that are mediated by a physiological response to infection or injury to tissues.

Ribeiro *et al.* (2016a) compared the effects of the uterine diseases (metritis) and non-uterine diseases (mastitis, lameness, digestive and respiratory problems) on reproduction of lactating dairy cows. Uterine and non-uterine diseases had similar impact on reproduction of dairy cows. Both type of disorder decreased pregnancy per breeding on day 45 after breeding, increased pregnancy loss after day 45 of gestation, and decreased calving per breeding (Fig. 4). Moreover, the two types of diseases have additive negative effects on reproductive outcomes. Cows that had both uterine and non-uterine diseases were 41% less likely to be pregnant on day 45 after breeding (adjusted odds ratio [AOR] = 0.59; CI = [0.47-0.75]), 3-times more likely to lose pregnancy after day 45 of gestation (AOR = 3.06; CI = [1.67-5.60]), and 60% less likely to calved from first breeding postpartum (AOR = 0.40; CI = [0.28-0.58]) compared with cows that did not have disease before breeding (Fig. 4). The effects of diseases on the development to morula and conceptus elongation were also similar between uterine and non-uterine diseases (Ribeiro *et al.*, 2016a).

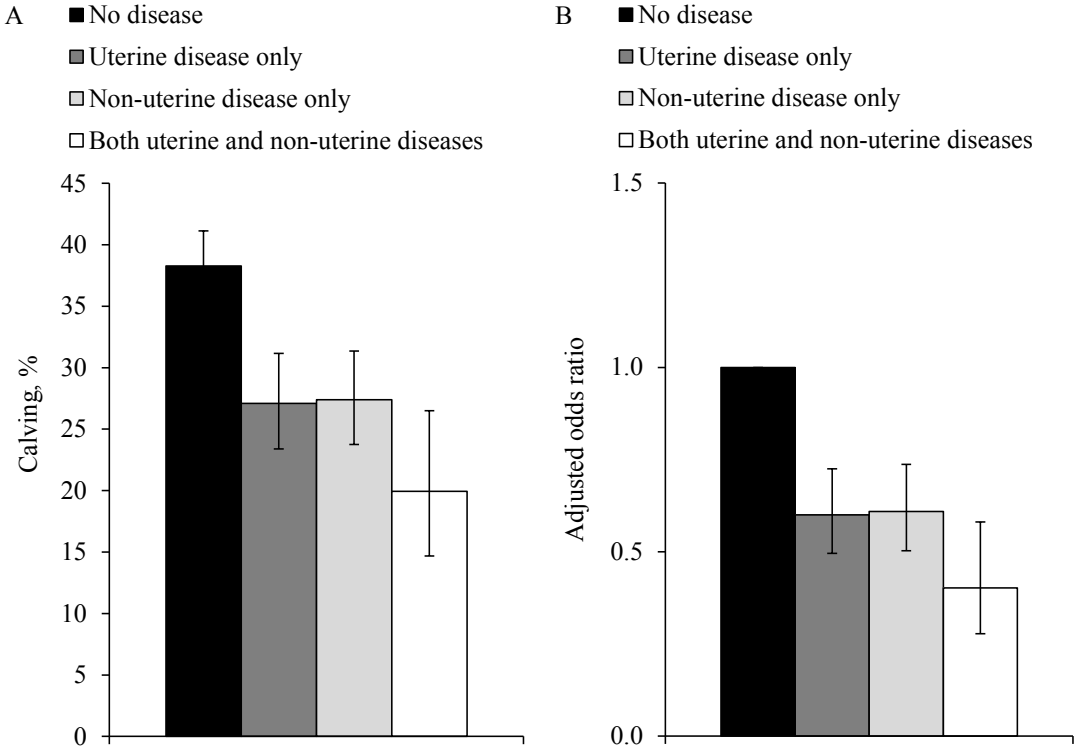


Figure 4. Adjusted means (Panel A) and adjusted odds ratio (Panel B) of the proportion of cows calving from first breeding postpartum according to the incidence of uterine and non-uterine diseases before breeding. Both uterine and non-uterine diseases significantly ($P < 0.01$) affected calving per breeding. The group of cows that did not have disease before breeding (no disease) were used as reference for comparison (adjusted odds ratio = 1). Error bars represent the 95% confidence limits. Information refers to data of 4,476 cows that were followed from first breeding postpartum until end of gestation (Ribeiro, 2015).

## Physiological responses to microbial infections and tissue injury

The similar impact of uterine and non-uterine diseases on reproduction has led us to the hypothesis that physiological responses to microbe infections and tissue injury, the common factor between the two types of diseases, is the major mediator of the consequences on reproduction. Microbial infections and tissue injury cause a series of physiological adaptations not only locally in the insulted tissue, but also systemically in the individual (Colditz, 2002). One of the first responses of the affected tissue to an insult is inflammation, which is mediated by cells of the innate immune system that secrete inflammatory molecules such as cytokines, chemokines, eicosanoids and vasoactive amines. These inflammatory mediators recruit more immune cells to the site of injury and induce production of plasma proteins with the purpose of containing the infection or tissue injury (Medzhitov, 2008). Nonetheless, inflammation has consequences for energy metabolism (Colditz, 2002), and, when exacerbated, may generate excessive oxidative stress and further tissue damage and dysfunction (Medzhitov, 2008).

In general, cattle affected by diseases have reduced appetite, increased body weight loss, and altered partition of nutrients (Gifford *et al.*, 2012). The reduced appetite after establishment of disease is caused by the communication between immune and the central nervous systems (Dantzer and Kelley, 2007). Inflammation also increases energy expenditure to mount a response against infection and, therefore, partitions resources away from production and reproduction (Romanyukha *et al.*, 2006; Kvidera *et al.*, 2017). Collectively, reduced nutrient intake, increased nutrient and energy expenditure and altered nutrient partition caused by inflammation worsen the nutrient balance of postpartum dairy cows.

## Indirect effects of disease on reproduction: anovulation and low body condition

The impact of inflammation on intake, expenditure, and partition of energy and nutrients cause compensative mobilization of body reserves, loss of body condition, and delayed resumption of estrous cyclicity postpartum (Ribeiro *et al.*, 2016a). Low body condition score (BCS) and anovulation at the onset of synchronization have been implicated individually with reduced fertility in dairy cows (Santos *et al.*, 2009). Therefore, it would be reasonable to speculate that the impact of diseases on reproduction is mediated indirectly by lower BCS at breeding and greater proportion of anovular cows at the onset of synchronization of estrous cycle.

To evaluate the hypothesis above, we collected information regarding health postpartum, estrous cyclicity before estrous cycle synchronization, BCS at AI, and conception risk at first AI postpartum in 2,190 cows (Ribeiro *et al.*, 2016a). As expected, cows with clinical diseases postpartum had greater proportion of anovular cows at the onset of synchronization (18.9 *vs*. 26.6%) and greater proportion of cows with low BCS at first AI postpartum (49.3 *vs*. 58.5%). Health status (disease or no disease), estrous cyclicity (anovular or cyclic), categorized BCS (low or adequate) and their interaction were then used concomitantly to predict conception risk of cows. All three predictors significantly reduced the probability of conception individually and did not interact with each other. The impact of disease on conception risk, therefore, was observed independently of estrous cyclicity and BCS of the cows. Thus, the results indicate that reduced BCS and increased incidence of anovulation caused by inflammatory diseases explain only a small portion of the impact of diseases on reproduction.

## Time of infection or tissue injury relative to time of breeding

Inflammatory mediators are produced by immune cells in the insulted tissue and, through systemic circulation, reach other organs including brain, ovaries and uterus. These molecules have the potential to disrupt GnRH and LH secretion, oocyte developmental competence, and embryonic survival in cattle (Hansen *et al.*, 2004). Studies infusing lipopolysaccharide in the uterus, mammary gland, or intravenously, resulted in reduced secretion of GnRH and LH (Peter *et al.*, 1989; Lavon *et al.*, 2008). Incubation of granulosa cells with lipopolysaccharide or tumour necrosis factor (TNF) α resulted in smaller production of estradiol (Williams *et al.*, 2008). Incubation of immature oocytes in maturation media with TNFα reduced the development to blastocysts after fertilization *in vitro* (Soto *et al.*, 2003). Moreover, incubation of bovine embryos with TNFα five days after fertilization *in vitro* increased apoptosis of blastomeres (Soto *et al.*, 2003). These studies exemplify multiple consequences of inflammation on reproductive tissues.

It is important, however, to put in prospective the time of disease occurrence relative to the time of breeding and embryo development. Approximately 75% of the cases of clinical inflammatory diseases occurred in the first three weeks postpartum. Because the typical voluntary waiting period in most farms is 50 to 60 days, the first breeding postpartum in most cows with diseases occurs six to eight weeks after clinical resolution of disease. Conventional inflammatory mediators are unlikely to be at high concentrations in systemic circulation by the time breeding (Qu *et al.*, 2014) and perhaps are not directly important for pregnancy establishment and maintenance.

To evaluate whether the time of disease occurrence relative to breeding was important to determine the consequences on fertility, we compared the impact of non-uterine diseases occurring before breeding with those occurring after breeding (up to day 42 of gestation) on calving per breeding. Uterine disease was included as covariate in the statistical model. Calving per breeding was similarly affected by disease before breeding and by disease after breeding. The odds of calving from first breeding postpartum was reduced 47% (AOR = 0.53; CI = [0.34 – 0.84]) and 41% (AOR = 0.59; CI = [0.38 – 0.93]) by diseases occurring before breeding and after breeding, respectively. The two

variables did not interact, and cows that had both types, diseases before and also after breeding, had a reduction of 68% in the odds of calving from first breeding postpartum (Fig. 5). Thus, disease affects fertility of dairy cows not only at the time of breeding or early pregnancy development but also in the period preceding breeding, which confirms long-lasting effects of disease on fertility of cattle.

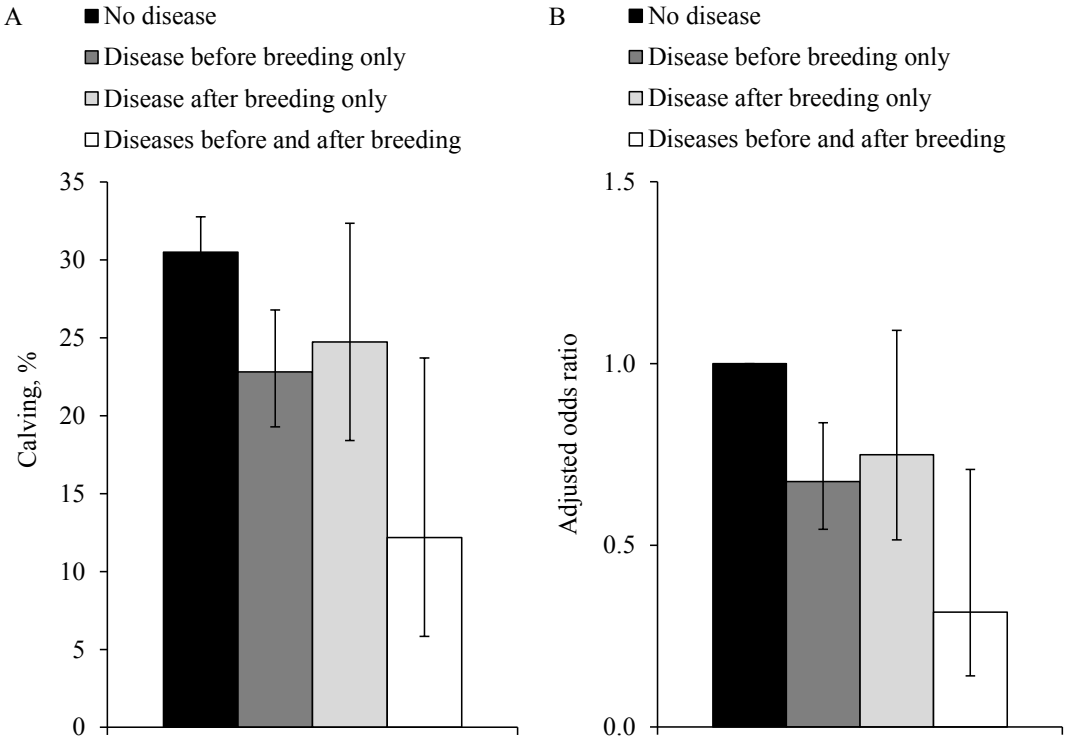


Figure 5. Adjusted means (Panel A) and adjusted odds ratio (Panel B) of the proportion of cows calving from first breeding postpartum according to the incidence of diseases before breeding and the incidence of diseases after breeding. Calving per breeding was significantly affected by disease before breeding ($P < 0.01$) and disease after breeding ($P = 0.02$), and these two variables did not interact ($P = 0.31$). The group of cows that did not have disease neither before nor after breeding (no disease) were used as reference for comparison (adjusted odds ratio = 1). Error bars represent the 95% confidence limits. Data from Ribeiro *et al.* (2016a).

**Oocyte developmental competence *vs.* uterine environment**

The interval from the activation of primordial follicles to the formation of preovulatory follicle is estimated to last 180 days (Fair, 2003), in which the majority of time would be spent in the pre-antral stages (138 days), and less time in the antral stages (42 days; Lussier *et al.*, 1987). During folliculogenesis, disease could potentially disturb the follicular environment and oocyte developmental competence without apparent effects on growth and ovulation (Bromfield *et al.*, 2015). Thus, a potential impact of postpartum disease on preantral or antral follicles is a plausible mechanism mediating the long-lasting effects of disease on reproduction.

If reduced oocyte developmental competence was the sole explanation for the long-lasting effects of postpartum diseases on reproduction, then fertility of cows in an embryo transfer (ET) program would not be affected by the occurrence of postpartum diseases. On the other hand, if diseases had an impact on fertility of cows receiving a viable embryo on day 7 of the cycle, then uterine environment should mediate at least part of the effects of disease on fertility of cattle. To test these hypotheses, information on the incidence of postpartum diseases, pregnancy and calving per breeding, and late pregnancy losses were collected in a large dairy farm using both AI and ET as part of the reproductive management for lactating cows (Ribeiro *et al.*, 2016a). Disease affected all reproductive outcomes, and the interaction with breeding technique was not significant (Tab. 1). Similar results were obtained when only uterine disease or only non-uterine diseases were considered, thereby suggesting that both types of disease have long-lasting effects on the uterine environment that impairs the ability to support pregnancy to term.

Furthermore, ET increased the proportion of cows calving from first breeding compared with AI (Tab. 1). The difference, however, was significant only in cows that had disease before breeding. The improvement in calving per breeding observed in cows that had disease when receiving ET suggests that oocyte quality and/or oviduct environment is also affected by disease. Supporting evidence for this interpretation is the slightly smaller change in adjusted odds ratios attributable to disease in cows receiving ET compared with those receiving AI (Tab. 1). Thus, reduced oocyte competence is a likely component in the carryover effects of disease in fertility of cows receiving AI, and impaired uterine environment is a reason for carryover effects of diseases in fertility of cows receiving AI and cows receiving ET.

Table 1. Reproductive outcomes of first breeding postpartum in dairy cows according to incidence of disease before breeding and breeding technique.

| | Adjusted mean ± SEM [1] | | | | P[2] | | | AOR (CI) [3] | |
|---|---|---|---|---|---|---|---|---|---|
| Item | No disease-AI | Disease-AI | No disease-ET | Disease-ET | DIS | BT | DIS*BT | Within AI | Within ET |
| Pregnant day 45 | 38.8 ± 1.8 | 31.0 ± 2.1 | 40.7 ± 1.7 | 35.9 ± 2.4 | < 0.01 | 0.12 | 0.37 | 0.71 (0.58-0.87) | 0.82 (0.65-1.02) |
| Calving | 32.9 ± 1.7 | 22.2 ± 1.9 | 35.9 ± 1.7 | 28.2 ± 2.2 | < 0.01 | 0.03 | 0.27 | 0.58 (0.46-0.73) | 0.70 (0.55-0.90) |
| Pregnancy loss | 12.4 ± 1.5 | 21.3 ± 3.1 | 11.1 ± 1.5 | 22.4 ± 3.4 | < 0.01 | 0.87 | 0.59 | 1.92 (1.24-2.98) | 2.30 (1.41-3.76) |

[1]Adjusted mean and standard error of the mean for cows that had or not disease before breeding and were bred by artificial insemination (AI) or embryo transfer (ET). [2]Probability values: DIS = disease; BT = breeding technique; DIS*BT = interaction between disease and breeding technique. [3]Adjusted odds ratio (confidence interval) for the effect of disease within cows bred by AI and within cows bred by ET. Data from Ribeiro *et al.* (2016a).

## Long-lasting effects of disease on uterine function

Conceptus cells sense changes in uterine environment and respond accordingly. Therefore, studying the transcriptome of conceptus cells could contribute to the discovery of a mechanism mediating long-lasting effect of inflammatory diseases on uterine biology. Ribeiro *et al.* (2016a) compared the transcriptome of conceptuses on day 16 of development from cows that had or did not have non-uterine diseases before AI. Five conceptuses recovered from cows that had non-uterine diseases before breeding were matched according to size with five conceptuses of cows that did not have disease before breeding and used for transcriptome analyses. Only a small number (n = 35) of transcripts were differently expressed between the two groups. Nonetheless, functional analysis of these transcripts revealed that changes in the transcriptome of conceptus cells recovered from cows that had diseases before breeding resemble an inflammatory response. Three proinflammatory molecules, lipopolysaccharide, IFN-γ and tumor necrosis factor were predicted to be potential upstream regulators of the changes in transcriptome. Moreover, the potential downstream consequences of these changes would include cell activation, particularly immune cells, and possibly problems with tissue rejection by immune system. These effects could result in rejection of the conceptus tissue by the maternal immune system and pregnancy loss. Nonetheless, it is not clear what specific alteration of the uterus would cause these inflammation-like changes in conceptus cells of cows that had postpartum diseases.

Lipids are important for elongation of the preimplantation conceptus (Ribeiro *et al.*, 2016b, c). They are used by trophectoderm cells for oxidation and generation of ATP, biosynthesis of membrane phospholipids and prostaglandins, cell signaling, and coordination of gene expression. Lipids accumulated in epithelial cells of the endometrium during diestrus are likely the most important source of fatty acids for utilization by the conceptus at the onset of elongation. Moreover, peroxisome proliferator-activated receptor (PPAR)-γ, a nuclear receptor activated by binding of fatty acids, seems to sense lipid composition in the histotroph and coordinate most of the lipid metabolism in trophectoderm cells (Ribeiro *et al.*, 2016b, c). The binding affinity of lipids to the ligand binding domain of PPARγ varies according to the biochemistry of the lipid molecule and modulates the activity of the transcription factor (McIntyre *et al.*, 2003; Itoh *et al.*, 2008). Thus, changing the composition of lipids in the endometrium can influence PPARγ activity and the biology of conceptus cells. Lipid composition of the endometrium at the time of breeding could be altered by long-lasting effects of postpartum diseases on energy metabolism of the lactating cow. Diseases worsen the negative energy balance of postpartum cows and increase the mobilization of fatty acids from adipose tissue (Ribeiro *et al.,* 2013), and excessive lipolysis postpartum may be sustained up to the time of breeding (Rukkwamsuk *et al.*, 1999; Contreras *et al.*, 2017). Adipose tissue derived-fatty acids, which are mostly saturated and monounsaturated fatty acids, are incorporated by multiple tissues (i.e. endometrium) and affect tissue physiology (Contreras *et al.*, 2017). Altered lipid composition of the endometrium could not only alter PPARγ activity in the conceptus but could also disturb the modulation of maternal immune cells in the endometrium at the time of conceptus elongation and early implantation. However, the contribution of altered composition of endometrial lipids to subfertility of cows with postpartum diseases still needs to be confirmed.

## Transgenerational effects of postpartum inflammatory diseases

Since inflammatory diseases have substantial impact on pregnancy survival in cattle, Carvalho *et al.* (2017) investigated whether the lasting effects of inflammatory diseases would be extended into postnatal life in pregnancies that survive until term. Incidence of inflammatory diseases in 5,085 cows was recorded from calving until first breeding postpartum, and cows that became pregnant to first breeding were followed until calving. Female calves were followed up to 305 days in milk of their first lactation, and data related to morbidity, mortality, culling, reproduction and milk production were recorded. A total of 1,211 cows calved from the first breeding. Out of those, 872 cows did not have any diseases postpartum in the previous lactation (H-DAM) and 339 cows had at least one disease postpartum in the previous lactation (D-DAM). Out of the 339 D-DAM, 300 had a single disease (SD-DAM) and 39 had multiple diseases (MD-DAM). The proportion of female calves born did not differ among groups and averaged 51.9%. Incidence of dystocia was greater in D-DAM compared with H-DAM (39.8 *vs.* 30.2%). Rate of morbidity, mortality, and culling before and after first calving, age at first AI, pregnancy after first AI, age at first calving, and milk production in the first lactation did not differ between heifers born from H-DAM and those born from D-DAM. Nonetheless, the incidence of diseases before first calving was smaller for MD-DAM heifers compared with SD-DAM and H-DAM heifers (26.3 *vs.* 62.2 *vs.* 57.4%). The rate of morbidity also was less for MD-DAM compared with H-DAM (hazard ratio = 0.35) and S-DAM (hazard ratio = 0.34) heifers. These results indicate that transgenerational effects of postpartum inflammatory diseases exist but only when multiple diseases occurred before breeding and the effects were limited to distinct susceptibility of daughters to diseases at young age.

## Strategies to alleviate the impact of postpartum diseases on reproduction

Prevention of postpartum inflammatory diseases is unquestionably the best approach to reduce the impact of diseases on fertility of cattle, and strategies to minimize the incidence of postpartum diseases were reviewed by others (LeBlanc *et al.*, 2006; Santos and Ribeiro, 2014). Nonetheless, understanding the mechanism mediating the impact of disease on

reproductive biology of cattle could lead to new strategies for mitigation of the negative consequences of diseases. Assuming that inflammation is the major mediator of subfertility in cows with postpartum diseases, control of inflammation during the clinical presentation of the disease could potentially mitigate the effects of inflammation on reproduction. McDougall *et al.* (2016) performed a randomized clinical trial testing the hypothesis that addition of a nonsteroidal anti-inflammatory drug (meloxicam) to antimicrobial treatment of clinical mastitis would improve subsequent fertility of dairy cows. Cows treated with meloxicam had greater conception risk in their first insemination postpartum and greater proportion of cows pregnant by day 120 after calving compared with the control group (Fig. 6). The results indicate that controlling inflammation during clinical presentation of an inflammatory disease might improve subsequent reproductive performance in dairy cows. A nutraceutical alternative for control of inflammation is reducing ratio of omega-6 to omega-3 fatty acids in the diet of postpartum cows (Greco *et al.*, 2015), which could also minimize the effects of inflammatory diseases on reproduction.

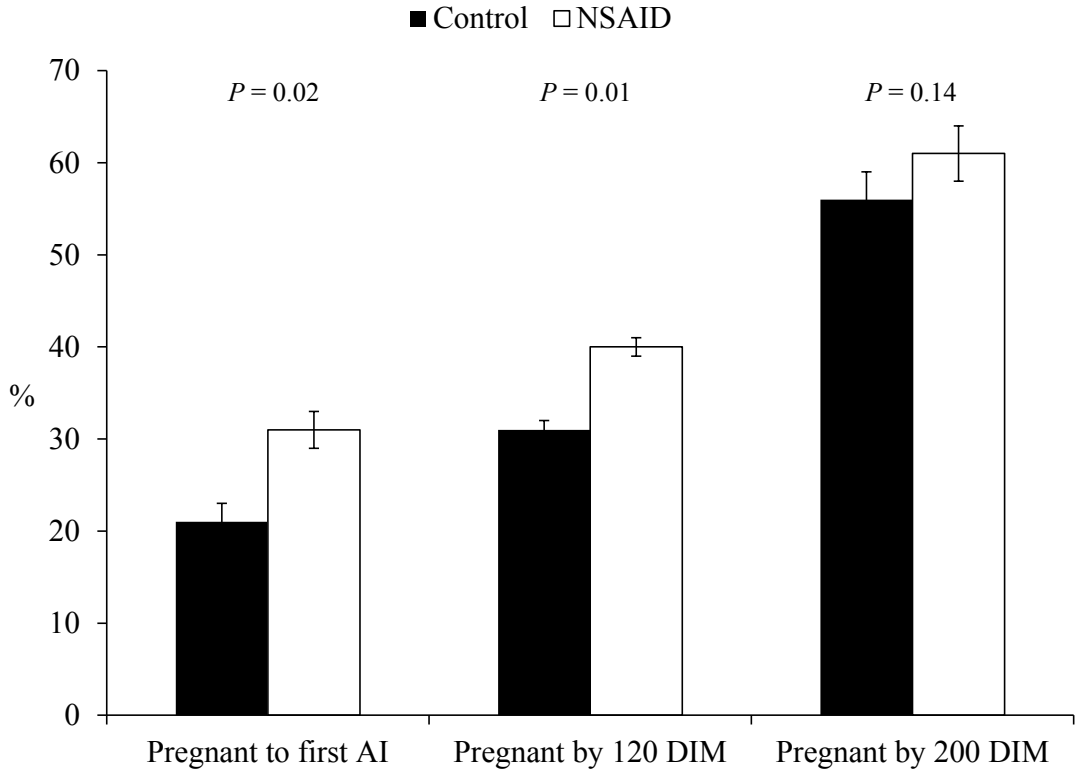


Figure 6. Proportion of dairy cows 1) pregnant to first AI, 2) pregnant by 120 days in milk (DIM) and 3) pregnant by 200 DIM according to treatment of clinical mastitis in a randomized clinical trial (McDougall *et al.*, 2016). Cows in the control group received only antimicrobial therapy and a placebo injection. Cows in the nonsteroidal anti-inflammatory drug (NSAID) group receive the same antimicrobial therapy of the control group plus a subcutaneous injection of 0.5 mg of meloxicam per kg of body weight. Error bars represent the standard errors of the means. Data from McDougall *et al.* (2016).

## Conclusions

Inflammatory diseases occurring before breeding are very prevalent in dairy cows and have long-lasting effects on subsequent pregnancy establishment and survival. The effect of diseases on fertility does not seem to be mediated by a single mechanism, rather a combination of multiple mechanisms that have additive effects, which include reduced BCS at the time of breeding, reduced developmental competence of oocytes, and altered uterine environment (Fig. 7). In pregnancies that survive to term, the long-lasting effects of diseases on developmental biology seem to be extended to the postnatal life of heifers and their susceptibility to diseases in early life. In addition to prevention of diseases, control of inflammation during clinical presentation of the disease mitigates their impact on reproductive biology of cattle. Because of the high incidence and the high impact on reproduction, inflammatory diseases postpartum represent an important problem in reproductive management of cattle and should always be considered when evaluating reproductive efficiency of herds and recommending management changes.

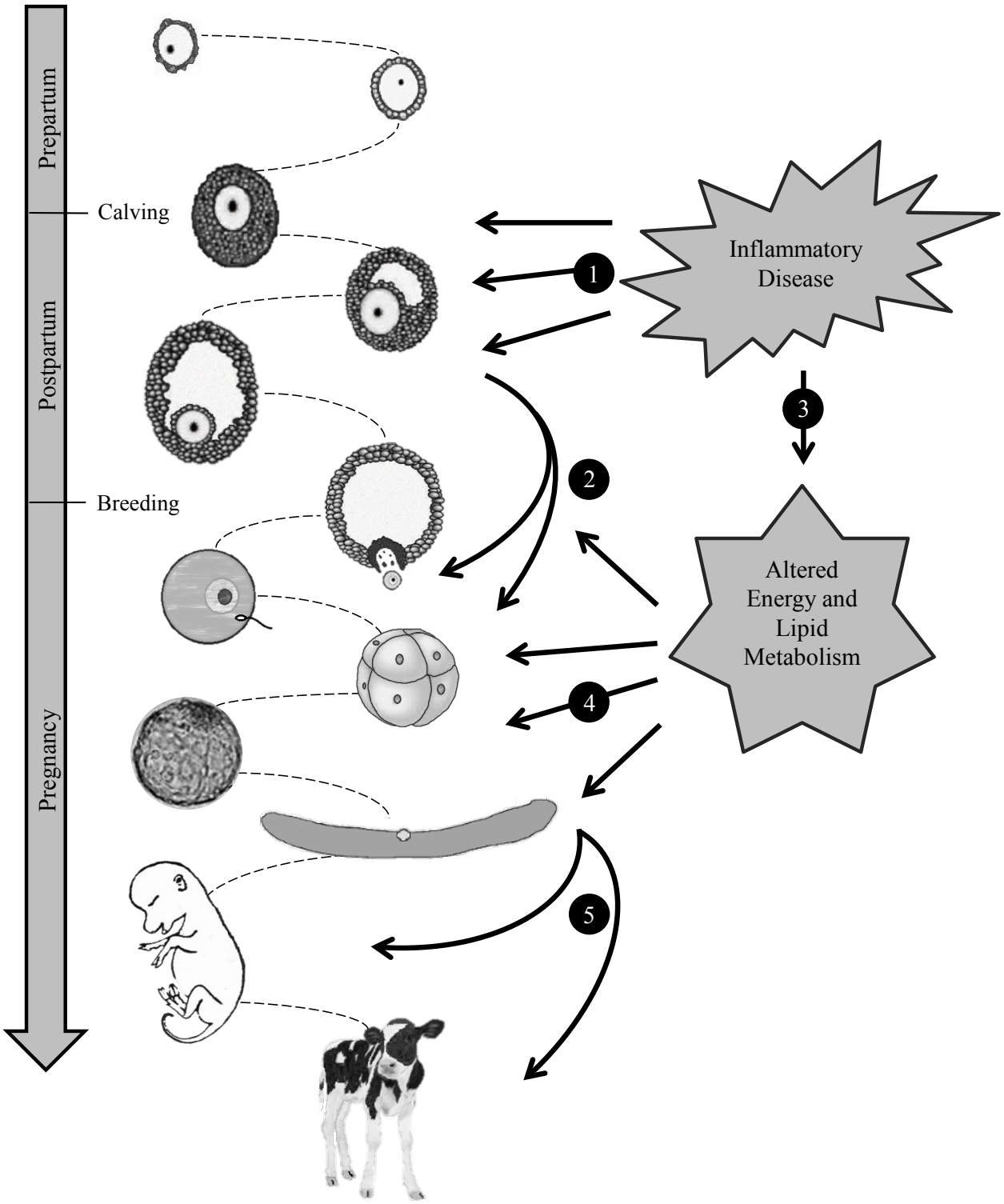


Figure 7. Schematic representation of putative long-lasting effects of postpartum inflammatory diseases on developmental biology and fertility of cows. Most clinical diseases occur in the first 3 weeks postpartum and cause inflammation. Inflammatory molecules secreted by innate immune cells in the insulted tissue reach the ovary through systemic circulation and alter ovarian follicle biology (1). Effects of inflammatory molecules on preantral and antral follicles reduce the future developmental competence of enclosed oocytes (2). Except for cows with chronical inflammation, concentration of inflammatory molecules in systemic circulation are not altered at the time of breeding, and the effects of postpartum diseases on uterine environment occur indirectly through lasting effects on energy and lipid metabolism of the cow (3). Altered metabolism of the cow during the time of embryonic development causes changes in uterine histotroph composition, impairs conceptus development and results in increased incidence of early pregnancy losses (4). Pregnancies that survive the embryonic period have altered programming of the developing fetus and placenta, which leads to increased incidence of late pregnancy losses and perhaps postnatal consequences in those that survive to term (5).

## References


**Bromfield JJ, Santos JEP, Block J, Williams RS, Sheldon IM**. 2015. Physiology and Endocrinology Symposium: Uterine infection: Linking infection and innate immunity with infertility in the high-producing dairy cow. *J Anim Sci*, 93:2021-2033.

**Carvalho MR, Peñagaricano F, Santos JEP, DeVries TJ, McBride B, Ribeiro ES**. 2017. Transgenerational effects of postpartum inflammatory diseases in dairy cows. *J Dairy Sci*, 100(E-suppl. 2):35-36.

**Colditz IG**. 2002. Effects of the immune system on metabolism: implication for production and disease resistance in livestock. *Livest Prod Sci*, 75:257-268.

**Contreras GA, Strieder-Barboza C, Raphael W**. 2017. Adipose tissue lipolysis and remodeling during the transition period of dairy cows. *J Anim Sci Biotech*, 8:41. doi: 10.1186/s40104-017-0174-4.

**Dantzer R, Kelley KW**. 2007. Twenty years of research on cytokine-induced sickness behavior. *Brain Behav Immun*, 21:153-160.

**De Vries A**. 2006. Economic value of pregnancy in dairy cattle. *J Dairy Sci*, 89:3876-3885.

**Fair T**. 2003. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci*, 78:203-216.

**Gifford CA, Holland BP, Mills RL, Maxwell CL, Farney JK, Terrill SJ, Step DL, Rich-ards CJ, Burciaga Robles LO, Krehbiel CR**. 2012. Growth and development symposium: Impacts of inflammation on cattle growth and carcass merit. *J Anim Sci*, 90:1438-1451.

**Greco LF, Neves Neto JT, Pedrico A, Ferrazza RA, Lima FS, Bisinotto RS, Martinez N, Garcia M, Ribeiro ES, Gomes GC, Shin JH, Ballou MA, Thatcher WW, Staples CR, Santos JEP**. 2015. Effects of altering the ratio of dietary n-6 to n-3 fatty acids on performance and inflammatory responses to a lipopolysaccharide challenge in lactating Holstein cows. *J Dairy Sci*, 98:602-617.

**Hansen PJ, Soto P, Natzke, RP**. 2004. Mastitis and fertility in cattle - possible involvement of inflammation or immune activation in embryonic mortality. *Am J Reprod Immunol*, 51:294-301.

**Itoh T, Fairall L, Amin K, Inaba Y, Szanto A, Balint BL, Nagy L, Yamamoto K, Schwabe JWR**. 2008. Structural basis for the activation of PPARγ by oxidized fatty acids. *Nat Struct Mol Biol,* 15:924-931.

**Kvidera SK, Horst EA, Abuajamieh M, Mayorga EJ, Sanz Fernandez MV, Baumgard LH**. 2017. Glucose requirements of an activated immune system in lactating Holstein cows. *J Dairy Sci*, 100:2360-2374.

**Lavon Y, Leitner G, Goshen T, Braw-Tal R, Jacoby S, Wolfenson D**. 2008. Exposure to endotoxin during estrus alters the timing of ovulation and hormonal concentrations in cows. *Theriogenology*, 70:956-967.

**LeBlanc SJ, Lissemore KD, Kelton DF, Duffield TF, Leslie KE**. 2006. Major advances in disease prevention in dairy cattle. *J Dairy Sci*, 89:1267-1279.

**Lussier JG, Matton P, Dufour JJ**. 1987. Growth rates of follicles in the ovary of the cow. *J Reprod Fertil*, 81:301-307.

**Medzhitov R**. 2008. Origin and physiological roles of inflammation. *Nature*, 454:428-435.

**McDougall S, Abbeloos E, Piepers S, Rao AS, Astiz, Van Werven T, Statham J, Pérez-Villalobos N**. 2016. Addition of meloxicam to the treatment of clinical mastitis improves subsequent reproductive performance. *J Dairy Sci*, 99:2026-2042.

**McIntyre TM, Pontsler AV, Silva AR, Hilaire AS, Xu Y, Hinshaw JC, Zimmerman GA, Hama K, Aoki J, Arai H, Prestwich GD**. 2003. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARγ agonist. *Proc Natl Acad Sci USA*, 100:131-136.

**Oliveira JF, Henkes LE, Ashley RL, Purcell SH, Smirnova NP, Veeramachaneni DNR, Anthony RV, Hansen TR**. 2008. Expression of ISGs in extrauterine tissues during early pregnancy in sheep is the consequence of endocrine IFN-s release from the uterine vein. *Endocrinology*, 149:1252-1259.

**Peter AT, Bosu WT, DeDecker RJ**. 1989. Suppression of preovulatory luteinizing hormone surges in heifers after intrauterine infusions of Escherichia coli endotoxin. *Am J Vet Res*, 50:368-373.

**Qu Y, Fadden AN, Traber MG, Bobe G**. 2014**.** Potential risk indicators of retained placenta and other diseases in multiparous cows. *J Dairy Sci,* 97:4151-4165.

**Ribeiro ES, Galvão K, Thatcher WW, Santos JEP**. 2012. Economic aspects of applying reproductive technologies to dairy herds. *Anim Reprod*, 9:370-387.

**Ribeiro ES, Lima FS, Greco LF, Bisinotto RS, Monteiro AP, Favoreto M, Ayres H, Marsola RS, Martinez N, Thatcher WW, Santos JEP**. 2013. Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. *J Dairy Sci*, 96:5682-5697.

**Ribeiro ES**. 2015. Molecular features of reproductive biology associated with fertility in lactating dairy cows. Gainesville, FL: University of Florida. PhD Dissertation,

**Ribeiro ES, Gomes GC, Greco LF, Cerri RLA, Vieira-Neto A, Monteiro PLJ Jr, Lima FS, Bisinotto RS, Thatcher WW, Santos JEP**. 2016a. Carryover effect of postpartum inflammatory diseases on developmental biology and fertility in lactating dairy cows. *J Dairy Sci* 99:2201-2220.

**Ribeiro ES, Greco LF, Bisinotto RS, Lima FS, Thatcher WW, Santos JEP**. 2016b. Biology of preimplantation conceptus at the onset of elongation in dairy cows. *Biol Reprod*, 94:97, 1-18.

**Ribeiro ES, Santos JEP, Thatcher WW**. 2016c. Role of lipids on elongation of the preimplantation conceptus in ruminants. *Reproduction*, 152:R115-R126.

**Rodgers JC, Bird SL, Larson JE, DiLorenzo N, Dahlen CR, DiCostanzo A, Lamb GC**. 2012. An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. *J Anim Sci*, 90:4055-4062.

**Romanyukhaa AA, Rudneva SG, Sidorov IA**. 2006. Energy cost of infection burden: An approach to understanding the dynamics of host-pathogen interactions. *J Theor Biol*, 241:1-13. doi:10.1016/j.jtbi.2005.11.004.

**Rukkwamsuk T, Kruip TAM, Meijer GAL, Wensing T**. 1999. Hepatic fatty acid composition in periparturient dairy cows with fatty liver induced by intake of a high energy diet in the dry period. *J Dairy Sci*, 82:280-287.

**Santos JEP, Thatcher WW, Chebel RC, Cerri RLA, Galvão KN**. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. *Anim Reprod Sci*, 82/83:513-535.

**Santos JEP, Rutigliano HM, Sá Filho MF**. 2009. Risk factors for resumption of postpartum cyclicity and embryonic survival in lactating dairy cows. *Anim Reprod Sci*, 110:207-221.

**Santos JEP, Bisinotto RS, Ribeiro ES, Lima FS, Greco LF, Staples CR, Thatcher WW**. 2010. Applying nutrition and physiology to improve reproduction in dairy cattle. *Soc Reprod Fertil Suppl*, 67:387-403.

**Santos JEP, Ribeiro ES**. 2014. Impact of animal health on reproduction of dairy cows. *Anim Reprod*, 11:254-269.

**Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth H**. 2009. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biol Reprod*, 81:1025-1032.

**Sordillo LM**. 2016. Nutritional strategies to optimize dairy cattle immunity. *J Dairy Sci*, 99:4967-4982.

**Soto P, Natzke RP, Hansen PJ**. 2003. Actions of tumor necrosis factor-a on oocyte maturation and embryonic development in cattle. *Am J Reprod Immunol*, 50:380-388.

**Williams EJ, Sibley K, Miller AN, Lane EA, Fishwick J, Nash DM, Herath S, England GCW, Dobson H, Sheldon IM**. 2008. The effect of Escherichia coli lipopolysaccharide and tumour necrosis factor alpha on ovarian function. *Am J Reprod Immunol*, 60:462-473.

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# Follicular environment and oocyte maturation: roles of local peptides and steroids

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## Abstract

A large amount of data on the mechanisms regulating cumulus-oocyte maturation in mammals has been generated in the last 20 years. It has been made clear that oocyte-secreted factors play a central role in the control of cumulus differentiation and oocyte developmental competence. However, more recent data indicate that cumulus-derived factors are also involved. In this mini-review, we have compiled and discussed data produced in our laboratory about the involvement of oocyte and cumulus-derived peptides, including fibroblast growth factors, bone morphogenetic protein 15, Kit ligand and natriuretic peptide C, in the regulation of cumulus metabolism and oocyte nuclear maturation. In addition, we discuss the interaction of follicular steroids with natriuretic peptide C in the control of meiosis progression.

**Keywords***:* oocyte, cumulus cells, intrafollicular peptides, steroids, *in vitro* maturation, cattle.

## Introduction

There is great interest to improve efficiency of *in vitro* maturation of oocytes (IVM) in animal species and humans as IVM has been considered the main technological bottleneck to improve embryo *in vitro* production following *in vitro* fertilization (IVF). It has been clearly demonstrated that current IVM systems do not adequately reproduce the follicular environment where the cumulus-oocyte complex (COC) physiologically differentiates, which compromises cumulus cells function and oocyte developmental competence (Rizos *et al.,* 2002; Brown *et al.*, 2017). Therefore, understanding the mechanisms that regulate COC differentiation is critical for the improvement of IVM systems.

The bidirectional interaction between the oocyte and cumulus cells is essential for oocyte developmental competence and constitutes a valuable parameter for improving IVM/IVF outcomes (Gilchrist, 2011). A lot of attention has been given to secreted paracrine factors as mediators of the oocyte-cumulus communication, mainly to oocyte secreted factors (OSF). There is robust evidence that OSF, particularly bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), both members of the transforming growth factor-ß (TGF-ß) superfamily, and fibroblast growth factors (FGF) regulate various aspects of cumulus cells differentiation such as expansion, metabolism, steroidogenesis and apoptosis (Eppig, 2001; Sugiura *et al.*, 2007; Gilchrist *et al.*, 2008; Caixeta *et al.*, 2013b). However, data obtained in cattle and pigs suggest that the importance of OSF in the control of cumulus expansion and metabolism may vary between species (Buccione *et al.,* 1990; Vanderhyden, 1993; Ralph *et al.*, 1995; Sutton *et al.*, 2003; Caixeta *et al.*, 2013b). Although the other direction of the oocyte-cumulus communication has been less explored, there is recent evidence that cumulus derived peptides regulate nuclear maturation and gene expression in the oocyte (Lima *et al.*, 2016).

Apart from secreted factors, oocyte-cumulus communication is also mediated by transzonal cytoplasmic projections (TZP), which are extensions of cumulus cells that cross the zona pellucida transporting ions, metabolites and regulatory molecules (Albertini *et al.*, 2001). The delivery of glucose metabolites and small regulatory molecules through gap junctions connecting the end of the TZPs with the ooplasm appears crucial for the control of meiosis, chromatin configuration, transcriptional activity and metabolism of the oocyte (Conti *et al.*, 2012; Luciano *et al.*, 2014; Gilchrist *et al.*, 2016; Brown *et al.*, 2017). In addition to the transport through gap junctions, recent studies indicate that TZPs can also deliver larger molecules such as RNA transcripts via micro-vesicles in a transport mechanism designated as the gametic synapse (Macaulay *et al.*, 2014). In fact, cumulus-derived RNA has been identified in oocyte polyribosomes suggesting that the gametic synapse can influence the translational activity of the oocyte (Macaulay *et al.*, 2016).

This paper aims to review and discuss some of the recent data on paracrine mediators of the oocyte-cumulus interaction, as well as mechanisms regulating periovulatory differentiation of cumulus cells and oocyte nuclear maturation with potential practical implications for IVM.

## Oocyte *vs.* cumulus secreted factors: who runs the show in the cow?

A large body of data produced in mice points to a leading role for the oocyte in the regulation of cumulus cells differentiation and metabolism (Matzuk *et al.,* 2002; Gilchrist *et al.*, 2008). However, studies using microsurgical removal of the oocyte from the COC and co-culture of oocytectomized COCs with secreting denuded oocytes indicate that OSF are needed for cumulus expansion in mice, but not in cattle, pigs or rats (Buccione *et al.*, 1990; Ralph *et al.*, 1995; Vanderhyden *et al.*, 2003). More recently, the same approach demonstrated that OSF also play a central role in the regulation of glycolytic activity of cumulus cells in mice (Sugiura *et al.*, 2005), whereas in cattle, utilization

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of oxygen, glucose, pyruvate and lactate by cumulus cells was not affected by removal of the oocyte (Sutton *et al.*, 2003). Taken together, these data point to differences between species with regard to the participation of the oocyte in cumulus differentiation, raising speculation that autocrine and paracrine signaling within the cumulus may be more influential in species other than the mouse. In mono-ovulatory mammals, the functional relevance of intra-cumulus TGF-ß signaling is controversial. Studies assessing the expression patterns of BMP15 and GDF9 in the COC are conflicting. While expression of BMP15 and GDF9 has been consistently detected in the oocyte, in cumulus cells it was observed in one but not all studies in cattle (Hosoe *et al.*, 2011; Crawford and McNatty, 2012).

Alternatively, FGF2 and Kit Ligand (KL) are two potential and less controversial cumulus-derived regulators of COC maturation. A microarray study pointed FGF2 as an important up-regulated gene in the predicted pathways activated by the LH surge to induce final differentiation of bovine cumulus cells (Assidi *et al.*, 2010). In addition, we have shown that transcription of FGFR2C and FGFR3C, two receptors efficiently activated by FGF2, is drastically and rapidly increased in cumulus cells from bovine COCs subjected to FSH-stimulated IVM, suggesting that sensitivity to FGF2 is enhanced with activation of the ovulatory cascade (Zhang *et al.*, 2006; Caixeta *et al.*, 2013a). Taken together, these studies indicate that FGF2 signaling is enhanced in preparation for ovulation and final COC maturation. In fact, recent data from our laboratory suggest the involvement of FGF2 in the regulation of meiosis progression, cumulus expansion and apoptosis (Buratini J.; 2017; Institute of Biosciences, São Paulo State University, Botucatu, SP, Brazil; unpublished data). Moreover, the involvement of FGF2 in the control of COC maturation is also consistent with our previous finding that FGF2 increases phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) in granulosa cells, two pathways known to regulate meiotic resumption and cumulus expansion (Jiang *et al.*, 2011; Prochazka *et al.*, 2012).

On the other hand, we have recently reported that mRNA levels of KL increase around 10 times in bovine cumulus cells during FSH-stimulated IVM and presented evidence of a role for KL in the regulation of meiosis progression under the influence of OSF in cattle (Lima *et al.*, 2016). These data are compiled and discussed in further details below. Therefore, whilst further studies are needed to address the importance of cumulus derived peptides for COC maturation, the data collected so far suggest that OSF are less influential in the cow than in the mouse and that an interaction of oocyte and cumulus derived factors likely runs the show in the cow.

## Oocyte secreted factors in the control of cumulus expansion and metabolism

Although OSF are not absolutely required for cumulus expansion in cattle, they appear to influence this process (Ralph *et al.*, 1995; Zhang *et al.*, 2010a; Caixeta *et al.*, 2013b). BMP15 and FGF10 are expressed by the oocyte and when added to the IVM medium they can enhance cumulus expansion and embryo production in cattle (Hussein *et al.*, 2006; Buratini *et al.*, 2007; Zhang *et al.*, 2010a; Crawford and McNatty, 2012). We have provided evidence that BMP15 and FGF10 control the expression of key genes in the ovulatory cascade. Supplementation of the IVM medium with BMP15 increased transcription of disintegrin and metalloprotease 10 (ADAM 10), ADAM 17, amphiregulin (AREG) and epiregulin (EREG) in cumulus cells from bovine COCs. Alternatively, treatment with FGF10 promptly increased mRNA levels of prostaglandin (PG)-endoperoxide synthase (PTGS2), and subsequently of pentraxin 3 (PTX3) and tumor necrosis factor alpha-induced protein 6 (TNFAIP6) in bovine cumulus cells (Caixeta *et al.*, 2013b). Therefore, BMP15 appears to enhance the ovulatory cascade right at its beginning by increasing production, cleavage and release of EGF-like factors, the last two events being a consequence of ADAM10 and ADAM17 activity (reviewed by Ben-Ami *et al.*, 2006). These effects of BMP15 are consistent with its positive impact on developmental competence in cattle (Hussein *et al.*, 2006) and with evidences of suboptimal EGF signaling in bovine COCs matured *in vitro* (Brown *et al.*, 2017). On the other hand, FGF10 would act downstream of EGF-like factors by increasing the expression of cross-linking proteins that stabilize the extracellular matrix (PTX3 and TNFAIP6). This appears to be at least in part mediated by the preceding prompt increase in PTGS2 expression, since PGE2 is required for TNFAIP6 expression (Ochsner *et al.*, 2003, reviewed by Russell and Robker, 2007). These findings are in agreement with the positive effect of FGF10 on embryo production following IVM/IVF and with higher expression of TNFAIP6 in cumulus cells from *in vivo* matured compared with *in vitro* matured bovine COCs (Tesfaye *et al.*, 2009; Zhang *et al.*, 2010a).

Despite the evidences that BMP15 and FGF10 act at different steps of the ovulatory cascade, they appear to act similarly with regard to their influence on glucose metabolism. They both increased glucose uptake without altering lactate production during IVM of bovine COCs, which was accompanied by increases in mRNA levels of glucose transporters (GLUT1 and GLUT4). Interestingly, BMP15 and FGF10 also increased mRNA abundance of glucosamine:fructose-6-$PO_4$ transaminases (GFPT1 and GFPT2) and hyaluronan synthase 2 (HAS2), which are rate-limiting enzymes in the hexosamine pathway of glucose metabolism that leads to the production of hyaluronic acid, the major component of the extracellular matrix (Sutton-McDowall *et al.*, 2010; Caixeta *et al.*, 2013b). Therefore, collectively, these data suggest that after the activation of the ovulatory cascade BMP15 and FGF10 direct the metabolism of glucose towards the synthesis of hyaluronic acid to support the formation of extracellular matrix for cumulus expansion.

Nevertheless, at earlier stages of COC maturation, before the activation of the ovulatory

cascade, the influence of BMP15 and FGFs on glucose metabolism may be different. BMP15 and FGF8 were shown to cooperate to increase the expression and activity of glycolytic enzymes phosphofructokinase (PFKP) and lactate dehydrogenase (LDHA) in cumulus cells from COCs arrested at the germinal vesicle (GV) stage in mice (Sugiura *et al.,* 2007). On the other hand, in bovine COCs undergoing IVM, the combination of BMP15 with FGF17, a member of the FGF8 superfamily also expressed by the bovine oocyte and capable of activating the same receptors that FGF8, did not alter PFKP mRNA levels in cumulus cells (Zhang *et al.*, 2006; Machado *et al.*, 2009, 2015). Therefore, additional studies dissecting the influence of species, COC developmental stage and culture conditions are needed for a clearer understanding of the roles of OSF in the regulation of glucose metabolism in the COC.

Like FGF10, FGF17 was shown to enhance expansion of bovine COCs during IVM (Machado *et al.*, 2015). However, FGF17 did not alter the expression of PTGS2, or any of the genes in the ovulatory cascade investigated [ADAM10, ADAM17, AREG, EREG, PTX3, TNFAIP6, VERS (versican) and HAS2]. Therefore, different FGFs appear to impact on cumulus expansion and differentiation through different mechanisms. Although no additional effect on cumulus expansion or meiosis progression was observed when FGF17 was combined with BMP15 during IVM, this combination increased mRNA levels of the nuclear progesterone receptor (nPR) in cumulus cells after IVM, as well as the number of cells in the inner cell mass (ICM) of blastocysts produced by IVF/IVC (Machado *et al.*, 2015). These data therefore suggest that FGFs and BMP15 interact during COC maturation to improve developmental competence, which may be at least in part a consequence of increased progesterone sensitivity. Previous studies using inhibitors of progesterone synthesis and nPR antagonists have elegantly demonstrated that progesterone signaling is crucial for cumulus expansion and oocyte developmental competence (Aparicio *et al.*, 2011).

## Oocyte and cumulus-derived factors in the regulation of nuclear maturation and cumulus-oocyte communication

A major and well recognized limitation of IVM is the asynchrony between oocyte nuclear and cytoplasmic maturation. Chromatin condensation is precipitated and transcriptional activity diminishes abruptly when the COC is removed from the follicle (Hyttel *et al.*, 1987; Lodde *et al.*, 2007). Therefore, pre-IVM cultures containing agents capable of delaying nuclear maturation such as natriuretic peptide precursor C (NPPC) and phosphodiesterase inhibitors have been proposed to improve the outcomes of IVM/IVF in cattle (Albuz *et al.*, 2010; Franciosi *et al.*, 2014), although these strategies have not yet provided consistent results in different breeds and laboratories (Gilchrist *et al*., 2015).

Robust studies using Nppc and Npr2 (natriuretic peptide receptor B) mutant mice first demonstrated the importance of NPPC signaling for meiotic arrest. A model has been proposed and widely accepted in which NPPC produced predominantly by mural granulosa cells activates natriuretic peptide receptor B (NPR2) on cumulus cells to induce production of cGMP, which is then transferred to the oocyte through gap junctions, deviating the activity of phosphodiesterase 3 from cAMP. This would maintain cAMP at levels required to prevent the synthesis of maturation promoting factor (MPF), thus holding the oocyte in meiotic arrest (Zhang *et al.*, 2010b; Conti *et al.*, 2012). Later studies demonstrated that NPPC is expressed by bovine cumulus cells, and that, like in the mouse, NPPC also inhibits germinal vesicle breakdown (GVBD) in cattle (Franciosi *et al.*, 2014; De Cesaro *et al.*, 2015). For meiosis resumption to occur, LH inhibits NPPC production by granulosa cells and reduces the flow of cGMP from the outer layers of the cumulus to the oocyte (Kawamura *et al.*, 2011; Shuhaibar *et al.*, 2015). Reduced gap junction functionality after the LH surge is believed to be a consequence of the production/secretion of EGF-like peptides that bind to the EGFR to induce mitogen activated protein kinase (MAPK) dependent phosphorylation of connexins, the main components of gap junctions (Conti *et al.*, 2012).

Therefore the influence of NPPC on nuclear maturation depends on the functionality of gap junctions between cumulus cells and the oocyte. The importance of gap junction mediated communication for meiotic arrest and developmental competence was unequivocally demonstrated by studies where chemically induced gap junction uncoupling led to chromatin condensation and decreased transcriptional activity in the bovine oocyte (Luciano *et al.*, 2011). And since these effects were neutralized by co-treatment with cilostamide, an oocyte specific phosphodiesterase inhibitor, it was concluded that the impact of gap junction functionality is mediated by intra-oocyte cAMP. This is in agreement with a later study from the same group reporting positive effects of NPPC and cilostamide on gap junction functionality in cattle (Franciosi *et al.*, 2014).

Interestingly, the influence of NPPC appears to be regulated by intrafollicular steroids. In mice, estradiol is required to maintain the ability of NPPC to stimulate cGMP production and to prevent GVBD in culture, and both estradiol and testosterone can increase mRNA levels of Npr2 in cumulus cells (Zhang *et al.*, 2011). The enhancement of NPPC action by steroids also occurs in cattle. We have recently demonstrated that intrafollicular steroids cooperate with NPPC to slow nuclear maturation and to increase gap junction mediated cumulus-oocyte communication in the bovine COC. More specifically, co-treatment with estradiol, progesterone and adrostenedione at physiological concentrations enhanced the ability of NPPC to inhibit GVBD and to increase the transfer of a dye from the oocyte to cumulus cells, which was accompanied by an increase in NPR2 mRNA levels. Therefore, the enhanced effects on nuclear maturation and gap junction functionality were interpreted as a consequence of greater NPPC signaling and cGMP production in the

presence of steroids. Moreover, the combination of NPPC with follicular steroids in a pre-IVM culture promoted improved embryo quality (assessed by total cell number), suggesting that this strategy may be useful to improve IVM/IVF outcomes (Soares *et al.*, 2017).

The NPPC system is also regulated by cumulus and oocyte-derived factors. We have recently reported evidence of a link between NPPC and KL under the influence of OSF in cattle. In mammals, Kit ligand is expressed by granulosa cells since very early stages of folliculogenesis and activates the receptor KIT on the oocyte and theca cells (Hutt *et al.*, 2006; Thomas and Vanderhyden, 2006). The roles of KL signaling in periovulatory COC differentiation have not been deeply investigated and are controversial; KL delayed 1st polar body extrusion in rats (Ismail *et al.*, 1997), but did the opposite in mice (Ye *et al.*, 2009). In cattle, first we demonstrated that mRNA levels of both isoforms of KL, KL1 and KL2, increase during the first 12 h of FSH-stimulated IVM in cumulus cells, suggesting that KL transcription is enhanced in preparation for ovulation. Secondly, we observed that KL supplementation during IVM of bovine COCs does not affect cumulus expansion, but enhances oocyte maturation as assessed by the percentage of oocytes reaching metaphase II. To investigate the mechanisms by which KL impacts on nuclear maturation, we assessed its effects on the expression of genes regulating meiosis in the bovine COC. Kit ligand did not alter mRNA levels of NPR2, but decreased mRNA abundance of NPPC in bovine cumulus cells. In addition, KL increased expression of Y-box binding protein 2 (YBX2) in the oocyte, a protein that regulates RNA stability and protein synthesis and is required for normal spindle formation (Medvedev *et al.*, 2011). Finally, we assessed whether the oocyte regulates KL expression in cumulus cells using the oocytectomy model, and observed mRNA levels around 5 times more abundant in oocytectomized compared with intact COCs at the end of IVM. The increase in KL expression was completely abrogated by co-culture with denuded oocytes, indicating that the influence of the oocyte on KL expression is mediated by OSF. Conversely and in agreement with the inhibitory influence of KL on NPPC expression described above, oocytectomy markedly decreased mRNA levels of NPPC in cumulus cells. The specific OSF that mediate the effects of KL on cumulus NPPC expression appear to vary between species and remain to be completely identified. In our studies, treatment with FGF10 during IVM decreased KL2 mRNA expression, suggesting that FGF10 may be one of these OSF in cattle (Lima *et al.*, 2016). Taken together, these data suggest that the oocyte and cumulus derived factors interact to control meiosis. It is tempting to speculate that an increase in cumulus KL expression overcoming the inhibitory effect of the oocyte through NPPC signaling may be part of the mechanisms leading to meiosis resumption in the periovulatory period in cattle.

## Concluding remarks

In this mini-review we compile data indicating that oocyte and cumulus derived factors interact to regulate cumulus differentiation, nuclear maturation and oocyte developmental competence in cattle. In addition, we present published evidence that steroids modulate the influence of cumulus-derived factors on meiosis progression and cumulus-oocyte communication. The data compiled herein widens our view of the mechanisms that regulate meiosis and cumulus function in cattle, and represent useful parameters for the improvement of IVM/IVF outcomes.

## References

**Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ**. 2001. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction*, 121**:**647-653.

**Albuz FK, Sasseville M, Lane M, Armstrong DT, Thompson JG, Gilchrist RB**. 2010. Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum Reprod*, 25**:**2999-3011.

**Aparicio IM, Garcia-Herreros M, O'Shea LC, Hensey C, Lonergan P, Fair T**. 2011. Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation. *Biol Reprod*, 84**:**910-921.

**Assidi M, Dieleman SJ, Sirard MA**. 2010. Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reproduction*, 140:835-852.

**Ben-Ami I, Freimann S, Armon L, Dantes A, Ron-El R, Amsterdam A**. 2006. Novel function of ovarian growth factors: combined studies by DNA microarray, biochemical and physiological approaches. *Mol Hum Reprod*, 12:413-419.

**Brown HM, Dunning KR, Sutton-McDowall M, Gilchrist RB, Thompson JG, Russell DL**. 2017. Failure to launch: aberrant cumulus gene expression during oocyte in vitro maturation. *Reproduction*, 153**:**R109-R120.

**Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ**. 1990. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol*, 138**:**16-25.

**Buratini J, Pinto MG, Castilho AC, Amorim RL, Giometti IC, Portela VM, Nicola ES, Price CA**. 2007. Expression and function of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 2B, in bovine follicles. *Biol Reprod*, 77:743-750.

**Caixeta ES, Machado MF, Ripamonte P, Price C, Buratini J**. 2013a. Effects of FSH on the expression of receptors for oocyte-secreted factors and members of the EGF-like family during in vitro maturation in cattle. *Reprod Fertil Dev*, 25:890-899.

**Caixeta ES, Sutton-McDowall ML, Gilchrist RB, Thompson JG, Price CA, Machado MF, Lima PF, Buratini J**. 2013b. Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-

oocyte complexes. *Reproduction*, 146:27-35.

**Conti M, Hsieh M, Zamah AM, Oh JS**. 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Mol Cell Endocrinol*, 356**:**65-73.

**Crawford JL, McNatty KP**. 2012. The ratio of growth differentiation factor 9: bone morphogenetic protein 15 mRNA expression is tightly co-regulated and differs between species over a wide range of ovulation rates. *Mol Cell Endocrinol*, 348:339-343.

**De Cesaro MP, Macedo MP, Santos JT, Rosa PR, Ludke CA, Rissi VB, Gasperin BG, Gonçalves PB**. 2015. Natriuretic peptides stimulate oocyte meiotic resumption in bovine. *Anim Reprod Sci*, 159:52-59.

**Eppig JJ**. 2001. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, 122:829-838.

**Franciosi F, Coticchio G, Lodde V, Tessaro I, Modina SC, Fadini R, Dal Canto M, Renzini MM, Albertini DF. Luciano AM**. 2014. Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes. *Biol Reprod*. 91:61. doi: 10.1095/biolreprod.114.118869.

**Gilchrist RB, Lane M, Thompson JG**. 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update*, 14:159-177.

**Gilchrist RB**. 2011. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. *Reprod Fertil Dev*, 23:23-31.

**Gilchrist RB, Zeng HT, Wang X, Richani D, Smitz J, Thompson JG**. 2015. Reevaluation and evolution of the simulated physiological oocyte maturation system. *Theriogenology*, 84:656-657.

**Gilchrist RB, Luciano AM, Richani D, Zeng HT, Wang X, Vos MD, Sugimura S, Smitz J, Richard FJ, Thompson JG**. 2016. Oocyte m.aturation and quality: role of cyclic nucleotides. *Reproduction*, 152:R143-157.

**Hosoe M, Kaneyama K, Ushizawa K, Hayashi KG, Takahashi T**. 2011. Quantitative analysis of bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) gene expression in calf and adult bovine ovaries. *Reprod Biol Endocrinol*, 9:33. doi: 10.1186/1477-7827-9-33.

**Hussein TS, Thompson JG, Gilchrist RB**. 2006. Oocyte-secreted factors enhance oocyte developmental competence. *Dev Biol*, 296:514-521.

**Hutt KJ, McLaughlin EA, Holland MK**. 2006. Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. *Mol Hum Reprod*, 12:61-69.

**Hyttel P, Xu KP, Smith S, Callesen H, Greve T**. 1987. Ultrastructure of the final nuclear maturation of bovine oocytes in vitro. *Anat Embryol (Berl)*, 176:35-40.

**Ismail RS, Dubé M, Vanderhyden BC**. 1997. Hormonally regulated expression and alternative splicing of kit ligand may regulate kit-induced inhibition of meiosis in rat oocytes. *Dev Biol*, 184:333-342.

**Jiang ZL, Ripamonte P, Buratini J, Portela VM, Price CA**. 2011. Fibroblast growth factor-2 regulation of Sprouty and NR4A genes in bovine ovarian granulosa cells. *J Cell Physiol*, 226:1820-1827.

**Kawamura K, Cheng Y, Kawamura N, Takae S, Okada A, Kawagoe Y, Mulders S, Terada Y, Hsueh AJ**. 2011. Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Hum Reprod*, 26:3094-3101.

**Lima PF, Ormond CM, Caixeta E, Barros RG, Price C, Buratini J**. 2016. Effect of kit ligand on natriuretic peptide precursor C and oocyte maturation in cattle. *Reproduction*, 152:481-489.

**Lodde V, Modina S, Galbusera C, Franciosi F, Luciano AM**. 2007. Large-scale chromatin remodeling in germinal vesicle bovine oocytes: interplay with gap junction functionality and developmental competence. *Mol Reprod Dev*, 74:740-749.

**Luciano AM, Franciosi F, Modina SC, Lodde V**. 2011. Gap junction-mediated communications regulate chromatin remodeling during bovine oocyte growth and differentiation through cAMP-dependent mechanism(s). *Biol Reprod*, 85:1252-1259.

**Luciano AM, Franciosi F, Dieci C, Lodde V**. 2014. Changes in large-scale chromatin structure and function during oogenesis: a journey in company with follicular cells. *Anim Reprod Sci*, 149:3-10.

**Macaulay AD, Gilbert I, Caballero J, Barreto R, Fournier E, Tossou P, Sirard MA, Clarke HJ, Khandjian É, Richard FJ, Hyttel P, Robert C**. 2014. The gametic synapse: RNA transfer to the bovine oocyte. *Biol Reprod*, 91:90. doi: 10.1095/biolreprod.114.119867.

**Macaulay AD, Gilbert I, Scantland S, Fournier E, Ashkar F, Bastien A, Saadi HA, Gagné D, Sirard MA, Khandjian É, Richard FJ, Hyttel P, Robert C**. 2016. Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation. *Biol Reprod*, 94:16. doi: 10.1095/biolreprod.114.127571.

**Machado MF, Portela VM, Price CA, Costa IB, Ripamonte P, Amorim RL, Buratini J**. 2009. Regulation and action of fibroblast growth factor 17 in bovine follicles. *J Endocrinol*, 202:347-353.

**Machado MF, Caixeta ES, Sudiman J, Gilchrist RB, Thompson JG, Lima PF, Price CA, Buratini J**. 2015. Fibroblast growth factor 17 and bone morphogenetic protein 15 enhance cumulus expansion and improve quality of in vitro-produced embryos in cattle. *Theriogenology*, 84:390-398.

**Matzuk MM, Burns KH, Viveiros MM, Eppig JJ**. 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science*, 296:2178-2180.

**Medvedev S, Pan H, Schultz RM**. 2011. Absence of MSY2 in mouse oocytes perturbs oocyte growth and maturation, RNA stability, and the transcriptome. *Biol Reprod*, 85:575-583.

**Ochsner SA, Russell DL, Day AJ, Breyer RM, Richards JS**. 2003. Decreased expression of tumor necrosis factor-alpha-stimulated gene 6 in cumulus cells of the cyclooxygenase-2 and EP2 null mice. *Endocrinology*, 144:1008-1019.

**Prochazka R, Blaha M, Nemcova L**. 2012. Signaling

pathways regulating FSH- and amphiregulin-induced meiotic resumption and cumulus cell expansion in the pig. *Reproduction*, 144:535-546.

**Ralph JH, Telfer EE, Wilmut I**. 1995. Bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor. *Mol Reprod Dev*, 42:248-253.

**Rizos D, Ward F, Duffy P, Boland MP, Lonergan P**. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev*, 61:234-248.

**Russell DL, Robker RL**. 2007. Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Hum Reprod Update*, 13:289-312.

**Shuhaibar LC, Egbert JR, Norris RP, Lampe PD, Nikolaev VO, Thunemann M, Wen L, Feil R, Jaffe LA**. 2015. Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles. *Proc Natl Acad Sci USA*, 112:5527-5532.

**Soares AC, Lodde V, Barros RG, Price CA, Luciano AM, Buratini J**. 2017. Steroid hormones interact with natriuretic peptide C to delay nuclear maturation, to maintain oocyte-cumulus communication and to improve the quality of in vitro-produced embryos in cattle. *Reprod Fertil Dev*. doi: 10.1071/RD16320.

**Sugiura K, Pendola FL, Eppig JJ**. 2005. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev Biol*, 279:20-30.

**Sugiura K, Su YQ, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ**. 2007. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development*, 134:2593-2603.

**Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB, Thompson JG**. 2003. Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. *Reproduction*, 126:27-34.

**Sutton-McDowall ML, Gilchrist RB, Thompson JG**. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction*, 139:685-695.

**Tesfaye D, Ghanem N, Carter F, Fair T, Sirard MA, Hoelker M, Schellander K, Lonergan P**. 2009. Gene expression profile of cumulus cells derived from cumulus-oocyte complexes matured either in vivo or in vitro. *Reprod Fertil Dev*, 21:451-461.

**Thomas FH, Vanderhyden BC**. 2006. Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. *Reprod Biol Endocrinol*, 4:19. doi: 10.1186/1477-7827-4-19.

**Vanderhyden BC**. 1993. Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). *J Reprod Fertil*, 98:219-227.

**Vanderhyden BC, Macdonald EA, Nagyova E, Dhawan A**. 2003. Evaluation of members of the TGFbeta superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis. *Reprod Suppl*. 61:55-70.

**Ye Y, Kawamura K, Sasaki M, Kawamura N, Groenen P, Gelpke MD, Rauch R, Hsueh AJ, Tanaka T**. 2009. Kit ligand promotes first polar body extrusion of mouse preovulatory oocytes. *Reprod Biol Endocrinol*, 7:26. doi: 10.1186/1477-7827-7-26.

**Zhang K, Hansen PJ, Ealy AD**. 2010a. Fibroblast growth factor 10 enhances bovine oocyte maturation and developmental competence in vitro. *Reproduction*, 140:815-826.

**Zhang M, Su YQ, Sugiura K, Xia G, Eppig JJ**. 2010b. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*, 330:366-369.

**Zhang M, Su YQ, Sugiura K, Wigglesworth K, Xia G, Eppig JJ**. 2011. Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro. *Endocrinology*, 152:4377-4385.

**Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM**. 2006. Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. *J Biol Chem* 281 15694-15700.

DOI: 10.21451/1984-3143-AR1004

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# Effects of nutrition on sexual development of bulls

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## Abstract

In the last decades a series of attempts have been made to improve reproductive performance of bulls via optimizing nutrition. Although an increase in energy uptake during the post-weaning period of calves led to a faster growing rate, it had no positive effects on sexual development. In contrast, a high-nutrition diet during the prepubertal period reduced the age at puberty of the bulls and increased the size/weight of the testis and the epididymal sperm reserves. This faster sexual development was associated with an increased transient LH peak, which seemed to be mediated by an increase in serum IGF-I concentrations. However, the exact mechanisms responsible for the interaction between nutrition and the subsequent development of calves are still not clear. Sexual development of bull calves depends not only on nutrition of the calves after birth but also on the feed intake of their mothers during pregnancy. A high-nutrition diet fed to the mother during the first trimester has negative effects on the reproductive performance of their offspring. In summary, growth, health and reproductive performance can be improved by nutrition, but further studies are necessary to obtain a better understanding about the mechanisms responsible for this phenomenon.

**Keywords**: nutrition, puberty, reproductive performance.

## Introduction

Because of the introduction of genomic selection in cattle breeding a few years ago, the relevance of the reproductive performance of bulls has increased tremendously. Using this new method, the breeders are able to obtain information about the genetic value of the bulls at either the embryonic period or immediately after birth. With this information, they may be able to obtain semen from bulls with high genetic value earlier. One factor that limits this goal of the breeders is the high variability in the onset of puberty and sexual maturation in bull calves (Brito *et al*., 2012).

It is well known that there is a relationship between body weight and sexual development (Brito *et al*., 2012). Therefore, a series of attempts have been made to improve the growth of bull calves. However, contradictory results have been obtained via the supplementation of feed. There are even reports that a high-energy feed intake during the pubertal period has negative effects on the health and reproductive performance of bulls (Coulter and Kozub, 1984; Coulter *et al*., 1987). In addition, there is now evidence that the nutritional differences in feed during the prepubertal period (Brito *et al*., 2007a, c, d) or even the feed intake of the mothers (Sullivan *et al*., 2010, Jaquiery *et al*., 2012) can affect the development of the calves during later stages of life. The aim of this paper is to give a review of the literature dealing with the effects of nutrition on sexual development of bull calves.

## Physiology of sexual maturation in bulls

For a better understanding of the effects of nutrition on sexual development it is important to be familiar with the physiological alterations occurring during this time period in bulls. An excellent overview of this topic has been given from Rawlings et al. (Rawlings *et al*., 2008). Therefore, only some aspects of the alterations occurring during sexual development in bulls will be described in this paper.

Testicular growth follows a sigmoidal pattern in bull calves, with small changes occurring up to 25 weeks of age, followed by a distinct increase in changes until puberty, and a slowing down of growth as the bull reaches sexual maturation (Abdel-Rauf, 1960; Macmillan and Hafs, 1968; Amann, 1983). In bulls, puberty is commonly defined as the time when the scrotal circumference (SC) is at least 28 cm and the ejaculate has a concentration of at least 50 million sperm/ml with ≥10% progressively motile sperm, and sexual maturation is defined as the first time when the ejaculate consists of ≥70% morphologically normal sperm (Wolf *et al*., 1965).

Based on the gonadotropin and testosterone concentrations in blood plasma, the reproductive development of bulls can be divided into three periods: the infantile, prepubertal and pubertal periods. During the infantile period, which lasts from birth to up to 8 weeks of age, there are low concentrations of both gonadotropins and testosterone (Amann *et al*., 1986; Rawlings *et al*., 2008). In the following prepubertal period, ranging from 8 to 20 weeks of age, a transient increase in gonadotropin concentration and a concurrent small increase in testosterone secretion occur (Amann and Walker, 1983; Barth *et al*., 2008; Rawlings *et al*., 2008). The concentration of LH starts to increase at 4 to 5 weeks and is at a maximum concentration from 12 to 16 weeks of age. It then falls, reaching a baseline at 25 weeks of age (Amann and Walker, 1983; Barth *et al*., 2008). The early postnatal increase in LH secretion is clearly triggered by an increase in the frequency of pulses of GnRH secretion (Rodriguez and Wise, 1989). High LH concentrations during the prepubertal period have a positive effect on sexual development (Secchiari *et al*., 1976). Calves with a higher LH secretion at this time period reach puberty earlier than calves with lower

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LH concentrations during the prepubertal period (Amann and Walker, 1983; Evans *et al*., 1995). Blood FSH concentrations are generally also elevated during the prepubertal period, but changes in FSH are less pronounced than the corresponding changes in LH. Calves that achieve a greater FSH concentration during calfhood are expected to develop larger testes and possibly reach puberty at an earlier age. The age at which SC first reaches 28 cm has been shown to occur earlier in FSH-treated calves than in saline-treated (control) calves. The concentration of FSH decreases to baseline levels by approximately 25 weeks of age (Miyamoto *et al*., 1989; Evans *et. al*., 1996; Bagu *et al*., 2006). Serum concentrations of testosterone increase slowly from birth to approximately 20 weeks of age; subsequently, testosterone concentrations increase rapidly until 35 weeks of age (Secchiari *et al*., 1976; Lacroix *et al*., 1977; Sundby and Velle, 1980; Miyamoto *et al*., 1989; Rawlings and Evans, 1995; Evans *et. al*., 1996). The subsequent distinct increase in testosterone concentrations after 20 weeks of age occurs during the period of rapid growth of the testes, but interestingly, it also occurs during a time period with low gonadotropin secretion (Bagu *et al*., 2006). The period of the most active spermatogenesis in bulls is at the end of the early postnatal increase in LH secretion and is at the time when FSH declines from its maximum concentration during the prepubertal period (Rawlings *et al*., 2008).

It has been hypothesized by Brito *et al*. (2007b) that endogenous metabolic hormones such as leptin, insulin, GH and IGF-I, which have distinctly altered concentrations during the pubertal period, could have distinct effects on the sexual development of bulls. The authors have shown characteristic alterations of the serum concentrations of leptin, insulin, GH, IGF-I and testosterone and moderate correlations of these hormones with body weight, backfat, SC and paired testes volume. Leptin, insulin, GH and IGF-I concentrations together accounted for 63% of the variation in SC and 59% of the variation in paired testes volume. Therefore, Brito *et al*. (2007b) assumed that these hormones might also be involved in testicular development during the pubertal phase.

## Effects of modifications in nutrition starting during the pubertal period

Most studies dealing with the effect of nutrition on sexual development have been performed after the weaning of beef bulls (Pruitt *et al*., 1986; Coulter *et al*., 1987; Mwansa and Makarechia**,** 1991; Ohl *et al*., 1996; Brito *et al*., 2012). As bulls are usually weaned at an age of approximately 7 to 8 months (Brito *et al*., 2012), this means that the modification of nutrition was started during the pubertal period after the transient increase in gonadotropin concentration. Most often a positive effect of feeding a high-nutrition diet during the pubertal period on SC and testis weight at 12 to 15 months of age but no effect or even negative effects on sperm production and semen quality was reported (Wolf *et al*., 1965; Secchiari *et al*., 1976; Amann *et al*., 1986). These deleterious effects of an excessive average daily gain (ADG) during the post-weaning period are likely due to fat deposition around the testicular vascular cones and the testes, thus, causing heat stress during spermatogenesis (Coulter *et al*., 1997). Furthermore, there is some evidence that excessive energy intake in young bulls may cause laminitis (Greenough *et al*., 1990), as well as abnormal bone and cartilage growth, resulting in stiffness and lameness. In a recent study, where beef bulls were fed diets with a low-, medium- or high-nutritional content from 6 to 16 months of age (Brito *et al*., 2012), no associations between ADG and the sexual development of the bulls could be detected. These observations are consistent with the hypothesis that the effects of a high-nutrition diet after the early increase in gonadotropin are negligible (Brito *et al*., 2007d; Rawlings *et al*., 2008).

## Effects of modifications in nutrition starting during the prepubertal period

Also Brito *et al*. (2007a, c, d) were among the first authors to investigate the effects of modifications to their nutrition on bull calves before weaning on their later reproductive performance. In several trials (Brito *et al*., 2007a, c, d) they clearly demonstrated that a high nutrition during the prepubertal period resulted in a more sustained increase in LH pulse frequency and bigger testicles at maturity. Therefore, LH secretion during the prepubertal period may prime testicular development and determine maximum adult testicular size (Barth *et al*., 2008). Circulating IGF-I concentrations increased constantly during the prepubertal and pubertal periods, indicating that IGF-I may be involved in regulating sexual development. The temporal association between GnRH/LH secretion and IGF-I concentration is a strong indicator for a regulatory role of IGF-I on GnRH secretion, but more studies should be conducted to determine whether IGF-I can indeed promote GnRH secretion in bulls (Barth *et al*., 2008). Nutrition also affected testosterone concentrations, which suggests effects on Leydig cell number, their function, or both. A consistent observation was that leptin, insulin, and GH concentrations did not differ among the groups with different nutrition levels during the period of the early gonadotropin increase and therefore were not involved in the differences in LH secretion. Therefore, the role of these hormones, if any, in regulating GnRH secretion is permissive. However, leptin and insulin had moderate to good correlations with SC and paired testes volume in some of the experiments, indicating that these hormones may promote testicular development (Barth *et al*., 2008).

## Effects of modifications in nutrition starting during the infantile period

There is very little information regarding the effects of a modification of nutrition starting during the infantile period. In one study (Bratton *et al*., 1956) carried out more than 60 years ago, restriction of feed intake in Holstein bull calves from 1 to 80 weeks of age had a tremendous negative effect on pubertal development. In recently published studies, Dance *et al*. (2015, 2016) examined the effects of early life nutrition on reproductive development in Holstein

bulls. Twenty-six Holstein bull calves were randomly allocated into 3 groups at approximately 1 week of age to receive either a low-, medium-, or high-nutrition diet from 2 to 31 weeks of age. Afterwards, all animals were fed the medium-nutrition diet. While there were no effects of nutrition on basal FSH concentrations, the increase in LH during the prepubertal period was advanced by 8 weeks (11 *vs.* 19 weeks) and mean LH concentrations were higher in bulls fed the high-nutrition diet than in those of the other 2 groups. Furthermore, bulls fed the high-nutrition diet had greater testosterone concentrations than those fed the low-nutrition diet from 11 to 27 weeks. Bulls fed the high-nutrition diet were younger at puberty and when they reached a SC of 28 cm than bulls fed the low-nutrition diet. In a similar study performed recently on Holstein-Friesian and Jersey bull calves between 3 to 49 weeks of age these results could be confirmed (Byrne *et al*., 2017). In addition, Dance *et al*. (2015) noticed at 72 weeks, that bulls fed the high-nutrition diet had greater paired testes weights. The estimated number of sperm produced by day in the bulls of the high-nutrition group was about 9% higher compared to the bulls of medium-nutrition group and about 30% higher than the number estimated in bulls of the low-nutrition group, while no differences in sperm quality were noticed depending on nutrition of the bulls (Dance *et al*., 2016). Overall, the results obtained by Dance *et al*. (2015, 2016) and Byrne *et al*. (2017) are consistent with the studies from Brito *et al*. (2007a, c, d), who modified the nutrition of beef bulls starting at the prepubertal period. All these studies provide clear evidence that nutritional modulation of bull calves before puberty has profound effects on reproductive development. Because Dance *et al*. (2015) and Byrne *et al*. (2017) modulated the nutrition during the infantile and prepubertal periods, it is unclear, however, whether a nutritional modulation is required for both periods to obtain these effects on reproductive development or whether a shorter time interval would be as effective.

**Effects of modifications in nutrition limited to the infantile period**

We conducted two own studies to characterize the effects of an *ad libitum* (AdL) feeding of milk within the first three weeks of life compared to the effects of an established restrictive feeding protocol on performance, health status, metabolism and the onset of reproductive activity in bull calves.

In the first experiment (Maccari *et al*., 2015; Prokop *et al*., 2015), 48 Holstein bull calves were randomly assigned to a group fed milk AdL for three weeks or to a group of restrictively fed calves. Calves were transferred from calf hutches into a group pen at either the second or third weeks of life. After the third week, the housing, feeding and management of calves from both groups were identical. Restricted amounts of milk replacer were offered, as well as a total mixed ration, concentrates and hay, and water was available AdL. The volume of milk replacer offered was reduced constantly from week 5 to 10 of life. Subsequent fattening was based on an established concentrate-based ration until slaughter at an age of eight mo. Average daily gains differed markedly: restrictively fed calves achieved an average gain of 380 g per d within the first three weeks of life, and the ADG of the AdL calves was threefold higher (1,280 g). As a result of the different feeding protocols within the first three weeks of life, some AdL-fed calves achieved a weight well above 80 kg by the fourth weeks, whereas most restrictively fed calves did not reach a body weight of 60 kg during this period. On average, AdL-fed calves were 20 kg heavier than the restrictively fed calves at an age of 22 days.

Calves fed restrictively in their first three weeks of life had lower testosterone plasma concentrations at an age of 10 weeks than AdL-fed calves, i.e. sexual development was accelerated by an intensified feeding within the first three weeks of life.

The effect of nutritional programming was obvious only at the time of slaughter in those calves that did not suffer from severe bronchopneumonia at some point. Calves with a history of pneumonia grew significantly less than healthy calves, and the effects of a higher level of nutrition were totally abolished.

We recently performed another study (Bollwein H; 2017; Vetsuisse-Faculty, University of Zurich, Zurich, Switzerland; unpublished results) for which the results are not yet complete but that would be useful to include here for the discussion of this issue. The objective of this study was to investigate whether a short-term postnatal nutritional trigger of 4 weeks affects daily weight gain, health status and the onset of puberty in Brown Swiss calves. Twenty-four bull calves were fed milk either restrictively (RES) or AdL for four weeks. Housing was identical throughout this initial feeding period and also thereafter, when calves were transferred to group pens. Feeding was similar for all calves from week 5 of life and beyond when a conventional ration for the fattening of bulls was offered. The reproductive performance of the calves was assessed by frequent measurements of SC. As soon as it reached 26 cm, electroejaculations were performed biweekly to determine the quantity and quality of the semen. There were clear differences in the results with respect to milk ingestion. The high inter-individual variance in the group of AdL-fed calves indicated that roughly one third of those calves did not consume more milk than calves fed according to the established restrictive feeding protocols while other AdL-fed calves ingested 12 L per d or more. Thus, we subdivided the group of AdL-fed calves into a group of calves with a substandard milk intake (designated as AdL-low) and calves with a greater-than-normal level of milk consumption (designated as AdL-high). In fact, AdL-high calves consumed roughly twice as much milk as the RES calves and calves in the AdL-low group, both in the first two weeks of life and in the subsequent two weeks under the different feeding protocols. In agreement with milk intake, daily weight gains were not markedly different between RES calves and AdL-low calves (Fig. 1). However, AdL-high calves achieved a ADG of 600 g by the first two weeks of life and gained almost 1,200 g/day in the subsequent two weeks. When we followed these bull calves until the end of the study,

AdL-high calves remained 30-40 kg heavier during the entire period than RES calves and AdL-low calves (Fig. 1). These results demonstrate the impact of energy intake in early calfhood on subsequent development.

With respect to reproductive performance, the onset of puberty based on the analysis of ejaculates did not differ between the groups (278 *vs*. 274 *vs*. 275 days) despite the considerable variance within each group. An SC of 28 cm was reached somewhat earlier (Fig. 1) in AdL-high calves (252 days) than in the AdL-low (271 days) and the RES calves (268 days). Testosterone plasma concentrations assessed between 6 and 13 months of age were significantly higher in AdL-high calves than in calves fed restrictively for the first four weeks of life. A significant difference also occurred in the proportion of morphologically normal sperm, which was higher in AdL-fed calves than in RES-fed calves between 10 and 16 months of age. In conclusion, there was a trend in the data suggesting that a high level of nutrition limited to the infantile period may also affect long-term reproductive performance but significant differences were rare. This might be due to the enormous inter-individual differences within each group. These differences also demonstrate that the onset of puberty is determined by a variety of factors and the intensity of pre-weaning milk feeding is only one of these factors. In addition, the number of animals analyzed in this study could simply be too small to demonstrate an effect of pre-weaning feeding intensity on the subsequent sexual performance of bull calves. Alternatively, the period of intensive milk feeding might have been too short to provoke more profound and long-lasting consequences. In that context, it should be taken into consideration that ADG was reduced markedly in all calves after transfer from individual hutches to a group pen. This setback may be harmful because it still falls in the critical period when the regulatory systems of the calves are responsive to epigenetic factors, and therefore, positive effects of events occurring during the first week of life may be abolished. Whether this speculation is valid should be investigated in forthcoming studies.

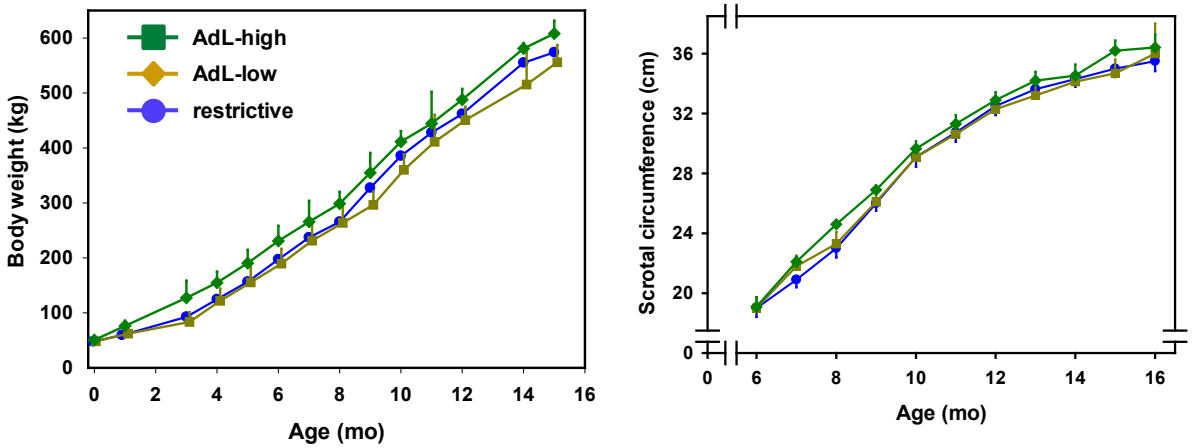


Figure 1. Differences in milk feeding intensity during the first four weeks of life induces long-lasting differences in weight gain and scrotal development in Brown Swiss calves. From Bollwein *et al*. (2016).

## Effects of modifications in nutrition during the fetal period

The hypothesis that in male calves the development of the testis and the hypothalamic–pituitary axis along with the associated synthesis of gonadotropins would be affected by maternal dietary intake and genotype and would correlate with IGF-I and leptin levels was proven by Sullivan *et al*. (2010). For this purpose, pregnant heifers were divided into two treatment groups stratified by body weight and genotype. For the first trimester of gestation, the groups were fed either high (H) or low (L) protein and energy diets. During the second trimester, half of the animals in each treatment group were switched to the alternate treatment group. This resulted in four treatment groups: high/high (HH), high/low (HL), low/high (LowH), low/low (LL). During the third trimester all heifers were fed a standard diet. Paired testicular weight was positively associated with the LowH nutritional group. There was a tendency for bull calves in the HL group to have smaller paired testicle volumes than those in the LowH group. Bull calves in the LL group had higher FSH than those in the HL group. There was a tendency for HL bull calves to have higher LH concentrations than LowH bull calves. Serum testosterone concentrations were not associated with nutritional group. Reduced circulating concentrations of FSH and lower paired testicular weights in prepubertal bull calves whose dams received higher dietary levels of protein and energy during early gestation suggest that the reproductive axis of beef bulls may be susceptible to nutritional perturbations *in utero* (Sullivan *et al*., 2010). According to their results, the authors of this study concluded that a compromised development of the hypothalamic-pituitary-gonadal axis in the early fetal stage may result in a reduced prepubertal gonadotrophin surge in male calves. Overall, the findings of this study suggest a deleterious effect of elevated dam dietary protein and energy in the first trimester of gestation on the reproductive development of their bull calves.

## Effects of modifications of nutrition during the periconceptional period

To the best of our knowledge up to know there have been no studies on the effect of nutrition around

the time of conception in cattle on the reproductive performance of their offspring. However, such investigations have been performed in sheep. Growth and body composition from birth to adulthood was measured in male and female singleton offspring of ewes that were undernourished before, before and after, or only after conception, and they measured carcass and organ weights after slaughter (Jaquiery *et al*., 2012). Five-year-old Romney ewes were randomly allocated to a control group or to one of three groups that were undernourished around the time of conception. Normally nourished controls (N) were fed a maintenance ration. The diets of the ewes in the three undernourished groups were adjusted to achieve and maintain a weight loss of 10-15% body weight from 61 days before mating to the time of mating (day 0; UN-61-0), from 61 days before to 30 days after mating (UN-61-30), or from 2 days before to 30 days after mating (UN-2-30). Thereafter, all animals were fed in the same way as the control group. Total gonadal weight was affected by periconceptional undernutrition in animals of both sexes but in opposite ways. UN males had heavier testes and UN females had lighter ovaries than N animals. As all time periods of undernutrition were associated with a similar effect on male body composition, it was assumed that the results were via a direct effect on the blastocyst or early embryo, and not on the ovum prior to conception, or via an indirect effect mediated by the altered adaptations to pregnancy observed in UN-61-0 and UN-61-30 groups. The authors of this study also mentioned that future investigations are necessary to obtain a better understanding of the signals in embryonic life that determine sex-related growth differences. However, it would be interesting if these effects were also observed in cattle.

## Conclusions

Sexual development of bull calves can be influenced by a modulation of nutrition during their infantile and prepubertal periods. However, the frequent practice of feed supplementation during the pubertal period after weaning does not seem to have positive effects on sexual development (Fig. 2). Interestingly, the development of bull calves is affected by their nutrition not only after but also before birth. In contrast to the positive effects of high feed intake by the bull calves during the first week after birth, a high-nutrition diet fed to the mother during the first trimester seems to have negative effects on the development and the reproductive performance of their offspring later in life. It has not yet been determined whether there is also an effect of periconceptional feed intake by the cows on the development of their male offspring, as has been reported in sheep. Several studies have demonstrated that modifications in feed intake have an influence on the hypothalamic-pituitary-gonadal axis, which might be mediated by serum IGF-I concentrations, but the exact mechanisms responsible for the interaction between nutrition and the subsequent development of offspring are not yet clear. Therefore, further studies are necessary to obtain a better understanding of the phenomenon of effects of nutrition on sexual development in bulls to be able to optimize the performance of young bulls.

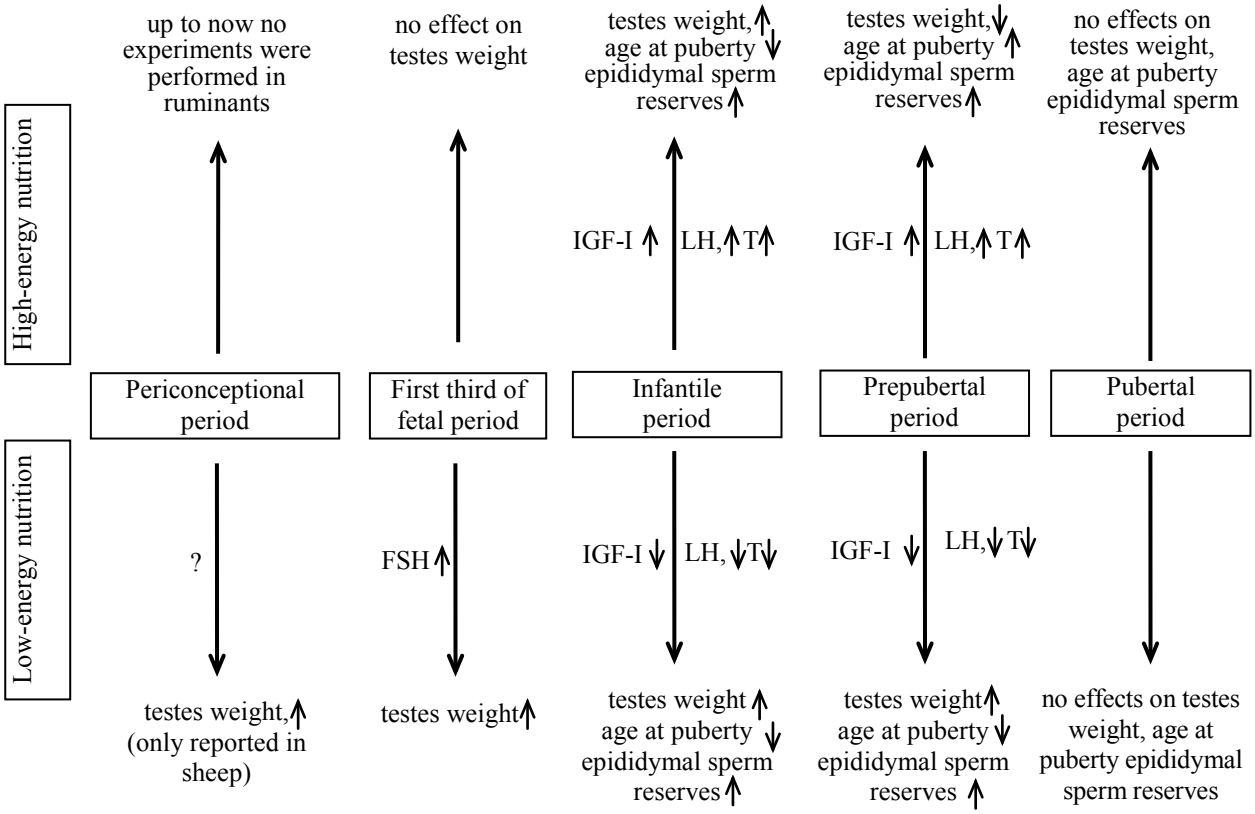


Figure 2. Scheme summarizing the nutritional effects through the different developmental stages (T = testosterone). From Bollwein *et al*. (2016).

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**References**

**Abdel-Raouf M**. 1960. The postnatal development of the reproductive organs in bulls with special reference to puberty (including growth of the hypophysis and the adrenals). *Acta Endocrinol*, 34(suppl. 49):1-109.

**Amann RP**. 1983. Endocrine changes associated with onset of spermatogenesis in Holstein bulls. *J Dairy Sci*, 66:2606-2622.

**Amann RP, Walker OA**. 1983. Changes in the pituitary-gonadal axis associated with puberty in Holstein bulls. *J Anim Sci*, 57:433-442.

**Amann RP, Wise ME, Glass JD, Nett TM**. 1986. Prepubertal changes in the hypothalamic-pituitary axis of Holstein bulls. *Biol Reprod*, 34:71-80.

**Bagu ET, Cook S, Gratton CL, Rawlings NC**. 2006. Postnatal changes in testicular gonadotropin receptors, serum gonadotropin, and testosterone concentrations and functional development of the testes in bulls. *Reproduction*, 132:403-411.

**Barth AD, Brito LF, Kastelic JP**. 2008. The effect of nutrition on sexual development of bulls. *Theriogenology*, 70:485-494.

**Bollwein H, Janett F, Kaske M**. 2016. Impact of nutritional programming on the growth, health, and sexual development of bull calves. *Domest Anim Endocrinol*, 56(suppl):180-190

**Bratton RW, Musgrave SD, Dunn HO, Foote RH, Henderson CR**. 1956. Semen production and fertility of young bulls raised on three different energy levels of feed intake. *J Anim Sci*, 15:1296-1297 (abstract)

**Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Boisclair YR, Ehrhardt RA, Kastelic JP**. 2007a. Effect of feed restriction during calfhood on serum concentrations of metabolic hormones, gonadotropins, testosterone, and on sexual development in bulls. *Reproduction*, 134:171-181.

**Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Mir PS, Kastelic JP**. 2007b. Circulating metabolic hormones during the peripubertal period and their association with testicular development in bulls. *Reprod Domest Anim*, 42:502-508.

**Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Mir PS, Kastelic JP**. 2007c. Effect of improved nutrition during calfhood on serum metabolic hormones, gonadotropins, and testosterone concentrations, and on testicular development in bulls. *Domest Anim Endocrinol*, 33:460-469.

**Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Mir PS, Kastelic JP**. 2007d. Effect of nutrition during calfhood and peripubertal period on serum metabolic hormones, gonadotropins and testosterone concentrations, and on sexual development in bulls. *Domest Anim Endocrinol*, 33:1-18

**Brito LF, Barth AD, Wilde RE, Kastelic JP**. 2012. Effect of growth rate from 6 to 16 months of age on sexual development and reproductive function in beef bulls. *Theriogenology*, 77:1398-1405.

**Byrne CJ, Fair S, English AM, Urh C, Sauerwein H, Crowe MA, Lonergan P, Kenny DA**. 2017. Effect of breed, plane of nutrition and age on growth, scrotal development, metabolite concentrations and on systemic gonadotropin and testosterone concentrations following a GnRH challenge in young dairy bulls. *Theriogenology*, 96:58-68.

**Coulter GH, Kozub GC**. 1984. Testicular development, epididymal sperm reserves and seminal quality in two-year-old Hereford and Angus bulls: effects of two levels of dietary energy. *J Anim Sci*, 59:432-440.

**Coulter GH, Carruthers TD, Amann RP, Kozub GC**. 1987. Testicular development, daily sperm production and epididymal sperm reserves in 15-mo-old Angus and Hereford bulls: effects of bull strain plus dietary energy. *J Anim Sci*, 64:254-260.

**Coulter GH, Cook RB, Kastelic JP**. 1997. Effects of dietary energy on scrotal surface temperature, seminal quality, and sperm production in young beef bulls. *J Anim Sci*, 75:1048-1052.

**Dance A, Thundathil J, Wilde R, Blondin P, Kastelic J**. 2015. Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls. *J Dairy Sci*, 98:987-998.

**Dance A, Thundathil J, Blondin P, Kastelic J**. 2016. Enhanced early-life nutrition of Holstein bulls increases sperm production potential without decreasing postpubertal semen quality. *Theriogenology*, 86:687-694

**Evans AC, Davies FJ, Nasser LF, Bowman P, Rawlings NC**. 1995. Differences in early patterns of gonadotrophin secretion between early and late maturing bulls, and changes in semen characteristics at puberty. *Theriogenology*, 43:569-578.

**Evans AC, Pierson RA, Garcia A, McDougall LM, Hrudka F, Rawlings NC**. 1996 Changes in circulating hormone concentrations, testes histology and testes ultrasonography during sexual maturation in beef bulls. *Theriogenology*, 46:345-357.

**Greenough PR, Vermunt JJ, McKinnon JJ, Fathy FA, Berg PA, Cohen RD**. 1990. Laminitis-like changes in the claws of feedlot cattle. *Can Vet J*, 31:202-208.

**Jaquiery AL, Oliver MH, Honeyfield-Ross M, Harding JE, Bloomfield FH**. 2012. Periconceptional undernutrition in sheep affects adult phenotype only in males. *J Nutr Metab*, 2012:123610. doi: 10.1155/2012/123610.

**Lacroix A, Garnier DH, Pelletier J**. 1997. Temporal fluctuations of plasma LH and testosterone in charolais bull claves during the first year of live. *Ann Biol Anim Biochim Biophys*, 17:1013-1019.

**Maccari P, Wiedemann S, Kunz HJ, Piechotta M, Sanftleben P, Kaske M**. 2015. Effects of two different rearing protocols for Holstein bull calves in the first 3 weeks of life on health status, metabolism and subsequent performance. *J Anim Physiol Anim Nutr*, 99:737-746.

**Macmillan KL, Hafs HD**. 1968. Gonadal and extra gonadal sperm numbers during reproductive development of Holstein bulls. *J Anim Sci*, 27:697-700.

**Miyamoto A, Umezu M, Ishii S, Furasawa T, Masaki J, Hasegawa Y, Ohta M**. 1989. Serum inhibin, FSH, LH and serum concentrations of FSH,LH and testosterone levels and testicular inhibin content in beef bulls from birth to puberty. *Anim Reprod Sci*, 20:165-178.

**Mwansa PB, Makarechian M**. 1991. The effect of postweaning levels of diet energy on sex drive and semen quality of young beef bulls. *Theriogenology*, 35:1169-1178.

**Ohl MW, Ott RS, Faulkner DB, Hornbuckle T, 2nd, Hess RA, Cmarik GF, Zinn GM**. 1996. Effects of rate of gain on scrotal circumference and histopathologic features of the testes of half-sibling yearling beef bulls. *Am J Vet Res*, 57:844-847.

**Prokop L, Kaske M, Maccari P, Lucius R, Kunz HJ, Wiedemann S**. 2015. Intensive rearing of male calves during the first three weeks of life has long-term effects on number of islets of langerhans and insulin stained area in the pancreas. *J Anim Sci*, 93:988-998.

**Pruitt RJ, Corah LR, Stevenson JS, Kiracofe GH**. 1986. Effect of energy intake after weaning on the sexual development of beef bulls. II. Age at first mating, age at puberty, testosterone and scrotal circumference. *J Anim Sci*, 63:579-585.

**Rawlings NC, Evans AC**. 1995. Androgen negative feedback during the early rise in LH secretion in bull calves. *J Endocrinol*, 145:243-249.

**Rawlings N, Evans AC, Chandolia RK, Bagu ET**. 2008. Sexual maturation in the bull. *Reprod Domest Anim*,43(suppl. 2):295-301.

**Rodriguez RE, Wise ME**. 1989. Ontogeny of pulsatile secretion of gonadotropin-releasing hormone in the bull calf during infantile and pubertal development. *Endocrinology*, 124:248-256.

**Secchiari P, Martorana F, Pellegrini S, Luisi M**. 1976. Variation of plasma testosterone in developing Friesian bulls. *J Anim Sci*, 42:405-409.

**Sullivan TM, Micke GC, Greer RM, Perry VE**. 2010. Dietary manipulation of Bos indicus x heifers during gestation affects the prepubertal reproductive development of their bull calves. *Anim Reprod Sci*, 118:131-139.

**Sundby A, Velle W**. 1980. Plasma concentration of testosterone in young bulls in relation to age, rate of weight gain and stimulation with human chorionic gonadotrophin. *J Endocrinol*, 86:465-469.

**Wolf FR, Almquist JO, Hale EB**. 1965 Prepuberal behavior and puberal characteristics of beef bulls on high nutrient allowance. *J Anim Sci*, 24:761-765.

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# Achievements and unmet promises of assisted reproduction technologies in large animals: a personal perspective

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## Abstract

This paper gives an overview of assisted reproductive technologies (ART) in livestock species coming from the author's direct experience and contribution to the development of several of them. The assessment is conducted on the basis of the progress achieved since the early eighties and the impact on the clinical/practical use of such procedures. Artificial insemination (AI) is still the leading technology used on a large scale in livestock with most favourable cost benefit ratio. All the other ARTs have niche applications compared to AI. Significant progress has been achieved in embryo culture, somatic cell nuclear transfer and on the identification of the many unknown variables affecting the success rate, while in areas such as superovulation, oocyte maturation, IVF, embryonic stem cells and cryopreservation progress has been limited or absent. It is the opinion of the author that ARTs have reached a plateau whereby only minimal improvement of efficiency can be achieved. Significant advances can only come from major breakthrough in the understanding of the underlying biological mechanisms.

**Keywords**: large animals, oocytes, embryos, stem cells, SCNT.

## Introduction

Assisted reproduction technologies (ARTs) as we know them today have been the result of a long process that started conventionally with the first artificial insemination performed by Spallanzani (1780) in a bitch and the first embryo transfer in rabbit performed by Heape (1890). The former laid down the principle and basic knowledge for the development of the artificial insemination (AI) industry on the male side while the latter marked the beginning of embryo technologies on the female side.

The theoretical and scientific basis for these developments came in fact much earlier (see (Cobb, 2012)for review) when William Harvey published his book De Generatione Animalium (Harvey, 1651) and when Leeuwenhoek discovered the spermatozoa (Leeuwenhoek, 1678).

It has been only in the second half of the last century that ARTs reached the high level of efficiency necessary to find a practical application in livestock breeding for diseases eradication and genetic selection and to serve as a model for clinical application in human clinics. Artificial insemination rapidly developed as a cost effective and reliable technique in the cattle industry (Vishwanath, 2003) where it is now the standard because of the limited amount of semen needed per individual female. However, in other species, like the pig or the horse, the requirement for higher amounts of semen per insemination dose did not allow the same widespread use of AI in breeding programmes. Moreover, significant research investments would be required still today to overcome sensitivity to cryopreservation and achieve further improvements.

Much less modest has been the impact of ARTs on the female germ line because the current state of the art allows to exploit only a very minute fraction of the oocyte pool present in the ovary to generate offspring.

In this paper I will review what were the expectations or the promises thirty years ago at the beginning of my scientific career, what has become a reality and what has remained undeveloped or has become an illusion based on a personal opinion.

## The sperm

Quantity and quality of semen per se has rarely been a limiting factor for artificial insemination in cattle both as frozen or refrigerated. In other livestock species the use of frozen semen is still highly variable and in general is associated with lower fertility, due to cryo-injuries of various kind (Bailey *et al*., 2000), and often requires laparoscope or deep intrauterine insemination. Advances in insemination with lower sperm numbers has come from the developments of sperm sexing by flow cytometry (Cran, 2007). It is now possible with the refinements introduced in the sexing procedure to achieve pregnancy rates in cattle comparable to non sexed semen, with a fraction of the number of spermatozoa used for conventional artificial insemination (Gonzalez-Marin *et al*., 2016). Methods of sperm sorting other than those based on DNA content have failed to become established in practice (Seidel, 2012) so far. Male germ cell transplantation (Brinster and Zimmermann, 1994) was also proposed as a way to increase sperm production, rescue particular genotypes or to alter the germ line (Dobrinski, 2005) but it has not progressed to a level of practical use for livestock breeding.

## The superovulation

The MOET (Multiple Ovulation and Embryo Transfer) is the most cost effective way to exploit the female genetics in cattle and small ruminants. The products that are used to induce superovulation have changed over the years as well as the average number of

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embryo produced (Bo and Mapletoft, 2014). What has changed over time however are the protocols that have become more user and animal friendly (Mapletoft and Bo, 2011). In the horse, the use of equine derived FSH induces a good superovulatory response that however does not correspond to the expected embryo recovery rate (Logan *et al*., 2007) probably due to the constrains typical of the anatomy of the horse ovary. Therefore horse superovulation is not being used in practice.

**The oocyte**

Despite being a finite number at birth, contrary to the sperm, primary oocytes are present in the order of tens of thousands (Lazzari *et al*., 1992) on livestock ovaries. In theory all of them could be harvested and recruited for maturation and fertilisation rather than being lost due to atresia. However the specific requirements, including sequential media and prolonged culture time, to grow a primordial oocyte to a stage of full competence for meiotic resumption and embryo development has been optimised only in mice (Eppig and O'Brien, 1996). In livestock species attempts have been made to grow ovarian follicles at various stages of development both in vitro and in xenografts but with very modest success (Silva *et al*., 2016). Only two calves have been produced from growing early antral follicles (Miyano and Manabe, 2007) and an improvement of blastocyst production could be obtained again by growing oocytes collected from early antral follicle cultured in vitro for 2 weeks (Makita *et al*., 2016), thus the supply of competent oocytes is still limited to the advanced antral follicles where the oocyte has completed the growth phase and acquired the full developmental competence.

The recovery of oocytes from pre-pubertal animals has been always proposed as a way to shorten the generation interval and increase the number of offspring from any given female. The advent of genomic selection, that allows the identification of superior genotypes soon after birth, has been an incentive to select oocyte donors at a very young age. For the same reason genetic screening has been optimised starting from embryo biopsies anticipating at the pre-implantation stage the identification of the required genotypes. In ruminants the competence of pre-pubertal oocytes is limited in the ability both to develop to blastocyst (Galli *et al*., 2001) and to establish pregnancy rates compared to adult donors (Ptak *et al*., 2006; Galli C. 2017; Galli and Lazzari; unpublished observations).

The protocols to mature in vitro competent oocytes capable of giving rise to offspring after fertilization, culture and embryo transfer has long been developed (Staigmiller and Moor, 1984; Gandolfi and Moor ,1987). It is well established that oocyte competence is correlated to follicular diameter (Galli and Moor, 1991), nevertheless even after the most accurate selection according to the state of the art and the best in vitro conditions, the average blastocyst production has remained fairly stable despite many scientific papers reporting every time few percentage points increase of blastocyst rates for any given treatment. After 30 years of developments of the *in vitro* technology, we still average at best 30% blastocyst rates in cattle under experimental conditions and half that in a clinical context (Galli *et al*., 2014b). The situation is even more inefficient in the buffalo and the horse where only 10% of the oocytes eventually develop into a transferable embryo (Galli *et al*., 2007, 2012). Nevertheless for these species this success rate is higher that with in vivo production of embryos by superovulation and embryo flushing.

If the oocytes are matured in vivo and harvested from pre-ovulatory follicles their developmental competence is higher (Scott *et al*., 2001; Rizos *et al*., 2002;) indicating that the majority of oocytes that we are using for in vitro maturation are either coming from regressing follicles (advanced atresia) or from growing follicles that are not yet ready for maturation and require more than the canonical in vitro maturation time necessary to reach metaphase II. The introduction of the concept of pre-maturation *in vitro* to allow time for the oocyte to complete cytoplasmic maturation before the resumption of meiosis has found no application because of the modest, if any, improvements (Dieci *et al*., 2013; Lodde *et al*., 2013;) despite the fact that such inhibited oocytes at least maintain their developmental competence (Ponderato *et al*., 2001) and allow better scheduling of the work. This approach has found application in the equine. It is remarkable that by simply holding equine oocytes at room temperature for 24 h maturation can be arrested without loss of viability (Choi *et al*., 2006; Galli *et al*., 2014a) and the same blastocyst rate can be obtained compared to freshly matured oocytes following in vitro production. This procedure allows shipping of oocytes from equine clinics where they are recovered, to a centralized in vitro production laboratory for ICSI and embryo culture, and returning to the same clinics the frozen blastocyst for transfer (Galli *et al*., 2016).

***In vitro* fertilization (IVF)**

This event critical for the successful trip of the oocyte towards becoming an embryo has been almost neglected in recent years and has not been the subject of much research. In humans IVF has become a reality after the first success obtained with the birth of Louise Brown (Steptoe and Edwards, 1978), celebrated also with the 2012 Nobel Prize to R. Edwards. This event was the turning point where human IVF began to be the model for livestock species. It was not until 1982 that the first success was reported in cattle (Brackett *et al*., 1982) and later became routine procedure with the use of heparin to capacitate bull spermatozoa (Parrish *et al*., 1986) and other ruminants but no significant progress has been made since then. Most of the bulls will fertilize in vitro under standard conditions but the variation can be high ranging from no fertilisation to polyspermy, both compromising embryo development. This problem has its origins both in the variable quality of the frozen semen batches, in the intrinsic genetic variability of the semen donors but also in the quality of the oocyte itself

and in its competence and ability to block polyspermy. In species other than ruminants IVF has remained an unreliable procedure. In pig the unresolved problem of polyspermy still dominates the field (Romar *et al*., 2016) but no solutions are on the horizon. Still porcine embryos can develop in vitro to reasonable rates (Grupen, 2014) and probably adjust to the diploid state during early embryo development. In the horse the situation has been the opposite: IVF does not work, in fact no reproducible advances have been made since the only successful IVF report (Palmer *et al*., 1991). Again the human model helped to overcome this limitation in the horse with the introduction of ICSI (Palermo *et al*., 1992) that is now largely used on a routine basis in human IVF. In the horse ICSI is currently the only option to obtain fertilization in vitro and luckily the horse is the livestock species where it gives consistent results (Lazzari *et al*., 2002a) like in humans. Given the paucity of oocytes that can be harvested from a mare, ICSI will remain the technique of choice even if IVF ever would become available in the future. With the introduction of ICSI *in vitro* embryo production in horses has become possible and it has been developed to a level of being used in the clinical practice (Galli *et al*., 2014b). ICSI would be of help also in cattle to use limited amount of sexed semen or semen of poor quality recovered from young bulls; however, so far results have been disappointing (Galli et al. 2003b), and the rates of blastocyst remains below those obtained by conventional IVF. The advent of piezo-ICSI could offer still some options that could be developed also for cattle or pigs.

**The preimplantation embryo**

Embryo development in vitro is the area where significant progress has been made in recent time. Forty years ago in vitro derived livestock embryos could be hardly kept alive in culture and the only successful report was from Tervit (Tervit *et al*., 1972). Then the use of embryo co-culture with oviductal cells (Gandolfi and Moor, 1987) was the beginning of a new era. To overcome the limits in early technology and lack of scientific knowledge, in vitro produced zygotes have been cultured for a long time in the surrogate sheep oviduct that ensured cryotolerance and viability after transfer to recipients comparable to that of in vivo generated embryos (Shehu *et al*., 1996; Lazzari *et al*., 2010). For many years and still now in some laboratories, co-culture with primary cells like oviductal or cumulus cells (Galli and Moor, 1989) or with established cell lines like BRL and Vero became the routine also in the human field (Menezo *et al*., 2012). The presence of a monolayer of somatic cells however required the use of complex media and the presence of serum to ensure the viability of the somatic cells. The cells function was to create a microenvironment with low oxygen, besides promoting detoxification and/or providing secretion of factors suitable for embryos to develop. It turned out however that serum (and probably some secreted factors) was detrimental for post implantation embryo development in ruminants and identified as primarily responsible for the Large Offspring Syndrome (LOS;Young *et al*., 1998). The presence of serum was also found detrimental for cryopreservation because it was responsible for the accumulation of lipids (Shehu *et al*., 1996; Lonergan and Fair, 2008). Therefore media formulations shifted towards a serum free and cell free solutions where the still undefined component remains only Bovine Serum Albumin. The medium used today by many laboratories is based on the SOF formulation of Tervit (Tervit *et al*., 1972) with the addition of amino acids and various energy substrates (Gardner *et al*., 1994) as well as the reduction of the level of oxygen to 5% by feeding nitrogen to the gas mixture to lower oxidative damage.

With the use of modified SOF medium the incidence of LOS was decreased (van Wagtendonk-de Leeuw *et al*., 2000) but still the presence of high levels of BSA is accountable for such problems (Lazzari *et al*., 2002b) and occasionally LOS is still reported. LOS is typical of ruminants. In the horse it has never been reported: the foals born out of *in vitro* embryo production are not oversize (Galli *et al*., 2007). This suggests that the problem is associated with the type of placentation and or placental abnormalities (Farin *et al*., 2006; Ptak *et al*., 2013).

The embryos that are selected for freezing or for transfer are chosen by experienced embryologists based essentially on morphological criteria. Attempt to develop more objective non-invasive measurements that could be use to select embryos for transfer have not found the way to the clinical use. Both the measurement of oxygen consumption (Lopes *et al*., 2007) or the amino acid turnover (Brison *et al*., 2004) were investigated for this purpose. Another aspect that is not taken into consideration is the embryo genotype. Usually beef breeds perform better in embryo production *in vitro* than Holstein, notoriously a breed with higher inbreeding. We have shown that crossbred embryos develop better that inbreed ones (Lazzari *et al*., 2011).

An important part of ARTs is the cryopreservation of embryos both for practical and commercial reasons. Despite the development of vitrification (Rall and Fahy, 1985), that found its application in research laboratory or in human clinics for oocyte cryopreservation (Vajta, 2013), the industry standard in livestock for both ruminants and horses embryos is still the slow cooling method (Willadsen *et al*., 1978). Embryos frozen in glycerol require very simple thawing procedures for cryoprotectant removal that any practitioner can perform on farm conditions. The development of direct transfer has further simplified the transfer of bovine embryos (Voelkel and Hu, 1992) allowing many more practitioners, without the ability to handle an embryo in a dish, to perform embryo transfer.

**The stem cells and somatic cell nuclear transfer**

At the beginning of my scientific career Steen Willadsen (AETE Pioneer Award recipient) had already cloned sheep and cattle using blastomeres of early stage

pre-implantation embryos (Willadsen, 1986) and companies were being set up by the breeding industry to exploit this technology. But soon it became evident that embryonic cloning had limitations (number of nuclei available, unpredictable genotype, etc.). It was from this limitation that the scientific community got interested in developing embryonic stem cells to have an unlimited source of nuclei for nuclear transfer. In collaboration with Martin Evans (later 2007, Nobel Prize for Medicine) we started a long and painful path in the attempt to establish embryonic stem cells in sheep and pig (Notarianni *et al*., 1991) and later on in cattle (Galli *et al*., 1994; Lazzari *et al*., 2006) but never succeeded to obtain naïve ESC lines and to date no one has reported success in this endeavour. At most we and others obtained ES-like cells that probably were not much different from somatic cells. Because of the difficulties in generating stable ESC lines the interest for nuclear transfer shifted towards this type of ES like cells (Galli *et al*., 1991, Campbell *et al*., 1996) and eventually to somatic cells (Wilmut *et al*., 1997, Galli *et al*., 1999). It turned out that somatic cells can be used for cloning animals and indeed surprisingly perform better that the supposedly less differentiated cells (Sung *et al*., 2006).

In the horse the development of in vitro maturation and embryo culture has also benefited the cloning of this species (Galli *et al*., 2003a) that fortunately, despite the low efficiency of development to term, as in other species, it turned out to be free of LOS and late pregnancy losses. In the pig the situation is intermediate and no dramatic phenotypes are reported (Kurome et al., 2013). The low efficiency is compensated by the transfer of many cloned embryos per recipients to ensure a high pregnancy rate and a reasonable number of newborn piglets.

## The recipient

The success of any ART procedure is measured at the end of the day on the birth of a viable offspring. Therefore the recipient that is selected for the transfer plays an important part in the successful outcome. Synchronization procedures have been greatly improved and simplified with the introduction of the Ov-Synch protocols (Thatcher *et al*., 2004; Baruselli *et al*., 2010) for cattle together with a better management of the health and nutrition of the recipient animals and the collection and registration of precise information in electronic formats. Maiden recipients are generally preferred and give the highest pregnancy rates across livestock species. However in cattle there is always a struggle to maintain the pregnancy rate close to 50% especially with cryopreserved embryos or embryos produced in vitro. This relatively low efficiency seems to be associated with the gradual decrease in fertility observed in cattle herds (Diskin *et al*., 2016) that hopefully will be reversed by genomic selection for fertility traits. On the contrary in vitro produced frozen-thawed equine embryos can achieve a consistent and remarkable pregnancy rates up to 60% with a foaling rate of 50% (Galli *et al*., 2007, 2016). Also in pig the use of gilts ensures after embryo transfer a pregnancy rate in excess of 50%.

## The unknowns of the laboratory

The outcome of ART procedures depends heavily, besides the gametes and the surrogate mothers mentioned above, also on two other components: the human factor and the laboratory set up. After 30 years in the profession I have meet and seen all sorts of people: the good, the bad, the ugly. Gametes and embryos are like little babies and they need a lot of care to be kept alive and thrive. Over the years this human factor has been and still is very important despite some technological advances and better equipment. Therefore it is crucial to find the right people to work in the laboratories moreover a long period of training, trial and testing is needed. Unfortunately these people are rarely found. The other critical aspects are the equipment, reagents and the disposables used in the various procedures. A great help has come from the enormous development of the human ART industry, in terms of varieties of supplies and technical solutions. However livestock gametes and embryos and in particular cattle are, in our experience, far more sensitive that mouse or human ones. This is why the most common quality control test used by the suppliers of disposable, chemicals or media, i.e. the MEA (mouse embryo assay; Punt-van der Zalm *et al*., 2009) does not detect toxicants or conditions that can affect cattle embryo development. For this reasons we have introduced our own testing for quality control using bovine embryos, both for disposables plastic, BSA and culture media once a new batch is prepared. A typical example of chemical that requires testing is mineral oil (Otsuki *et al*., 2007). After several years there is still an issue in discussion forums about its toxicity and the use of paraffin oil instead of mineral oil. We are not using oil at all for long term culture. Only during cloning or ICSI procedures we cover the micromanipulation drops with mineral oil but exposure is limited to short time. The reason is that even batches that we tested and found suitable for embryo culture, over time became toxic.

## The unknowns of the biology

What has emerged with the implementation of ART in livestock, in particular in ruminants, is the incidence of some abnormalities that might result in low pregnancy rates, high pregnancy losses and abnormal offspring with a higher birth weight that can cause dystocia (Lazzari *et al*., 2002b). Several factors can contribute to this phenomenon and the causes of these perturbations are likely to be a response to stressors (Thompson *et al*., 2002) . Sub-optimal in vitro environment, inadequate culture medium and untested disposable material can contribute to alteration of cellular parameters such as pH and redox state ultimately affecting embryo development. Factors such as diet and metabolic conditions can be involved in epigenetic effects while specific procedure such as somatic cell nuclear transfer, can directly affect the

methylation status of the cloned embryos. These findings in the animal models have fuelled concerns for human ART (Thompson *et al*., 2002) where these alterations might have far greater impact on the health of the resulting babies at birth and later on during adult life on the incidence of diseases such as diabetes type 2, cardiovascular diseases and obesity (Chen and Heilbronn, 2017). This issue will be addressed more fully in the near future with the increasing amount of data collected and analysed from ART adult offspring. In livestock species the long term effects impact essentially on the efficiency of the technology while animal welfare questions emerge mainly in ruminants and in relation to pregnancies and offspring derived from somatic cell nuclear transfer procedures. On a positive note these epigenetic alterations do not appear to be transmitted to the progeny (Tamashiro *et al*., 2002; Shimozawa *et al*., 2002).

## Conclusions

ARTs have come a long way in the last 30 years both in animals and humans and are well established in the clinical practice. Progress has been slow but steady especially in the area of embryo culture (Table 1). It seems that we have now reached a plateau with only small margins for improvement because of the intrinsic biological and/or technical limitation /variation of the source of gametes and of in vitro conditions. We have to live with that unless a major breakthrough occurs in our understanding of the underlying biological mechanisms.

Table 1. Current status of assisted reproductive technologies in livestock, a personal opinion.

| | progress | practical use | research activity |
|---|---|---|---|
| | last 30 years | present | present |
| AI | + | ++++ | + |
| MOET | + | +++ | - |
| Oocyte | ++ | +++ | + |
| IVF | + | +++ | + |
| embryo culture | ++++ | +++ | +++ |
| cryopreservation | + | +++ | + |
| embryonic stem cells | - | - | + |
| SCNT | +++ | + | ++ |
| recipient animals | ++ | +++ | + |
| unknowns | +++ | +++ | ++ |
| | ++++ intense | +++ moderate | ++ low |
| | + minimal | - absent | |

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## References

**Bailey JL, Bilodeau JF, Cormier N**. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl*, 21:1-7.

**Baruselli PS, Ferreira RM, Sa Filho MF, Nasser LF, Rodrigues CA, Bo GA**. 2010. Bovine embryo transfer recipient synchronisation and management in tropical environments. *Reprod Fertil Dev*, 22:67-74.

**Bo GA, Mapletoft RJ**. 2014. Historical perspectives and recent research on superovulation in cattle. *Theriogenology*, 81:38-48.

**Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA**. 1982. Normal development following in vitro fertilization in the cow. *Biol Reprod*, 27:147-158.

**Brinster RL, Zimmermann JW**. 1994. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA*, 91:11298-11302.

**Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ**. 2004. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod,* 19:2319-2324.

**Campbell KH, McWhir J, Ritchie WA, Wilmut I**.

1996. Sheep cloned by nuclear transfer from a cultured cell line. *Nature*, 380:64-66.

**Chen M, Heilbronn LK**. 2017. The health outcomes of human offspring conceived by assisted reproductive technologies (ART). *J Dev Orig Health Dis*, 8:388-402.

**Choi YH, Love LB, Varner DD, Hinrichs K**. 2006. Holding immature equine oocytes in the absence of meiotic inhibitors: effect on germinal vesicle chromatin and blastocyst development after intracytoplasmic sperm injection. *Theriogenology*, 66:955-963.

**Cobb M**. 2012. An amazing 10 years: the discovery of egg and sperm in the 17th century. *Reprod Domest Anim*, 47(suppl. 4):2-6.

**Cran DG**. 2007. XY sperm separation and use in artificial insemination and other ARTs. *Soc Reprod Fertil Suppl*, 65:475-491.

**Dieci C, Lodde V, Franciosi F, Lagutina I, Tessaro I, Modina SC, Albertini DF, Lazzari G, Galli C, Luciano AM**. 2013. The effect of cilostamide on gap junction communication dynamics, chromatin remodeling, and competence acquisition in pig oocytes following parthenogenetic activation and nuclear transfer. *Biol Reprod*, 89:68. doi: 10.1095/biolreprod.113.110577.

**Diskin MG, Waters SM, Parr MH, Kenny DA**. 2016. Pregnancy losses in cattle: potential for improvement. *Reprod Fertil Dev*, 28:83-93.

**Dobrinski I**. 2005. Germ cell transplantation and testis tissue xenografting in domestic animals. *Anim Reprod Sci*, 89:137-145.

**Eppig JJ, O'Brien MJ**. 1996. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod*, 54:197-207.

**Farin PW, Piedrahita JA, Farin CE**. 2006. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology*, 65:178-191.

**Galli C, Moor RM**. 1989. Co-culture: the future for embryo development in vitro. *Acta Eur Fertil*, 20:231-235.

**Galli C, Laurie S, Lazzari G, Moor R**. 1991. Nuclear transplantation (by electrofusion) of cultured embryonic cells with Met II cytoplasts in the sheep. *In*: XLVII Annual Conference, Italian Society of Veterinary Science, 1991, Brescia, Italy. Brescia: SISvet. pp. 435-439.

**Galli C, Moor RM**. 1991. Development of immature bovine oocytes into viable embryos in vitro. *Bull Assoc Anat (Nancy)*, 75:67-71.

**Galli C, Lazzari G, Flechon JE, Moor RM**. 1994. Embryonic stem cells in farm animals. *Zygote*, 2:385-389.

**Galli C, Duchi R, Moor RM, Lazzari G**. 1999. Mammalian leukocytes contain all the genetic information necessary for the development of a new individual. *Cloning*, 1:161-170.

**Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G**. 2001. Embryo production by ovum pick up from live donors. *Theriogenology*, 55:1341-1357.

**Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G**. 2003a. Pregnancy: a cloned horse born to its dam twin. *Nature*, 424:635.

**Galli C, Vassiliev I, Lagutina I, Galli A, Lazzari G**. 2003b. Bovine embryo development following ICSI: effect of activation, sperm capacitation and pre-treatment with dithiothreitol. *Theriogenology*, 60:1467-1480.

**Galli C, Colleoni S, Duchi R, Lagutina I, Lazzari G**. 2007. Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Anim Reprod Sci*, 98:39-55.

**Galli C, Duchi R, Lazzari G, Lagutina I, Turini P, Crotti G, Colleoni S, Angel J, Berdugo J**. 2012. Pregnancies and calves after transfer of in vitro produced river buffalo embryos after cryopreservation. *Reprod Fertil Dev*, 24:190-191.

**Galli C, Colleoni S, Turini P, Crotti G, Dieci C, Lodde V, Luciano AM, Lazzari G**. 2014a. Holding equine oocytes at room temperature for 18 hours prior to in vitro maturation maintains their developmental competence. *J Equine Vet Sci*, 34:174-175.

**Galli C, Duchi R, Colleoni S, Lagutina I, Lazzari G**. 2014b. Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology*, 81:138-151.

**Galli C, Colleoni S, Claes A, Beitsma M, Deelen C, Necchi D, Duchi R, Lazzari G, Stout T**. 2016. Overnight shipping of equine oocytes from remote locations to an ART laboratory enables access to the flexibility of Ovum Pick Up-ICSI and embryo cryopreservation technologies. *J Equine Vet Sci*, 41:82. doi: http://dx.doi.org/10.1016/j.jevs.2016.04.084.

**Gandolfi F, Moor RM**. 1987. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *J Reprod Fertil*, 81:23-28.

**Gardner DK, Lane M, Spitzer A, Batt PA**. 1994. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod*, 50:390-400.

**Gonzalez-Marin C, Lenz RW, Gilligan TB, Evans KM, Gongora CE, Moreno JF, Vishwanath R**. 2016. SexedUltra, a new method of processing sex sorted bovine sperm improves post-thaw sperm quality and in vitro fertility. *Reprod Fertil Dev*, 29:204. doi: 10.1071/RDv29n1Ab191.

**Grupen CG**. 2014. The evolution of porcine embryo in vitro production. *Theriogenology*, 81:24-37.

**Harvey W**. 1651. *Exercitationes de Gener- atione Animalium*. Amsterdam: Jansson.

**Heape W**, 1890: Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. *Phil Trans R Soc Lond B Biol Sci* 48, 457-459.

**Kurome M, Geistlinger L, Kessler B, Zakhartchenko V, Klymiuk N, Wuensch A, Richter A, Baehr A, Kraehe K, Burkhardt K, Flisikowski K, Flisikowska T, Merkl C, Landmann M, Durkovic M, Tschukes A, Kraner S, Schindelhauer D, Petri T, Kind A, Nagashima H, Schnieke A, Zimmer R, Wolf E**. 2013. Factors influencing the efficiency of generating genetically engineered pigs by nuclear transfer: multi-factorial analysis of a large data set. *BMC Biotechnol*, 13:43. doi: 10.1186/1472-6750-13-43.

**Lazzari G, Galli C, Moor RM**. 1992. Centrifugal elutriation of porcine oocytes isolated from the ovaries of newborn piglets. *Anal Biochem*, 200:31-35.

**Lazzari G, Crotti G, Turini P, Duchi R, Mari G, Zavaglia G, Barbacini S, Galli C**. 2002a. Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. *Theriogenology*, 58:709-712.

L**azzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C**. 2002b. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. *Biol Reprod*, 67:767-775.

**Lazzari G, Colleoni S, Giannelli SG, Brunetti D, Colombo E, Lagutina I, Galli C, Broccoli V**. 2006. Direct derivation of neural rosettes from cloned bovine blastocysts: a model of early neurulation events and neural crest specification in vitro. *Stem Cells*, 24:2514-2521.

**Lazzari G, Colleoni S, Lagutina I, Crotti G, Turini P, Tessaro I, Brunetti D, Duchi R, Galli C**. 2010. Short-term and long-term effects of embryo culture in the surrogate sheep oviduct versus in vitro culture for different domestic species. *Theriogenology*, 73:748-757.

**Lazzari G, Colleoni S, Duchi R, Galli A, Houghton FD, Galli C**. 2011. Embryonic genotype and inbreeding affect preimplantation development in cattle. *Reproduction*, 141:625-632.

**Leeuwenhoek A**. 1678. Observationes D. Anthonii Lewenhoeck, de natis e` semini genitali animalcules. *Phil Trans R Soc Lond B Biol Sci*, 12:1040-1043.

**Lodde V, Franciosi F, Tessaro I, Modina SC, Luciano AM**. 2013. Role of gap junction-mediated communications in regulating large-scale chromatin configuration remodeling and embryonic developmental competence acquisition in fully grown bovine oocyte. *J Assist Reprod Genet*, 30:1219-1226.

**Logan NL, McCue PM, Alonso MA, Squires EL**. 2007. Evaluation of three equine FSH superovulation protocols in mares. *Anim Reprod Sci*, 102:48-55.

**Lonergan P, Fair T**. 2008. In vitro-produced bovine embryos: dealing with the warts. *Theriogenology*, 69:17-22.

**Lopes AS, Madsen SE, Ramsing NB, Lovendahl P, Greve T, Callesen H**. 2007. Investigation of respiration of individual bovine embryos produced in vivo and in vitro and correlation with viability following transfer. *Hum Reprod*, 22:558-566.

**Makita M, Ueda M, Miyano T**. 2016. The fertilization ability and developmental competence of bovine oocytes grown in vitro. *JReprod Dev*, 62:379-384.

**Mapletoft RJ, Bo GA**. 2011. The evolution of improved and simplified superovulation protocols in cattle. *Reprod Fertil Dev*, 24:278-283.

**Menezo YJ, Servy E, Veiga A, Hazout A, Elder K**. 2012. Culture systems: embryo co-culture. *Methods Mol Biol*, 912:231-247.

**Miyano T, Manabe N**. 2007. Oocyte growth and acquisition of meiotic competence. *Soc Reprod Fertil Suppl*, 63:531-538.

**Notarianni E, Galli C, Laurie S, Moor RM, Evans MJ**. 1991. Derivation of pluripotent, embryonic cell lines from the pig and sheep. *J Reprod Fertil Suppl*, 43:255-260.

**Otsuki J, Nagai Y, Chiba K**. 2007. Peroxidation of mineral oil used in droplet culture is detrimental to fertilization and embryo development. *Fertil Steril*, 88:741-743.

**Palermo G, Joris H, Devroey P, Van Steirteghem AC**. 1992: Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet (London)*, 340:17-18.

Palmer E, Bezard J, Magistrini M, Duchamp G. 1991. In vitro fertilization in the horse. A retrospective study. *J Reprod Fertil Suppl* ,44:375-384.

**Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL**. 1986. Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology*, 25:591-600.

**Ponderato N, Lagutina I, Crotti G, Turini P, Galli C, Lazzari G**. 2001. Bovine oocytes treated prior to in vitro maturation with a combination of butyrolactone I and roscovitine at low doses maintain a normal developmental capacity. *Mol Reprod Dev*, 60:579-585.

**Ptak G, Matsukawa K, Palmieri C, Della Salda L, Scapolo PA, Loi P**. 2006. Developmental and functional evidence of nuclear immaturity in prepubertal oocytes. *Hum Reprod*, 21:2228-2237.

Ptak GE, D'Agostino A, Toschi P, Fidanza A, Zacchini F, Czernik M, Monaco F, Loi P. 2013. Post-implantation mortality of in vitro produced embryos is associated with DNA methyltransferase 1 dysfunction in sheep placenta. *Hum Reprod*, 28:298-305.

**Punt-van der Zalm JP, Hendriks JC, Westphal JR, Kremer JA, Teerenstra S, Wetzels AM**. 2009. Toxicity testing of human assisted reproduction devices using the mouse embryo assay. *Reprod Biomed Online*, 18:529-535.

**Rall WF, Fahy GM**. 1985. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature*, 313:573-575.

**Rizos D, Ward F, Duffy P, Boland MP, Lonergan P**. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev*, 61:234-248.

**Romar R, Funahashi H, Coy P**. 2016. In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years. *Theriogenology*, 85:125-134.

**Scott TJ, Carnevale EM, Maclellan LJ, Scoggin CF, Squires EL**. 2001. Embryo development rates after transfer of oocytes matured in vivo, in vitro, or within oviducts of mares. *Theriogenology*, 55:705-715.

**Seidel GE Jr**. 2012. Sexing mammalian sperm - Where do we go from here? *J Reprod Dev*, 58:505-509.

**Shehu D, Marsicano G, Flechon JE, Galli C**. 1996. Developmentally regulated markers of in vitro-produced preimplantation bovine embryos. *Zygote*, 4:109-121.

**Shimozawa N, Ono Y, Kimoto S, Hioki K, Araki Y, Shinkai Y, Kono T, Ito M**. 2002. Abnormalities in cloned mice are not transmitted to the progeny. *Genesis*,

34:203-207.
**Silva JR, van den Hurk R, Figueiredo JR**. 2016. Ovarian follicle development in vitro and oocyte competence: advances and challenges for farm animals. *Domest Anim Endocrinol*, 55:123-135.
**Spallanzani L**, 1780: Dissertazioni di fisica animale e vegetabile. In Modena, MDCCLXXX. La Società Tipografica.
**Staigmiller R, Moor R**. 1984. Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. *Gamete Res*, 9:221-229.
**Steptoe PC, Edwards RG**. 1978. Birth after the reimplantation of a human embryo. *Lancet (London*), 2:366.
**Sung LY, Gao S, Shen H, Yu H, Song Y, Smith SL, Chang CC, Inoue K, Kuo L, Lian J, Li A, Tian XC, Tuck DP, Weissman SM, Yang X, Cheng T**. 2006. Differentiated cells are more efficient than adult stem cells for cloning by somatic cell nuclear transfer. *Nat Genet*, 38:1323-1328.
**Tamashiro KL, Wakayama T, Akutsu H, Yamazaki Y, Lachey JL, Wortman MD, Seeley RJ, D'Alessio DA, Woods SC, Yanagimachi R, Sakai RR**. 2002. Cloned mice have an obese phenotype not transmitted to their offspring. *Nat Med*, 8:262-267.
**Tervit HR, Whittingham DG, Rowson LE**. 1972. Successful culture in vitro of sheep and cattle ova. *J Reprod Fertil*, 30:493-497.
**Thatcher WW, Bartolome JA, Sozzi A, Silvestre F, Santos JE**. 2004. Manipulation of ovarian function for the reproductive management of dairy cows. *Vet Res Commun*, 28(suppl. 1):111-119.
**Thompson JG, Kind KL, Roberts CT, Robertson SA, Robinson JS**. 2002. Epigenetic risks related to assisted reproductive technologies: short- and long-term consequences for the health of children conceived through assisted reproduction technology: more reason for caution? *Hum Reprod*, 17:2783-2786.
**Vajta G**. 2013. Vitrification in human and domestic animal embryology: work in progress. *Reprod Fertil Dev*, 25:719-727.
**van Wagtendonk-de Leeuw AM, Mullaart E, de Roos AP, Merton JS, den Daas JH, Kemp B, de Ruigh L**. 2000. Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. *Theriogenology*, 53:575-597.
**Vishwanath R**. 2003. Artificial insemination: the state of the art. *Theriogenology*, 59:571-584.
**Voelkel SA, Hu YX**. 1992. Use of ethylene glycol as a cryoprotectant for bovine embryos allowing direct transfer of frozen-thawed embryos to recipient females. *Theriogenology*, 37:687-697.
**Willadsen S, Polge C, Rowson LE**. 1978. The viability of deep-frozen cow embryos. J Reprod Fertil, 52:391-393.
**Willadsen SM**. 1986. Nuclear transplantation in sheep embryos. *Nature*, 320:63-65.
**Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH**. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385:810-813.
**Young LE, Sinclair KD, Wilmut I**. 1998. Large offspring syndrome in cattle and sheep. *Rev Reprod*, 3:155-163.

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# Postpartum uterine infection and endometritis in dairy cattle

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## Abstract

After parturition, uterine involution, regeneration of the endometrium, return of ovarian cyclic activity, and the control of pathogenic bacteria in the uterus is required before cows are likely to conceive again. However, pathogenic bacteria often cause uterine disease in modern dairy cattle, leading to decreased productivity and reduced fertility. This review aims to provide an overview of postpartum uterine infection and disease in dairy cattle. Metritis and endometritis are the main postpartum clinical conditions; although, subclinical endometritis is an emerging issue. Postpartum uterine disease is associated with the isolation of *Escherichia coli, Trueperella pyogenes,* and anaerobic pathogenic bacteria. Sensing of bacteria or their pathogen-associated molecules, such as lipopolysaccharide, by the innate immune system generates inflammatory responses. Endometrial inflammation includes increased expression of complement, calgranulins, interleukins and acute phase proteins, as well as the chemotaxis of neutrophils and macrophages to the site of infection. Uterine disease is also characterised by tissue damage, including endometrial cytolysis caused by the cholesterol-dependent cytolysin, pyolysin. The responses to pathogens are energetically expensive, and depletion of the key cellular nutrients, glucose or glutamine, impairs inflammatory responses by endometrial tissues. For sustainable intensification of the dairy industry over the next 50 years, it is vital to understand why high-milk-yield cows are so susceptible to uterine pathology and develop new ways to prevent uterine disease.

**Keywords**: cow, immunity, infertility, metritis, ovary, uterus.

## Introduction

Bacterial infections of the endometrium that cause uterine disease are common in modern dairy cattle after parturition, and lead to decreased productivity and subfertility (Sheldon *et al.*, 2009). The rising incidence of postpartum metritis and endometritis over the last 50 years has generated interest in better understanding the characteristics of the diseases and the impact of the disease on animal health. There has also been a parallel increase in understanding of the mechanisms underlying uterine disease in dairy cattle. Here we provide an overview of postpartum uterine infection and disease in dairy cattle.

## Definitions of uterine diseases

The definitions of the various uterine diseases in the literature varied considerably until 2006, when a series of definitions were formulated, with consensus amongst about 20 international experts and referees prior to publication (Sheldon *et al.*, 2006). The initial definitions are now widely used, as set out in 2006 or with minor modifications (Sheldon *et al.*, 2009; de Boer *et al.*, 2014).

The two main postpartum clinical conditions are metritis and endometritis. Metritis is most common within 10 days of parturition, and is characterized by an enlarged uterus containing a watery red-brown fluid to viscous off-white purulent uterine discharge, which often has a fetid odour. The severity of metritis is categorized by the signs of the animal's health, from mild disease to toxaemia. The incidence of metritis varies between breed, country and herd, but in a study of the records from 97,318 cows in the USA, the lactation incidence of metritis, including retained placenta, was 21% (Zwald *et al.*, 2004). Clinical endometritis is defined as the presence of a purulent discharge detectable in the vagina 21 days or more post partum, or mucopurulent discharge detectable in the vagina after 26 days post partum. The incidence of clinical endometritis is around 10 to 20%, with variation between breed, country and herd; a typical study reported that 16.9% of 1,865 cows were affected in Canada (LeBlanc *et al.*, 2002). One of the determinants of the likelihood of uterine disease is the incidence of risk factors. These risk factors can be divided into factors that are associated with damage to the uterus, metabolic stress, or deficits in hygiene. Interestingly, the latter is the least important in the majority of epidemiological models that quantify the risk factors for uterine disease (Dubuc *et al.*, 2010; Potter *et al.*, 2010; Sheldon, 2014). The risk factors most frequently associated with uterine infection are those that likely lead to some trauma to the endometrium, including stillbirth, twins, male and beef-sire calves, dystocia, caesarean section operation, and retained placenta (Hussain *et al.*, 1990; Peeler *et al.*, 1994; Dubuc *et al.*, 2010; Potter *et al.*, 2010).

The diagnosis of metritis and clinical endometritis should include an inspection of the contents of the female genital tract by speculum or insertion of a clean-gloved-hand into the vagina (Sheldon, 2004; Sheldon *et al.*, 2006; de Boer *et al.*, 2014). Whilst somewhat invasive, examination of the vagina carries little risk of further microbial contamination of the uterus in postpartum dairy

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cattle (Sheldon *et al.*, 2002a). Manual examination of the vagina also facilitates collection of fluid from the vagina to evaluate the presence and odour of pus, which can be used to score the severity of disease and predict the likely success of treatment (Sheldon *et al.*, 2006). Vaginal examination also allows the operator to detect damage to the wall of the vagina and cervix, indicative of obstetric injuries, vaginitis, and cervicitis. However, as with any clinical examination, the evaluation of uterine disease is subjective and there is inter- and intra-operator variation (Sannmann and Heuwieser, 2015).

The absence of pus in the postpartum genital tract does not mean that the tract is normal. The importance of subclinical endometritis has emerged over the last 15 years, with the realisation that cytological evidence of inflammation of the endometrium is associated with reduced fertility (Kasimanickam *et al.*, 2004; Gilbert *et al.*, 2005). The cause of subclinical endometritis is not yet clear, and may include resolving bacterial infections, immune-pathology without pathogenic bacteria, or even aberrations of postpartum tissue regeneration and repair. Subclinical endometritis is characterized by inflammation of the endometrium that results in a significant reduction in reproductive performance in the absence of signs of clinical endometritis. Subclinical disease is defined by the proportion of polymorphonuclear neutrophils (PMNs) exceeding operator-defined thresholds, usually about 5% of cells in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of clinical endometritis, about 35 to 40 days post partum (Sheldon *et al.*, 2006; de Boer *et al.*, 2014).

Pyometra is characterized by the accumulation of purulent or mucopurulent material within the uterine lumen and distension of the uterus, in the presence of a closed cervix and an active corpus luteum. Postpartum pyometra is uncommon, with an incidence rate of less than 2%, and is thought to be caused by the growth of pathogenic bacteria within the uterine lumen after the formation of the first corpus luteum (Noakes *et al.*, 1990). Although there is functional closure of the cervix, the lumen is not always completely occluded and pus may occasionally discharge through the cervix into the vaginal lumen. Pyometra is sonographically characterised by mixed echodensity fluid in the uterine lumen with distension of the uterus, and a corpus luteum in an ovary (Sheldon *et al.*, 2006).

## The postpartum period

Uterine disease reflects a disturbance of the normal postpartum period, which usually lasts about 40 days, and is defined as the time between parturition and completion of uterine involution (Sheldon, 2004). After parturition, four concomitant events need to be completed before cows are likely to be able to conceive again: uterine involution, regeneration of the endometrium, return of ovarian cyclic activity, and the control of pathogenic bacteria in the uterus (Sheldon, 2004; Sheldon *et al.*, 2006). Failure to resist the growth of pathogenic microbes in the endometrium commonly results in uterine disease.

### *Uterine involution*

Involution is the term used to describe the physical reduction in size of the uterus and cervix after parturition. Involution is thought to be driven by uterine muscular contractions, turnover of the extracellular matrix, necrosis and sloughing of the uterine caruncles, and regeneration of the endometrium (Gier and Marion, 1968). It is often difficult to insert a hand through the cervix 24 h after parturition, and it only admits two fingers by 96 h postpartum. By about 2 weeks post partum, the entire genital tract is palpable per rectum in normal animals; although, the previously gravid horn can still be identified because it is wider and longer than the previously non-gravid horn, and this difference is evident up to 4 weeks postpartum (Okano and Tomizuka, 1987; Tian and Noakes, 1991b; Risco *et al.*, 1994). In parallel with the changes in dimensions, the weight of the uterus decreases from about 9 kg at parturition to 1 kg by 30 days postpartum (Gier and Marion, 1968).

Uterine involution can be monitored by repeated estimation of the size of the uterus, using transrectal palpation or transrectal ultrasonography (Okano and Tomizuka, 1987; Sheldon *et al.*, 2000, 2003). It should be noted that dimensions estimated by transrectal palpation are often about 1 to 2 cm greater than ultrasound measurements; presumably because operators include the thickness of the rectal wall when using transrectal palpation. The changes in uterine horn diameter are almost imperceptible by 4 weeks postpartum, and are probably complete by 6 weeks. In the literature, the time to completion of uterine involution is often reported, but this endpoint is difficult to estimate in clinical practice. On the other hand, factors that delay uterine involution are important because completion of involution is associated with fertility (Fonseca *et al.*, 1983). The factors that delay involution include dystocia, hypocalcaemia, retained placenta, metritis, and endometritis.

### *Regeneration of the endometrium*

The epithelium of the endometrium is often damaged during parturition, the caruncular tissue sloughs as part of the physiological process of the puerperium, and there is considerable tissue remodelling during the postpartum period (Gier and Marion, 1968; Wagner and Hansel, 1969; Tian and Noakes, 1991a). It is thought that the endometrium takes 3 to 4 weeks to fully recover the normal tissue architecture, and it is assumed that a normal endometrium is important for fertility.

### *Return of ovarian cyclic activity*

Within a few days of parturition, circulating steroid hormone concentrations decrease to basal values, and there is an increase in plasma FSH concentration, with subsequent recurrent increases in FSH concentrations every 7 to 10 days (Crowe *et al.*, 1998; Duffy *et al.*, 2000). The first postpartum dominant follicle, with a diameter >8 mm, is usually

selected about 10 days after parturition. This dominant follicle may ovulate to form the first postpartum corpus luteum, the dominant follicle may undergo atresia with subsequent emergence of a second dominant follicle, or it may abnormally persist as an ovarian cyst (Savio *et al.*, 1990; Stagg *et al.*, 1995; Beam and Butler, 1997). The fate of the first postpartum dominant follicle depends on LH pulse frequency, and failure to ovulate is usually a consequence of inadequate LH pulse frequency and reduced ovarian follicle estradiol (Beam and Butler, 1999; Duffy *et al.*, 2000; Cheong *et al.*, 2016). In dairy cattle, metabolic stress - most often negative energy balance - is the main cause of reduced LH pulse frequency, although a range of other factors can impact ovarian cyclic activity (Cheong *et al.*, 2016).

**Microbes that cause uterine disease**

Postpartum uterine disease is associated with the isolation of pathogenic bacteria, particularly *Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum*, *Prevotella* and *Bacteroides* (Elliott *et al.*, 1968; Griffin *et al.*, 1974; Huszenicza *et al.*, 1991; Noakes *et al.*, 1991). Indeed, *T. pyogenes, F. necrophorum* and *Prevotella* act synergistically to increase the likelihood and the severity of endometritis (Ruder *et al.*, 1981; Olson *et al.*, 1984). More recent studies using aerobic and anaerobic culture confirm the importance of *E. coli*, *T. pyogenes* and anaerobic bacteria (Dohmen *et al.*, 2000; Sheldon *et al.*, 2002b; Williams *et al*., 2005; Westermann *et al.*, 2010). Novel endometrial pathogenic *E. coli* have been isolated from animals with uterine disease (Sheldon *et al.*, 2010); and, *T. pyogenes* is associated with the severity of endometrial pathology and clinical disease (Bonnett *et al.*, 1991; Westermann *et al.*, 2010). The link between *T. pyogenes* and disease may be explained by the cholesterol-dependent cytolysin pyolysin (PLO) secreted by *T. pyogenes*, which causes cytolysis particularly of endometrial stromal cells (Amos *et al.*, 2014; Preta *et al.*, 2015).

The role of *E. coli* and *T. pyogenes* is highlighted by infusing *E. coli* and *T. pyogenes* into the uterus of naïve cows to create animal models of endometritis (Ayliffe and Noakes, 1982; Amos *et al.*, 2014). In addition, vaccines containing components of *E. coli*, *F. necrophorum* and/or *T. pyogenes* protect animals against postpartum uterine disease (Nolte *et al.*, 2001; Machado *et al.*, 2014). However, metagenomics techniques have found associations between uterine disease and bacteria that are not readily cultured by standard techniques (Machado *et al.*, 2012; Santos and Bicalho, 2012; Peng *et al.*, 2013; Knudsen *et al.*, 2015; Wagener *et al.*, 2015). Whilst some of the studies find *E. coli*, *T. pyogenes* and the expected anaerobic bacteria, others report finding *Bacteroidetes* and *Firmicutes*. There remains a gap in understanding how "uncultureable" bacteria contribute to the pathogenesis of uterine disease. A consistent finding among most microbiology studies is that anaerobic bacteria are more abundant in the diseased endometrium than in healthy uteri. Perhaps this is not surprising as the endometrium is a microaerophilic environment, with tissue damage likely reducing the oxygen tension further. Taken together the evidence is that *E. coli*, *T. pyogenes* and anaerobic bacteria are probably the main pathogens causing the clinical signs of postpartum uterine disease (Fig. 1).

One note of caution about our understanding of microbes in the endometrium is that recent evidence counters the traditional view that the uterus is sterile outside the postpartum period. There is evidence from studies using fluorescent probes for bacteria and from 16S ribosomal RNA gene sequencing, that there is a sparse microbiome in the uterus, even during pregnancy (Karstrup *et al.*, 2017; Moore *et al.*, 2017). The bacteria include *Trueperella, Fusobacteria and Prevotella* species, but the abundance of these bacteria is a small fraction of those present in animals with postpartum uterine disease. Whilst postpartum uterine bacteria may also derive from the vagina, skin and the environment, it is possible that the pathogenic bacteria present in the uterus before parturition grow and cause pathology after parturition.

**Host defence against infections of the uterus**

The host has a range of defences against microbial contamination of the uterus and infection of the endometrium. Whilst the animals' environment is heavily contaminated with bacteria, the vulva, vagina and cervix provide anatomical barriers to ascending infections, except during parturition (Fig. 1). Whether the resident flora of the vagina or the pH of the vagina might also compete with pathogens to limit disease is a contentious matter. However, there is a range of antimicrobial peptides, glycoproteins and mucins in the vagina, cervix and uterus, that counter bacterial contamination and restrain bacterial growth (Davies *et al.*, 2008; Chapwanya *et al.*, 2013; Kasimanickam *et al.*, 2014).

Of course, microbial invasion of the female genital tract is not unnoticed. Adaptive immune responses are evident, with increase abundance of antibodies (Dhaliwal *et al.*, 2001); which, concur with the ability to vaccinate against uterine pathogens (Nolte *et al.*, 2001; Machado *et al.*, 2014). A recent advance in knowledge has been about the role of innate immunity in the female genital tract (Fig. 1). Innate immunity depends on the binding of pathogen-associated molecular patterns from microbes to pattern recognition receptors in host cells. There is a range of pattern recognition receptors found in the plasma membrane or cytoplasm of mammalian hematopoietic cells. The two most widely investigated pattern recognition receptor families are the Toll-like receptors and components of the inflammasome (Moresco *et al.*, 2011; Lamkanfi and Dixit, 2014). The Toll-like receptors bind components of bacteria, such as lipopolysaccharide, lipopeptides and nucleotides, which leads to production of inflammatory mediators; typically interleukin (IL)-6 and IL-8. Similarly, pathogen-associated molecules that reach intracellular compartments activate the inflammasome. However, the inflammasome can also be activated by a

range of generalized cell perturbations, including the ion fluxes that are associated with pore-forming toxins secreted by bacteria. Activation of the inflammasome typically leads to cleavage of pro-IL-1β and secretion of the mature form of IL-1β (Lamkanfi and Dixit, 2014). The Toll-like receptor system is present and active in the cells of the endometrium, both epithelium and stroma, as well as in bovine hematopoietic cells (Herath *et al.*, 2006; Cronin *et al.*, 2012, 2016; Turner *et al.*, 2014). However, endometrial cells secrete little IL-1β protein, and so inflammasome activity may be more important in hematopoietic cells.

The innate immune system provides a non-specific and rapid response to pathogens and damage. However, excessive inflammation leads to immunopathology or septic shock, and so innate immunity is carefully calibrated. A series of checks and balances are in place to scale inflammation to meet the level of microbial threat, and to limit inflammation when infections are cleared (Blander and Sander, 2012). One example in the bovine endometrium, is the role of STAT3 to regulate the secretion of IL-6 and IL-8 in stromal cells (Cronin *et al.*, 2016). Another example is the apical secretion of IL-6 and IL-8 from bovine endometrial epithelial cells, toward the invading pathogens in the uterine lumen and away from the underlying stromal cells (Healy *et al.*, 2015).

Beyond recognition of microbes, one of the features of infection is tissue damage, which in the endometrium is often caused by secretion of pyolysin by *T. pyogenes* (Amos *et al.*, 2014; Preta *et al.*, 2015). Damaged cells release damage-associated molecular patterns, such as nuclear and cytoplasmic molecules that are not normally encountered in the extra-cellular compartment (Kono and Rock, 2008). Some pattern recognition receptors, primarily in hematopoietic cells, sense damage-associated molecular patterns, leading to inflammatory responses. Damaged endometrial tissue cells, primed with LPS, produce the damage-associated molecular patterns, IL-1α, which is normally retained in the cytoplasm of healthy cells (Healy *et al.*, 2014). Furthermore, endometrial stromal cells express the receptor for IL-1 and generate inflammatory responses to IL-1α, including secretion of more IL-6 (Healy *et al.*, 2014).

Innate immunity is an evolutionary ancient system and so it is not surprising that it is integrated with other cellular homeostatic and metabolic pathways (Kotas and Medzhitov, 2015). Dairy cattle are under metabolic stress after parturition, with reduced concentrations of nutrients and changes in metabolic hormones, including reduced abundance of glucose, glutamine and insulin-like growth factor 1 (Chagas *et al.*, 2007; Kerestes *et al.*, 2009). Negative energy balance may impair the inflammatory response and clearance of bacteria from the endometrium, leading to chronic endometritis (Esposito *et al.*, 2014). Certainly, the response to pathogen molecules is energetically expensive *in vivo* and *in vitro* (Turner *et al.*, 2016; Kvidera *et al.*, 2017). A striking example is that animals use >1 kg of glucose in the first 12 h after challenge with LPS (Kvidera *et al.*, 2017). Furthermore, the depletion of the key cellular nutrients, glucose or glutamine, reduces inflammatory responses by endometrial tissues *in vitro* (Turner *et al.*, 2016; Noleto *et al.*, 2017). If metabolic stress compromises the ability of animals to respond sufficiently to pathogens, this may result in persistence of infections and chronic inflammation.

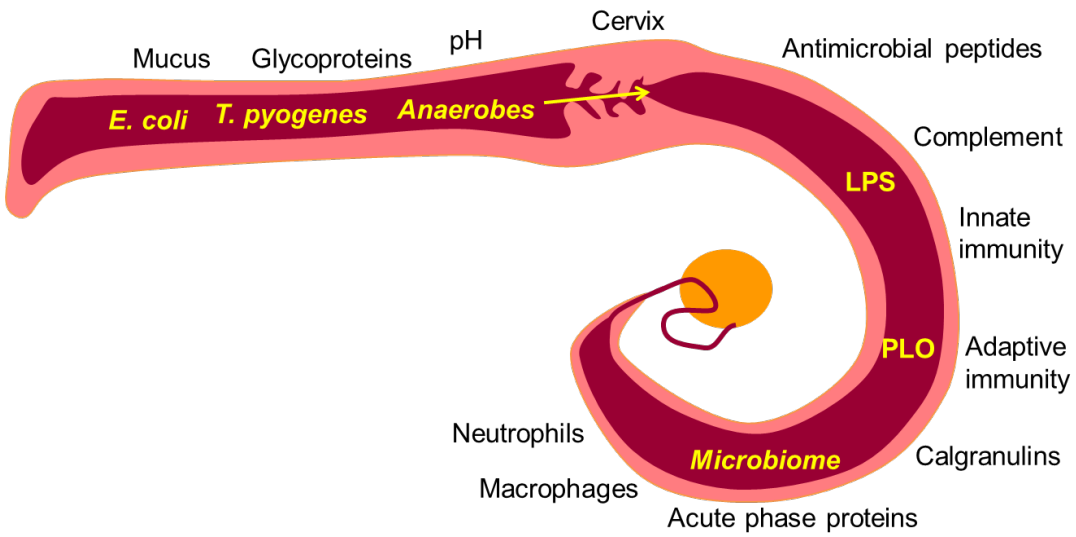


Figure 1. Schematic outline of factors contributing to postpartum uterine health. After parturition the anatomical barriers of the vulva, vagina and cervix are breached, introducing bacteria into the uterus, including pathogens, along with bacteria that constitute the uterine microbiome. However, tissue factors such as mucus, glycoproteins, the pH of the genital tract, and antimicrobial peptides help counter bacterial invasion. If bacteria or their pathogen-associated molecules, such as lipopolysaccharide (LPS), are sensed by the innate or adaptive immune systems then an inflammatory response ensues, including increased expression of complement, calgranulins and acute phase proteins, and chemotaxis of neutrophils and macrophages to the site of infection. As well as inflammation, uterine disease is characterised by tissue damage, including cytolysis caused by the cholesterol-dependent cytolysin, pyolysin (PLO).

## Impact of uterine disease on animal health and fertility

Clinical uterine disease has a marked impact on reproductive health in cattle, causing subfertility and infertility. In a meta-analysis of records from more than 10,000 animals, there was evidence that postpartum metritis caused subfertility by increasing the time to first insemination by 7.2 days, reducing conception rate to first insemination by 20%, and increasing the calving to conception interval by 18.6 days (Fourichon *et al.*, 2000). Similarly, clinical endometritis increased the interval to first insemination by 11 days, and delayed conception by 32 days, compared with animals that did not have endometritis (Borsberry and Dobson, 1989). Although less common than subfertility, uterine disease also cause infertility. Cows with clinical endometritis between 20 and 33 days post partum were 1.7 times more likely to be culled for reproductive failure than cows without endometritis (LeBlanc *et al.*, 2002).

Pathology in the endometrium is likely to be detrimental to fertilization and conception. In addition, extension of infection or inflammation to the oviduct likely disrupts the delicate balance of the immune systems that are required for fertilization (Marey *et al.*, 2016). However, an important observation for mechanisms that perturb fertility, is that postpartum uterine infection also impacts fertility after resolution of the clinical disease (Borsberry and Dobson, 1989). Several mechanisms may underlie the wider effects of uterine infection on fertility, beyond the tubular genital tract. First, there is evidence that bacterial infections disrupt the endocrine signalling in the hypothalamic-pituitary-gonadal axis, and the secretion of gonadotrophins (Karsch *et al.*, 2002). Secondly, uterine infections disrupt ovarian follicle growth and function, with smaller and less steroidogenic ovarian follicles (Sheldon *et al.*, 2002b). Finally, uterine infections may reduce oocyte quality, with increased rates of meiotic arrest and germinal vesicle breakdown failure (Bromfield and Sheldon, 2011). Oocyte development lasts about 120 days, between the primordial follicle stage to ovulation of a cumulus-oocyte complex. Thus, in cows inseminated 60 to 120 days post partum, the oocytes that are ovulated may have been exposed to pathogen molecules and inflammatory mediators throughout the postpartum period, if the animal had uterine disease. Therefore, limiting uterine disease is not only important for the affected animals, but also for their offspring. Further discussion of the mechanisms linking uterine disease and reproductive biology are published elsewhere (Sheldon *et al.*, 2014; Bromfield *et al.*, 2015).

## Outstanding questions

Whilst there is a clear understanding of the clinical aspects and implication of postpartum uterine disease, and some of the mechanisms of pathology, there are important outstanding questions. The most obvious question is why are modern high-milk-yield cows so susceptible to metritis and endometritis? Allied to this, is what can be done to prevent uterine disease? Answering these questions is vital for sustainable intensification of the dairy industry over the next 50 years.

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## References

**Amos MR, Healey GD, Goldstone RJ, Mahan S, Duvel A, Schuberth HJ, Sandra O, Zieger P, Dieuzy-Labaye I, Smith DGE, Sheldon IM**. 2014. Differential endometrial cell sensitivity to a cholesterol-dependent cytolysin links *Trueperella pyogenes* to uterine disease in cattle. *Biol Reprod*, 90:54,1-13.

**Ayliffe TR, Noakes DE**. 1982. Effects of exogenous oestrogen and experimentally induced endometritis on absorption of sodium benzylpenicillin from the cow's uterus. *Vet Rec*, 110:96-98.

**Beam SW, Butler WR**. 1997. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. *Biol Reprod*, 56:133-142.

**Beam SW, Butler WR**. 1999. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. *J Reprod Fertil Suppl*, 54:411-424.

**Blander JM, Sander LE**. 2012. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nat Rev Immunol*, 12:215-225.

**Bonnett BN, Martin SW, Gannon VP, Miller RB, Etherington WG**. 1991. Endometrial biopsy in Holstein-Friesian dairy cows. III. Bacteriological analysis and correlations with histological findings. *Can J Vet Res*, 55:168-173.

**Borsberry S, Dobson H**. 1989. Periparturient diseases and their effect on reproductive performance in five dairy herds. *Vet Rec*, 124:217-219.

**Bromfield JJ, Sheldon IM**. 2011. Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. *Endocrinology*, 152:5029-5040.

**Bromfield JJ, Santos JE, Block J, Williams RS, Sheldon IM**. 2015. Physiology and Endocrinology Symposium: Uterine infection: linking infection and innate immunity with infertility in the high-producing dairy cow. *J Anim Sci*, 93:2021-2033.

**Chagas LM, Bass JJ, Blache D, Burke CR, Kay JK, Lindsay DR, Lucy MC, Martin GB, Meier S, Rhodes FM, Roche JR, Thatcher WW, Webb R**. 2007. Invited review: New perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. *J Dairy Sci*, 90:4022-4032.

**Chapwanya A, Meade KG, Doherty ML, Callanan JJ, O'Farrelly C**. 2013. Endometrial epithelial cells are potent producers of tracheal antimicrobial peptide and serum amyloid A3 gene expression in response to *E.*

*coli* stimulation. *Vet Immunol Immunopathol*, 151:157-162.

**Cheong SH, Sa Filho OG, Absalon-Medina VA, Pelton SH, Butler WR, Gilbert RO**. 2016. Metabolic and endocrine differences between dairy cows that do or do not ovulate first postpartum dominant follicles. *Biol Reprod*, 94:18, 1-11.

**Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM**. 2012. Toll-Like receptor 4 and MyD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. *Biol Reprod*, 86:51, 1-9.

**Cronin JG, Kanamarlapudi V, Thornton CA, Sheldon IM**. 2016. Signal transducer and activator of transcription-3 licenses Toll-like receptor 4-dependent interleukin (IL)-6 and IL-8 production via IL-6 receptor-positive feedback in endometrial cells. *Mucosal Immunol*, 9:1125-1136.

**Crowe MA, Padmanabhan V, Mihm M, Beitins IZ, Roche JF**. 1998. Resumption of follicular waves in beef cows is not associated with periparturient changes in follicle-stimulating hormone heterogeneity despite major changes in steroid and luteinizing hormone concentrations. *Biol Reprod*, 58:1445-1450.

**Davies D, Meade KG, Herath S, Eckersall PD, Gonzalez D, White JO, Conlan RS, O'Farrelly C, Sheldon IM**. 2008. Toll-like receptor and antimicrobial peptide expression in the bovine endometrium. *Reprod Biol Endocrinol*, 6:53.

**de Boer MW, LeBlanc SJ, Dubuc J, Meier S, Heuwieser W, Arlt S, Gilbert RO, McDougall S**. 2014. Invited review: systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows. *J Dairy Sci*, 97:3983-399.

**Dhaliwal GS, Murray RD, Woldehiwet Z**. 2001. Some aspects of immunology of the bovine uterus related to treatments for endometritis. *Anim Reprod Sci*, 67:135-152.

**Dohmen MJ, Joop K, Sturk A, Bols PE, Lohuis JA**. 2000. Relationship between intra-uterine bacterial contamination, endotoxin levels and the development of endometritis in postpartum cows with dystocia or retained placenta. *Theriogenology*, 54:1019-1032.

**Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ**. 2010. Risk factors for postpartum uterine diseases in dairy cows. *J Dairy Sci*, 93:5764-5771.

**Duffy P, Crowe MA, Boland MP, Roche JF**. 2000. Effect of exogenous LH pulses on the fate of the first dominant follicle in postpartum beef cows nursing calves. *J Reprod Fertil*, 118:9-17.

**Elliott L, McMahon KJ, Gier HT, Marion GB**. 1968. Uterus of the cow after parturition: bacterial content. *Am J Vet Res*, 29:77-81.

**Esposito G, Irons PC, Webb EC, Chapwanya A**. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. *Anim Reprod Sci*, 144:60-71.

**Fonseca FA, Britt JH, McDaniel BT, Wilk JC, Rakes AH**. 1983. Reproductive traits of Holsteins and Jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate and days open. *J Dairy Sci*, 66:1128-1147.

**Fourichon C, Seegers H, Malher X**. 2000. Effect of disease on reproduction in the dairy cow: a meta-analysis. *Theriogenology*, 53:1729-1759.

**Gier HT, Marion GB**. 1968. Uterus of the cow after parturition: involutional changes. *Am J Vet Res*, 29:83-96.

**Gilbert RO, Shin ST, Guard CL, Erb HN, Frajblat M**. 2005. Prevalence of endometritis and its effects on reproductive performance of dairy cows. *Theriogenology*, 64:1879-1888.

**Griffin JFT, Hartigan PJ, Nunn WR**. 1974. Non-specific uterine infection and bovine fertility. I. Infection patterns and endometritis during the first seven weeks post-partum. *Theriogenology*, 1:91-106.

**Healy LL, Cronin JG, Sheldon IM**. 2014. Endometrial cells sense and react to tissue damage during infection of the bovine endometrium via interleukin 1. *Sci Rep*, 4:7060, 1-9. doi:10.1038/srep07060.

**Healy LL, Cronin JG, Sheldon IM**. 2015. Polarized epithelial cells secrete interleukin 6 apically in the bovine endometrium. *Biol Reprod*, 92:151, 1-12.

**Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE, Sheldon IM**. 2006. Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. *Endocrinology*, 147:562-570.

**Hussain AM, Daniel RCW, O'Boyle D**. 1990. Postpartum uterine flora following normal and abnormal puerperium in cows. *Theriogenology*, 34:291-302.

**Huszenicza G, Fodor M, Gacs M, Kulcsar M, Dohmen MJW, Vamos M, Porkolab L, Legl T, Bartyik J, Lohuis JACM, Janosi S, Szita G**. 1991. Uterine bacteriology, resumption of cyclic ovarian activity and fertility in postpartum cows kept in large-scale dairy herds. *Reprod Domest Anim*, 34:237-245.

**Karsch FJ, Battaglia DF, Breen KM, Debus N, Harris TG**. 2002. Mechanisms for ovarian cycle disruption by immune/inflammatory stress. *Stress*, 5:101-112.

**Karstrup CC, Klitgaard K, Jensen TK, Agerholm JS, Pedersen HG**. 2017. Presence of bacteria in the endometrium and placentomes of pregnant cows. *Theriogenolgy*, 99:43-47.

**Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH**. 2004. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology*, 62:9-23.

**Kasimanickam R, Kasimanickam V, Kastelic JP**. 2014. Mucin 1 and cytokines mRNA in endometrium of dairy cows with postpartum uterine disease or repeat breeding. *Theriogenology*, 81:952-958 e2.

**Kerestes M, Faigl V, Kulcsar M, Balogh O, Foldi J, Febel H, Chilliard Y, Huszenicza G**. 2009. Periparturient insulin secretion and whole-body insulin responsiveness in dairy cows showing various forms of ketone pattern with or without puerperal metritis. *Domest Anim Endocrinol*, 37:250-261.

**Knudsen LR, Karstrup CC, Pedersen HG, Agerholm JS, Jensen TK, Klitgaard K**. 2015. Revisiting bovine

pyometra--new insights into the disease using a culture-independent deep sequencing approach. *Vet Microbiol*, 175:319-324.

**Kono H, Rock KL**. 2008. How dying cells alert the immune system to danger. *Nat Rev Immunol*, 8:279-89.

**Kotas ME, Medzhitov R**. 2015. Homeostasis, inflammation, and disease susceptibility. *Cell*, 160:816-827.

**Kvidera SK, Horst EA, Abuajamieh M, Mayorga EJ, Fernandez MV, Baumgard LH**. 2017. Glucose requirements of an activated immune system in lactating Holstein cows. *J Dairy Sci*, 100:2360-2374.

**Lamkanfi M, Dixit VM**. 2014. Mechanisms and functions of inflammasomes. *Cell*, 157:1013-1022.

**LeBlanc SJ, Duffield TF, Leslie KE, Bateman KG, Keefe GP, Walton JS, Johnson WH**. 2002. Defining and diagnosing postpartum clinical endometritis and its impact on reproductive performance in dairy cows. *J Dairy Sci*, 85:2223-2236.

**Machado VS, Bicalho ML, Meira Junior EB, Rossi R, Ribeiro BL, Lima S, Santos T, Kussler A, Foditsch C, Ganda EK, Oikonomou G, Cheong SH, Gilbert RO, Bicalho RC**. 2014. Subcutaneous immunization with inactivated bacterial components and purified protein of Escherichia coli, *Fusobacterium necrophorum* and *Trueperella pyogenes* prevents puerperal metritis in Holstein dairy cows. *PLOS One*, 9:e91734.

**Machado VS, Oikonomou G, Bicalho ML, Knauer WA, Gilbert R, Bicalho RC**. 2012. Investigation of postpartum dairy cows' uterine microbial diversity using metagenomic pyrosequencing of the 16S rRNA gene. *Vet Microbiol*, 159:460-469.

**Marey MA, Yousef MS, Kowsar R, Hambruch N, Shimizu T, Pfarrer C, Miyamoto A**. 2016. Local immune system in oviduct physiology and pathophysiology: attack or tolerance? *Domest Anim Endocrinol*, 56(suppl):S204-211.

**Moore SG, Ericsson AC, Poock SE, Melendez P, Lucy MC**. 2017. Hot topic: 16S rRNA gene sequencing reveals the microbiome of the virgin and pregnant bovine uterus. *J Dairy Sci*, 100:4953-4960.

**Moresco EM, LaVine D, Beutler B**. 2011. Toll-like receptors. *Curr Biol*, 21:R488-93.

**Noakes DE, Wallace LM, Smith GR**. 1990. Pyometra in a Friesian heifer: bacteriological and endometrial changes. *Vet Rec*, 126:509.

**Noakes DE, Wallace L, Smith GR**. 1991. Bacterial flora of the uterus of cows after calving on two hygienically contrasting farms. *Vet Rec*, 128:440-442.

**Noleto PG, Saut JP, Sheldon IM**. 2017. Short communication: Glutamine modulates inflammatory responses to lipopolysaccharide in ex vivo bovine endometrium. *J Dairy Sci*, 100:2207-2212.

**Nolte O, Morscher J, Weiss HE, Sonntag H**. 2001. Autovaccination of dairy cows to treat post partum metritis caused by Actinomyces pyogenes. *Vaccine*, 19:3146-3153.

**Okano A, Tomizuka T**. 1987. Ultrasonic observation of postpartum uterine involution in the cow. *Theriogenology*, 27:369-376.

**Olson JD, Ball L, Mortimer RG, Farin PW, Adney WS, Huffman EM**. 1984. Aspects of bacteriology and endocrinology of cows with pyometra and retained fetal membranes. *Am J Vet Res*, 45:2251-2255.

**Peeler EJ, Otte MJ, Esslemont RJ**. 1994. Inter-relationships of periparturient diseases in dairy cows. *Vet Rec*, 134:129-132.

**Peng Y, Wang Y, Hang S, Zhu W**. 2013. Microbial diversity in uterus of healthy and metritic postpartum Holstein dairy cows. *Folia Microbiol (Praha)*, 58:593-600.

**Potter T, Guitian J, Fishwick J, Gordon PJ, Sheldon IM**. 2010. Risk factors for clinical endometritis in postpartum dairy cattle. *Theriogenology*, 74:127-134.

**Preta G, Lotti V, Cronin JG, Sheldon IM**. 2015. Protective role of the dynamin inhibitor Dynasore against the cholesterol-dependent cytolysin of Trueperella pyogenes. *FASEB J*, 29:1516-1528.

**Risco CA, Drost M, Thatcher WW, Savio J, Thatcher MJ**. 1994. Effects of calving-related disorders on prostaglandin, calcium, ovarian activity and uterine involution in postpartum dairy cows. *Theriogenology*, 42:183-203.

**Ruder CA, Sasser RG, Williams RJ, Ely JK, Bull RC, Butler JE**. 1981. Uterine infections in the postpartum cow: II Possible synergistic effect of *Fusobacterium necrophorum* and *Corynebacterium pyogenes*. *Theriogenology*, 15:573-580.

**Sannmann I, Heuwieser W**. 2015. Technical note: Intraobserver, interobserver, and test-retest reliabilities of an assessment of vaginal discharge from cows with and without acute puerperal metritis. *J Dairy Sci*, 98:5460-5466.

**Santos TM, Bicalho RC**. 2012. Diversity and succession of bacterial communities in the uterine fluid of postpartum metritic, endometritic and healthy dairy cows. *PLOS One*, 7:e53048.

**Savio JD, Boland MP, Hynes N, Roche JF**. 1990. Resumption of follicular activity in the early postpartum period of dairy cows. *J Reprod Fertil*, 88:569-579.

**Sheldon IM, Noakes DE, Dobson H**. 2000. The influence of ovarian activity and uterine involution determined by ultrasonography on subsequent reproductive performance. *Theriogenology*, 54:409-419.

**Sheldon IM, Noakes DE, Rycroft AN, Dobson H**. 2002a. Effect of postpartum manual examination of the vagina on uterine bacterial contamination in cows. *Vet Rec*, 151:531-534.

**Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU, Dobson H**. 2002b. Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. *Reproduction*, 123:837-845.

**Sheldon IM, Noakes DE, Rycroft AN, Dobson H**. 2003. The effect of intrauterine administration of estradiol on postpartum uterine involution in cattle. *Theriogenology*, 59:1357-1371.

**Sheldon IM**. 2004. The postpartum uterus. *Vet Clin North Am Food Anim Pract*, 20:569-591.

**Sheldon IM, Lewis GS, LeBlanc S, Gilbert RO**. 2006. Defining postpartum uterine disease in cattle. *Theriogenology*, 65:1516-1530.

**Sheldon IM, Cronin J, Goetze L, Donofrio G,**

**Schuberth HJ**. 2009. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biol Reprod*, 81:1025-1032.

**Sheldon IM, Rycroft AN, Dogan B, Craven M, Bromfield JJ, Chandler A, Roberts MH, Price SB, Gilbert RO, Simpson KW**. 2010. Specific strains of Escherichia coli are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLOS One*, 5:e9192.

**Sheldon IM**. 2014. Genes and environmental factors that influence disease resistance to microbes in the female reproductive tract of dairy cattle. *Reprod Fertil Dev*, 27:72-81.

**Sheldon IM, Cronin JG, Healey GD, Gabler C, Heuwieser W, Streyl D, Bromfield JJ, Miyamoto A, Fergani C, Dobson H**. 2014. Innate immunity and inflammation of the bovine female reproductive tract in health and disease. *Reproduction*, 148:R41-R51.

**Stagg K, Diskin MG, Sreenan JM, Roche JF**. 1995. Follicular development in long-term anoestrous suckler beef cows fed two levels of energy postpartum. *Anim Reprod Sci*, 38:49-61.

**Tian W, Noakes DE**. 1991a. A radiographic method for measuring the effect of exogenous hormone therapy on uterine involution in ewes. *Vet Rec*, 129:463-466.

**Tian W, Noakes DE**. 1991b. Plasma 3 methyl-histidine concentrations and uterine involution in the post partum cow. *Vet Rec*, 128:109-110.

**Turner ML, Cronin JC, Healey GD, Sheldon IM**. 2014. Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1 and TLR6. *Endocrinology*, 155:1453-1465.

**Turner ML, Cronin JG, Noleto PG, Sheldon IM**. 2016. Glucose availability and AMP-activated protein kinase link energy metabolism and innate immunity in the bovine endometrium. *PLOS One*, 11:e0151416.

**Wagener K, Prunner I, Pothmann H, Drillich M, Ehling-Schulz M**. 2015. Diversity and health status specific fluctuations of intrauterine microbial communities in postpartum dairy cows. *Vet Microbiol*, 175:286-293.

**Wagner WC, Hansel W**. 1969. Reproductive physiology of the post partum cow. l. Clinical histological findings. *J Reprod Fertil*, 18:493-500.

**Westermann S, Drillich M, Kaufmann TB, Madoz LV, Heuwieser W**. 2010. A clinical approach to determine false positive findings of clinical endometritis by vaginoscopy by the use of uterine bacteriology and cytology in dairy cows. *Theriogenology*, 74:1248-1255.

**Williams EJ, Fischer DP, England GCW, Dobson H, Pfeiffer DU, Sheldon IM**. 2005. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the inflammatory response to endometritis in cattle. *Theriogenology*, 63:102-117.

**Zwald NR, Weigel KA, Chang YM, Welper RD, Clay JS**. 2004. Genetic selection for health traits using producer-recorded data. I. Incidence rates, heritability estimates, and sire breeding values. *J Dairy Sci*, 87:4287-4294.

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# COST-Action GEMINI and EPICONCEPT: what we learned after 8 years?

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## Abstract

Scientific societies have a major role in facilitating and disseminating scientific discoveries. Here, we are all members of societies related to reproductive biology, such as AETE (European Association of Embryo Transfer), SRF (Society for Reproduction and Fertility) or ESHRE (European Society of Human Reproduction and Embryology). However, many of you may be unfamiliar with COST Actions. These are atypical, EU-funded temporary societies, that can have a huge impact upon the lives and careers of their members. The objective of the present paper was to capture the influence that one specific COST Action, EPICONCEPT, and to a lesser extent also the earlier COST Action GEMINI, has had on European scientists involved in animal reproduction and embryo transfer. We discuss the intrinsic value of belonging to EPICONCEPT, we focus on how EPICONCEPT advanced the careers of the scientists involved and the lessons learned. We conclude that such specific short-lived societies as granted by COST can be the basis of permanent collaborative ties and networking within Europe. Moreover, EPICONCEPT has been a very useful tool to raise awareness about epigenetics among animal scientists and breeders.

**Keywords**: COST, epigenetics, periconception environment, researcher.

## Introduction

Young scientists are not always aware of the importance of attending different conferences and the value of engaging in networking activities. My[1] former boss told me: "If you attend a conference it will benefit your network". But do we actually need a network to be successful in science and to advance in our career? I can now wholeheartedly say: "Yes" to this question.

The network that was of major importance to me, and also to many other European researchers, was EPICONCEPT, short for "Epigenetics and Periconception Environment".

EPICONCEPT really started about ten years ago. I received an e-mail from a scientist, Prof Alireza Fazeli, that was at that time only affiliated to the University of Sheffield in the UK. It read: "Dear Dr. Van Soom, we may have never met before, but I know from your published work that you are interested in sperm-oviduct interaction. I plan to apply for a COST Action that is focusing on this broader topic. Are you willing to take part in this Action and if so, can you give me the names of other people who may also intend to join? If you are interested, we can also talk on the phone, so I can explain the background."

I had never heard of a COST Action before, so I agreed to talk on the phone to learn more. I asked Alireza if this was a way to raise European money for research. "No", he said, "you can get money for organizing conferences and workshops, for visiting each other's laboratories and for exchanging PhD students."

At first, I did not really think that the COST Action was going to be a useful approach at all, but I agreed to contribute nonetheless. I produced a list of emails from people involved in research regarding oviducts and spermatozoa, and thought that would be the last thing that I would ever hear from it. Little did I know! Alireza Fazeli, together with many others, turned from an unknown person into a dear lifelong friend. I will review in this personal testimony, how EPICONCEPT affected our lives and careers, and we will point out what we have learned from EPICONCEPT.

## History of COST Action

The COST-Action website (http://www.cost.eu/), states **"**COST is a unique means for European researchers, engineers and scholars to jointly develop their own ideas and new initiatives across all fields of science and technology through trans-European networking of nationally funded research activities."

The first COST Action I was involved in was GEMINI (Maternal Interaction with Gamete and Embryo 2008-2012). Until then, I had been mainly involved with bovine embryos and how they interact with their environment (the Petri dish), but during this action, we learned about maternal interaction in insects, fish, reptiles (Holt and Lloyd, 2010) and even apprehended information on *in silico* models (Burkitt *et*

[1]Just for the reader's information, wherever in this manuscript the words "I/me/my" are used, it refers to my own (Ann Van Soom) experiences. However, whenever "we" is used it will refer to my coauthor and/or the wider COST community.

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*al*., 2011). I started writing (opinion) papers with other European scientists, we exchanged students with other labs, we organized workshops and meetings and we were inspired by all these contacts and communications: it broadened our view, it encouraged us to apply for more nationally funded projects and we were able to start common research with other EU-groups, with our own funding of course. The annual meetings and workshops increased the bonds we had and they created the sense of belonging to a large scientific family.

COST Actions can be a very useful tool, and we used it very well: GEMINI turned out to be important for my career and also for many young investigators and their supervisors. Scientific societies, including COST Actions, have a major role in facilitating scientific discoveries and disseminating them (Bahr, 2008). To use a metaphor: The network that is created by a COST Action is like a spider web, connecting different people with sticky threads, and when a new fly is caught, it is signaling by its movement its presence to the central spider, who can easily catch it for a presentation at one of the upcoming conferences. The outstanding feature of COST is that it provides a platform to young investigators, to researchers that are underrepresented (many of whom are female), to researchers from countries within and outside Europe that is very different from the "Old Boy Network" (see Merriam Webster: an informal system in which wealthy men with the same social and educational background help each other), which is not always in favor of young researchers belonging to a minority to present his or her research.

In this digital age, one could wonder if the network that is provided by COST and other societies could not be replaced by a Facebook page, or by twitter, or by a comparable social media connection. The answer is probably "No". A questionnaire which was filled in by trainee members of the Society for Study of Reproduction provided indeed more evidence that people need to interact in a personal way, to connect and to stay current (Table 1). Meeting in an informal manner removes many of the prejudices people may have when they receive an unsolicited e-mail from somebody they have not met before. Contrary to the common expectation, a discussion at a poster session or even having a drink at the bar with another scientist can be the start of a lifelong scientific collaboration!

So we can conclude that COST Actions are indeed useful, contrary to what I expected after my first contact with Alireza Fazeli. During this period, my career moved on to the fast track. I applied for many more grants than before and as a result, I was also successful in achieving more funding. At the end of GEMINI, I even decided to apply for a second COST action, EPICONCEPT, as the Chair this time. The topic was on epigenetics, and although I was not a molecular biologist, I had always been intrigued by genetics, by evolution, by Lamarck and Darwin, and the link it had with embryology, as in the discredited theory of Ernest Haeckel, where he stated that ontogeny is a recapitulation of phylogeny. EPICONCEPT would give me the opportunity to delve deeper into this topic. I considered the fact that I was not a geneticist to be an advantage, since I had to make the topic understandable for non-geneticists. This is often the key to success: convey your message in a simple, understandable way, both to other scientists and to the general public. That too, I learned during the COST Actions.

Table 1. Survey filled in by trainees that were member of Society for the study of Reproduction (SSR) (adapted from Bahr 2008)

| Question | Most popular answer | Second important answer |
|---|---|---|
| *What is the primary reason for joining a scientific society?* | To attend annual meeting | To share knowledge with other researchers |
| *What is the value of belonging to a scientific society?* | To interact with people who share common interest and to meet experts in the field | To stay informed about the latest advances in the field |
| *What can scientific societies do to advance trainees' scientific careers?* | To facilitate networking and collaboration | To hold annual meetings |

**Why is epigenetics interesting when you are working with embryos?**

So why did I think EPICONCEPT was an interesting line of research? Many things had evolved in the field of assisted reproduction since the birth of the first test-tube baby, Louise Brown, in 1978. Cattle were the first species, after the human, in which transfers of *in vitro* produced embryos were performed on a large scale during the 1990s. Coinciding with the first reports of the birth of the first cloned calves and sheep, troubling anecdotal reports emerged of congenital abnormalities associated with cloned animals and later in a broader perspective, also of abnormal offspring born after *in vitro* culture of ruminant embryos (Willadsen *et al*., 1991; Van Soom *et al*., 1994; Walker *et al*., 1996). The most obvious characteristic of the abnormal offspring was an overgrowth phenotype, and thus the syndrome was termed "Large Offspring Syndrome" or "Abnormal Offspring Syndrome" (for review see Farin *et al*., 2010).

As early as 1998 it was hypothesized that the mechanism was probably related to changes in DNA-methylation of imprinted genes, which were imposed upon the embryo by its exposure during a critical period to a perturbing environment (Young *et al*., 1998).

Also in humans there were similar reports on the influence of the intrauterine or perinatal environment on fetal development. Barker postulated that a baby with a low birth weight has a higher risk to

suffer from cardiovascular disease as an adult (Barker *et al*., 1989). This hypothesis was later called the "Developmental Origins of Health and Disease" or DOHAD hypothesis, and by the mid-1990s the concept that late-onset diseases are related with earlier prenatal events, was well established (Barker, 1995; Grace and Sinclair, 2009). Barker studied mainly fetal undergrowth, but also fetal overgrowth has been reported in humans. Assisted reproduction, which is currently accounting for 5-6 % of the live birth rates in Belgium, has indeed been associated with increased risk of imprinting diseases such as Beckwith-Wiedemann syndrome, which is a fetal overgrowth syndrome (Owen and Segars, 2009). Both "Large Offspring Syndrome" in cattle and the "Developmental Origins Of Adult Health And Disease" hypothesis in humans are reflections of the fact that small changes in the environment to which the embryo is exposed can either lead to obvious phenotypical changes in the neonate (oversized calf, Beckwith-Wiedemann baby) or to more subtle, long-term programming effects, which can lead to impaired health during adulthood (Sinclair and Singh, 2007; Fig. 1).

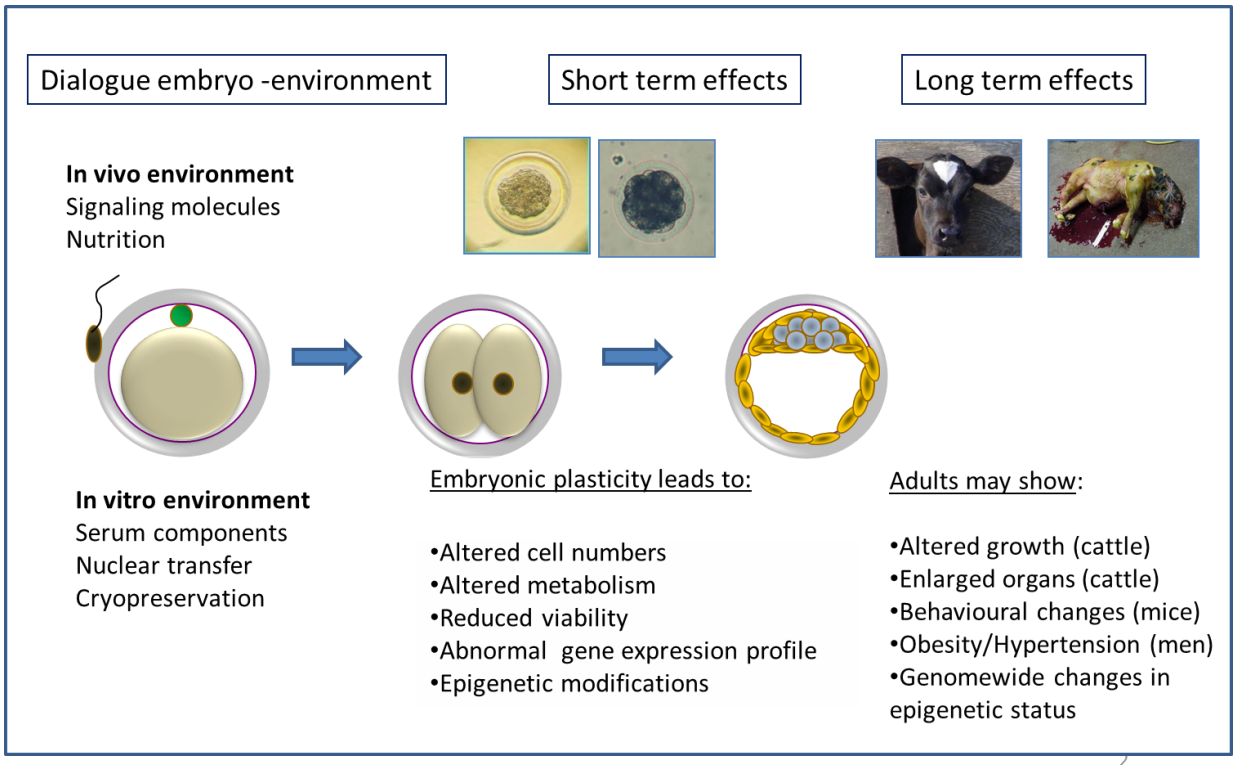


Figure 1. An unusual environment to which the embryo is exposed will lead to short and long term effects, both of which are caused by epigenetic modifications and which in some cases can be transgenerational. At present, these effects have been shown to be induced by *in vitro* embryo culture in mice, man and cattle.

Such an important concept (DOHAD) called for more in depth research. The field of environmental epigenetics, which was closely related to the concept of Developmental Origins Of Health And Disease, was studied extensively by using various animal models. These models provided a means to understand how environmental factors, which are present at periconception, may induce heritable changes in gene expression and as such, can cause diseases that cannot be explained by conventional genetic mechanisms (Rosenfeld, 2010). These changes were called epigenetic changes: in Epiconcept , we aimed to apply our animal models to the search for an answer on how environment affects offspring health and performance in the adulthood (Van Soom *et al*., 2010, 2013, 2014). Understanding the epigenetic mechanisms involved in embryonic development will help to address such issues as a) the risks associated with stress, illness or dietary restrictions and metabolic imbalances during the peri-conceptional period, which is including prenatal and early postnatal life (Mossa *et al*., 2013; Fleming *et al*., 2015; Velazquez, 2015); b) the effects of maternal and paternal nutritional status/stress on epigenetic programming through the germline; and c) transgenerational effects where, in future, greater emphasis in livestock species should be placed on traits of agricultural importance (Gonzalez-Recio *et al*., 2012; Opsomer *et al*., 2017).

Epigenetic changes may be less harmful than genetic mutations since they are reversible. Understanding the healthy settings of the periconception environment that avoid deleterious epigenetic changes will allow to potentially improve this environment to attain the ideal conditions to which breeding animals and embryos should be exposed in order to prevent epigenetic mutations to occur. The periconception environment encompasses ontogenesis and the organs and tissues in which gametogenesis, embryogenesis, implantation and placentation take place. Although sexual reproduction is globally robust, it is also a vulnerable process. Gametes and embryos are especially vulnerable to epigenetic changes. Most epigenetic marks are systematically

erased in the preimplantation embryo and in the primordial germ cells in order to down-regulate the inheritance of epigenetic (acquired) information between generations, and appear again later on. Likewise, epigenetic processes are responsible for laying down the gender-specific imprinting that allows for gender-specific gene expression, which is of paramount importance for embryonic development and placentation.

**What have we learnt during EPICONCEPT**

Parental stress before, during and after conception (i.e. the periconception period), induces epigenetic changes in gametes and embryos. Such epigenetic changes may adversely affect the future health, development, productivity and fertility of those offspring. While there is increasing evidence for this in agricultural species, most of this knowledge is derived from epidemiological studies in humans and controlled studies in laboratory animals. In EPICONCEPT, time frames and mechanisms during which the gametes and early embryo are susceptible to epigenetic modifications were investigated in livestock in order to optimize their health and productivity. The objectives were to:

1. Develop an epigenomic toolbox for large scale screening of epigenetic changes in gametes and embryos.
2. Define the factors that can influence the epigenetic profile during the periconceptional period of gametes and embryos.
3. Define the time-window during which most epigenetic changes take place
4. Define the range of the optimal periconception environments to ensure healthy offspring.
5. Compare the susceptibility of different species (livestock, poultry, fish) and different model systems (*in vivo vs*. *in vitro*) to epigenetic disturbances.

We achieved these objectives by discussing these topics at our conferences and workshops. Here, we need to acknowledge the generosity of fellow scientists who were not members of Epiconcept, but who travelled from around the world to participate in our meetings, to share their knowledge with us and whose presence meant that we were exposed to cutting edge science and methodologies. As material output we published several review papers on the topic (Brevini *et* al., 2014; Gutierrez-Adan *et al*., 2014; O'Doherty and McGettigan, 2014; Salvaing *et al*., 2014; Anckaert and Fair, 2015), in a Research Front entitled Epigenetics and Periconception environment in Reproduction, Fertility and Development (Editors: Ann Van Soom and Alireza Fazeli), and a book entitled Periconception in Physiology and Medicine (Editors: Alireza Fazeli and William V. Holt), which is in press by Springer.

Public engagement activities were carried out during the COST Action to inform the general public on the importance of the epigenome via the periconception environment in future food production, health and welfare. We communicated via organized Weeks and Nights of Science at our universities, we raised awareness and had an impact on young students even at the level of the secondary school pupils. We used our website (cost-epiconcept.eu) to inform scientists and the public and we produced a facebook page (www.facebook.com/Epiconcept-COST-Action-1381626895453232/?fref=ts ) to interact with scientists, stakeholders, clinicians and practitioners to improve gamete and embryo handling and animal husbandry and breeding. We informed different companies and invited them to our workshops and courses, to learn about the possible impact of gamete and embryo handling on later life. A major success story from our action was to convince stakeholders, such as companies involved in semen freezing and cattle breeding by artificial insemination, to become involved in new research EU-projects on this topic.

**Final conclusions**

With this short review I wanted mainly to point out how important interaction is between scientists. We have not only got to know each other better during Epiconcept, we have also become like friends and family. Some of us are still collaborating, either in an EU Project, or in a project based on national funding or as a member of a new Cost Action, Cell-fit (https://www.facebook.com/COST-Action-16119-CellFit-1660173010901682/ ). I do not have a final message for you, suggesting to do this or that to prevent epigenetic changes to occur, in order to prevent diseased offspring resulting from your research. But I do think that we have raised awareness, that scientists now know that the addition of certain ingredients, such as serum, to the culture medium can have far reaching consequences, and that the introduction of novel techniques should be carefully investigated for subsequent epigenetic effects. But since epigenetics is part of life, and since we are all influenced by our environment; it is also important to realize that we cannot prevent this interaction with the environment. We should live healthy lives and we need to expose our gametes and embryos to a healthy environment, but we should not be terrified or reluctant towards change.

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**References**

**Anckaert E, Fair T**. 2015. DNA methylation reprogramming during oogenesis and interference by reproductive technologies: studies in mouse and bovine models. *Reprod Fertil Dev*, 27:739-754.

**Bahr JM**. 2008. Are scientific societies serving the need of graduate and postdoctoral students? *Reprod Fertil Dev*, 20:19-22.

**Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ**. 1989. Weight in infancy and death from ischaemic heart disease. *Lancet*, 2:577-580.

**Barker DJ**. 1995. Fetal origins of coronary heart disease. *Br Med J*, 311:171-174.

**Brevini T A L, Pennarossa G, Maffei S, Gandolfi F**. 2015. Phenotype switching through epigenetic conversion. *Reprod Fertil Dev*, 27:776-783.

**Burkitt M, Walker D, Romano DM, Fazeli A**. 2011. Computational modelling of maternal interactions with spermatozoa: potentials and prospects. *Reprod Fertil Dev*, 23:976-989.

**Farin CE, Farmer WT, Farin PW**. 2010. Pregnancy recognition and abnormal offspring syndrome in cattle. *Reprod Fertil Dev*, 22:75-87.

**Fleming TP, Watkins AJ, Sun C, Velazquez MA, Smyth NR, Eckert JJ**. 2015. Do little embryos make big decisions? How maternal dietary protein restriction can permanently change an embryo's potential, affecting adult health. *Reprod Fertil Dev*, 27:684-692.

**González-Recio O, Ugarte E, Bach A**. 2012. Trans-generational effect of maternal lactation during pregnancy: a Holstein cow model. *PLoS One*, 7:e51816.

**Grace KS, Sinclair KD**. 2009. Assisted reproductive technology, epigenetics, and long-term health: a developmental time bomb still ticking. *Semin Reprod Med*, 27:409-416.

**Gutierrez-Adan A, White CR, Van Soom A, Mann MRW**. 2014. Why we should not select the faster embryo: lessons from mice and cattle. *Reprod Fertil Dev*, 27:765-775.

**Holt WV, Lloyd RE**. 2010. Sperm storage in the vertebrate female reproductive tract: how does it work so well? *Theriogenology*, 73:713-722.

**Mossa F, Carter F, Walsh SW, Kenny DA, Smith GW, Ireland JL, Hildebrandt TB, Lonergan P, Ireland JJ, Evans AC**. 2013. Maternal undernutrition in cows impairs ovarian and cardiovascular systems in their offspring. *Biol Reprod*, 88:92. doi: 10.1095/biolreprod.

**O'Doherty AM, McGettigan P**. 2014. Epigenetic processes in the male germline. *Reprod Fertil Dev*, 27:725-738.

**Opsomer G, Van Eetvelde M, Kamal M, Van Soom A**. 2016. Epidemiological evidence for metabolic programming in dairy cattle. *Reprod Fertil Dev*, 29:52-57.

**Owen CM, Segars JH Jr**. 2009. Imprinting disorders and assisted reproductive technology. *Semin Reprod Med*, 27:417-428.

**Rosenfeld CS**. 2010. Animal models to study environmental epigenetics. *Biol Reprod*, 82:473-488.

**Salvaing J., Li Y., Beaujean N., O'Neill C**. 2014. Determinants of valid measurements of global changes in 5′-methylcytosine and 5-hydroxymethylcytosine by immunolocalisation in the early embryo. *Reprod Fertil Dev*, 27:755-764.

**Sinclair KD, Singh R**. 2007. Modelling the developmental origins of health and disease in the early embryo. *Theriogenology*, 67:43-53.

**Van Soom A, Mijten P, Van Vlaenderen I, Van den Branden J, Mahmoudzadeh AR, de Kruif A**. 1994. Birth of double-muscled Belgian Blue calves after transfer of in vitro produced embryos into dairy cattle. *Theriogenology*, 41:855-867.

**Van Soom A, Vandaele L, Peelman LJ, Goossens K, Fazeli A**. 2010. Modeling the interaction of gametes and embryos with the maternal genital tract: from in vivo to in silico. *Theriogenology*, 73:828-837.

**Van Soom A, Vandaele L, Goossens K, Heras S, Wydooghe E, Kamal MM, Van Eetvelde M, Opsomer G, Peelman L**. 2013. Epigenetics and the periconception environment in ruminants. *Proc Belg Royal Acad* Med, 2:1-23.

**Van Soom A, Peelman L, Holt WV, Fazeli A**. 2014. An introduction to epigenetics as the link between genotype and environment: a personal view. *Reprod Domest Anim*, 49(suppl. 3):2-10.

**Velazquez MA**. 2015. Impact of maternal malnutrition during the periconceptional period on mammalian preimplantation embryo development. *Domest Anim Endocrinol*, 51:27-45.

**Walker S.K, Hartwich KM, Seamark R**. 1996. The production of unusually large offspring following embryo manipulation: concepts and challenges. *Theriogenology*, 45:111-120.

**Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G, McDermand D**. 1991. The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology* ,35:161-170.

**Young LE, Sinclair KD, Wilmut I**. 1998. Large offspring syndrome in cattle and sheep. *Rev Reprod*, 3:155-163.

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# Number of oocytes retrieved per donor during OPU and its relationship with *in vitro* embryo production and field fertility following embryo transfer

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## Abstract

The association of OPU-IVEP is an important instrument to drive genetic progress. *In vitro* embryo production (IVEP) has remarkably expanded in the last decade compared to *in vivo* embryo production. Because of the high repeatability of oocyte retrieval within oocyte-donors, studies exploring the relationship between the number of oocytes recovered per OPU section with IVEP efficiency, as well as with field fertility (pregnancy results following embryo transfer; P/ET) are extremely important to guide cow-donor selection and optimize field reproduction efficiency and the herd's genetic gain. Based on this rationale, our group conducted a retrospective analysis of a large database comprising IVEP records from several cattle breeds, including *Bos indicus* and *Bos taurus* for either beef or dairy purposes. A total of 205,140 oocytes recovered from 7,906 OPU procedures of 6,902 donors (5,227 beef and 1,675 dairy) of Brazilian farms were analyzed. Beef breeds analyzed were Nelore (*Bos indicus*) and Senepol (*Bos taurus*) and dairy breeds were Gyr (*Bos indicus*) and Holstein (*Bos taurus*). According to our analysis, the IVEP in beef cattle had a great improvement throughout the last years, with a remarkable increase in numbers of pregnancies per OPU compared to late 90's (averaging only 1 pregnancy per OPU in 1998 vs 2,4 in 2014). As for the distribution of oocytes retrieved, both *Bos indicus* beef (Nelore = 27.2) and dairy (Gyr = 23.8) breeds seem to yield greater average numbers of oocytes per OPU compared to *Bos taurus* (Senepol = 21.8; Holstein = 19.3). Despite these differences across genetic groups, outstanding donors can be found in all breeds and the number of oocytes retrieved per donor seems consistent across time. For both beef cattle breeds studied, it appears that number of oocytes retrieved at OPU had a negative but minor effect on both cleavage and blastocyst rates, especially for Senepol breed. Conversely, in dairy breeds the number of oocytes recovered per OPU had essentially no effect on cleavage rates, but we captured a trend for lower blastocyst rates with greater numbers of oocytes per OPU. For both, beef and dairy breeds the number of blastocyst per OPU was greater when higher number of oocytes were recovered per OPU, regardless of genetic group. Pregnancy rate following ET in Nelore breeds was lower in donors with greater amounts of oocytes retrieved per OPU. In contrast, in the Senepol breed and both dairy breeds (Gyr and Holstein) pregnancy rates after ET seems to increase when the number oocytes recovered per OPU increases. In addition, the semen utilized had a major impact of IVEP efficiency: top ranking sires yielded outstanding blastocyst rates, while poor performers produced very low blastocyst rates. The season of the year also had effect on IVEP, with *Bos indicus* breeds showing less variation in IVEP results throughout the year. In conclusion, despite the evolution of IVEP in the last two decades, the number of oocytes recovered per OPU had a minor effect both on blastocyst rate and pregnancy rates after ET. However as more oocytes are collected, the number of produced blastocysts improves. Thus, it seems important to identify donors with greater oocyte recovery-per-OPU potential, especially in cattle breeds yielding fewer oocytes per OPU, such as Holstein, to assure greater IVEP efficiency. It is also clear that cattle breed, semen used during IVEP and season of the year can potentially influence IVEP and field fertility results. A holistic approach controlling the quality of the performed OPU, consistency in lab routines, as well as selecting donors with high genetic value (through genomics) and greater oocyte population (through AMH assays or ultrasound) are highly advisable.

**Keywords**: antral follicle population, pregnancy rate, cattle, bovine.

## Introduction

The association of reproductive efficiency and genetic selection is strategic for the success of dairy and beef industries. Reproductive technologies, such as ovum-pick-up (OPU) and *in vitro* embryo production (IVEP) can rapidly enhance genetics of cattle through both the female and male linage. However, the outcomes of these techniques are highly impacted by individual physiological characteristics of the animal, such as the high variability in ovarian antral follicle population (AFP; Ireland *et al*., 2011; Pontes *et al*., 2011). For example, a number of recent studies have

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shown the strong correlation between blood circulating concentrations of anti-Müllerian hormone (AMH), AFP and IVEP in cattle of different genetic groups (Baldrighi *et al*., 2014; Batista *et al*., 2014; Guerreiro *et al*., 2014). In addition, a large variability of AFP among oocyte donors has also been reported. Despite of that, the high repeatability of oocyte retrieval within oocyte-donors allows identification of donors with enhanced potential for oocyte production (Boni *et al*., 1997; Burns *et al*., 2005; Ireland *et al*., 2007, 2008; Baruselli *et al*., 2015; Monteiro *et al*., 2017). Thus, studies further exploring the relationship between the number of oocytes recovered per OPU section (highly correlated with AFP) and with IVEP efficiency, as well as with field fertility (pregnancy results following embryo transfer; P/ET), are extremely important to guide decision making in terms of donors selection to ultimately fasten evolution on embryo production technology.

Based on this rationale, our group has conducted a retrospective analysis of a large database from a Brazilian commercial IVEP enterprise in order to elucidate the effects of the number of oocytes recovered per OPU on IVEP efficiency and pregnancy results following ET. The database encompassed several cattle breeds, including *Bos indicus* and *Bos taurus* of either beef or dairy purposes. This database comprised a total of 205,140 oocytes recovered from 7,906 OPU procedures of 6,902 donors (5,227 beef and 1,675 dairy) from 2001 to 2017, in which all OPU/IVEP/ET were conducted in Brazil. Briefly, the OPUs were performed by 97 veterinarians and IVEP was done by the same IVEP enterprise. Beef breeds analyzed included Nelore (*Bos indicus*) and Senepol (*Bos taurus*) breeds and for the dairy breeds were utilized data from Gyr (*Bos indicus*) and Holstein (*Bos taurus*) breeds. The beef cattle database included 163,549 oocytes (154,386 from 5612 OPU of 5,048 Nelore donors and 9,163 from 421 OPU of 179 Senepol donors). For dairy breeds it included 41,591 oocytes, in which 28,584 were from 1,200 OPU performed in 956 Gyr cows and 13,007 from 673 OPU done in 719 Holstein cows.

### Statistical analyses and data editing

The total amount of oocytes recovered per OPU was divided in quartiles within each cattle breed. Then, the dataset was edited employing two criteria. The first criterion was to restrict the cleavage to eliminate biologically abnormal results. Thus, the final dataset for cleavage rates that were lower than 10% and greater than 90% were excluded from final analysis. Similarly, the second criterion was to restrict the blastocyst rate, eliminating rates that were lower than 10% and greater than 90%.

Statistical analyses were performed using the GLIMMIX procedure utilizing the Statistical Analysis System (SAS, Version 9.4 for Windows; SAS Inst., Cary, NC). The variables evaluated were the total number of COCs recovered, cleavage rate (number of cleaved zygotes per total number of COCs recovered), blastocyst rate (number of blastocysts produced per total number of COCs recovered), pregnancy rate (number of pregnancies per total number of blastocysts transferred). The binomial distribution was assumed for categorical response variables. Continuous data were tested for normality of the residues using the Guided Data Analysis, and transformed when necessary. The fixed effects included in the model were quartile of total amount of oocytes (Q1, Q2, Q3 and Q4), year of OPU (2001 to 2017), season of OPU, technician who performed the OPU and meaningful two-way interactions. The number of COCs cultured (CIV) was considered as a covariate when required. The interaction of semen and donor was included as a random effect. Statistical differences with $P < 0.05$ were considered significant.

Correlations between cleavage rate, blastocyst rate and pregnancy rate with total oocytes recovered were determined using Proc CORR of SAS. Logistic regression curves were created using the coefficients provided by the interactive data analysis from SAS and the formula $y = \exp(\alpha \times x + b)/ [1 + \exp(\alpha \times x + b)]$, where y = success probability of pregnancy, cleavage or blastocyst; exp = exponential; $\alpha$ = slope of the logistic equation; b = intercept of the logistic equation; and x = total oocytes recovered.

The HPMIXED procedure of SAS through the best linear unbiased prediction (BLUP) analysis was utilized to rank sires from all breeds in terms of blastocyst success as well as field fertility following ET.

### OPU/IVEP procedure: a remarkable progress over the last decades

The OPU-IVEP combined is an important tool to drive genetic progress. *In vitro* embryo production has expanded remarkably in the last decade when compared to *in vivo* embryo production; and lately accounts for 40.6% of the total embryo production in the world (1,275,874 embryos; Perry, 2014). The outstanding growth of IVEP seen in the last decade is a consequence of a number of factors, such as the significant improvement of *in vitro* culture procedures and the successful use of sex-sorted semen in the IVEP programs enabling the manipulation of the proportion of male and female embryos produced (Blondin, 2015). The evolution of IVEP through the last decades is clearly shown in Table 1 (data analyzed for all breeds). It is obvious the great improvement IVEP, with the remarkable increase in numbers of pregnancies per OPU, improving nearly 2 times in later years compared to late 90's.

Table 1. Evolution of OPU/IVEP from 1998 to 2014.

| Year | OPU (n) | MIV | % CLIVED | % BLAST | % P/ET | PREG/OPU |
|---|---|---|---|---|---|---|
| 1998 | 56 | 11.2 | 55.2% | 22.5% | 40.4% | 1.0 |
| 1999 | 510 | 8.7 | 71.3% | 25.9% | 39.9% | 0.9 |
| 2000 | 1182 | 13.7 | 68.4% | 30.2% | 39.6% | 1.6 |
| 2001 | 2556 | 14.9 | 87.9% | 30.2% | 39.7% | 1.8 |
| 2002 | 4116 | 20.3 | 72.1% | 24.5% | 39.9% | 2.0 |
| 2003 | 5430 | 20.5 | 72.8% | 26.6% | 39.8% | 2.2 |
| 2004 | 3731 | 21.7 | 69.1% | 25.6% | 42.3% | 2.3 |
| 2005 | 995 | 20.0 | 70.9% | 32.6% | 41.0% | 2.7 |
| 2006 | 968 | 20.4 | 71.2% | 32.9% | 35.5% | 2.4 |
| 2007 | 1025 | 24.6 | 69.1% | 32.6% | 37.2% | 3.0 |
| 2008 | 1171 | 20.3 | 66.7% | 32.2% | 38.6% | 2.5 |
| 2009 | 1580 | 15.4 | 69.4% | 28.8% | 41.5% | 1.8 |
| 2010 | 703 | 20.0 | 70.0% | 27.3% | 41.8% | 2.3 |
| 2011 | 378 | 18.9 | 65.6% | 26.9% | 41.7% | 2.1 |
| 2012 | 320 | 21.1 | 61.8% | 20.7% | 44.4% | 1.9 |
| 2013 | 157 | 20.6 | 66.4% | 27.3% | 42.0% | 2.4 |
| 2014 | 485 | 16.0 | 69.1% | 32.0% | 47.3% | 2.4 |
| Total | 25,363 | 19.2 | 72.2% | 27.8% | 40.0% | 2.1 |

**Variation in oocyte retrieval during OPU procedures**

Several research groups have reported a great variation in oocyte retrieval per OPU across breeds (Pontes *et al*., 2010; Gimenes *et al*., 2015; Sales *et al*., 2015). Data shown in Fig. 1 corroborate with such findings and shows the wide distribution of oocytes retrieved across main beef and dairy cattle breeds receiving OPU/IVEP in Brazil in the last two decades. Altogether, both beef and dairy breeds having *Bos indicus* blood seem to yield greater average numbers of oocytes per OPU (Beef: Nelore = 27.5 ± 0.3 *vs*. Senepol = 21.8 ± 0.7; Dairy: Gyr = 23.8 ± 0.5 *vs*. Holstein = 19.3 ± 0.6). This information is very critical to IVEP routine, since the average number of viable embryos produced during IVEP is highly correlated with initial numbers of oocytes sent to *in vitro* embryo production. As a result, it seems far more important to identify donors with greater oocyte recovery-per-OPU potential in some breeds such as Holstein for example though ultrasound selection or AMH assay. This may determine IVEP success in some cattle breeds yielding fewer oocytes per OPU.

Despite the great differences in total of oocytes retrieved per OPU procedure across breeds, outstanding donors can be found in all breeds and the number of oocytes retrieved per individual donor seems consistent across time. In fact, it was previously shown the occurrence of high within-cow repeatability in oocyte production over time (Boni *et al*., 1997; Ireland *et al*., 2007, 2008; Baruselli *et al*., 2015; Monteiro *et al*., 2017). However, contrasting results were shown across genetic groups. For *Bos taurus*, the number of recovered oocytes seemed fairly constant, even up to 32 consecutive OPU sessions (Petyim *et al*., 2003). Conversely, in *Bos indicus* cattle, decreased numbers of recovered oocytes following consecutive OPU sessions has been reported (Gimenes *et al*., 2015); it appears to be an issue particularly within donors with high numbers of cumulus-oocyte complex (COC) retrieved at the beginning of the program (Monteiro *et al*., 2017). Despite that, donors classified as high COC resulted in increased blastocyst production per OPU. More importantly, the high-repeatability efficiency in terms of COC retrieved (shown in Table 2 below) is a remarkable finding that has major implications to IVEP labs worldwide (Monteiro *et al*., 2017).

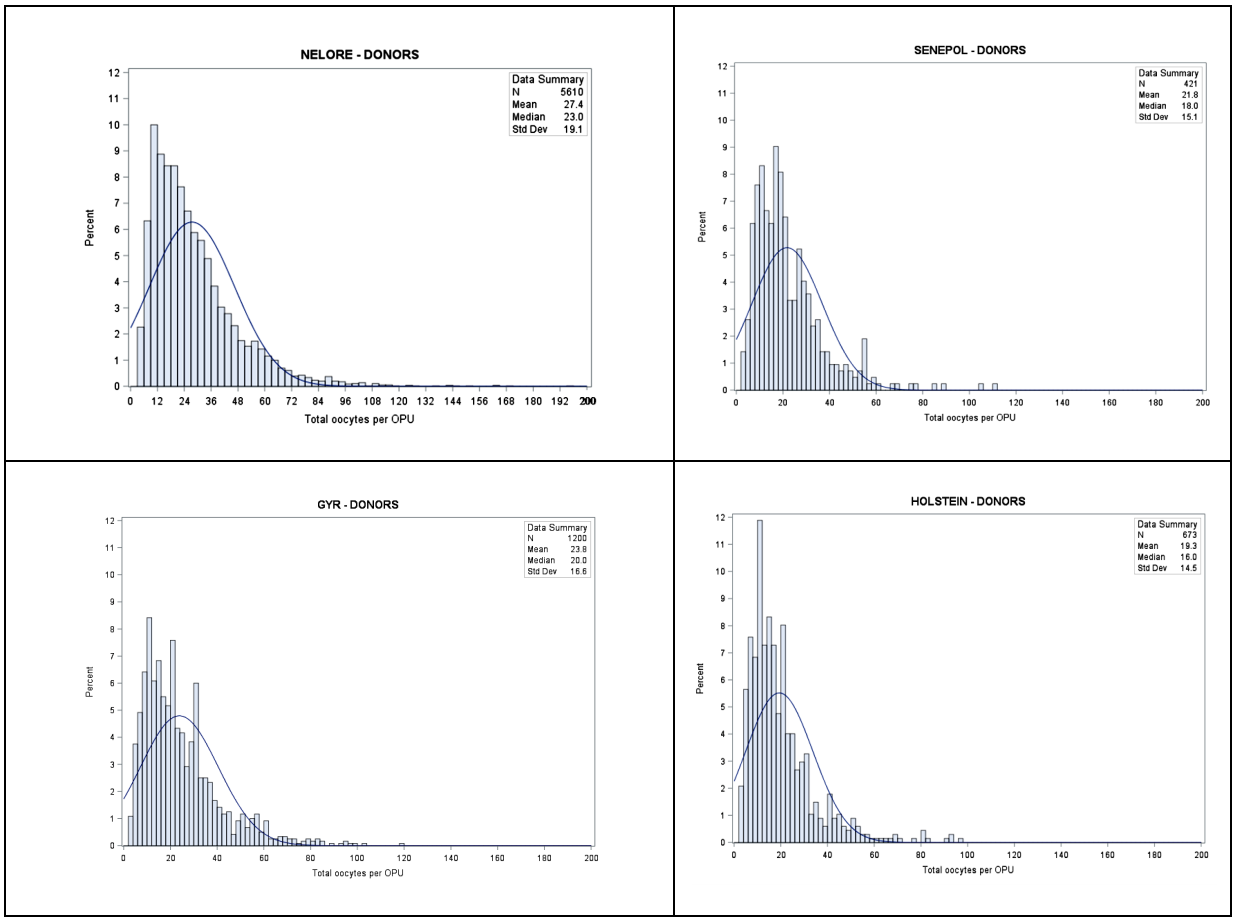


Figure 1. Distribution of oocytes retrieved per OPU in beef (*Bos indicus* = Nelore; and *Bos taurus* = Senepol – upper panels) and in dairy breeds (*Bos indicus* = Gyr; and *Bos taurus* = Holstein – lower panels).

Table 2. Repeatability of OPU/IVEP in *Bos indicus* (Nelore) oocyte donors.

| Variable | Repeatability |
|---|---|
| Recovered COC | 0.81 |
| Cultured COC | 0.81 |
| Blastocyst | 0.79 |
| Cultured COC rate | 0.55 |
| Blastocyst rate | 0.69 |

Adapted from Monteiro *et al*., 2017.

**Number of oocytes retrieved at OPU: impact on *in vitro* embryo production**

In the following sessions, we have described the impact of the total number of oocytes retrieved per OPU on embryo viability during the IVEP, as well as pregnancy results following ET. This is an important topic for field vets, mainly because studies utilizing a limited number of donor-cows yielded mixed findings in the currently available scientific literature. Thus, this topic warrants further investigations utilizing large number of records to more clearly draw final conclusions in this subject, as we describe below.

*Beef breeds – impact of number of oocytes per OPU on embryo viability*

Tables 3 and 4, as well Fig. 2 and 3, are summarizing our findings for beef breeds (*Bos indicus* represented by Nelore breed and *Bos taurus* represented by Senepol breed) in terms of the relationship between number of oocytes recovered during OPU and its relationship to IVEP efficiency. Altogether, it appears that number of oocytes retrieved at OPU had a minor effect on both cleavage and blastocyst rates (Table 3 and 4), except by a minor negative trend for blastocyst rate in the Senepol breed (Table 4 and Fig. 3B). In Nelore, only a tendency (P = 0.08; Table 3) was observed for reduction of the cleavage rate as the number of recovered oocytes increased.

As expected, the number of blastocyst per OPU is greater for beef donors with higher number of oocytes recovered per OPU, regardless of genetic group (Table 3 and 4).

Table 3. Effect of retrieved numbers of oocytes per OPU from Nelore (*Bos indicus*) donors on IVEP.

| Variable | Quartile (Lower) | Intermediate Quartile (Lower) | Intermediate Quartile (Superior) | Quartile (Superior) | P-value |
|---|---|---|---|---|---|
| Quartile, n | 1403 | 1403 | 1403 | 1403 | - |
| Oocytes recovered per OPU | 9.4[d] (13246/1403) | 18.1[c] (25376/1403) | 28.6[b] (40119/1403) | 53.9[a] (75645/1403) | < 0.001 |
| Cleavage rate, % | 68.80% (8089/11756) | 66.70% (14900/22337) | 66.20% (23582/35634) | 65.10% (42714/66860) | 0.08 |
| Blastocyst rate, % | 45% (3634/8089) | 45.70% (6841/14900) | 46.10% (10948/23582) | 44.40% (19001/42714) | 0.23 |
| Blastocyst per OPU | 2.6[d] (3604/11756) | 4.9[c] (6841/22337) | 7.8[b] (10948/35634) | 13.5[a] (19001/66860) | < 0.001 |

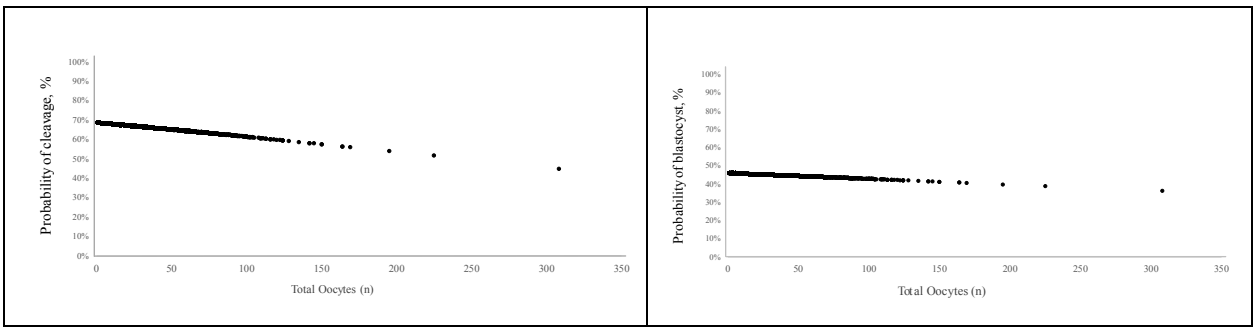


Figure 2. Probability of cleavage rate (A) and blastocyst rate (B) as a function of numbers of retrieved oocytes per OPU in Nelore (*Bos indicus*) donors (from 154,386 fertilized oocytes). [Logit(PROB_CLEAVAGE) = 0.7833-0.0032*Total oocytes; P < 0.001]; [Logit (PROB_BLASTOCYST= -0.1539-0.0013*Total oocytes; P = 0.01].

Table 4. Effect of retrieved numbers of oocytes per OPU from Senepol (*Bos taurus*) donors on IVEP.

| Variable | Quartile (Lower) | Intermediate Quartile (Lower) | Intermediate Quartile (Superior) | Quartile (Superior) | P-value |
|---|---|---|---|---|---|
| Quartile, n | 105 | 105 | 105 | 106 | - |
| Oocytes recovered per OPU | 7.9[d] (827/105) | 14.8[c] (1552/105) | 22.3[b] (2339/105) | 41.9[a] (4445/106) | <.0001 |
| Cleavage rate, % | 58.2%[b] (481/827) | 61.5%[ab] (955/1552) | 66.6%[a] (1558/2339) | 61%[ab] (2710/4445) | 0.001 |
| Blastocyst rate, % | 33%[a] (273/827) | 27.2%[b] (422/1552) | 28.1%[b] (658/2339) | 26.6%[c] (1181/4445) | <.0001 |
| Blastocyst per OPU | 2.6[d] (273/105) | 4.1[c] (422/105) | 6.3[b] (658/105) | 11.2[a] (1181/106) | <.0001 |

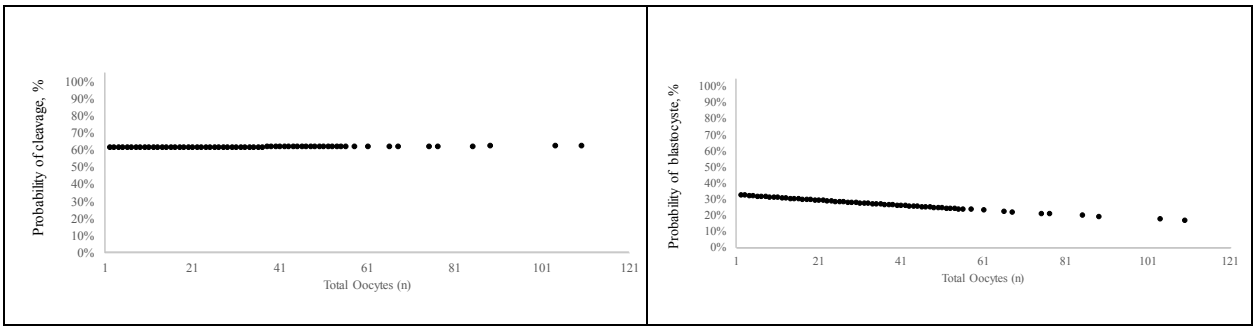


Figure 3. Probability of cleavage rate (A) and blastocyst rate (B) as a function of the number of retrieved oocyte per OPU in Senepol (*Bos taurus*) donors (from 9,163 fertilized oocytes). [Logit(PROB_CLEAVAGE) = 0.486+0.0004*Total oocytes; P = 0.8598]; [Logit(PROB_BLASTOCYST)= -0.7093-0.008*Total oocytes; P = 0.0011].

*Dairy breeds – impact of number of oocytes per OPU on embryo viability*

In dairy breeds, we considered that the number of oocytes recovered per OPU had essentially no effect on cleavage rates, and that differences observed might possibly be explained by lab artifacts such as greater number of oocytes to be handled at the time. However, we did capture lower blastocyst rates with increasing numbers of oocytes per OPU for both genetic groups (Fig. 4 and 5), although still minor and with debatable biological importance to field veterinarians.

As similarly found in beef breeds, the number of blastocyst per OPU is greater for dairy donors with higher number of oocytes recovered per OPU, regardless of genetic group (Table 4 and 5).

Table 5. Effect of retrieved numbers of oocytes per OPU from Gyr (*Bos indicus*) donors on IVEP.

| Variable | Quartile (Lower) | Intermediate Quartile (Lower) | Intermediate Quartile (Superior) | Quartile (Superior) | P-value |
|---|---|---|---|---|---|
| Quartile, n | 300 | 300 | 300 | 300 | - |
| Oocytes retrieved per OPU | 8.0$^{d}$ (2399/300) | 15.7$^{c}$ (4696/299) | 24.9$^{b}$ (7487/301) | 46.7$^{a}$ (14002/300) | <.0001 |
| Cleavage rate, % | 57.4%$^{b}$ (1376/2399) | 57.3%$^{b}$ (2704/4717) | 59.2%$^{a}$ (4420/6033) | 58.1%$^{b}$ (8136/14002) | <.0001 |
| Blastocyst rate, % | 30.56%$^{a}$ (734/2399) | 29.5%$^{b}$ (1392/4717) | 28.5%$^{c}$ (2127/7466) | 28.2%$^{d}$ (3951/14002) | <.0001 |
| Blastocyst per OPU | 2.5$^{d}$ (734/300) | 4.6$^{c}$ (1392/300) | 7.1$^{b}$ (2127/300) | 13.2$^{a}$ (3951/300) | <.0001 |

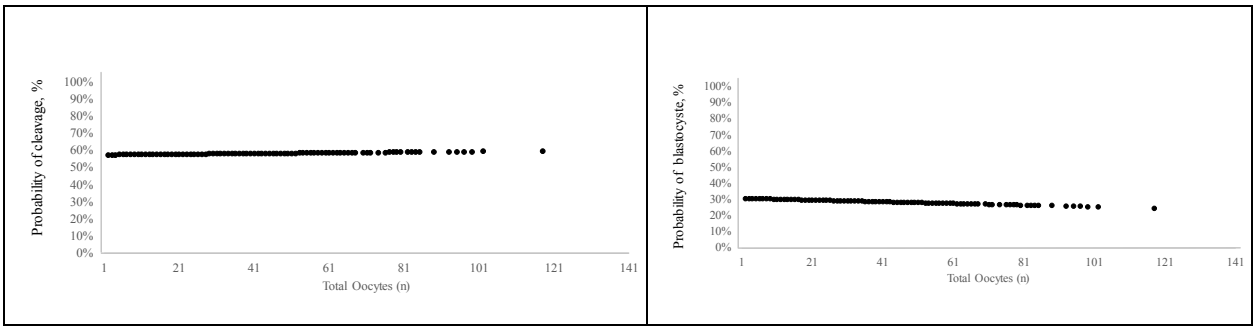


Figure 4. Probability of cleavage rate (A) and blastocyst rate (B) as a function of the number of retrieved oocytes per OPU in Gyr (*Bos indicus*) donors (from 28,584 fertilized oocytes). [Logit(PROB_CLEAVAGE) = 0.304+0.0008*Total oocytes; P = 0.5203]; [Logit(PROB_BLASTOCYSTE) = -0.8177-0.0026 *Total oocytes; P = 0.0305].

Table 6. Effect of retrieved numbers of oocytes per OPU from Holstein (*Bos taurus*) donors on IVEP.

| Variable | Quartile (Lower) | Intermediate Quartile (Lower) | Intermediate Quartile (Superior) | Quartile (Superior) | P-value |
|---|---|---|---|---|---|
| Quartile, n | 168 | 168 | 168 | 169 | - |
| Oocytes recovered per OPU | 6.7$^{d}$ (1120/168) | 12.5$^{c}$ (2098/168) | 19.4$^{b}$ (3256/168) | 38.7$^{a}$ (6533/169) | <.0001 |
| Cleavage rate, % | 50.9%$^{b}$ (570/1120) | 53.9%$^{ab}$ (1130/2098) | 57.8%$^{a}$ (1883/3256) | 52.7%$^{ab}$ (3441/6533) | <.0001 |
| Blastocyst rate, % | 26.1%$^{a}$ (292/1120) | 26.2%$^{a}$ (550/2098) | 24.8%$^{b}$ (806/3256) | 20.3%$^{c}$ (1324/6533) | <.0001 |
| Blastocyst per OPU | 1.7$^{d}$ (292/168) | 3.3$^{c}$ (550/168) | 4.8$^{b}$ (806/168) | 7.8$^{a}$ (1324/168) | <.0001 |

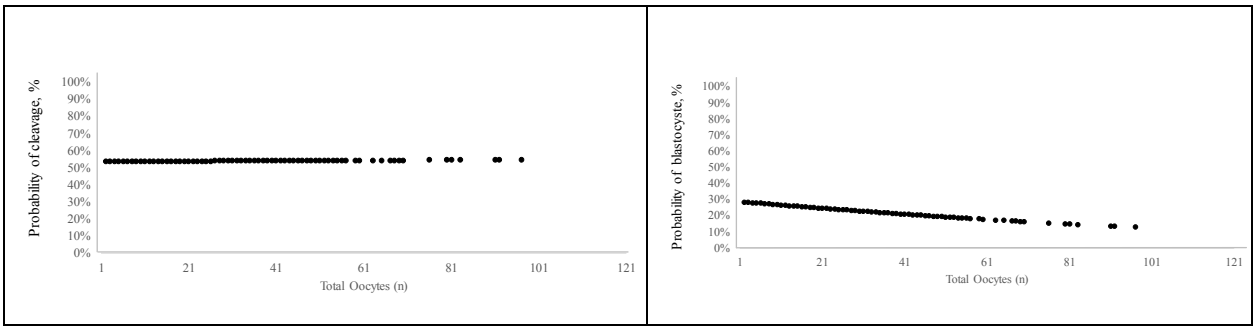


Figure 5. Probability of cleavage rate (A) and blastocyst rate (B) as a function of the number of retrieved oocyte of Hostein (*Bos taurus*) donor (from 13,007 fertilized oocytes). [Logit(PROB_CLEAVAGE) = 0.147+0.0004*Total oocytes; P = 0.7946]; [Logit(PROB_BLASTOCYSTE) = -0.9127-0.0106*Total oocytes; P < 0.0001].

**Number of oocytes retrieved at OPU: impact on field conception results**

*Beef breeds – impact of number of oocytes per OPU on pregnancy results following ET.*

Pregnancy results following ET in Nelore breeds was lower in donors with greater amounts of oocytes retrieved per OPU (lower quartile versus the others; Table 8 and Fig. 6). These differences were captured due to the large amount of recorded OPUs in the Nelore breed, and its biological significance is debatable, since it might be related to constrains in trying to transfer larger numbers of embryos on the same day. For example, in terms of utilizing recipients in the field, it is likely that a greater percentage of recipients are used when greater amounts of oocytes need to be transferred. Field vets tend to utilize recipients more aggressively in these cases even perhaps with less than ideal CL quality or synchronization with embryo age and days post-ovulation been less than ideal in some recipients. Still, these constrains are common in the field and the data reported herein should represent more realistically expected results in the field.

In contrast, in the Senepol breed, pregnancy increased in a positive fashion in relation to oocytes recovered per OPU (Table 7 and Fig. 6). This could possibly be related to the smaller sample size in the lower quartile (n = 215 transfers), and we do not have a clear explanation for these results otherwise.

Table **7**. Effect of retrieved numbers of oocytes per OPU from Nelore and Senepol donors on pregnancy results following embryo transfer.

| Variable | Quartile (Lower) | Intermediate Quartile (Lower) | Intermediate Quartile (Superior) | Quartile (Superior) | P-value |
|---|---|---|---|---|---|
| Nelore - Pregnancy rate, % | 44.2%[a] (187/424) | 40.3%[b] (302/750) | 39.9%[b] (414/899) | 38.6%[b] (387/1003) | 0.0010 |
| Senepol - Pregnancy rate, % | 10.0%[b] (21/215) | 17.6%[a] (59/337) | 19.8%[a] (101/508) | 19.5%[a] (164/841) | 0.0015 |

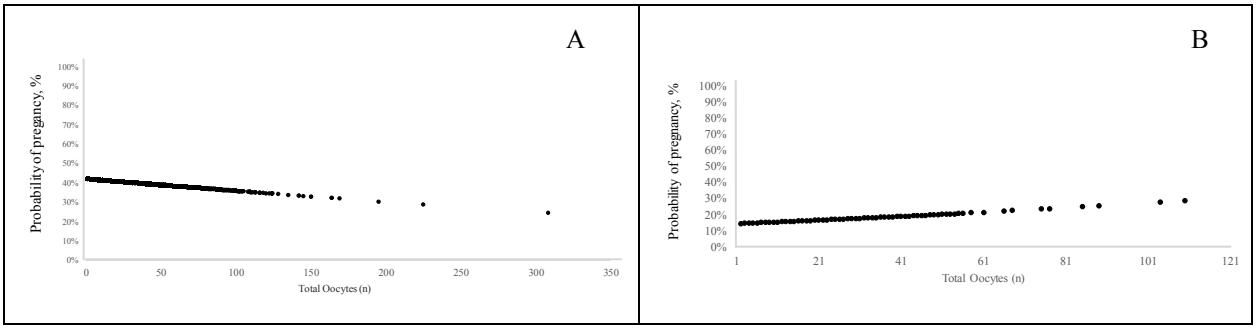


Figure 6. Probability of recipient pregnancy rate as a function of the number of retrieved oocyte per OPU in Nelore (*Bos indicus*; n = 3,076; A) [Logit(PROB_PREGNANCY) = -0.3163-0.0026*Total Oocytes; P < 0.0001] and (B) Senepol (*Bos taurus;* n = 1,901) donors [Logit(PROB_PREGNANCY) = -1.7818+0.008*Total oocytes; P = 0.0793].

*Dairy breeds - impact of number of oocytes per OPU on pregnancy results following ET.*

For dairy breeds, the overall pregnancy results after ET followed a slight positive trend as the number of oocytes recovered per OPU increased (Table 8). The only difference observed was for Holstein donors, when an increase on pregnancy after ET was observed only when comparing the lower quartile to upper quartiles, similarly as for Senepol breed (Table 7 and 8).

Additionally, the analysis of probability indicated improved pregnancy results after ET when the number of oocytes recovered per OPU increased in Gyr donors and a small trend for the same behavior for Holstein donors (Fig. 7).

Table 8. Effect of retrieved numbers of oocytes per OPU from Gyr (*Bos indicus*) and Holstein (*Bos taurus*) donors on pregnancy results following embryo transfer.

| Variable | Quartile (Lower) | Intermediate Quartile (Lower) | Intermediate Quartile (Superior) | Quartile (Superior) | P-value |
|---|---|---|---|---|---|
| Gyr - Pregnancy rate, % | 25.5% (139/543) | 26.6% (267/1001) | 27.1% (420/1549) | 29.9% (893/2982) | 0.29 |
| Holstein - Pregnancy rate, % | 16.9%$^{b}$ (32/188) | 27.2%$^{a}$ (113/418) | 25.9%$^{ab}$ (171/660) | 25.9%$^{ab}$ (252/971) | 0.0002 |

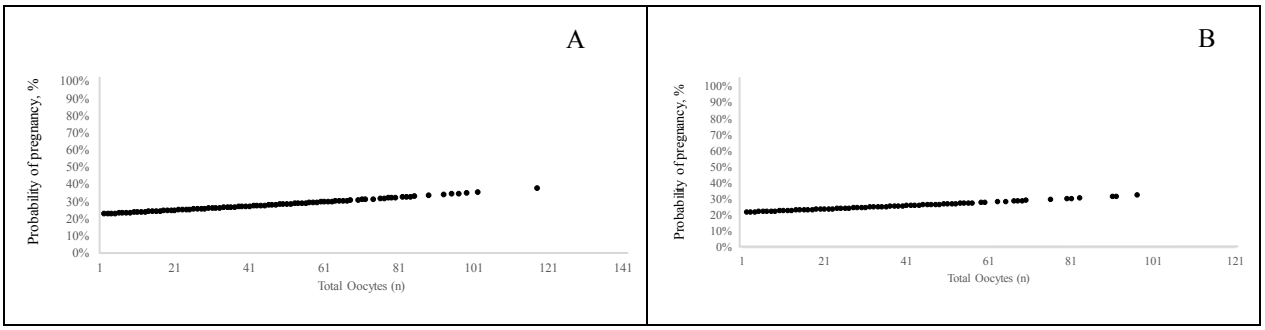


Figure 7. Probability of recipient pregnancy rate as a function of the number of retrieved oocyte per OPU in Gyr (*Bos indicus*; n = 6,075; A) [Logit(PROB_PREGNANCY) = -1.2181+0.0061*Total oocytes; P = 0.0133] and (B) Holstein (*Bos taurus*; n = 2,237) donors [Logit(PROB_PREGNANCY) = -1.2637+0.0056*Total oocytes; P = 0.1140].

**Other nuisance factors altering IVEP efficiency**

*Effect of IVF-sire used during IVEP*

Despite of cattle breed, as expected, the semen utilized also had a major impact of IVEP efficiency. As shown in Fig. 8, top ranking sires used in Nelore (*Bos indicus*) donors yielded outstanding blastocyst rates consistently averaging over 30% (18 sires out of 48). In contrast, poor performers (5 out of 48 sires) produced very low blastocyst rates that were lower than 20%. These results highlight the importance of pruning out sires with inadequate results in blastocyst formation during IVEP procedures.

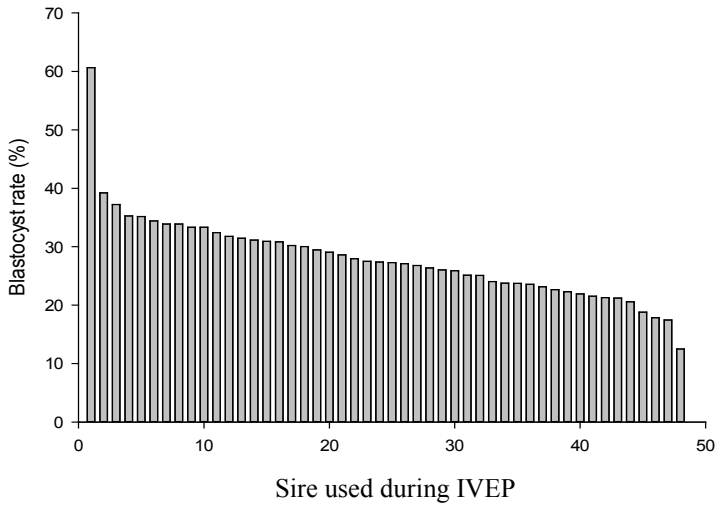


Figure 8. Blastocyst rate (%) for 48 Nelore sires used in at least 50 OPU sessions during IVEP from Nelore (*Bos indicus*) donors.

*Effect of season on IVEP efficiency*

In general, season also had effect on IVEP, with *Bos indicus* breeds showing less variation in IVEP results throughout the year. In other hand, *Bos taurus* breeds had greater variation in blastocyst rates throughout the differing seasons, which might be accounted by heat stress and possible interactions with feed/pasture quality (Fig. 9). The great susceptibility of *Bos taurus* oocytes to heat stress was previously demonstrated by several groups including ours (Al-Katanani *et al*., 2002; Ferreira *et al*., 2011, 2013, 2016; Paula-Lopes *et al*., 2003; Roth, 2008; Roth *et al*., 2000). Although less intense, the heat stress was also shown to negatively affects oocyte competence in *Bos indicus* cattle (Paula-Lopes *et al*., 2003; Hansen, 2004; Torres-Júnior *et al*., 2008). Thus, variation in temperature-humidity index in which cattle are exposed to, as well as feed quality to avoid, for example, issues with subclinical acidosis or postpartum ketosis or even high blood-urea levels need to be considered during IVEP.

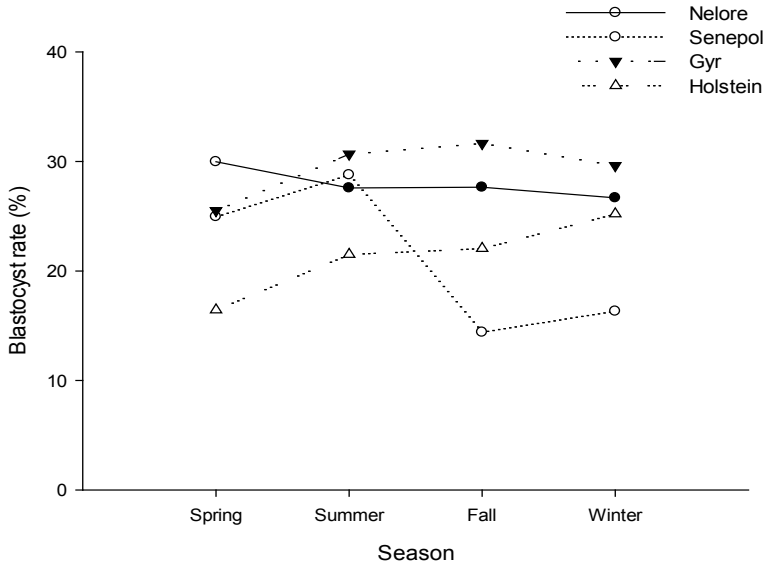


Figure 9. Blastocyst rate (%) throughout the year for beef donors [Nelore (*Bos indicus*), Senepol (*Bos taurus*)], and dairy donors [Gyr (*Bos indicus*) and Holstein (*Bos taurus*)].

## Closing remarks & current barriers to OPU/IVF in cattle

In conclusion, IVEP has improved a lot in the last two decades, with greater progress attained particularly in beef breeds. Despite of that, the number of oocytes recovered per OPU had a minor effect both on embryo viability (measured by blastocyst rate success) and on pregnancy rates following ET. It is clear though that other factors such as cattle breed, semen used during IVEP, season, as well as other cow-nuisance variables not considered in the current study such as body condition score or level of negative energy balance experienced by the donor will undeniably alter IVEP and field fertility results. A holistic approach controlling OPU and lab variation, as well as selecting donors with greater genetic value (through genomics) and oocyte population (through AMH assays or ultrasound) are highly advisable.

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## References

**Al-Katanani YM, Paula-Lopes FF, Hansen PJ**. 2002. Effect of season and exposure to heat stress on oocyte competence in Holstein cows. *J Dairy Sci*, 85:390-396.

**Baldrighi JM, Sá Filho MF, Batista EOS, Lopes RNVR, Visintin JA, Baruselli PS, Assumpção MEOA**. 2014. Anti-Mullerian hormone concentration and antral ovarian follicle population in murrah heifers compared to Holstein and Gyr kept under the same management. *Reprod Domest Anim*, 49:1015-1020.

**Baruselli PS, Batista EOS, Vieira LM, Souza AH**. 2015. Relationship between follicle population, AMH concentration and fertility in cattle. *Anim Reprod*, 12:487-497.

**Batista EOS, Macedo GG, Sala RV, Ortolan MDDV, Sá Filho MF, Del Valle TA, Jesus EF, Lopes RNVR, Rennó FP, Baruselli PS**. 2014. Plasma antimullerian hormone as a predictor of ovarian antral follicular population in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. *Reprod Domest Anim*, 49:448-452.

**Blondin P**. 2015. Status of embryo production in the world. *Anim Reprod*, 12:356-358.

**Boni R, Roelofsen M, Pieterse M, Kogut J, Kruip T**. 1997. Follicular dynamics, repeatability and predictability of follicular recruitment in cows undergoing repeated follicular puncture. *Theriogenology*, 48:277-289.

**Burns DS, Jimenez-Krassel F, Ireland JLH, Knight PG, Ireland JJ**. 2005. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. *Biol*

*Reprod*, 73:54-62.

**Ferreira RM, Ayres H, Chiaratti MR, Ferraz ML, Araújo AB, Rodrigues CA, Watanabe YF, VirequeAA, Joaquim DC, Smith LC, Meirelles FV, Baruselli PS**. 2011. The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. *J Dairy Sci*, 94:2383-2392.

**Ferreira RM, Lima FA, Veras MB, Torres FP, Guida TG, Viechnieski S, Ayres H, Chiaratti MR, Baruselli PS**. 2013. Effect of heat stress and repeat breeding on P/AI of high-producing Holstein cows. *Anim Reprod*, 10:484. (abstract).

**Ferreira RM, Chiaratti MR, Macabelli CH, Rodrigues CA, Ferraz ML, Watanabe YF, Smith LC, Meirelles FV, Baruselli PS**. 2016. The infertility of repeat-breeder cows during summer is associated with decreased mitochondrial DNA and increased expression of mitochondrial and apoptotic genes in oocytes. *Biol Reprod*, 94:66. doi:10.1095/biolreprod. 115.133017.

**Gimenes LU, Ferraz ML, Fantinato-Neto P, Chiaratti MR, Mesquita LG, Sá Filho MF, Meirelles FV, Trinca LA, Rennó FP, Watanabe YF, Baruselli PS**. 2015. The interval between the emergence of pharmacologically synchronized ovarian follicular waves and ovum pickup does not significantly affect in vitro embryo production in *Bos indicus*, *Bos taurus*, and *Bubalus bubalis*. *Theriogenology*, 83:385-393.

**Guerreiro BM, Batista EOS, Vieira LM, Sá Filho MF, Rodrigues CA, Netto AC, Silveira CRA, Bayeux BM, Dias EAR, Monteiro FM, Accorsi M, Lopes RNVR, Baruselli PS**. 2014. Plasma anti-Mullerian hormone: an endocrine marker for in vitro embryo production from *Bos taurus* and *Bos indicus* donors. *Domest Anim Endocrinol*, 49:96-104.

**Hansen PJ**. 2004. Physiological and cellular adaptations of zebu cattle to thermal stress. *Anim Reprod Sci*, 82/83:349-360.

**Ireland JJ, Ward F, Jimenez-Krassel F, Ireland JLH, Smith GW, Lonergan P, Evans ACO**. 2007. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum Reprod*, 22:1687-1695.

**Ireland JJ, Smith GW, Scheetz D, Jimenez-Krassel F, Folger JK, Ireland JLH, Mossa F, Lonergan P, Evans ACO**. 2011. Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Müllerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in. *Reprod Fertil Dev*, 23:1-14.

**Ireland JLH, Scheetz D, Jimenez-Krassel F, Themmen APN, Ward F, Lonergan P, Smith GW, Perez GI, Evans ACO, Ireland JJ**. 2008. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biol Reprod*, 79:1219-1225.

**Monteiro FM, Batista EOS, Vieira LM, Bayeux BM, Accorsi M, Campanholi SP, Dias EAR, Souza AH, Baruselli PS**. 2017. Beef donor cows with high number of retrieved COC produce more in vitro embryos compared with cows with low number of COC after repeated ovum pick-up sessions. *Theriogenology*, 90:54-58.

**Paula-Lopes FF, Chase Jr CC, Al-Katanani YM III, CEK, Rivera RM, Tekin S, Majewski AC, Ocon OM, Olson TA, Hansen PJ**. 2003. Genetic divergence in cellular resistance to heat shock in cattle : differences between breeds developed in temperate versus hot climates in responses of preimplantation embryos , reproductive tract tissues and lymphocytes to increased culture temperature. *Reproduction*, 125:285-294.

**Perry G**. 2014. 2013 Statistics of embryo collection and transfer in domestic farm animals. *Embryo Transfer Newslett*, 32(4):14-26.

**Petyim S , Bå ge R , Hallap T, Bergqvist AS, Rodriguez-Martinez H, Larsson B**. 2003. Two different schemes of twice-weekly ovum pick-up in dairy heifers: effect on oocyte recovery and ovarian function. *Theriogenology*, 60:175-188.

**Pontes JHF, Silva KCF, Basso AC, Rigo AG, Ferreira CR, Santos GMG, Sanches BV, Porcionato JPF, Vieira PHS, Faifer FS, Sterza FAM, Schenk JL, Seneda MM**. 2010. Large-scale in vitro embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and indicus-taurus dairy cows using sexed sperm. *Theriogenology*, 74:1349-1355.

**Pontes JHF, Melo Sterza FA, Basso AC, Ferreira CR, Sanches BV, Rubin KCP, Seneda MM**. 2011. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (*Bos indicus*) donors. *Theriogenology*, 75:1640-1646.

**Roth Z, Meidan R, Braw-Tal R, Wolfenson D**. 2000. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. *J Reprod Fertil*, 120:83-90.

**Roth Z**. 2008. Heat stress, the follicle, and its enclosed oocyte: mechanisms and potential strategies to improve fertility in dairy cows. *Reprod Domest Anim*, 43(suppl. 2):238-244.

**Sales JNS, Iguma LT, Batista RITP, Quintão CCR, Gama MAS, Freitas C, Pereira MM, Camargo LSA, Viana JHM, Souza JC, Baruselli PS**. 2015. Effects of a high-energy diet on oocyte quality and in vitro embryo production in *Bos indicus* and *Bos taurus* cows. *J Dairy Sci*, 98:3086-3099.

**Torres-Júnior JRS, Pires MFA, Sá WF, Ferreira AM, Viana JHM, Camargo LSA, Ramos AA, Folhadella IM, Polisseni J, Freitas C, Clemente CAA, Sá Filho MF, Paula-Lopes FF, Baruselli PS**. 2008. Effect of maternal heat-stress on follicular growth and oocyte competence in *Bos indicus* cattle. *Theriogenology*, 69:155-166.

A001 TAI/FTET/AI

# Pregnancy rates in beef heifers synchronized with the J-Synch protocol and inseminated with conventional or sexed semen

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Two experiments were performed to evaluate pregnancy rates in beef heifers synchronized with the J-Synch protocol and Fixed-time AI (FTAI) with sexed or non-sexed semen. In both experiments cycling Angus or Angus cross-bred heifers weighting 270 to 320 kg and with a body condition score of 2.5 to 3.5 (scale of 1 to 5) were randomly allocated to be inseminated with Ultra-Sexed 4M semen (4 million sperm per dose, Sexing Technologies, Argentina) or non-sexed (conventional) semen from two bulls (one bull in each experiment). All heifers received a progesterone (P4) device (DIB 0.5 g P4, Zoetis, Argentina) and 2 mg estradiol benzoate (Gonadiol, Zoetis) on Day 0. On Day 6 heifers in Experiment 1 received 500 µg of cloprostenol (PGF, Ciclase DL, Zoetis) and DIB removal and heifers in Experiment 2 also received 300 IU eCG (Novormon 5000, Zoetis) at the same time. All heifers were also tail-painted at the time of DIB removal and observed for signs of estrus (i.e. >30% of the tail-paint rubbed off). In Experiment 1, heifers with the tail-paint rubbed off by 72 h after DIB removal were FTAI at that time with either sexed or non-sexed semen. Those not showing estrus by 72 h received 100 µg of gonadorelin acetate (GnRH, Gonasyn gdr, Zoetis) at that time and were also FTAI with either sexed or non-sexed semen 12 h later (i.e. 84 h). In Experiment 2, FTAI was performed as is Experiment 1, except that estrus observation was recorded also at 60 h after DIB removal. Pregnancy was diagnosed by ultrasonography 30 days after FTAI. Data was analyzed by logistic regression. In Experiment 1, there were 57.9% (206/356) of heifers in estrus at 72 h and an overall pregnancy rate of 45.5% (82/180) for sexed semen and 62.5% (110/176) for conventional semen ($P < 0.01$). Furthermore, pregnancy rates were higher in heifers that showed estrus and were FTAI at 72 h (62.1%, 128/206) than those that didn't showed estrus and were FTAI at 84 h (42.7%, 64/150; $P<0.01$). In Experiment 2, there were 75.8% (185/244) of heifers in estrus but there were no differences in pregnancy rates between sexed semen (59.3%, 73/123) and conventional semen (67.7%, 82/121; $P=0.14$). Pregnancy rates in heifers FTAI with sexed semen tended to be ($P<0.09$) higher for those in heat at 60 h and FTAI at 72 h (71.4%, 30/42) than those in heat at 72 h and FTAI at the same time (53.8%, 28/52) and those not in heat and FTAI at 84 h (51.7%, 15/29). In heifers FTAI with conventional semen pregnancy rates were higher ($P<0.05$) in those in heat at 60 h and FTAI at 72 h (76.3%, 29/38) and those in heat and FTAI at 72 h (69.8%, 37/53) than in those not in heat and FTAI at 84 h (53.3%, 16/30). In conclusion, the combination of tail painting for estrus detection and FTAI can be successfully applied to inseminate beef heifers with sexed semen synchronized with a J-Synch protocol, and pregnancy rates in those heifers showing estrus are higher than in those not showing estrus.

A002 TAI/FTET/AI

# Efficacy of a short protocol (6 days) using EB to induce ovulation compared with a conventional protocol using ECP on pregnancy rate in heifers

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The objective of the study was to evaluate the performance of a short FTAI protocol (long proestrous) combined with estradiol benzoate (EB) as inductor of ovulation compared to a conventional protocol using estradiol cypionate (ECP) on pregnancy rate in Bos taurus beef heifers. Three replicates of the experiment were conducted (A, n=68; B, n=63; C, n=57) totalizing 188 Aberdeen Angus heifers, with body condition score (BCS) range between 3 to 4 (1-5 scale) and 15 to 16 months old. On D0 ultrasonography (US; Honda HS 101V – 5 MHz) was performed to evaluate ovarian structures (OST): corpus luteum (68.6%) or follicles ≥10mm (31.4%); and determine BCS. All heifers were homogenously distributed based on theirs OST to two different groups: 1) 6D36EB (n=96) or 2) 7DECP (n=92). On that day all heifers received 2 mg of EB (Bioestrogen®, Biogénesis Bagó, Argentina) i.m. along with an intravaginal device containing 0,558 g of progesterone (IVD; Cronipres® Monodosis, Biogénesis Bagó, Argentina). The IVD were removed on D6 (6D36EB) or D7 (7DECP) and all heifers received an i.m. administration of 0,150 mg of D-Cloprostenol (Enzaprost® D-C, Biogénesis Bagó, Argentina). To induce ovulation: 1 mg of EB was administered 36 h after IVD withdrawal (6D36EB); while 0,5 mg of ECP (Croni-Cip®, Biogénesis Bagó, Argentina) was administered at IVD removal (7DECP). Time of AI was 72 h (6D36EB) or 48 h (7DECP) after IVD removal. FTAI was performed by one veterinarian (the same for all replicates) and semen from three bulls was used (one for each replicate). Pregnancy diagnosis was done by US between 40 and 44 days after FTAI. Pregnancy rate was analyzed by logistic regression (InfoStat, UNC, 2015. Argentina). There was no effect of Replicate, Bull, OST or their interactions (P>0,1). Non-statistical differences were observed for pregnancy rates between 6D36EB [55.2% (53/96)] and 7DECP [54.3% (50/92); P>0.1]. In conclusion, short IVD treatment (long proestrous) combined with EB for inducing ovulation (6D36EB protocol) achieved similar pregnancy rates than conventional FTAI protocol (7DECP).

A003 TAI/FTET/AI

# Utilizing day 24 pregnancy associated glycoprotein (PAG) concentrations to diagnosis pregnancy in dairy cattle

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Dairy producers are limited to day 28 to 30 of gestation as the earliest time point for accurate pregnancy diagnosis due to the effectiveness of most ultrasound and chemical based methods, including pregnancy associated glycoproteins (PAG) tests. The objective of the current study was to determine if early gestation circulating PAG levels at day 24 could be used to diagnose pregnancy in dairy cattle undergoing embryo transfer. In vitro produced embryos were transferred into estrus synchronized Holstein × Gir crossbred cows and heifers on day 7 following ovulation. Experiment 1 utilized only cows (n=101) determined to be pregnant on day 24 of gestation following timed embryo transfer (TET) by (1) increased PAG concentration, (2) vascularized CL and (3) progesterone concentration >1 ng/ml. Crossbred heifers (n= 111) and cows (n=242) were used in experiment 2. In both experiments, blood was collected at day 24 for PAG analysis as well as day 31 for confirmation of pregnancy. Final pregnancy confirmation occurred on day 60 via transrectal ultrasonography. Serum concentrations of PAG were quantified using an in house PAG ELISA with polyclonal antibodies raised against PAGs expressed early in gestation. Following TET in experiment 1 of the 101 cows diagnosed as pregnant on day 24, 77 cows were identified as still pregnant on day 31 of gestation (77%) using ultrasound and PAG testing. Experiment 2 had an overall pregnancy rate at day 31 of 33.7% of total embryos transferred. Mean circulating PAG concentration at day 24 differed between animals identified as pregnant and non-pregnant by ultrasound at day 31 in both experiments (experiment 1, 2.9635± 0.262 ng/mL vs 0.94619± 0.168 ng/mL and experiment 2, 1.962 ± 0.261 ng/mL vs 0.707 ± 0.114 ng/mL). Concentration of PAG between pregnant and non-pregnant animals in experiment 1 and 2 was significant ($p \leq 0.06$). A predictive cutoff value for diagnosing pregnancy was identified at 2.50 ng/mL for 90% confidence using a ROC curve. Only animals that were pregnant at day 31 were analyzed in late embryo mortality analysis (heifers, n= 54; cows, n=159), defined as pregnancy loss between day 31 and 60. Between day 31 and 60, 39 (11 in experiment 1 and 28 in experiment 2) animals experienced late embryo mortality. Circulating concentrations of PAG were not significantly different at day 24 of gestation in animals that maintained pregnancy until day 60 than animals that lost pregnancy between day 31 and 60 (late embryo mortality, LEM) ($P > 0.10$); however, in experiment 2 the mean concentration of pregnant animals were numerically higher (2.043± 0.167 ng/mL) than animals that experienced LEM (1.327 ± 0.251 ng/mL). In summary, early gestation circulating PAG concentration may have application in diagnosing pregnancy at day 24 gestation and more work is needed to determine the potential of early gestation PAGs predicting embryonic loss in dairy cattle.

A004 TAI/FTET/AI

# Pregnancy rates in timed artificial insemination in Nelore cows using estradiol benzoate as ovulation inducer associated with eCG or FSHp

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In different tropical areas of the world, as in the case of the Brazilian Amazon, beef production is made using zebu cattle (Bos taurus indicus) due to its greater resistance to the humid tropical climate and its adaption to grazing in extensive management systems (Baruselli, P.S. Animal Reproduction Science, v. 82-83, p. 479–86 2004). The participation of the Amazon region in the production of beef in Brazil, has been increasing year after year.In this respect, reproductive biotechnologies (RB) have been used and have significantly increased the CR through the use of protocols for Timed Artificial Insemination (TAI) which has proven efficient to ensure improvement of the sustainability of cattle ranching (Neves, K. A. L. Papers do NAEA, v. 330, p. 1-19, 2014). The use of gonadotropins could improve LH support and considering that in the literature there are still gaps with respect to the use of equine chorionic gonadotropin (eCG) and follicle stimulating hormone (FSHp), the aim of the present study was to test protocols of TAI using hormone EB as ovulation inducer, combined with eCG or FSHp in multiparous Nelore cows. Multiparous suckled Nelore cows (n= 559) at 40-60 days postpartum and average BCS of 3.17 (1-5 points scale, were used). Cows were kept on pasture (Brachiaria brizanhta var. Marandu) with ad libitum access to water and mineral supplement during the experimental period. On D0 all cows received a PRID (Sincrogest® Ouro Fino® 1g P4) and 2.0 mg i.m. EB (Gonadiol®; Intervet/Schering-Plough, Brazil). On D8 PRID were removed and administered 4.0mg of dinoprost tromethamine (Lutalyse® Pfizer® 4mg PGF2α) i.m and, in the same day, cows were assigned according BCS in groups EB-eCG (n= 279) and received 300 IU i.m. (Novormon®, Syntex, Buenos Ayres, Argentina) or EB-FSHp (n= 280) and received 10mg i.m. (Folltropin®, Bioniche, Canada). On D9 1mg of EB im (Gonadiol®; Intervet/Schering-Plough, Brazil) were administered. Insemination was performed 56h after PRID removal. Statistical analyses were performed using the Statistical Analysis Systems for Windows version 8.2 (SAS 2001). The Chi-square procedure was used to determine significant differences between groups. Treatment differences are considered significant at $P < 0.01$.To evaluate the diameter of largest follicle (LF), ovulatory follicle (OF) and ovulation (OV) rates the GLM procedure with SNK adjustment was used to determine significant differences among groups. The largest follicle, maximum diameter of the LF, maximum diameter of the ovulatory follicle, ovulation and interval P4 device removal to ovulation did not differ between groups (eCG =13,5mm, FSHp=12,6mm). In the present experiment the eCG treatment increased CR compared with FSHp (65.23% versus 48.57%) P=0,001. The use of eCG at the time of PRID removal in a TAI program improves the CR and these treatments may facilitate the application of genetic improvement programs more efficiently in Bos taurus indicus herds under Amazonian tropical conditions.

A005 TAI/FTET/AI

# Effect of different gonadotropins on the final follicular growth and CL development in Nelore cows Submitted to FTAI protocols

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The aim of this study was to evaluate the effect of eCG, FSH or hCG on follicular growth rate, ovulatory follicle size, CL volume and circulating P4 concentration post ovulation, as well as the number of large and small luteal cells in cows submitted to protocol of FTAI. Sixteen non-lactating Nelore cows with body condition score (BCS) of 2.7 ± 0.4 (scale of 1 to 5) were used. At the beginning of the protocol (D0) all cows received 2 mg i.m. of estradiol benzoate (Gonadiol, Zoetis, Brazil), 25 mg i.m. of PGF2α (Lutalyse, Zoetis, Brazil), and an intravaginal P4 device (CIDR, Zoetis, Brazil). All cows received 25 mg i.m. of PGF2α on D7 and had CIDR removal + 25 mg i.m. PGF2α on D8. On D7 cows were randomly assigned into four groups: Control = No Gonadotropin treatment; eCG = 300 IU i.m. of eCG (Novormon, Zoetis, Brazil) on D7; FSH = 20 mg of FSH on D7, D8, and D9 (divided into two treatments of 10 mg i.m., 12 h apart; Folltropin-V, Bioniche, Canada); hCG = 200 IU i.m. of hCG (Chorulon, MSD, Brazil) on D7 and 100 IU i.m. of hCG on D8 and D9. Ultrasound examinations and blood sampling were performed daily until the seventh d post ovulation and on this day CL biopsies were performed for histological analysis. Two Latin squares were performed, totaling eight replicates. Data were analyzed by PROC GLIMMIX of SAS and the results are presented as least squares means ± SE following the group order Control, eCG, FSH, and hCG. As expected, there was no difference in follicle diameter (mm) on D7 (7.7 ± 0.3, 7.9 ± 0.3, 7.7 ± 0.3, 7.5 ± 0.3; P>0.05). However, on D10 the follicle diameter of the groups eCG, FSH and hCG were larger than Control (11.3±0.3, 12.5±0.3, 12.5±0.3, 12.6±0.3; P < 0.05) with a greater follicular growth rate between D7-10 (1.2±0.3; 1.5±0.3, 1.6±0.3, 1.7±0.3; P=0.03). Similarly, the diameter of the ovulatory follicle (12.9±0.3, 13.6±0.3, 13.6±0.3, 13.8±0.3; P<0.05) was smaller for Control, but seven d later, the CL volume (3876.1±261, 4529.6±256, 3797.4±308, 4443.7±278; P>0.05) and plasma P4 concentration (2.9±0.3, 3.6±0.3, 2.9±0.3, 3.5±0.3; P>0.05) were not different among groups. Nevertheless, the groups treated with gonadotropins had a greater number of large (224x107±610, 468x107±558, 586x107±588, 519x107±541; P < 0.01) and small luteal cells (238x108±688, 478x108±629, 484x108±717, 555x108±579; P < 0.01) than the Control group. In conclusion, all gonadotropin treatments, eCG, FSH, or hCG, were effective in increasing the follicular growth rate between D7-10 and consequently the follicular diameter on D10 and ovulatory follicle diameter. In addition, treatment with different gonadotropins increased the number of large and small luteal cells, however did not affect CL volume and P4 concentration.
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A006 TAI/FTET/AI

# Addition of a second dose of Prostaglandin (PGF2α) to a timed AI protocol influences pre-ovulatory follicle and pregnancy per AI in anovular dairy cows

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Objectives were to determine the effects of a second PGF2α in a timed AI protocol on LH pulsatility, pre-ovulatory follicle characteristics, and pregnancy per AI (PAI) in anovular cows. In experiment 1, 56 Holstein cows had the estrous cycle synchronized to start the timed AI without a corpus luteum (CL). All cows were assigned to a synchronization protocol: d -11, 2 mg of estradiol (E2) benzoate and a progesterone (P4) insert; d -4, 25 mg of dinoprost; d -2, 1 mg of E2 cypionate and removal of the P4 insert. On d -11, cows were blocked by milk yield and parity and randomly assigned to 1PGF, 25 mg of dinoprost on d -4 and 5 mL of saline on d -2; or 2PGF, 25 mg of dinoprost on -4 and -2 d. Blood was sampled from d -11 to 0 and assayed for P4. Jugular catheters were placed and blood was sampled every 15-min from 1 h before to for 6 h after treatments, and every 2 h thereafter for 58 h. Plasma samples were assayed for concentrations of LH and PGF2α metabolite (PGFM). The pre-ovulatory follicle was aspirated on d 0 and fluid assayed for E2 and P4. In experiment 2, 454 lactating anovular Holstein cows (no CL on d -11 and -4) were randomly assigned to either 1PGF or 2PGF and subjected to timed AI. Pregnancy was diagnosed on d 58 after AI. Rectal temperature (RT) was measured on d 0 and 7, according to the average, the cows were classified as RT below (normothermic) or above 39.0oC (hyperthermic). Continuous data were analyzed by mixed models with the fixed effects of treatment, time, and interaction, and the random effects of block and cow nested within treatment. Categorical data were analyzed by logistic regression with a model that included the fixed effects of treatment, RT and interaction. Although both groups had subluteal concentrations of P4 between d-11 and d0, we detected a statistical difference between groups, the concentration of P4 from d-11 to 0 was greater (P=0.04) for 1PGF than 2PGF (0.59 vs. 0.45 ± 0.06 ng/mL). 2PGF increased (P<0.001) concentrations of PGFM in plasma (47.0 vs. 702.8 ± 25.1 pg/mL) starting immediately after treatment and lasted at least 6 h. 2PGF reduced (P=0.05) the number of LH pulses/6 h (4.5 vs. 3.9 ± 0.2). Relative to treatment, the beginning of LH surge (22.4 vs. 19.3 ± 2.1 h) and the hour when the peak of LH surge was detected (29.0 vs. 28.0 ± 1.8 h) did not differ between 1PGF and 2PGF, but duration of the surge was longer (P=0.04) for 2PGF than 1PGF (13.1 vs. 15.5 ± 0.8 h). Cows in 2PGF had larger (P=0.05) pre-ovulatory follicle diameter (12.3 vs. 14.4 ± 0.8 mm) with greater (P=0.02) estradiol concentration in the follicular fluid in all aspirated follicles (115 vs. 262 ± 39 ng/mL) or in estrogenic follicles (161 vs. 372.8 ± 28 ng/mL). 2PGF increased (P=0.04) ovulation after AI in all cows (75.3 vs. 83.1%). Also, in cows with RT≤39.0, 2PGF increased (P<0.03) PAI in all cows (15.7 vs. 30.7%) or synchronized cows (19.5 vs. 35.1%), but not in cows with RT>39.0 (all cows, 10.0 vs. 9.5%; synchronized cows, 14.8 vs. 12.2%). Treatment with a second dose of PGF2α improved PAI in normothermic anovular cows because of increased ovulation and improved pre-ovulatory follicle characteristics.

A007 TAI/FTET/AI

# Vaginal temperature in Holstein cattle: Effect on productive and reproductive traits and genome contribution to thermotolerance

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The aim of the experiment was to evaluate vaginal temperature on productive and reproductive traits and potential genetic contribution to thermomtolerance. Lactating Holstein cows n = 641 (209 primíparous 36,9 ± 6,54 kg milk/d; 432 multíparous 43,9 ± 9,77 kg milk/d) had vaginal temperature monitored using thermometers, attached to an intravaginal device (CIDR® 1,9 g de P4, Zoetis, SP, Brasil) as part of a timed-AI protocol and recorded vaginal temperature every 10 minutes for 3 days. Ambient temperature and relative humidity were monitored using an external thermometer placed inside the barn. The data were analyzed with SAS 9.4 using Pearson correlation, ANOVA and logistic regression. Heat stress was calculated based on the percentage of time the cow spent with a vaginal temperature ≥39,1°C (PCT). Cows were classified using the 75th percentile threshold (HighPCT and LowPCT) for PCT and the median value for milk, which were different for primiparous and multiparous. There was a low correlation between THI and milk production with PCT (r=0,01) indicating a large variation in thermoregulation. Multiparous LowPCT (22,4 and 13,9%) and HighPCT (12,6 and 9,7%) significantly reduced P/AI on day 30 and 52 post-AI (P<0.01) and no interactions with parity, body condition score and THI were observed. Coat color and skin thickness did not influenced PCT.

Illumina® BovineSNP50 v2 genotypes were obtained for 467 cows (258 lactating cows and 209 heifers). Animals were distributed in 61 different contemporary groups and a total of 194,406 IVT records were obtained, with an average of 416 ± 50 measurements per animal (min = 133, max = 867). All animals had call rate (CR) greater than 90%. A total of 39,549 single nucleotide polymorphism (SNP) markers were selected for analysis with PLINK v.1.90. These markers presented a minimum CR of 95% and a minor allele frequency (MAF) of at least 5%. The 39,549 SNPs explained 32.9% of the variance in IVT, suggesting that genomic variation accounts for a substantial portion of the thermotolerance, but no individual loci contributing with 1% or more of the marked additive genetic variance, indicating that the trait is highly polygenic.

In summary, there is a large variability on how individual cows respond to heat stress, but a sub-population of animals is able maintain lower PCT under similar production and environmental conditions, which resulted in improved P/AI.

A008 TAI/FTET/AI

# Reproductive efficiency of Nelore cows submitted to 7-d FTAI protocols initiated with estradiol benzoate or GnRH and with or without gnrh at the time of AI

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This study assessed the response of Nelore (*Bos indicus*) cows to 7-d fixed-time AI (FTAI) protocols using progesterone (P4) intravaginal device (IVD) and initiating with buserelin acetate (GnRH) or estradiol benzoate (EB). In addition, treatment with GnRH concomitant with FTAI was evaluated. A total of 820 cows (primiparous, multiparous and non-lactating) on pasture system was completely randomized assigned to four treatments. At the beginning of the protocol (D0) cows either received GnRH (G; 20.0 μg) or EB (B; 2.0 mg), and at the time of AI cows were treated with GnRH (G; 10.0 μg) or not treated (0), resulting in four groups: G0, GG, B0, and BG. Simultaneously with these treatments on D0, all cows received an intravaginal P4 device (1.0 g) that was removed 7 d later (D7). Also on D7, all cows were treated with sodium cloprostenol (PGF; 530 μg) and estradiol cypionate (EC; 0.5 mg). Cows from G0 and GG received eCG (300 IU) and an additional PGF on D6, whereas B0 and BG received the eCG treatment on D7. FTAI was performed on D9 by the same technician, and seven sires were used. All hormones were from Globalgen Vet Science, Jaboticabal, Brazil. Body condition score (BCS) was evaluated on D0, and at D7 all cows had chalk applied on their tailhead to evaluate estrous behavior at AI. Ovarian ultrasonography was performed on D0, D6, D7, D9, and 8 d after AI. Statistical analyses were performed using GLIMMIX and MIXED of SAS 9.3 (LSM ± SEM; $P \leq 0.05$). Overall pregnancy per AI (P/AI) was noteworthy (62.7%; 514/820), although there were no differences among groups [57.8 (122/205); 67.6 (144/208); 60.0 (128/207), and 58.0% (120/200); B0, BG, G0, and GG, respectively]. The BCS at the onset of the protocols was not different among groups (3.0 ± 0.28), and cows with BCS ≥ 3 had greater P/AI than cows with BCS < 3 [65.7 (354/533) vs. 55.7% (160/287)]. Non-lactating and multiparous cows had greater P/AI than primiparous [65.5$^{a}$ (81/116) vs. 62.1$^{a}$ (327/513) vs. 54.3%$^{b}$ (106/191)]. More cows treated with GnRH on D0 were detected in estrus at the time of AI than cows receiving EB [66.7 (269/404) vs. 55.0% (227/411)], and cows detected in estrus had greater P/AI [(66.2 (344/496) vs. 51.3% (168/319)]. Furthermore, there was a tendency ($P < 0.10$) for treatment with GnRH at the time of AI to increase P/AI of cows not detected in estrus [from 46.2% (77/164) to 57.0% (91/155)]. Related to cows detected in estrus, there was no improvement on fertility due to GnRH treatment at the time of AI [(66.1 (171/244) vs. 66.4% (173/252), for not treated vs. GnRH-treated cows, respectively]. A greater percentage of cows ovulated at the onset of the protocol if treated with GnRH than EB [71.5 (83/114) vs. 28.6% (35/116)]. Follicle diameter on D7 was larger for cows from group GnRH than EB [(10.8 ± 0.2 (n = 61) vs. 9.6 ± 0.3 (n = 62)]; however, treatment did not affect follicle diameter at AI [12.8 ± 0.3 (n = 61) vs. 12.4 ± 0.2 (n = 65)]. Ovulation rates after AI were not different among treatments [93.1% (297/319)]. In conclusion, 7-d FTAI protocols initiating with GnRH or EB achieved similar and relatively high fertility in Nelore cows. Moreover, treatment with GnRH at AI tended to increase P/AI in cows not detected in estrus.

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A009 TAI/FTET/AI

# Ratio of thawed semen viability and gestation rate in a fixed-time artificial insemination program (TAI) in cattle

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The aim of this study was to evaluate the relationship of thawed bovine semen and pregnancy rates in Nelore females after artificial insemination in fixed time (TAI). Thirty seven conventional semen straws were used from 16 Nelore bulls and 3 bulls Aberdeen Angus breed, that had proven fertile. Four thousand, one hundred and seventy one Nelore cows were inseminated, distributed in groups of 150 animals, according to the TAI program established on farm. All females (4171) were subjected to same TAI protocol and were inseminated by same technical team. The straws were thawed at 37°C, evaluated by light microscopy as morphology (DEF), motility (MOT), and the CASA system (MT, PM, VAP, VSL, LIN, STR) were used for sperm kinetics. The integrity of spermatic membrane (MEMB) and mitochondrial function (MITO) were evaluated through flow cytometry. The mean results were: DEF 12.7±6.5, MOT 45.3 ±6.6, MEM 44.4±11.4, MITO 47.6±8.8, MT 39.6±11.8, PM 29.8±9.1, VAP 97.5±11.5, VSL 80,3±9.1, LIN 49.1±4.9, STR 81.5±4.0. Pregnancy rate was 50.8±12.9. Statistical analysis has been done, and the Pearson correlation, positive correlation was found between the variables motility and progressive motility ($R = 0.940$, $p < 0.001$); path velocity, progressive velocity ($R = 0.869$, $P < 0.001$); linearity and straightness ($R = 0.928$, $p < 0.001$). However, there was no direct correlation between any of seminal analysis and fertility outcomes (pregnancy rate). Through Cluster analysis and combinations between semen evaluations, it was verified formation of four good quality clusters, with combination of progressive speed, mitochondrial activity and pregnancy rate. It is concluded that no parameter considered in isolation influence the result of the pregnancy rate and the results of semen analysis are not enough to predict the fertilizing capacity of frozen semen.

A010 TAI/FTET/AI

# Daily ovarian dynamics in Nelore heifers and non-lactating multiparous cows submitted to progesterone (P4)-based FTAI protocols

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Three experiments were performed to understand ovarian dynamics of Nelore cows and heifers during P4-based FTAI protocols differing in: initial treatments to synchronize follicle waves, protocol lengths (5, 7, or 9 d), and final ovulation induction treatments. For Exp1, cows were assigned to the groups: EB-9-EC [n = 20; D0: 2 mg EB and an intravaginal device (IVD) with 1 g P4; D9: 0.5 mg estradiol cypionate (EC), 0.526 mg sodium cloprostenol (PGF), 300 IU eCG and IVD removal]; G-7-2-G [n = 19; D0: 20 µg buserelin acetate (GnRH) and IVD; D6: PGF and 300 IU eCG; D7: PGF and IVD withdrawal; D9: 10 µg GnRH]; or 5-EC-2-G (n = 20; D0: only IVD; D5: EC, PGF, eCG, and IVD withdrawal; D7: 10 µg GnRH). For Exp2, cows were assigned to protocols: EB-7-EC-2-G [n = 26; same protocol as EB-9-EC, but D9 treatments were performed on D7, and GnRH (10 µg) was given on D9]; or G-7-EC-2-G (n = 30; same protocol as G-7-2-G, but with EC on D7). For Exp3, heifers were assigned to protocols: EB-7-EC-2-G (n = 22; same protocol as EB-7-EC-2-G from Exp2); G-7-2-G (n = 22; same protocol as G-7-2-G from Exp1); or 5-EC-2-G (n = 22; same protocol as 5-EC-2-G from Exp1). Doses of EB (1.5 mg), eCG (200 IU) and the IVD (0.5 g P4) were different for Exp3. All hormones were from Globalgen Vet Science. Ovarian ultrasonography was performed daily from D0 until 4 d after IVD withdrawal. Continuous variables were analyzed using PROC MIXED and binomial variables using PROC GLIMMIX of SAS ($P \leq 0.05$; tendency = $0.05 < P < 0.1$). In Exp1, only G-7-2-G cows ovulated to treatment on D0 (40.2%) with earlier follicular wave emergence in G-7-2-G and 5-EC-2-G than EB-9-EC ($0.9^{b}$, $1.1^{b}$, and $2.6^{a}$ d). Synchronization rate (SR) of follicular wave emergence and growth rate of the ovulatory follicle (GROF) was lower for 5-EC-2-G, compared to other groups (SR: 5-EC-2-G = $8.4^{b}$, EB-9-EC = $95.9^{a}$, and G-7-2-G = $87.7\%^{a}$; and GROF: 5-EC-2-G = $0.8^{b}$, EB-9-EC = $1.2^{a}$, and G-7-2-G = $1.2^{a}$ mm/d). Maximum diameter of the ovulatory follicle (DOF) was larger for G-7-2-G than 5-EC-2-G and tended to be larger than EB-9-EC ($14.4^{a}$, $12.9^{b}$, and $13.1^{b}$, mm). Ovulation rate and time to ovulation at end of the protocol was similar for all groups: 86.4% and 21.9 h. For Exp2, ovulation to treatments on D0 was greater in G-7-EC-2-G than EB-7-EC-2-G (39.5 vs 12.2%), although follicular wave emergence (2.4 d), SR (89.3%), GROF (1.1 mm/d), DOF (12.6 mm), ovulation rate (83.9%), and time to ovulation (23.6 h) at the end of the protocol did not differ between groups. In Exp3, ovulation rate to treatments on D0 was greater for G-7-2-G compared to other groups ($65.2^{a}$, $6.9^{b}$, and $0.0\%^{b}$ for G-7-2-G, EB-7-EC-2-G, and 5-EC-2-G, respectively). For heifers treated with 5-EC-2-G, SR was lower ($19.1^{b}$, $95.8^{a}$, and $75.7\%^{a}$; 5-EC-2-G, EB-7-EC-2-G, and G-7-2-G, respectively), and the DOF tended to be larger than EB-7-EC-2-G ($13.4^{A}$, $11.9^{B}$, and $12.7 \pm 0.5^{AB}$ mm; 5-EC-2-G, EB-7-EC-2-G, and G-7-2-G, respectively). Moreover, 5-EC-2-G ovulated earlier at the end of the protocol ($13.0^{a}$, $19.3^{b}$, and $22.7^{b}$ h; 5-EC-2-G, EB-7-EC-2-G, and G-7-2-G, respectively). However, follicular wave emergence (2.2 d), GROF (1.1 mm/d) and ovulation rate at the end of the protocol (75.8%) did not differ among groups. In conclusion, each of these protocols has distinct ovarian dynamics and is promising for FTAI in beef cattle. Of particular interest, protocols initiated only with IVD did not synchronize follicle wave emergence but had a similar ovulation rate at the end.

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A011 TAI/FTET/AI

# Reproductive performance of Nelore heifers submitted to a 7-d P4-based FTAI protocol using either estradiol benzoate or GnRH at the beginning and with or without GnRH at the time of AI

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Reproductive variables were evaluated in Bos indicus heifers submitted to progesterone (P4)-based fixed-time AI (FTAI) protocols. We hypothesized that using buserelin acetate (GnRH) instead of estradiol benzoate (EB) at the start of a 7-d protocol would increase fertility in heifers. In addition, treatment with GnRH at the time of AI would also improve pregnancy per AI (P/AI). Therefore, 462 Nelore heifers [26.4 ± 2.0 mo old, body condition score of 2.9 ± 0.1 (5-point scale), and body weight of 299.2 ± 20.3 kg; mean ± SD], in a pasture system, were used. All heifers were evaluated by transrectal ultrasonography to determine presence of corpus luteum (CL), and heifers without CL were submitted to a protocol for induction of cyclicity based on insertion of a previously used intravaginal P4 device (D-24) followed by device withdrawal and estradiol cypionate (EC; 0.5 mg) administered i.m. (D-12). After 12 d (D0), heifers were assigned to four groups in a completely randomized design: B0 (n = 116), BG (n = 115), G0 (n = 115), and GG (n = 116). On D0, all heifers received an intravaginal P4 device (0.5 g) for 7 d and were treated with EB (1.5 mg; B) or GnRH (20 μg; G). On D7, concomitant with device withdrawal, heifers received cloprostenol sodium (PGF2α; 0.530 mg), EC (0.5 mg) and had the base of their tailhead painted with tail-chalk. Heifers from G groups also received an extra PGF2α and eCG (200 IU) treatment on D6, while B heifers received eCG on D7. At the time of AI (48 h after device withdrawal), only BG and GG groups received GnRH (10 μg), and all heifers were evaluated for estrus based on disappearance of the tail-chalk. Hormones used for synchronization were from Globalgen Vet Science, Jaboticabal, Brazil. Ultrasound evaluations were performed on D0, D6 and D7 to determine presence of CL, and on D7 and D9 for diameter of the dominant follicle (DF) and pre-ovulatory follicle (OF), respectively (~20% of the heifers from each group). Pregnancy diagnosis was performed 40 d after AI. Statistical analyses were performed by GLIMMIX and MIXED of SAS (LSM ± SEM; $P \leq 0.05$). At the beginning of the study, 50 heifers were cycling (detected with CL) and 412 were not. The protocol for induction of cyclicity resulted in 75.6% of heifers with a CL on D0. Independent of cyclicity all heifers underwent FTAI. Ovulation rate after D0 was greater in G than B heifers [71.2 (163/229) vs. 17.1% (39/228)]. The diameter of the DF at device withdrawal was larger in G than B [10.8 ± 2.8 (n = 38) vs. 9.5 ± 1.8 mm (n = 42)]. Despite that, the diameter of the OF was not influenced by treatment (11.8 ± 2.6 mm; n = 74). For heifers submitted to protocol for induction of cyclicity, overall percentage of heifers detected in estrus at the end of the FTAI protocol was 76.9%, resulting in greater estrus status for G than B groups [84.9 (174/205) vs. 68.9% (142/206)]. Regarding P/AI, there was no difference between heifers submitted or not to the cyclicity induction protocol. Therefore, data were combined, and there was no treatment effect (B0 = 50.9 vs. BG = 54.2 vs. G0 = 54.0 vs. GG = 60.4%). Estrus status and GnRH at FTAI did not influence fertility. In summary, the cyclicity induction protocol resulted in about 75% of heifers with CL at the onset of a FTAI protocol. Moreover, treatment with GnRH on D0 increased percentage of heifers with CL and DF size at device withdrawal, and increased percentage of heifers in estrus at FTAI but did not significantly alter P/AI compared to heifers treated with EB on D0.

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A012 TAI/FTET/AI

# The administration of buserelin acetate at the moment of TAI in multiparous Nelore cows with low or absent estrous expression enhances the pregnancy per AI

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The objective was to evaluate the effect of the IM administration of GnRH (buserelin acetate) on the pregnancy per AI (P/AI) of Nelore (Bos indicus) females with low or absent estrous expression, submitted to a timed artificial insemination (TAI) hormonal protocol. A total of 315 multiparous (BCS=2.88±0.01) and 175 primiparous (BCS=3.06±0.02) cows, from a commercial farm located at Porto Murtinho city (MS state – Brazil) were enrolled in the study. At Day 0 (D0), all females received an ultrassound examination in order to check the presence of a corpus luteum (CL) and received: 0.530 mg IM sodium cloprostenol (PGF; Sincrocio, Ourofino Saude Animal – only when a CL was present), 2.0 mg IM estradiol benzoate (Sincrodiol, Ourofino Saude Animal) and an intravaginal progesterone releasing device, previously used during 8 days (Sincrogest dispositivo, Ourofino Saude Animal). At Day 8 (D8) the devices were removed and it was administered 1.0 mg IM estradiol cypionate (Sincrocp, Ourofino Saude Animal), 0.530 mg IM PGF and 300IU IM equine chorionic gonadotrophin (SincroeCG, Ourofino Saude Animal). At this moment, all cows were marked with a chalk (Raidl-Maxi; RAIDEX – Germany) at the tail head region. The TAI were performed 48 hours after P4 device removal (D10) using conventional semen. Previously to TAI, the estrous expression was checked, as total removal of the chalk mark was considered positive heat [61.8% (303/490) of positive heat] and females from this group were removed from the experiment. Cows that still had any chalk mark were considered as negative heat and were randomly alocatted in two groups: Control (no adicional treatment) and GnRH [administration of 10 µg IM buserelin acetate (Sincroforte, Ourofino Saude Animal) at TAI]. Data were analized using the GLIMMIX procedure of the SAS® 9.4 software. Similar P/AI was observed among sires (P=0.45) and no interaction sire*insemination technician (P=0.32) was found. However, there was an interaction treatment*category [Control x multiparous = 22.9%b(14/61), Control x primiparous = 25.0%b(8/32), GnRH x multiparous = 41.7%a(25/60), GnRH x primiparous = 17.6%b(6/34); P = 0.04). Still, there was an insemination technician effect (A = 19.6%b, B = 24.6%ab, C = 31.7%ab e D = 48.3%a; P=0.01). Thus, it is concluded that the administration of buserelin acetate at the moment of TAI is a considerable strategy to improve P/AI in multiparous Nelore cows that were negative for heat detection.

A013 TAI/FTET/AI

# Can insemination difficulty at timed-AI affect reactivity and/or fertility of Brangus females?

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Temperament of cattle and stress caused by AI are less studied factors, but they can also affect reproductive programs. The objective of this study was to evaluate temperament (Te), chute exit period (ExP), AI difficulty AI (DifAI) and time for AI accomplishment (tAI) of Brangus heifers submitted to the same Timed-AI protocol. Management of 112 animals was monitored, being 28 cows and 84 heifers. For each animal, cattle reactivity was recorded from 1 to 5, according to behavioral characteristics (Te1 = very calm animals in the chute and Te5 = very temperamental) and time of chute exit at D9 (shortly after progesterone implant removal) and D11 (shortly after Timed-AI). In addition, on D11, tAI was recorded and the technician defined a score regarding to the difficulty of performing AI procedure (1 for mild, 2 for moderate and 3 for high degree of AI difficulty). Pregnancy rate (PR) were compared between groups using Fisher's exact test (P=0.05) in GraphPad INSTAT program. Overall PR was 43% for cows (12/28) and 43% for heifers (36/84). Hence data from these animals were analyzed togheter. No effect of BCS (2.5-2.75=29%, n=24; 3.0-3.5=49%, n=47; 3.75-4.0=44%, n=41; P=0.2830), nor of AI technician (technician1=45%, n=22; technician2=42%, n=59; technician3=41%, n=31; P = 0.9632) was observed. Similarly, no temperament effect on PR was observed in D9 (Te1=39%, n=79; Te2-3=51%, n=33; P=0.2352) or D11 (Te1=40%, n=92; Te2-3=53%, n=20; P = 0.3232). No Te4-5 animals were found since this farm adopts low stress cattle handling and selection for docility. Te1 animals left the chute more slowly (P<0.001) than Te2-3 animals, in both passages (D9 and D11). The mean ExP on D9 was 4:14±01:39sec for Te1 and 02:38±1:06sec for Te2-3. On D11, Te1=03:43±02:47sec and Te2-3=01:59±1:15sec in D11. When DifAI was analyzed, it was interesting to note a trend to greater (P=0.0799) PR in animals with lower difficulty in performing AI procedure (DifAI1=47%, n=78) compared to animals that presented moderate or high difficulty of AI (Df2e3=32%, n=34). Comparing these two groups, lower (P<0.001) time was necessary for completing AI procedure for DifAI1 (tAI=00:34:40±00:23:00min) than for DifAI2-3 (tAI=1:45:00±1:05:00min). However, difficulty of AI did not seem to influence the behavior of the animals, since no difference (P=0.5232) was observed in the percentage of Te1 (calm animals) for DifAI1 (85%) or DifAI2-3 (79%) group. The degree of difficulty in AI did not increase the reactivity of Brangus heifers, but in ANIMALS WITH GREATER DIFFICULTY IN INSEMINATION, LOWER PREGNANCY RATES CAN BE EXPECTED AFTER TIMED-AI.

A014 TAI/FTET/AI

# Alternative of cost reduction in the execution of TAI in beef cattle: Preliminary results

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The objective of this study was to evaluate the use and cost of injectable progesterone in ovulation synchronization protocols. The experiment was carried out at Fazenda Santana (Valença-RJ), where 82 nulliparous Nellore heifers, 39% cyclic, with ages and average weights of 19 months and 315 kg, were randomly assigned to two groups: Control Group (n = 41) and P4i Group (n = 41) so that the groups had the same number of cyclic animals. The animals had ad libitum access to water and selective mineral supplementation. The treatments started on a random day of the estrus cycle of heifers, this day being considered Day Zero (D0) where heifers of the Control Group received an intravaginal device containing 1.9 g of progestogen (CIDR®, Zoetis, Brazil) and 2 mg im estradiol benzoate (SINCRODIOL®, Ourofino, Brazil). Eight days later (D8), the device was removed and 150 μg of cloprostenol im (VETEGLAN®, Hertape, Brazil), 1 mg of estradiol cypionate (ECP®, Zoetis, Brazil) and 300 IU of eCG (NOVORMON ®, Zoetis, Brazil). In the P4i Group, heifers received 150 mg of injectable progesterone im (SINCROGEST injectable® - Ouro Fino, Brazil) at D0 instead of the placement of the device. The other procedures and drug administrations were similar to the Control Group. In D8, heifers received an estrous identification sticker (BoviFlag) for evaluation of estrus manifestation. In both groups, the TAI was performed 48 hours after withdrawal of the progesterone device. Ultrasonographic examinations (Mindray DP-2200 Vet) were performed at the time of the TAI to measure the diameter of the dominant follicle (DFAI) and at D20 to evaluate ovulation rate by corpus luteum observation. The DFAI evaluation was performed by analysis of variance and the means were compared by the T test, with a significance level of 5%. Ovulation rates, estrus manifestation (EM) and number of females with presence of dominant follicle ≥ 8 mm in the TAI were analyzed using the chi-square test with significance level of 5%. The EM rate was higher in heifers of the Control Group [92% (35/38) for the Control Group and 67% (24/36) for the P4i Group; P = 0.006]. However, there was no difference between the experimental groups for the DFAI variables (Control Group - 10.2 ± 1.6mm and P4i Group - 10.9 ± 2.0mm, P = 0.08), ovulation rate - 82.9% (34/41) and P4i Group - 70.7% (29/41); P = 0.19] and presence of dominant follicle ≥8 mm in the FTAI [Control Group - 87.8% (36/41) and P4i Group - 87.8% (36/41); P = 1.0]. In the analysis of the cost of the hormones used, there was a 19.9% reduction with the use of injectable P4 (R$ 13.36). It is concluded that P4i presented similar results to intravaginal progesterone in the synchronization of ovulation of Nellore heifers and promoted a reduction in protocol cost. However, new studies with higher numbers of animals and use of different ovulation inducers are necessary to consolidate the hypothesis.

A015 TAI/FTET/AI

# Amplitude of the LH peak after short and long protocol in Oeste Paulista sheeps during the breeding season

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Several opportunities have been identified as attractive for the expansion of sheep production in Brazil, with emphasis on genetic improvement through artificial insemination (AI). The genetic improvement can be facilitated by the use of estrus synchronization and ovulation for fixed-time artificial insemination (IATF). The aim of the present study was to study the LH surge and evaluate the pregnancy rate using a short (6 days) and long term progestagen protocol (12 days) followed by AI in sheep (Texel x Santa Inês) on Oeste Paulista, inside the breeding season. In the experiment 36 sheep were used (paternal lineage Texel-Te x maternal lineage Santa Inês-SI), aged between 24 and 48 months, and had an average body condition score of 3.0. The sheep were divided into two groups according to time of permanence of a 1st use progesterone release vaginal device (Easy-Breed CIDR®, Pfizer, Brazil). In the 6 day group (n = 18), at a random stage of the estrous cycle, the sheep received the insertion of the CIDR (D0). On the day of removal of the implant (D6), 0.075 mg of cloprostenol (Veteglan®, HertapeCalier, Brazil) and 300 UI of equine chorionic gonadotropin were administered intramuscularly (eCG, Novormon®, MSD Saúde Animal, Brazil). The 12 day group (n = 18) received the same protocol as the G-6; however the permanence of the CIDR was 12 days. Approximately 50 hours after removal of the CIDR, the sheep were inseminated with frozen semen at a fixed time by laparoscopy. The pregnancy diagnosis was performed 40 days after the artificial insemination. Blood samples were taken every 4 hours, 10 animals per group, to measure plasma concentrations of LH and progesterone through radioimmunoassay (RIA). The data for the LH surge were analyzed using the Kruskal-Wallis test and, in case of significant differences, the means were compared through the Student Newman Keuls (SNK). The chi-squared test was used for the pregnancy rate. All analyzes were performed in the BioEstat program 5.0 and the level of significance was 5% ($p < 0.05$). The amplitude of LH did not differ ($p > 0.05$) in the G-12 (45.31 ± 15.41 ng/mL) compared to the G-6 (36.81 ± 18.89 ng/mL). The LH surge occurred earlier ($p < 0.05$) in the G-12 (30.86 ± 8.86 h) when compared with the G-6 (43.11 ± 6.57 h) and the end of the peak was earlier in G-12 (34.86 ± 8.86 h) than the G-6 (46.22 ± 7.51 h). There were no differences ($p > 0.05$) in pregnancy rates between the groups G-6 61.11% and G-12 66.66%. It was concluded that the time of a permanence of progesterone implant influence the LH surge on the Oeste Paulista. The short protocol delays the start, occurrence and the end of the LH surge. There was no significant difference in the pregnancy rate between groups. Therefore, the use of short duration protocol during the breeding season allows the reuse of the implant reducing costs.

A016 TAI/FTET/AI

# Effect of the delay at TAI in Nelore cows (*Bos indicus*) using low fertility bulls semen

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The objective of the present study was to evaluate the effect of delaying TAI on the pregnancy rate of Nelore cows (Bos indicus) inseminated with low fertility bulls' semen. The hypothesis of this experiment is that the delay of TAI using low fertility bulls' semen increases pregnancy rate. A total of 522 suckled Nellore cows (Bos indicus), from three differents farms in Jacarezinho – PR, Ribeirão Claro – PR and Paraíso das Águas – MS, were used. On a random day of the estrus cycle (D0) cows received 2 mg of estradiol benzoate i.m. (EB; Fertilcare Sincronização®, Vallée) and an intravaginal P4 device (1.2g of P4; Fertilcare 1200®, Vallée). On the eighth day of protocol (D8), the P4 device was removed, and of 300IU of equine corionic gonadotropin (eCG; Folligon®, MSD Saúde Animal), 0.530mg of cloprostenol sodium (PGF2α; Ciosin®, MSD Saúde Animal) and 1mg estradiol cipionate (EC; Fertilcare Ovulação®, Vallée) were administered. On the tenth day of protocol (D10), cows were allocated in two groups (G): G48h, inseminated 48 hours after P4 device removal; and G54h, inseminated 54 hours after P4 device removal. To every five cows that were inseminated in the morning (G48h), the next five were put aside to be inseminated on (G54h). Six Aberdeen Angus sires were used, three of them considered with high fertility and three with low fertility. The pregnancy diagnosis was done using ultrasonography (Mindray, DP10) 30 days after TAI. Data were analized using the GLIMMIX precedure of SAS. The semens' fertility influenced the pregnancy rate at TAI [high = 49.2% (128/260); low = 35.9% (94/262); P=0.002]. However, no effect was verified regarding the moment of TAI [48 hours = 41.4% (109/263); 54 hours = 43.6% (113/259); P=0.66] as well as fertility*moment interaction (P=0.55) on the pregnancy rate at TAI. Therefore, the delay in TAI with low fertility bulls did not increase pregnancy rate, not confirming the initial hypothesis of the present experiment.
Acknowledgments: Alta Genétics Ltda, FIRMASA.

A017 TAI/FTET/AI

# Evaluation of the performance and effectiveness of a semen extender (Prosêmen®) in fixed time artificial Insemination and *in vitro* embryo production

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The aim of the present study was to evaluate the effectiveness of a commercial extender (Prosêmen®, Prosêmen Indústria e Comércio de Produtos Veterinários, Cornélio Procópio, Paraná, Brazil) for commercial semen after thawing in a FTAI program, as well as to evaluate its efficiency in the IVEP. For better evaluation of the extender, the rate of oocyte fertilization and total and progressive sperm motility rates at different times after thawing were compared. For all the steps, two experimental groups were compared: control (CONT; without extender) versus extender (PROS). In the PROS group for each 0.5 µL ampoule, two straws (0.25 µL) of semen were diluted after thawing and packed into four straws for use. In the CONT group the semen was thawed in the same manner, but without the use of the extender. The performance and dilution efficacy (PROS) versus non-diluted (CONT) were compared in: a) pregnancy rate to FTAI; B) IVEP; C) rate of in vitro fertilization by counting the pronuclei in an epifluorescence microscope; D) Computer Assisted Sperm Analyzer (CASA) of total and progressive sperm motility at 0 hour, 10 minutes, 3, 6, 8 and 12 hours after thawing. Statistical analysis was performed by the Chi-square test for FTAI, IVEP and oocyte fertilization. Motility data were compared through analysis of variance and T-test and between the different moments after thawing, variance analysis was used with time-repeated measures. All statistical analyzes were performed in Minitab® statistical software 16.1.1 ($P \leq 0.05$). No difference was found in the pregnancy rate at FTAI (CONT 55.48% and PROS 52.11%, $p = 0.567$). In IVEP there was also no difference in cleavage rates (78.52% and 81.56%, $p = 0.147$), blastocysts (39.48% and 36.10%, $p = 0185$) and hatching (41.97% and 47.28%, $p = 0.228$) for CONT and PROS, respectively. There was no difference in the oocyte fertilization rate between groups (CONT 65.22% and PROS 61.76%, $p = 0.751$). The percentage of total and progressive motility were similar, with difference observed only after 3 hours of thawing with best results in PROS. However, after 6 hours of thawing the CONT group had best motility. Despite the difference in motility observed after 3 and 6 hours, there was no differences in the rate of oocyte fertilization, pregnancy rate at FTAI and IVEP when the extender was used. In conclusion, the use of the semen extender (Prosêmen®) demonstrated results similar to the group without extender in the biotechniques tested. Therefore, considering the fractionation of the semen straw, we suggest that the use of this extender can reduce the total number of straws, reducing the cost per pregnancy without affecting the efficiency of biotechniques.

A018 TAI/FTET/AI

# Evaluation of mineral and vitamin supplementation (Adapter Kit MIN and VIT, Biogénesis Bagó) in improving fertility in primiparous Nelore

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The strategic supplementation of vitamins and minerals during the pre-IATF period has been associated with improved reproductive performance in beef cows. The lack of selenium, Zinc and Copper, can delay the development and entry of puberty into zebu females and consequently a delay in the genetic program of the properties. The objective of this study was to compare the use of mineral and vitamin supplementation (Adapter Kit MIN and VIT, Biogénesis Bagó) during the mating season in nelore primiparous animals (n = 376) in order to improve fertility. The experiment was conducted at Fazenda Agropecuária Farroupilha, in Paracatu-MG. The experimental groups were G1) 1 dose of Adapter® 20 before and another at the beginning of the IATF protocol (n = 196); G2) control (placebo, n = 180). In this way, the animals (n = 376) were randomly distributed between the groups. The hormonal protocol used was: D0 = progesterone intravaginal device insertion (Cronipres® Mono Dose with 1 g of P4 + application of 2 mg of BE (Bioestrogen®, Biogenesis Bagó, Brazil); D8.5 = Remove P4 device + application of 300 IU of eCG (Ecegon®, Biogenesis Bagó, Brazil), + 75 µg of D-Cloprostenol (PGF2α, Croniben®, Biogenesis Bagó, Brazil) + 1mg BE (Bioestrogen® , Biogenesis Bagó, Brazil). In the D10 the IATF was performed in the morning. Cyclicity rate and pregnancy rate (TP) were evaluated by ultrasonography (Mindray M5 Vet, with linear probe of 5.0 MHz). The evaluation of Prenhez was performed 30 and 60 days after IATF. The data were submitted to frequency analysis by PROC FREQ and logistic regression analysis by PROC LOGISTIC, using the program Statistical Analyzes System (SAS, 9.3), adopting Significance level of 5%. There was an increase in the rate of cyclicity at the D0 of the protocol (G1 = 56.6, G2 = 47.7 P = 0.04), at the pregnancy rate at 30 (G1 = 53.6%, G2 = 45.6% P = 0.03) in heifers of the treated group. There was also a higher rate of cyclicity in treated heifers that were empty at diagnosis (G1 = 62.6, G2 = 48.9 P = 0.01) as well as at the 60 day pregnancy rate (G1 = 52.1%, G2 = 43.9%, P = 0.04). Therefore, strategic supplementation with Adapter Kit (Biogénesis Bagó) 20 days before and at the beginning of the protocol was efficient in improving the cycling and the pregnancy rate to IATF of Nellore heifers.

A019 TAI/FTET/AI

# Evaluation of mineral and vitamin supplementation (Kit Adaptador MIN and VIT, Biogénesis Bagó) in improving fertility in primiparous Nelore

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The strategic supplementation of vitamins and minerals during the pre-IATF period has been associated with improved reproductive performance in beef cows. The lack of selenium, Zinc and Copper occurs in most of the animals, throughout the national territory, especially in periods of higher metabolic growth and lactation. The objective of this study was to compare the use of mineral and vitamin supplementation (Adapter Kit MIN and VIT, Biogénesis Bagó) during the mating season in nelore primiparous animals (n = 532) in order to improve fertility increases. The experiment was conducted at Fazenda Agropecuária Farroupilha, in the municipality of Paracatu-MG. The experimental groups were G1) 1 dose of Adapter® 20 before and another at the beginning of the IATF protocol; G2) control (placebo). In this way, the animals (n = 532) were randomly distributed between the groups. The hormonal protocol used was: D0 = intravaginal progesterone device insertion (Cronipres® Mono Dose with 1 g of P4) + application of 2 mg of BE (Bioestrogen®, Biogenesis Bagó, Brazil); D8.5 = Remove P4 device + application of 300 IU of eCG (Ecegon®, Biogenesis Bagó, Brazil), + 75 μg of D-Cloprostenol (PGF2α, Croniben®, Biogenesis Bagó, Brazil) + 1mg BE (Bioestrogen® , Biogenesis Bagó, Brazil). In the D10 the IATF was performed in the morning. Cyclicity rate and pregnancy rate (TP) were evaluated by ultrasonography (Mindray M5 Vet, with linear probe of 5.0 MHz). The evaluation of Prenhez was performed 30 and 60 days after IATF. The data were submitted to frequency analysis by PROC FREQ and logistic regression analysis by PROC LOGISTIC, using the program Statistical Analyzes System (SAS, 9.3), adopting Significance level of 5%. There was no significant difference in the D0 cycling rate of the protocol (G1 = 52.4, G2 = 49.7), although gains in cycling were higher in the treated group. The pregnancy rate at 30 (G1 = 56.7%, G2 = 52%) and at 60 days (G1 = 56.1%, G2 = 50.3%) was higher in cows treated with Adaptaor Kit ($P < 0.05$ ). However, unlike other studies conducted, there was no difference in the rate of cyclicity in the supplemented cows that were evaluated for resynchronization at the 30 day diagnosis (G1 = 55.2%, G2 = 54.3%), which may be linked to low Rate of the treated group before the first supplementation. Cows receiving injectable supplementation of the adapter had a greater follicular diameter (G1 = 16.3 mm, G2 = 13.74 mm) evaluated at the time of FTAI ($P < 0.05$). TAI/FTET/AI

A020 TAI/FTET/AI

# Evaluation of mineral and vitamin supplementation (Adapter Kit MIN and VIT, Biogénesis Bagó) in the improvement of fertility in embryo recipients

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The strategic supplementation of vitamins and minerals during the pre-TETF period has been associated with improved reproductive performance and rate of recovery in embryo recipient heifers. The lack of selenium, Zinc and Copper occurs in most of the animals, throughout the national territory, especially in periods of higher metabolic growth and lactation. The aim of this study was to compare the use of mineral and vitamin supplementation (Adapter Kit MIN and VIT, Biogénesis Bagó) during the next embryo transfer program in cross heifers (Indicus X Taurus) (n = 286) with To improve cyclicity and fertility. The experiment was conducted at the Paineiras farm, in the municipality of Mogi Mirim-SP. The experimental groups were G1) 1 dose of Adapter® 20 before and another at the beginning of the TETF protocol; G2) control (placebo). In this way, the animals (n = 286) were randomly distributed between the groups. The hormonal protocol used was: D0 = P4 intavaginal device insertion ( Cronipres® Mono Dose with 1 g of P4 )+ application of 2 mg of BE (Bioestrogen®, Biogenesis Bagó, Brazil); D8.5 = remove p4 device + application of 300 IU of eCG (Ecegon®, Biogenesis Bagó, Brazil), + 75 µg of D-Cloprostenol (PGF2α, Croniben®, Biogenesis Bagó, Brazil) + 1mg BE (Bioestrogen® , Biogenesis Bagó, Brazil). In D17 embryo transfer was performed in the morning. Cyclicity rate and pregnancy rate (TP) were evaluated by ultrasonography (Mindray M5 Vet, with linear probe of 5.0 MHz). The evaluation of Prenhez was performed 30 and 60 days after IATF. The data were submitted to frequency analysis by PROC FREQ and logistic regression analysis by PROC LOGISTIC, using the program Statistical Analyzes System (SAS, 9.3), adopting Significance level of 5%. There was no significant difference in the D0 cycling rate of the protocol (G1 = 53.5, G2 = 57.5). The rate of recovery for TETF was greater in the treated group (G1 = 86.4%, G2 = 81.3%), as well as the pregnancy rate 30 (G1 = 58.3%, G2 = 50.8%) and At 60 days after TETF (G1 = 55.1%, G2 = 44.2%). The cyclicity rate of empty heifers at diagnosis was greater in the supplemented group (G1 = 83.2%, G2 = 62.7). Therefore, strategic supplementation with Adapter Kit (Biogénesis Bagó) 20 days before and at the beginning of the protocol was efficient in improving the results of TETF protocols in cross heifers.

A021 TAI/FTET/AI

# Evaluation of the strategic use of GnRH (Gonaxal ®, Biogenesis Bagó) in the improvement of fertility in beef suclked cows

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Correct execution and manipulation of follicular development at the most appropriate times of the estrous cycle can generate better results in IATF protocols in beef cows. The objective of this study was to compare the Ovulation and Synchronization rate in fixed time artificial insemination (FTAI) and resynchronization protocols in nelore cows receiving strategic supplementation with Gonaxal® (Biogenesis Bagó). The experiment was conducted at Agropecuária Farroupilha Farm, in Paracatu City (Brazil) and São Paulo Farm in a city of Varjão de Minas. The experimental groups were G1) Control; G2) 1 dose of Gonaxal at the beginning of the IATF protocol; G3) 1 dose of Gonaxal at TAI moment; G4) 1 dose of Gonaxal at the beginning of the protocol and another dose at the FTAI moment. In this way, the animals (n = 1180) with and without corpus luteum at the beginning of the protocol were equaly distributed between the groups. The baseline hormonal protocol used was: D0 = Cronipres® Mono Dose intravaginal device with 1 g of Progesterone (P4)+ application of 2 mg of estradiol benzoate (EB) (Bioestrogen®, Biogenesis Bagó, Brazil); D8.5 = removed intravaginal device + application of 300 IU of eCG (Ecegon®, Biogenesis Bagó, Brazil), + 75 µg of D-Cloprostenol (PGF2α, Croniben®, Biogenesis Bagó, Brazil) + 1mg EB (Bioestrogen® , Biogenesis Bagó, Brazil). In the D10 the IATF was performed in the morning. Cyclicity rate and pregnancy rate (TP) were evaluated by ultrasonography (Mindray M5 Vet, with linear probe of 5.0 MHz). The evaluation of Pregnancy rates was performed 30 and 60 days after FTAI. The data were submitted to frequency analysis by PROC FREQ and logistic regression analysis by PROC LOGISTIC, using the program Statistical Analyzes System (SAS, 9.3), adopting a significance level of 5%. The pregnancy rate at 30 days (G1 = 51.5%, G2 = 57.7%, G3 = 62.9%, G4 = 64.7%) and at 60 days (G1 = 49%, G2 = G4 = 63.5%) was higher in treated cows receiving Gonaxal, mainly on day of FTAI ($P < 0.05$). There was no difference in follicular diameter, measured at the time of FTAI between the experimental groups (G1 = 15.2mm, G2 = 14.9mm, G3 = 16.1mm, G4 = 15.5mm). However, when ovulation rate was assessed, it was also higher in treated cows receiving Gonaxal, especially on day of FTAI ($P < 0.05$) (G1 = 82.3%, G2 = 87.2%, G3 = 89.6 G4 = 94.5%). Therefore, the strategic use of Gonaxal® (Biogénesis Bagó) in the FTAI protocols of beef suckled cows was efficient to improve the pregnancy rate; Especially when the cows receive at FTAI moment.

A022 TAI/FTET/AI

# Evaluation of the pregnancy rates in primiparous buffaloes submitted to FTAI with refrigerated semen vs. frozen during the unfavorable breeding station

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The buffaloes present high incidence of anestrous during the unfavorable reproductive season, which increases the conception interval and, consequently, negatively affects the reproductive performance. To overcome these limitations, IATF protocols have been used, associated with ovulation synchronization, making this biotechnology applicable to the field. The objective was to compare the reproductive efficiency of milk buffaloes submitted to FTAI with refrigerated and frozen semen (diluted in Botu-Bov®). This experiment was carried out in Oliveira/MG, Brazil Latitude 20° 41'45" South and Longitude 44º 49' 37" West, in the period of March 2017. Semen collections were performed with artificial vagina, using 3 bulls following the parameters recommended by CBRA (2013). For this, 90 primiparous buffalo females with the following means: age 3.3 years; Weight 435.8 kg; ECC = 3.3 (1-5) and with 75.6 days postpartum were used. The buffaloes were randomly distributed into three groups: G1 (n = 30), G2 (n = 30) and G3 (n = 30), subdivided, half inseminated with refrigerated semen and half with frozen semen. The three groups were inseminated with semen of 1 bull each, and the ejaculate was fractionated in two aliquots (½ refrigerated/24hs and ½ frozen both with dose of 50x106 total SPTZ/dose). In the afternoon (T) D0 (14: 00hs), the animals received 2.0 mg im of BE (Estrogin®, Farmavet, SP, Brazil) and atrial implantation (CRESTAR® 3.0 mg P4, MSD, Brazil). In D9 (T) withdrawn from the implant and 400UI im eCG application (Folligon® 5000, MSD, Brazil) + 0.530 mg PGF2α Cloprostenol im (Sincrocio®, Ourofino, SP, Brazil). In D10 (T) applied 1.0 mg i.e. of BE (Estrogin®) and in D12 (8:00 hs) AI. Thirty days after AI, the animals were submitted to the diagnosis of gestation by ultrasonography. The results were submitted to the 5% chi-square test. The total pregnancy rate was 50.0% (45/90), 55.6$^{a}$% (25/45) and 44.4$^{a}$% (20/45) for refrigerated and frozen semen ($p>0.05$), respectively. It is concluded that the use of refrigerated semen in the IATF is one of the alternatives to improve the pregnancy rate during the unfavorable reproductive season.

A023 TAI/FTET/AI

# Evaluation of morphological characteristics of corpus luteum from pregnant and non-pregnant cows after 20 days of FTAI (Fixed-time Artificial Insemination)

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The corpus luteum (CL) is a highly vascularized structure, which increases concomitantly with the production of P4 that follows ovulation and it leads an early identification of pregnant females. Thus, it was aimed to perform a morphological evaluation of the corpus luteum, 20 days after FTAI doing a correlation and identification of early pregnancy by Doppler Ultrasonography (USG). For that, 49 Nelore cows were used with a protocol for synchronization of estrus and ovulation, on the day labeled as day minus 10 (D-10) they received a new progesterone (P4) intravaginal device (CIDR®, Zoetis, São Paulo, Brazil) which contains 19,4 g of P4 associated with 2 mg of Estradiol Benzoate (GONADIOL®, Zoetis, São Paulo, Brasil) i.m. On the day minus 2 (D-2) this P4 device was removed following an administration of 12,5 mg of Dinoprost Tromethamine (PGF2α) (LUTALYSE®, Zoetis, São Paulo, Brazil) i.m and 300 UI of eCG i.m (eCG, NOVORMON ®, MSD Saúde Animal, São Paulo, Brazil). In addition, 48h to 54h after removal of the P4 device, these animals were inseminated using cryopreserved doses of a semen from a unique Nelore bull. The luteal parameters were measured 20 days after FTAI (Mindray Z5, Shenzhen, China) by USG on B-mode and Color Doppler in order to determine CL diameter (CLD cm), CL area (CLA cm2), CL volume (CLV) and the ratio of CL area (CLR %). Corpora luteal images were stored and analyzed using an USG (color Doppler mode). Following 34 days after FTAIs, the definite pregnancy diagnoses (PD) of the animals were performed using USG on B-mode having as a positive diagnosis (PD) the visualization of the embryo and its feasibility with the presence of heart beating. Statistical analysis was performed using the analysis of variance (ANOVA) and using Tukey test on Statistical Package for Social Science (SPSS, version 19) program ($p<0,05$). It was observed in the first line of outcomes that there was significant difference for all evaluated parameters, with the mean numbers of pregnant cows of CLD 1,87±0,24 cm, CLA 2,71±0,67cm², CLV 1,37±0,56 cm², CLR 50,77±20,90% and non-pregnant cows were ,50±0,29 cm, 1,75±0,73 cm², 0,51±0,54 cm², 24,57±21,47%, respectively. During the evaluation of pregnant and non-pregnant groups, it was detected the averages of 1,68±0,26 cm for CLD, 2,23±0,70 cm² for CLA, 0,96±0,55 cm² for CLV and 37,67±21,18% for the proportion of the vascularization area in relation to the total CL area. It was observed statistical difference ($P<0,05$) in all parameters among the experimental groups. In view of that, there is a significant difference among CL from pregnant and non-pregnant cows, it was concluded that possibly the USG color Doppler mode technique can be used for morphological evaluations of the CL of cows, showing differences between pregnant and non-pregnant cows, and can be used as a tool for early diagnosis at 20 days.

A024 TAI/FTET/AI

# Evaluation of E2/P4 protocols with or without pre-synchronization

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The objective of this study was to compare if in protocols that initiate with GnRH+EB the pre-synchronization (PS) improve fertility. This study used a total of 665 lactating Holstein cows. At 31±0.56 DIM the cows were divided in 3 groups (d -28 of experimental design): (I.GnRH) an intravaginal P4 insert containing 1.9 g of P4 (CIDR, Zoetis, São Paulo, Brazil), 2.0 mg estradiol benzoate (EB, i.m.; 2.0 mL of Estrogin, Farmavet, São Paulo, SP, Brazil) and GnRH (i.m. 2.0 mL of Cystorelin®, Merial, SP, Brazil) on d -11, 25 mg (i.m.) dinoprost tromethamine (PGF; 5.0 mL of Lutalyse, Zoetis, Brazil) on d -4, CIDR withdrawal, 1.0 mg (i.m.) of estradiol cypionate (0.5 mL of E.C.P., Zoetis, Brazil) and PGF on d -2, and TAI on d 0; (II. PS + GnRH) a pre - synchronization beginning with CIDR insertion + EB at d -28, PGF+ECP and CIDR removal at d -21, and starting the same protocol as GnRH treatment at d -11; (III. PS + EB) the same protocol as "PS + GnRH", regardless without GnRH treatment at d-11.The PROC GLIMMIX was used to evaluate the binomial variables and PROC MIXED to evaluate continuous variables. Was considered significant when $P<0.05$ and trend when $P<0.1$. The proportion of cows with CL at d -28 not differ between treatments ($P = 0.23$). At d -11 there was a higher proportion of cows with CL in the PS groups (78.4% [326/416];$P < 0.01$), compared to cows not PS (60.6% [151/249]). However, the circulating P4 concentration not differ (no PS = 2.53±0.14 vs. PS = 2.76±0.11 ng/mL; $P=0.19$). There were differences in the distribution of classes of circulating P4 concentration at d -11, the group of not PS presented more cows with circulating P4 concentration <1.0 and ≥ 5.0 ng/mL, the group of PS there were more cows between 1.0 and 4.99 ng/mL. Ovulation of the protocol was not affected by treatment (88.4%; $P= 0.20$). There were no effects of treatment on P/AI at the 32d (GnRH = 38.2% [96/249], PS+GnRH = 42.1% [88/209], PS+EB = 40.6% [84/207]; $P = 0.68$), at 60d (GnRH = 31.7% [79/249], PS+GnRH = 35.9% [75/209], PS+EB = 32.4% [67/207]; $P = 0.61$) and on pregnancy loss between 32 and 60d (GnRH = 16.8% [17/96], PS+GnRH = 14.8% [13/88], PS+EB = 20.2% [147/84]; ($P = 0.64$). Analyzing only cows that ovulate to the protocol, no effect was observed on P/AI at 32, 60d and pregnancy loss. There were no effects of treatments and interactions between treatment and heat stress ($P = 0.78$), parity ($P = 0.84$), milk yield ($P = 0.81$), presence or absence of a CL on d -11 ($P = 0.22$), and circulating P4 concentration at d -11 ($P = 0.17$). There were effects these variables to reduce P/AI such as: cows with heat stress (≥ 39.1ºC; $P < 0.01$), lower BCS (< 2.75; $P <0.01$), multiparous compared to primiparous cows ($P < 0.01$), cows without compared to with a CL on d -11 ($P <0.01$) and circulating P4 concentration at d -11 ($P < 0.01$). A tendency of interaction ($P = 0.07$) between treatment and CL presence at d -28 was observed, in cows without CL at d -28 both PS treatments improve the P/AI (GnRH = 28% [35/125], PS+GnRH = 36.8% [42/114], PS+EB = 34.9% [38/109]) and in cows with CL the PS treatments decrease the P/AI compared to GnRH treatment (GnRH = 38.6% [34/88], PS+GnRH = 32.2% [19/59], PS+EB = 23.5% [16/68]). The circulating P4 concentration have a linear effect (higher P4 = higher P/AI; $P<0.01$) on P/AI at 32d. In conclusion, there was no effect of PS on P/AI. However, PS increased P/AI in cows that did not have a CL present at the beginning of the experiment. PS treatments can be used for animals that do not have a CL present in the postpartum period in order to improve P/AI.

A025 TAI/FTET/AI

# Evaluation of sexed or conventional semen in Holstein cows in lactation submitted to TAI protocols

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The aim of this study was to evaluate the results of the use of sexed or conventional semen, from the same bull, in lactating cows submitted to TAI protocols based on GnRH + E2 / P4. All cows were synchronized with the following TAI protocol: 2mg estradiol benzoate im (Gonadiol®, Zoetis, SP, Brazil) + 100 mg gonadorelin diacetatetetrahydrate im (Cystorelin®, Merial, SP, Brazil) administered concomitantly with 1.9 mg P4 intravaginal device (CIDR®, Zoetis, SP, Brazil); In D-4, 25 mg of dinoprost i.m (Lutalyse®, Zoetis, SP, Brazil) was administered; In D-2, 25 mg of dinoprost i.m (Lutalyse®, Zoetis, SP, Brazil) + 1.0 mg i.m. estradiol cypionate (ECP, Zoetis, SP, Brazil); In D0 TAI. The ovaries of the animals were evaluated by ultrasonography on D-11 to determine the presence of CL and in D0 the diameter of the largest follicle and the rectal temperature were measured. In D-2 the animals had the base of the tail marked with a marker stick for evaluation of estrus expression performed at D0. Production lots were divided by parity, days postpartum and milk production. Six bulls were used, with 50% of the inseminations of each bull being sexed semen and 50% with conventional semen. The cows were randomly assigned to receive the IATF. Pregnancy diagnosis was performed on D30 and D59 after AI. The relation of the pregnancy rate between sexed and conventional semen was evaluated. In order to evaluate the binomial variables we used the PROC GLIMMIX of the SAS and the continuous ones the MIXED. Significance was considered when $P \leq 0.05$ and tendency when $0.05 < P \leq 0.10$].The mean AI to AI (193 x 197 ± 121), milk production (31 x 31.5 ± 7.1), number of inseminations (3.1 x 3.2 ± 1.7), ECC (3, 1 x 3.1 ± 0.3), temperature at D0 (38.8 x 38.8 ± 0.4); Conventional and sexed semen, respectively, were the same among treatments; As well as estrous expression of 94%. Conventional semen had a higher pregnancy rate at 30 days [Conv = 34.7% (176/506) vs. Sex = 24.2% (118/487); P <0.01) and at 60 days of gestation (Conv = 28.0% (141/506) vs. Sex = 19.4% (94/487); P <0.01] in relation to sexed semen. There was no difference in the loss of pregnancy [Conv = 19.9% (35/176) vs. Sex = 20.3% (24/118); P> 0.10]. Interaction between treatment and order was detected. There was no treatment effect between Primiparas at 30 days [Conv = 34.3% (50/146) vs. Sex = 30.6% (41/134); P = 0.50], but in the Multiparas, pregnancy at 30 days was lower when sexed semen was used [Conv = 35% (126/360) vs. Sex = 21.8% (77/353); P <0.01]. It was detected a bull's effect on pregnancy (P <0.01); Bull 01 (Conv = 26.9% (18/67) vs. Sex = 22.2% (14/63) P = 0.57); Bull 02 (Conv = 38.0% (41/108) vs. Sex = 24.0% (25/104) P = 0.02); Bull 03 (Conv = 21.0% (12/57) vs. Sex = 22.4% (13/58) P = 0.87); Bull 04 (Conv = 34.2% (38/111) vs. Sex = 20.4% (21/103) P = 0.02); Bull 05 (Conv = 46.3% (50/108) vs. Sex = 36.6% (37/101) P = 0.12); Bull 06 (Conv = 30.9% (17/55) vs. Sex = 13.7% (8/58) P = 0.04). The relation between sexed and conventional semen was higher in primiparous than in multiparous (89% in primiparous and 62% in multiparous), and this proportion was bull dependent and ranged from 40% to 106%.The detection of bulls with a higher rate between sexed and convencional semen would allow more intense use of sexed semen in lactating cows.

A026 TAI/FTET/AI

# Evaluation of the J-Synch protocol associated with eCG and estrous detection in beef heifers

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The J-Synch protocol was developed for prolonging proestrus lenght, aiming to enhance follicular development, oestradiol concentration and promoting the ovulation of a larger follicle. The aim of this study was evaluate the J-Synch protocol associated with eCG and estrous detection. The experiment was carried out with 295 heifers Braford (Bos taurus x Bos indicus). The heifers were randomly allocated into one of two groups: ECP or J-Synch. On Day 0, all heifers received 2 mg of estradiol benzoate (Sincrodiol, Ourofino®, Cravinhos, Brazil) and an intravaginal device with 1 g of progesterone (Sincrogest, Ourofino®, Cravinhos, Brazil). The heifers in the ECP group received 500 µg of cloprostenol (Sincrocio, Ouro Fino®, Cravinhos, Brazil) on Day 7. On Day 9, the device was removed and the heifers received 1 mg of estradiol cypionate (SincroCP, Ouro Fino®, Cravinhos, Brazil) and 200 IU eCG (SincroECG, Ouro Fino®, Cravinhos, Brazil). Estrous detection was performed on Day 10 during an hour. The heifers in estrous (n=62) were inseminated 12 hours later and those not (n=84) were inseminated in fixed-time 48 hours after the progesterone device was removed. For heifers in the J-Synch group the progesterone device was removed on Day 6, and the heifers also received 500 µg cloprostenol and 250 IU eCG in the same day. Estrous detection was performed on Day 8 during an hour. The heifers standing estrus (n=39) were inseminated after 12 hours and those not (n=110) were inseminated in fixed-timed 72 hours after the device was removed. All heifers in J- Synch group received 10 µg GnRH (Sincroforte, Ourofino®, Cravinhos, Brazil) at the moment of insemination. The results were analyzed by Chi-square test. Overall pregnancy rate did not differ (P=0.68) between J-Synch (60.40%) and ECP groups (50.68%). In the ECP group, more heifers standing estrus before time of FTAI than J-Synch group (P=0.003). In the ECP group, the pregnancy rate was higher (P=0.001) for heifers demonstrating estrous (66.13%) than those not (39.28%). However, the same was not observed for heifers in the J-Synch protocol (P=0.58). Comparing only heifers demonstrating estrous, pregnancy rate was not different (P=0.83) between ECP and J-Synch groups. In conclusion, heifer in the J-Synch protocol had a pregnancy rate similar to heifer in the ECP protocol, and no difference was observed in the pregnancy rate for heifers demostrating estrous.

A027 TAI/FTET/AI

# Animal temperament and difficulty of insemination on Timed-AI fertility of Nellore cows and heifers

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In order to investigate if stress caused by AI can affect a reproductive program, this study aimed to evaluate temperament (Te), chute exit period (ExP), AI difficulty (DifAI) and time for AI accomplishment (tAI) of Nelore females at Timed-AI. Management of 165 Nelore females was monitored, being 93 multiparous and 72 heifers. For each animal, cattle reactivity was recorded from 1 to 5, according to behavior characteristics (Te1 = very calm animals in the chute and Te5 = very temperamental animals) and time of chute exit both on D9 (shortly after progesterone implant removal) and on D11 (shortly after Timed-AI). In addition, on D11, time for AI accomplishment was recorded and the technician defined a score (1 to 3) regarding to the difficulty of performing AI procedure (1 for mild, 2 for moderate and 3 for high degree of AI difficulty). Only animals with BCS between 3 and 4 (between 1 to 5) were included in the study. The two AI technicians shifted between them at each 5 animals entering the chute. Pregnancy rate (PR) was compared between groups using Fisher's exact test (P=0.05) in GraphPad INSTAT program. Overall pregnancy rate (PR) was 40%, being 35% for multiparous and 46% for heifers (P = 0.09). No effect of BCS and AI technitian on PR was observed (P>0,05). No effect (P>0.05) of D9 temperament on PR was observed, neither for multiparous (Te2=36%, n=33; Te3=38%, n=44; Te4-5=25%, n=16), heifers (Te2=0 animals; Te3=50%, n=38; Te4-5=41%, n=34) nor for both categories assessed togheter (Te2=36%, n=33; Te3=44%, n=82; Te4-5=36%, n=50). Similarly, no effect (P>0.05) of D11 temperament on PR was observed, neither for multiparous (Te2=30%, n=27; Te3=43%, n=46; Te4-5=25%, n=20), heifers (Te2 = 0 animals; Te3=47%, n=17; Te4-5=45%, n=55) nor for both categories assessed togheter (Te2=30%, n=27; Te3=44%, n=63; Te4-5=40%, n=75). In all analyzes cited above, Te2 animals left the chute more slowly (P<0.001) than Te3 animals, and these were slower (P<0.001) than Te4-5 animals, in both passages (D9 and D11). The mean ExP of all females at D11 was 05:20±01:08sec for Te2 (n=27), 03:40±1:16sec for Te3 (n=63) and 02:43±00:55sec for Te4-5 (n=75). Assessing DifAI for multiparous and heifers together, a tendency for higher (P = 0.09) PR was observed in animals with lower difficulty in performing AI procedure (DifAI 1 = 42%, n = 143 A; DifAI 2-3 = 27%, n = 22 B). Comparing these two groups, lower (P <0.001) time was necessary for completing AI procedure for DifAI 1 (tAI=17:31±06:02sec) than for DifAI2-3 (tAI=30:10±15:45 sec). Spliting animals categories, the reduction observed in PR regarding to DifAI was only numerical (multiparous: DifAI1=37%, n=76; Dif2-3=29%, n=17; Heifers: DifAI1=48%, n=62; Dif2-3=20%, n=10). Animal behavior in the chute, assessed by reactivity and escape time, did not affect PR of Nelore females in this study, but those animals in which difficulty and/or time for AI accomplishment is higher, lower fertility at Timed-AI may be expected.

A028 TAI/FTET/AI

# Dairy buffaloes present a similar pregnancy rate when submitted to be and P4 based protocol for FTAI during the breeding and non-breeding seasons

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The objective of the present study was to compare the pregnancy rate of dairy buffaloes submitted to BE and P4 based protocol for FTAI during the breeding (autumn and winter, n=359) and non-breeding (spring and summer; n=483) seasons. At a random stage of the estrous cycle (D0; afternoon, 16h), 842 buffaloes received an intravaginal progesterone device (0.5g of P4; Primer® Monodose, Agener União Saúde Animal, Brazil) and 2.0mg im of Estradiol Benzoate (BE; FertilCare® Sincronização, Vallée, Brazil). In D9 (16h), females received 0.53mg im of Cloprostenol sodium (PGF2α, Ciosin®, MSD Animal Health, Brazil) and 400IU im of eCG (Folligon®, MSD Animal Health), followed by P4 removal. After 24h (D10, 16h), the ovulation was induced by the injection of 1.0mg im of BE (FertilCare® Sincronização, Vallée). All animals were submitted to FTAI 64h after administration of $PGF_{2\alpha}$ (D12; morning, 8h). The pregnancy diagnosis was made 30 days later (D42; Mindray DP2200Vet). The pregnancy rate was analyzed by the GLIMMIX procedure of SAS®. There was no difference in the pregnancy rate of buffaloes submitted to FTAI during the breeding [45.1% (162/359)] and non-breeding [47.4% (229/483); P=0.51] seasons. In conclusion, dairy buffaloes present a similar pregnancy rate when submitted to BE and P4 based protocol for FTAI during the breeding and non-breeding seasons.

A029 TAI/FTET/AI

# Comparison of two protocols to increase circulating progesterone concentration before timed artificial insemination in lactating dairy cows

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Two treatments designed to increase circulating progesterone concentration (P4) during preovulatory follicle development were compared. One treatment utilized 2 intravaginal P4 implants (CIDR) and the other utilized GnRH treatment at beginning of the protocol. Lactating Holstein cows were randomly assigned to receive time artificial insemination (TAI) following one of two treatments (n = 379 breedings): (GnRH) d-11 2mg of estradiol benzoate (EB, 2.0 mL of Estrogin®, Farmavet, SP-Brazil) + 100 µg of GnRH (2.0 mL of Cystorelin®, Merial, SP) + an intravaginal P4 insert containing 1.9 g of P4 (CIDR®, Zoetis, SP-Brazil); D -4 25 mg of PGF (5.0 mL of Lutalyse®, Zoetis, SP-Brazil); D-2 withdrawal of CIDR + PGF + 1mg ECP (0.5 mL of ECP®, Zoetis, SP-Brazil), d0 TAI; (2CIDR) d-11 2mg EB (Estrogin®, Farmavet, SP-Brazil) + 2CIDR; D-4 PGF (Lutalyse®, Zoetis, SP-Brazil) + removal of a CIDR; D-2 withdrawal of the remaining CIDR + PGF + 1mg ECP (ECP®, Zoetis, SP-Brazil), d0 TAI. Cows with temperature ≥39.1ºC at the time of TAI were considered in heat stress. The diagnosis of gestation was performed 30 days after AI, where pregnancy by insemination (P/AI) was calculated dividing the number of pregnant cows by the number of inseminated cows. The PROC GLIMMIX was used to evaluate the binomial variables and the PROC MIXED to evaluate the continuous variables, significance was considered when P <0.05 and tendency when P <0.1. There was no difference between treatments in P/AI (GnRH = 31.6% [65/206] vs. 2CIDR 33.0% [57/173]; P = 0.77). There was no interaction between treatment and parity order in P/AI (GnRH = Primiparous 35.1% [27/77]; Multiparous 29.5% [38/129] vs. 2CIDR = Primiparous 26.2% [16/61]; Multiparous 36.6% [41/112]; P = 0.11), presence of corpus luteum at the beginning of the protocol (GnRH = no CL 29.3% [22/75]; CL 32.8% [43/131] vs. 2CIDR = no CL 24.1% [13/54]; CL 40.0% [44/119]; (P=0.36), days in lactation (P = 0.68) and heat stress (GnRH = ≤39.1ºC 34.1% [56/164]; >39.1ºC 20.5% [8/39] vs. 2CIDR = ≤39.1ºC 36.0% [49/136]; >39.1ºC 21.6% [8/37]; P=0.92). Parity order (Primiparous = 31.2% [43/138] vs. Multiparous = 32.8% [79/241]; P= 0.74), days at lactation (P = 0.23) and presence of corpus luteum at the beginning of the protocol (no CL = 27.1% [35/129] vs. CL = 34.8% [87/250]; P=0.13) had no effect on P/AI, however, cows under heat stress presented lower P/AI (<39.1 = 34.8% vs. ≥39.1 = 21.1%; P = 0.02). Both protocols resulted in similar fertility, but probably due to different physiological changes, treatment with GnRH probably increased the proportion of cows with corpus luteum at the time of the first PGF and the protocol with 2 CIDR probably increased the concentration of progesterone in all cows.

A030 TAI/FTET/AI

# Comparison of estradiol benzoate and cypionate in the induction of ovulation of Girolando cows submitted to timed artificial insemination

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Among the main esters used for timed artificial insemination (TAI) programs in bovine females, estradiol benzoate (EB) and estradiol cypionate (EC) are more frequently used because of their low cost. However, both are different in terms of use because they have different half-lives. In order to reduce the management in TAI protocols, the present study evaluated the follicular dynamics in dairy cows treated with EB or EC as inducers of ovulation. For this purpose, 59 Girolando cows, lactating, with a mean body condition score of 3.06±0.49 (1-5 scale) were used. On a random day of the estrous cycle, the animals received intravaginal implant containing 1g of progestogen (DIB®, Coopers, São Paulo, Brazil) and an intramuscular injection (im) of 2mg of EB, being this day considered D0. At D8, the implant was removed and 500µg of cloprostenol (CIOSIN®, Intervet Schering Plow Animal Health, Sao Paulo, Brazil) was administered in all females. Cows were then randomly allocated into two treatments: Group EB (n = 33) and Group EC (n = 26). The animals of the EC Group received 1mg of EC (im) (ECP®, Pfizer, São Paulo, Brazil) at the moment of implant removal, while the EB Group cows received 1mg of EB (im) (ESTROGIN® , Agroline, São Paulo, Brazil) 24 hours later (D9). After implant removal, ultrasound evaluations were performed every 12 hours up to ovulation. The following parameters were evaluated: ovulatory follicle diameter - OF (mm); Ovulation rate - OR (%), implant withdrawn / ovulation interval - WOI (hours) and pregnancy rate (PR). For statistical analysis, the chi-square test was used for OR and PR and the "T" test was used for OF and WOI, and a P value of 5% was considered as significant. The results for the groups EB and EC were, respectively: OF: 14.10 ± 0.60 mm and 13.42 ± 0.55 mm; OR: 89.47% and 74.41%; WOI: 68.52 ± 1.70 hours and 63.30 ± 2.05 hours and PR: 54.16% and 64.51%. There was no difference ($p>0.05$) between treatments for any of the parameters evaluated. The results of the present study corroborate those of França et al. (Rev. Bras. Anim. Saúde, v.16, n.4, p.958-965, 2015), who did not observe a difference between benzoate and estradiol cypionate in the synchronization of ovulation of Girolando cows. As Freitas et al. (Anim. Reprod., v.10, n.3, p.401, 2013), who also found no difference for both ovulation inducers in Holstein cows. The results of this study suggest that it is possible to substitute EB for EC in the TAI of Girolando cows, reducing management but maintaining the same efficiency.

A031 TAI/FTET/AI

# Correlations between plasma progesterone and ultrasonographic characteristics of the corpus luteum in fixed-time inseminated Nelore cows

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Ultrasound evaluation of the corpus luteum (CL) may be a practical and real-time method for determining luteal functionality in bovine females. The objective of this study was to evaluate the correlations between plasma progesterone concentrations (P4) and CL size and blood perfusion during its development, maintenance and regression in inseminated cows. For this purpose, Nelore cows (n = 22) had the ovulation synchronized by a hormonal protocol based on estradiol/P4 and were inseminated at fixed time (day 0; D0). Blood samples were collected and B-mode and Doppler ultrasonography (MyLab30 VetGold; Esaote) were performed at D8, 12, 15, 18 and 20. Plasma concentrations of P4 were measured by RIA. At each ultrasonographic examination, the size of CL and luteal cavity (diameter, area and volume) and blood perfusion (peripheral, total and vascularized area) were measured. Volume was estimated from the mean diameter considering the formula for a sphere (4/3 $\pi r3$) and the area was calculated by the tracing function. Pearson correlations were calculated between P4 concentrations and all variables of luteal size and blood perfusion for each day (SAS 9.2 program, SAS). The luteal cavity was observed in 55, 32, 23, 14 and 9% of the cows in the D8, 12, 15, 18 and 20, respectively. There was no difference in the proportion of the cavity in relation to the diameter (27.2 ± 1.8%), area (8.9 ± 1.0%) or CL volume (3.2 ± 0.6%) among D8, 12, 18 and 20. For the CL development phase (D8), significant correlations ($P < 0.05$) with P4 concentrations were observed only for the total area ($r = 0.767$), area without cavity ($r = 0.720$), total diameter ($r = 0.620$), vascularized luteal area ($r = 0.544$), and the cavity volume ($r = 0.212$), area ($r = 0.490$) and diameter ($r = 0.462$).In the medium and late diestrus (D12 and 15) only significant correlation ($P < 0.05$) was observed at D15 with total area and area without cavity ($r > 0.601$), diameter and total volume, and volume and diameter without cavity ($r > 0.434$). For the luteal regression phase (D18 and 20), only moderate correlation ($P < 0.05$) was observed at D18 for the area without cavity ($r = 0.478$) and high correlation ($P < 0.05$) on D20 with several variables (vascularized area [$r = 0.850$], peripheral and total blood perfusion [$r = 0.820$], area without cavity [$r = 0.775$], total area [$r = 0.762$], diameter and volume without cavity [$r > 0.712$], total volume [$r = 0.712$] and total diameter [$r = 0.688$]). However, when analyzed separately for pregnant and non-pregnant cows, significant correlations ($P < 0.05$) were only observed among the variables for the non-pregnant cows, which presented similar values in relation to the analysis with all cows (volume, area, diameter without cavity [$r > 0.735$], vascularized area, peripheral and total perfusion [$r > 0.658$]). In conclusion, CL area may be the best characteristic to evaluate luteal functionality during its development (D8), whereas after the maternal recognition period (D20 post-insemination) the blood perfusion evaluated by Doppler mode shows greater correlation than the luteal size end-points.

A032 TAI/FTET/AI

# Productive and reproductive correlations that influence the pregnancy rate of 14 month old Nellore heifers submitted to 3 FTAI in 48 days using color Doppler ultrasonography

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The objective of this study was to evaluate the correlation between productive and reproductive characteristics during an IATF protocol in the fertility of 14-month-old Nellore heifers. The work was carried in a commercial farm in Camapuã. To that, 631 heifers aging 14.4±0.92 months, weighting 272.9±20.4kg and BCS 3.3±0.28 were submitted to the following TAI protocol: D-10, insertion of an auricular implant with 3mg of Norgestomet (Crestar®, MSD, Brazil) and 1mg of EB (Fertilcare Sincronização®, Vallée) IM. After 8 days (D-2) the implant was removed and 200IU of eCG (Folligon®, MSD), 0.265mg Cloprostenol Sodic (PGF; Ciosin®, MSD) and 0.5mg EC (Fertilcare Ovulação®, Vallée ). The 1st TAI was performed 48h (D0) after implant removal. After 14d (D14), all inseminated heifers were resynchronized with an auricular implant and 50mg of P4 (Afisterone®, Hertape) IM. After 8d (D22), the pregnancy diagnosis was performed by ultrasonography (US) Color Doppler (M5vet®, Mindray). Heifers with an area of CL≥2cm² and/or ≥25% CL blood flow (BF) were diagnosed as pregnant and underwent implant removal without further treatment. Those diagnosed as non-pregnant (CL ≤ 2cm² and/or ≤ 25% of BF) underwent removal of the implant and treatment with 200IU of eCG, 0.265mg of PGF and 0.5mg of EC, with the 2nd IATF 48h after removal of the implant (D24). In D38 the heifers were submitted to the same resynchronization protocol, with a diagnosis of gestation of the 2nd IATF in D46 and the 3rd IATF in the D48. Heifers considered pregnant by US Doppler on D22 were reexamined by US B-mode on the D30 to verify the occurrence of false positive. False-positive heifers were inseminated for the second time on D48. Weight, BCS, and age at D0, and diameter of the largest follicle (DF) by US at D-2 and D0 were evaluated. The data were analyzed by the PROC CORR of SAS. The TAI pregnancy rate was 42.8% (270/631); 2nd TAI = 34.1% (107/314) and 3rd TAI = 34.3% (59/172). The false positive rate was 14.8% (47/317) and the pregnancy rate of these heifers for the second time inseminated in the D48 was 40.4% (19/47), increasing 2% in the pregnancy rate of D48 (3rd TAI + false positive, 35.6%, 78/219). The pregnancy rate at the end of the 48d breeding season was 72.1% (455/631). The diameter of the DF in D-2 was 9.22 ± 2.02 and on D0 10.59 ± 2.04 mm. There was a correlation of pregnancy 30d after TAI with weight ($R^2$ = 0.09, P = 0.03), age ($R^2$ = 0.07, P = 0.06), BCS ($R^2$ = 0.07, P=0,09, DFD0 ($R^2$ = 0.20, P <0.0001) and DFD-2 ($R^2$ = 0.11, P = 0.007). Moreover, negative correlation was observed between the incidence of false positive and weight ($R^2$ = -0.15, P = 0.009), age ($R^2$ = -0.10, P = 0.07), DFD0 ($R^2$ = -0, 15, P = 0.01) and DFD-2 (R 2 = -0.10, P = 0.10). It is concluded that it is possible to obtain pregnancy rates >70% in Nelore heifers at 14 months of age and that there is a positive correlation between weight, age, BCS, DFD0 and DFD-2 with the pregnancy rate 30d after FTAI and negative between Weight, age, DFD0, DFD-2 and the occurrence of false positives.

Acknowledgement: Engano farm.

A033 TAI/FTET/AI

# Diagnosis of gestation through proteins associated with gestation in *Bos indicus* heifers

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Early diagnosis of pregnancy is essential for a better reproduction management, and of fundamental importance to economic viability of livestock farming, because, after the diagnosis is possible to use biotechnology of reproduction and reinseminate non pregnant animals in a short period of time. With the increasing rise of biotechnologies such as TAI (timed artificial insemination), embryo transfer (TE), or by implantation of embryos from IVF, also is necessary the improvement of pregnancy diagnostic techniques. Glycoproteins associated with pregnancy in cattle (bPAGs) or pregnancy specific protein B (bPSPB) are secreted primarily by Trophoblastic binucleated cells, and secreted by placental tissue, therefore, are direct indicators of pregnancy. The present work was carried out in a commercial cattle ranch located in the city of Barra de Guabiraba-PE (Lat 08°25'12", Long 35°39'29"), where 69 nulliparous Nellore heifers with equivalent weight and age, raised under grazing with mineral supplementation and water ad libitum, were subjected to the same protocol of TAI which consisted of 2 mg of Estradiol Benzoate (EB) IM and insertion of 1g of intravaginal Progesterone (P4) device in D0, removing the implant in D8, followed by an application of 300 IU of equine chorionic gonadotropin (eCG) and 150 µg of Prostaglandin (PGF[G1] ). As ovulation inductor, 1 mg of Estradiol Cipionate (EC) was used and after 48 hours the AI was performed. On D25 after TAI, blood of all heifers were collected, centrifuged and serum obtained, identified, frozen and referred to laboratory where the test was carried out for detection of proteins associated with pregnancy by visual immunoassay method. At D32 after TAI, ultrasound examination was performed, as a gold test to validate the results obtained with the ELISA visual test (IDEXX®), which revealed 25 animals pregnant and 44 not pregnant. As results we had a 96% sensitivity (24/25) while the specificity was 100% (44/44). It was concluded that bPAGs pregnancy test using the ELISA visual Kit (IDEXX®), it is a practical test due to its quickness (20 minutes) and is a very good test based upon standards of Statistical Kappa Analysis specificity and sensitivity for diagnosis of pregnancy on the D25 after TAI in nulliparous Nellore heifers.

A034 TAI/FTET/AI

# Diagnosis and early treatment of uterine infections to optimize implantation of ovulation synchronization protocols

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In order to improve reproductive efficiency, many dairy farms have adopted ovulation synchronization protocols and strategies to reduce the calving-to-conception interval. For the success of these protocols it is essential that, at the end of the voluntary waiting period, the cows present healthy uterus and free of infectious or inflammatory conditions. A hipótese foi que o diagnóstico precoce e tratamento das doenças uterinas reduzem o tempo destas vacas estarem aptas à reprodução e a taxa de descartes involuntários. The hypothesis was that the early diagnosis and treatment of uterine diseases decrease the time for cows are be able to reproduce and reducing involuntary culling. The objective of this study was to evaluate the effect of early diagnosis of uterine infections in postpartum dairy cows, such as retained placenta (RP), metritis and clinical endometritis associated with a single treatment of 3.3 mg/kg, IM, ceftiofur hydrochloride (Lactofur®, Ourofino Saude Animal, Brazil), on the interval between calving and release for reproductive management. A total of 168 calving (01/2015 - 12/2015) of Holstein and Girolando dairy cows from a farm in Uberlândia-MG under a semi-confinement system and three daily milking were followed. The animals were evaluated at the time between partum up to 7º DIM (M1), 10º-15º DIM (M2), 25º-35º DIM (M3) and then weekly until release for reproduction. A general physical examination and specific of the female genital tract was performed by transrectal palpation and ultrasonography, intrauterine fluid assessment (IUF), and vaginal discharge (metricheck). Cows diagnosed with RP, metritis and endometritis were treated and monitored weekly until complete macroscopic uterine involution (symmetric horns; no IUF; clean vaginal discharge). The prevalence of RP was 23.2% (39/168) and for metritis of 38.1% (64/168), with 24.4% (41/168) being mild metritis and without the presence of severe metritis or death. The prevalence of clinical endometritis was 33.3% (56/168). The protocol allowed to release 96.4% (162/168) of the cows for breeding in 40.6 ± 16.2 DIM (95%C.I.- 38.1-43.1). There was a significant difference between days of release for reproduction in the presence or absence of RP (45.5 ± 17.8 versus 39.2 ± 15.4), metritis (47.6 ± 20.3 versus 36.6 ± 11.5), and clinical endometritis (57.6 ± 18.0 versus 33.0 ± 6.8). It was concluded that the implantation of an early diagnosis to uterine diseases and the single treatment with ceftiofur hydrochloride was efficient in reducing involuntary culling and the time of the dairy cows to be able to reproduce.

A035 TAI/FTET/AI

# Different doses of equine chorionic gonadotropin in FTAI protocol on *Bos taurus* beef heifers: impact on ovarian response, occurrence of estrus and pregnancy rate

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This study evaluated the effect of different doses (200, 300 or 400 IU) of equine chorionic gonadotrophin (eCG) in FTAI protocols on follicular growth, occurrence of estrus and pregnancy rate in *Bos taurus* beef heifers (Angus, Brangus and Braford). Were utilized 1080 heifers, 24 months old and a body condition score of 2.90 ± 0.02 (1-extremely thin and 5-obese) from 8 commercial beef farms located in two distinct regions of Rio Grande do Sul State, Brazil At the onset of the synchronization protocol (D0), heifers were evaluated in cyclicity (CLD0) and received an intravaginal P4 device (CIDR®, Zoetis, Campinas, SP, Brazil) and 2 mg of estradiol benzoate IM (Gonadiol®, Zoetis, Brazil). On Day 7, were administrated 12.5 mg of dinoprost tromethamine IM (Lutalyse®, Zoetis, Brazil) and on Day 9 (D9), the P4 device was removed and administered 0.5mg of estradiol cypionate IM (E.C.P.®, Zoetis, Brazil). At this moment, heifers were homogeneous distribution by ovarian cyclicity in to three treatments: 200 IU (n=387), 300 IU (n=357) or 400 IU (n=336) of eCG. The females had their tail-heads painted with chalk paint (Raidl-Maxi, Raidex GmbH, Dettingen / Erms, Germany) at the time of removed of P4 device. Females without mark at the time of AI were considered as displayed estrous. The FTAI was 48h later P4 device removal (D11). Additionally, a sample of heifers (n=213) the evaluated in D9 and D11 of largest follicle diameter (LF) and on day 17 the corpus luteum (CL) diameter was measured. The pregnancy diagnosis was evaluated on day 41. Statistical analyses were performed using the GLIMMIX procedure SAS, the averages were compared by Tukey-Kramer test ($P<0.05$). The eCG treatment did not influence the follicular growth (1.52mm/day, 1.40mm/day, 1.75mm/day; P=0.22); LF diameter in D11 (13.41mm±0.29, 13.10mm±0.34, 13.57mm±0.38; P=0.79); estrus occurrence (86.5%±1.73, 84.8%±1.89, 84.8%±1.96; P=0.92); pregnancy rates (52.2%±2.54, 49.77%±2.63, 51.48%±2.73; P=0.46) and CL diameter (18.60±0.57, 17.92±0.67 and 18.93±0.5; P=0.29), respectively, for to dose 200, 300 and 400 IU. This study shows that eCG reduction dose to 200 IU can be used in IATF programs in Bos taurus heifers, without detriment to ovarian response, estrus and pregnancy rate. Acknowledgment: Zoetis Brasil, PPGCA/Unipampa, CAPES, Agrop. Pitangueira, Estância Renascer, Santa Camila, Âncora, Posto Branco, Baviera e Fepagro.

A036 TAI/FTET/AI

# Follicular dynamics in Nelore heifers subjected to TAI protocol using Primer® PR or Primer® Monodose

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This study aimed to evaluate the dynamics of follicular growth in 14 to 16 months old Nelore heifers, synchronized with two different intravaginal P4 device releasers (P4DR). Twenty one Nelore heifers were used (BCS=3,65), distributed in two different experimental groups, G1) Primer® Monodose (0.5g of progesterone, Agener União – Saúde Animal, São Paulo, SP; n=10), and G2) Primer® PR (0.36g of progesterone, Agener União; n=11). All heifers received a norgestomet ear device (Crestar®, MSD Saúde Animal, São Paulo, SP), followed by IM treatment of cloprostenol (0,150 mg of D-clorprostenol, PROLISE®, Agener União) on D-8; on D-1, heifers received cloprostenol treatment (PROLISE®, Agener União) and the ear device was removed. On D0, they received P4DR and 2 mg of EB (RIC-BE®, Agener União). On D8, the P4DR was removed and heifers were treated with 0.6 mg of Estradiol Cipionate, 200 IU of eCG (Folligon, MSD) and cloprostenol (PROLISE®, Agener União). From D0 to D8, transrectal ultrasonography (Mindray® DP-2200Vet) was performed every 24 hours to evaluate the emergence of a new wave of follicular growth using 7.5 MHz frequence. After P4DR removal (D8 to D13) ultrasonography evaluations were made every 12 hours until ovulation time. Variables analyzed in this study: emergence and dispersion of new wave of follicular growth, DF diameter on D8 and D10, follicular growth rate during treatment (beginning of the wave until P4DR removal), ovulation rate and ovulation time. Data were analyzed by GLIMMIX procedure of SAS. It was observed that there was no difference for the day of emergence of new wave of follicular growth (3.8 ± 0.1 for G1 and 3.7 ± 0.2 days for G2; P = 0.76). The DF diameter on D8 was 7.4 ± 0.3 mm for G1 and 7.8 ± 0.4 mm for G2 (P = 0.56). On D10, the DF diameter was 9.5 ± 0.5 mm for G1 and 9.7 ± 0.5 mm for G2 (P = 0.64). The follicular growth rate was 0.88 ± 0.04 mm for G1 and 0.89 ± 0.06 mm for G2 (P= 0.92). Ovulation rate was 80% (8/10) for G1 and 91% (10/11) for G2 (P=0.52). Ovulation happened, on average, 75 ± 2.0 hours after P4DR removal for G1 and 78 ± 3.7 hours for G2 (P = 0.51). It was not observed any difference within groups for no one of the variables analyzed. It is possible to conclude that the use of PRIMER® PR is an alternative to the PRIMER® Monodose to synchronize ovulation in Nelore heifers.
Acknowledgments: Agener União – Saúde Animal.

A037 TAI/FTET/AI

# Immunohistochemical distribution of estrogen receptor alpha (ERα) in the uterus of sows under different hormonal protocols

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The use of fixed-time artificial insemination (FTAI) in sows aims to minimize errors, labor related to estrus detection,estrus cycle variation, interval of ovulation (KNOX, Theriogenology, v.75, p.308-19, 2011), and semen doses per sow in each estrous,consequently, decreasing production cost. Studies on effects of the exogenous ovarian stimulation in sows are scarce and many questions of the possible effects of its use remain unanswered. The uterus is one of the most affected organs under the influence of steroidal hormones. The hormonal changes are regulated by estrogen, mainly ERα and progesterone receptors (Sukjumlong et al., 2009). The distribution of these receptors in the uterus could be related to the ideal environment for the embryo development. The aim of this study was to evaluate the effects of different FTAI protocols on the distribution and quantification of ERα in the uterus of sows using immunohistochemistry (IHC). Thirty-eight sows were randomly assigned into groups: control, eCG (eCG IM 600UI at weaning), GnRH56h (600UI eCG IM at weaning, 50 mcg GnRH IM 56h after eCG) and GnRH80h (600UI eCG IM at weaning, 50 mcg GnRH IM 80h after the eCG). At day 6.5 after AI, animals were euthanised and samples of the uterus were fixed in 10% neutral buffered formalin for 48 hours and routinely processed for histology/IHC. Tissue sections were incubated with a primary antibody (ERα, #SC-7207, rabbit polyclonal, 1:200, Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour, followed by secondary antibody incubation with UV LP HRP polymer (Thermo Fisher Scientific, Fremont, California, USA) for 15 minutes, and visualized using a chromogen complex 3, 3'-diaminobenzidine. For each section, 10 randomly selected high power fields (400x) of the following areas of the uterus were examined for nuclear immunolabeling: superficial epithelium, endometrial stroma, endometrial glands and myometrium. The IHC reactivity was scored as follows: (-) absent, (+) ≤ 30% of nuclear immunolabeling in each area; (++) 31-60% of nuclear immunolabeling in each area, (+++) > 60% of nuclear immunolabeling in each area. Data were analyzed by One-way ANOVA and Tukey test ($P \leq 0.5$). The results are written as mean ± SD as follows for control, eCG, GnRH56h and GnRH80h groups, respectively: superficial epithelium (0.33 ± 0.16, 0.22 ± 0.14, 0.20 ± 0.13 and 0.50 ± 0.26 (P=0.66); endometrial stroma (1.1 ± 0.35, 1.55 ± 0.57, 1.6 ± 0.4, 1.5 ± 0.34) (P=0.78); endometrial glands (1.77 ± 0.49, 2.44 ± 0.37, 2.00 ± 0.33, 2.3 ± 0.30) (P=0.61), and myometrium (0.33 ± 0.16, 0.66 ± 0.16, 0.60 ± 0.16; 0.7 ± 0.15 (P= 0.39). No significant differences were observed among experimental groups for any of the evaluated uterine areas. The estrus cycle synchronization using the proposed AI protocols does not interfere with distribution of ERα in the uterus 6.5 days after AI.

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A038 TAI/FTET/AI

# Effect of prostaglandin administration at the moment of TAI in dairy buffalo submitted to the synchronization of ovulation during the non-breeding season: Preliminary results

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It was evaluated the effect of prostaglandin administration ($PGF_{2\alpha}$) at the moment of FTAI in dairy buffalo submitted to the synchronization of ovulation during the non-breeding season (spring to summer; 24º 26' 15" South and 47º 48' 45" West). The hypothesis is that $PGF_{2\alpha}$ administration at the moment of FTAI increases the synchronization of ovulation, as well as ovulation rate. At random stage of the estrous cycle (D0), 27 dairy buffalo received an intravaginal progesterone device (P4; Sincrogest®, Ourofino Agronegócio, Brazil) and 2mg im of estradiol benzoate (EB, Benzoato HC®, Hertape Calier Saúde Animal S.A., Brazil). In D9, the animals received 0.53mg im of $PGF_{2\alpha}$ (sodium cloprostenol, Sincrocio®, Ourofino Agronegócio, Brazil) and 400IU im of eCG (Folligon®, MSD Saúde Animal, Brazil), followed by P4 removal. After 24h (D10), the ovulation was induced by the application of 1mg im of EB (Benzoato HC®) and 32h later, all buffalo were subjected to FTAI (D11). The animals were submitted to ultrasonographic examinations (Mindray DP2200Vet, China) in D0 to check ovarian activity, in D9 and D10 to measure the follicular diameter (Ø) and from D11 to D14 (12/12h for 60h) to check the moment of ovulation and the ovulatory follicle Ø (OF). The buffalo which present follicles < 9mm in D10 (n=4) were removed from the experiment. The remaining females were divided according to weight, calving number, days postpartum, body condition score, ovarian activity and the largest follicle Ø verified in D10 into two groups: Control (n=11) and $PGF_{2\alpha}$ (n=12). The buffalo of the $PGF_{2\alpha}$ group received 0.53mg im of $PG_{F2\alpha}$ (Sincrocio®, Ourofino Agronegócio, Brazil) at the moment of FTAI. In D19 and D41, the animals were submitted to ultrasonographic examinations (Mindray DP2200Vet) for the measurement of CL Ø and to access the pregnancy rate, respectively. The statistical analysis was performed by GLIMMIX of the SAS®. There was no difference between the experimental groups (Control vs. $PGF_{2\alpha}$) for the analyzed variables: OF Ø (15.4±0.5 vs.14.9±0.6 mm; P=0.52); moment of ovulation (76.7±2.4 vs. 74.7±2.0 h, P=0.58); ovulation rate [81.8% (9/11) vs. 91.7% (11/12); P=0.46]; CL Ø (18.7±0.6 vs. 17.6±0.7 mm; P=0.29) and pregnancy rate [45.4% (5/11) vs. 58.3% (7/12); P=0.55]. According to the conditions of the present study, it was concluded that the $PGF_{2\alpha}$ administration at the moment of FTAI does not increases the synchronization of ovulation and the ovulation rate in dairy buffalo during the non-breeding season. New studies are needed to assess the pregnancy rate.

A039 TAI/FTET/AI

# Effect of the application of gonatoreline at the time of FTET

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The objective of this study was to evaluate the effect of the application of gonadorelin at the time of FTET on conception rates at 30 (DG30), at 60 (DG60) days of gestation and at gestational losses (PGest) in PIVE's embryo recipients. It were evaluated 1,311 FTET performed in 11 commercial farms, located in the Uberlândia-MG region, from July / 2015 to July / 2016. The recipients were randomly divided into two groups: Control (n = 624): without gonadorelin and treated (n = 687): application of 0.1mg gonadorelin at the time of FTET. We also analyzed the effects of variables categorized into: FTET seasons (summer, winter, autumn and spring), farms (1 to 11), laboratory (1 to 3), recipient breed (nellore, girolando and undefined race), embryo breed (girolando, gir and nelore), embryo stage (initial blastocyst, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst), ovarium structures (compact CL, cavity CL and CL plus dominant follicle), CL quality (1, 2 and 3) and type of estrus (natural or protocol), and the interactions. The synchronization protocol used was: D0: intravaginal progesterone device (CIDR®, Zoetis, São Paulo-SP) and intramuscular application of 2.0 mg of estradiol benzoate (Sincrodiol®, Ourofino, Cravinhos- SP); D7: intramuscular application of 0.526mg sodium cloprostenol (Sincrocio®, Ourofino, Cravinhos-SP); D9: intramuscular application of 0.526mg sodium cloprostenol (Sincrocio®, Ourofino, Cravinhos-SP) + 1mg of estradiol cypionate (E.C.P.®, Zoetis, São Paulo-SP) + withdrawal of the intravaginal device; D18: TE + intramuscular application of 0.1mg gonadorelin (Fertagyl®, MSD, Cruzeiro-SP) or not. The data were evaluated by multivariate logistic regression using the GLIMMIX procedure of SAS version 9.2. In the final logistic regression model, some variables were removed based on the Wald criterion for P> 0.20. The interaction effects were not detected. The effects of treatment was detected on DG30 (40 vs. 45%, P = 0.03), DG60 (37 vs. 43%, P = 0.01) and tendency on PGest (7 vs. 4%, P = 0, 09). The season affected DG30 (P = 0.04) and DG60 (P = 0.01). Farm effect was detected in DG30 (ranging from 33 to 67%, P <0.0001) and DG60 (ranging from 31 to 67%, P <0.0001). It was also detected tendency of effect of recipient category on DG30 (P = 0.06; ranging from 40% up to 47%) and DG60 (P = 0.06: ranging from 38% up to 47%). It was concluded that Fertagyl® treatment at FTET increased the conception rate in both DG30 and DG60 and the farm has a strong effect on the success of FTET.

A040 TAI/FTET/AI

# Effect of body condition and application of PGF-2α on day zero of the IATF protocol in crossbred buffaloes

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The objective of this work was to evaluate the efficiency of the use of PGF-2α (d-Cloprostenol) at the beginning of the protocol of artificial insemination at fixed time (IATF) in buffaloes. The experiment was carried out at the Rotak farm, located in the municipality of Viseu, State of Pará, during the period from December 2016 to January 2017, an unfavorable season in the region. Composed of 104 mongrel animals of the Murrah and Mediterranean breeds, suitable for breeding, and divided into two groups. In group 1 (G1), with 48 females with a mean body condition score (ECC) of 2.72 ± 0.2, a 4-treatment protocol, received 0.15 mg of PGF on day zero (D0) (IMP), intravenous progesterone (P4) device of nomodose (Primer®, Tecnopec, São Paulo, Brazil), and 2 mg of Estradiol Benzoate - BE (Sincrodiol®, Ouro Fino, São Paulo, Brazil) of form IMP; On the 9th day (D9) the P4 implant was withdrawn and 400 IU of equine chorionic gonadotrophin - eCG (Sincro eCG®, Ouro Fino, São Paulo, Brazil) and 2 mL of PGF-2α (Sincio®, Gold Fino, São Paulo, Brazil) both via IMP; On day 11 (D11), 0.025 mg of gonadotrophin releasing hormone - GnRH (Gestran Plus®, Tecnopec, São Paulo, Brazil) was applied and on day twelve (D12) the IATF was performed. In G2, with 56 buffaloes and ECC of 2.96 ± 0.2, they received the same G1 protocol, without application of d-Cloprostenol at day zero (D0). The statistical analysis of variance (ANOVA) and Tukey's test were used, adopting the significance level of 5%. The G1 pregnancy rate was 27% (13/48) and G2 was 44% (25/56). A statistically significant difference was observed between the groups ($P < 0.05$) and it could be observed that the treatment without prostaglandin At the beginning of the protocol was more effective. Of the buffaloes with ECC <2.75, 28% (21/73) were involved and those with ECC> 2.75 were 54% (17/31) ($P < 0.05$), showing that ECC influenced the rate of Final pregnancy Therefore, the use of d-Cloprostenol at the beginning of the protocol in the unfavorable season did not increase the pregnancy rate and the ECC of the buffaloes was determinant in the results.

A041 TAI/FTET/AI

# Effect of equine chorionic gonadotrophin on follicular and luteal functionality in Murrah buffaloes cows submitted to TAI protocols

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The objective of this study was to evaluate the effect of equine chorionic gonadotrophin (eCG) on follicular and luteal development and vascularization and on plasma concentration of progesterone during a TAI protocol in Murrah buffaloes cows. Twenty Murrah buffaloes cows were randomly divided into two groups, group with eCG (WITH ECG, n = 20) and group without eCG (WITHOUT ECG, n = 20), in a cross-over design. On the first day of the hormonal protocol (Day 0), cows received an intravaginal P4 device (Sincrogest, Ourofino, Cravinhos, SP, Brazil) plus 2.0 mg/IM of estradiol benzoate (Sincrodiol, Ourofino, Cravinhos, SP, Brazil). On Day 9, the P4 device was removed, all cows received 0.150 mg/IM of PGF2a (Croniben, Biogéneses Bagó, Buenos Aires, Argentina), and 400 IU/IM of eCG (Folligon, MSD Saúde Animal São Paulo, SP, Brazil) was administered in the animals of WITH ECG group. On Day 11, all cows received 10 µg/IM of buserelin acetate (Sincroforte, Ourofino, Carvinhos, SP, Brazil). After the intravaginal P4 device withdrawal, color Doppler ultrasonography was performed daily, using 7.5 MHz linear transducer, 1.4 KHz PRF and 75 Mz wall filter, until day 16 to evaluate the development and irrigation of the dominant follicle up to ovulation and the luteal development and irrigation during luteogenesis, and thereafter every 3 days until day 32 to evaluate the luteal development and irrigation during luteogenesis and luteolysis. Simultaneously with ultrasonography, blood samples were collected to measure a plasmatic P4 concentration by radioimmunoassay. For statistical analysis, analysis of variance (ANOVA) was used to evaluate the means of the variables between the groups and Pearson's correlations, considering $p < 0.05$. Considering ovulation as day 0, the WITH ECG group presented greater follicle perimeter irrigation (16.93 ± 0.7 mm and 13.54 ± 0.5 mm, p = 0.018, 11.05 ± 0.4 mm and 9.77 ± 0.5 mm, p = 0.03) and also a higher percentage of the follicle perimeter irrigation (40.55 ± 0.5% and 33.11 ± 0.5%, p = 0.025, 28.27 ± 0.55% and 22.52 ± 0.47%, p = 0.03) on days -1 and -2, respectively. The WITH ECG group showed a greater diameter of CL on day 3 (16.03 ± 0.39 mm and 14.053 ± 0.39 mm, p = 0.00092), as well as higher CL irrigated area on days 1 (64.6 ± 2.1 $mm^2$ and 50.59 ± 4.11 $mm^2$, p = 0.008), 2 (94.15 ± 4.13 $mm^2$ and 70.63 ± 2.77 $mm^2$, p = 0) , 3 (115.9 ± 5.02 $mm^2$ and 90.76 ± 3.03 $mm^2$, p = 0.0084), 7 (135.9 ± 5.34 $mm^2$ and 115.3 ± 4.06 $mm^2$ , P = 0.0048) and 11 (137.1 ± 4.62 $mm^2$ and 114.2 ± 4.09 $mm^2$, p = 0.0037). Plasmatic progesterone concentration was higher in the WITH ECG group on days 3 (2.52 ± 0.37 ng/mL and 2.27 ± 0.23 ng/mL, p = 0.048), 7 (4.33 ± 0, 59 ng/mL and 3.09 ± 0.35 ng/mL, p = 0.0054) and 11 (4.45 ± 0.37 ng/mL and 3.28 ± 0.24 ng/mL, p = 0.0063). Positive correlations were observed between CL diameter and irrigation with plasmatic P4 concentration (0.52 and 0.75, $p < 0.0001$, respectively), as well as between the size of the follicle and the size of CL (0.54, $p < 0.0001$) between follicle size and CL irrigation (0.55, $p < 0.0001$), between follicle irrigation and CL size (0.78, $P < 0.0001$), between follicle irrigation and CL irrigation (0.64, $p < 0.0001$) and between follicle irrigation and plasmatic P4 concentration (0.59, $p < 0.0001$). Therefore, the use of eCG during TAI protocol in Murrah buffaloes cows favors follicle irrigation, increasing CL´s size and irrigation, raising plasmatic P4 concentrations, presenting potential to obtain better reproductive rates in buffaloes herds.

A042 TAI/FTET/AI

# Effect of long-acting injectable progesterone on the induction of puberty and pregnancy rate of Nelore heifers submitted to FTAI

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The objective of this study was to evaluate the effect of long-acting injectable P4 on the induction of puberty and pregnancy rate of Nelore heifers submitted to FTAI. Nelore heifers (n=457) with 22.1±3.5 months and 294±25.6 kg were classified by ultrasonography on D-22 in pubertal (i.e. with CL, PB, n=232) or prepubertal (i.e. absence of CL; PP, n=225), and received 150 mg of long-acting P4 (iP4; im, Sincrogest Injetável®, Ouro Fino) or not (NoiP4), making a factorial arrangement 2x2. On D-12, only heifers treated with iP4 received 150 μg of D-cloprostenol (PGF, i.m, Croniben®, Biogenesis Bagó) and 1 mg of estradiol benzoate (i.m, EB; Bioestrogen®, Biogenesis Bagó). On D0, presence of CL was evaluated and all heifers were synchronized according to the J-Synch protocol: D0: intravaginal release P4 device (1 g, Cronipres Monodose®, Biogenesis Bagó) + 2 mg EB and 75 μg PGF; D6: removal of the P4 device + 150 μg PGF. Animals detected in estrus at 48 hours after implant removal were inseminated 12 h after or at fixed time on D9 with application of 10.5 μg of GnRH analogue (i.m., Gonaxal®, Biogenesis Bagó). The pregnancy diagnostic was performed 30 days after FTAI. The data were analyzed using the PROC GLIMMIX (SAS, 9.3). There was interaction ($P < 0.01$) between iP4 and pubertal status on the presence of CL in D0. The PP-NoiP4 group had a lower proportion of CL in D0 (16.9% [22/137]) than the PP-iP4 group (73.7% [99/143]). PB heifers presented higher proportions of CL on D0 (iP4: 93.8% [83/89] and NoiP4: 86.7% [76/88]), independent of iP4 treatment. There was interaction ($P<0.01$) between iP4 and pubertal status on the expression of estrus. The PP-iP4 group (23.2% [33/143]) had higher estrus expression than PP-NoiP4 group (6.5% [9/137]) and similar to PB heifers treated (22, 4% [20/89]) or not with iP4 (23.2% [21/88]). There was no interaction ($P > 0.10$) between iP4 and pubertal status on the pregnancy rate. However, the pregnancy rate of the PP-NoiP4 group (38.6% [52/137]) was numerically lower than the PP-iP4 group (45.1% [63/143]), which was similar to PB heifers (NoiP4: 48.9% [44/88] and iP4: 48.7% [44/89]). A second analysis was performed considering the 4 groups: PB: heifers with CL on D-22 and/or D0, regardless of treated or not with iP4; PP-NoiP4: heifers without CL on D-22 and D0, not treated with iP4; PP-iP40: heifers without CL on D-22 did not respond to iP4 on D0 and PP-iP41: heifers without CL on D-22 responded to iP4 and presented CL on D0. In this analysis, there was a group effect ($P < 0.01$) on the pregnancy rate. The PP-iP41 group had a higher pregnancy rate (50.5% [50/99]) than the PP-iP40 group (29.6% [13/44]) and PP-NoiP4 (33.9% [39/115]). Response to treatment with iP4 (PP-iP41 group) guaranteed pregnancy rate similar to PB heifers (50.8% [101/199]). In conclusion, pre-synchronization with long-acting P4 in pre-puberal heifers induces puberty and allows a pregnancy rate similar to heifers that are already puberal at the beginning of the protocol.

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A043 TAI/FTET/AI

# Effect of Fosfosal® supplementation on pregnancy rate at FTAI of suckled Nelore cows

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The objective of the present study was to evaluate the effect of Fosfosal® (Virbac, São Paulo – SP, Brasil) treatment during a fixed time aritificial insemination (FTAI) protocol on pregnancy rate of suckled Nellore cows. For this experiment, 752 cows were used (542 multiparous and 210 primiparous), from four commercial beef farms located on the state of Paraná - BR. All the animals were evaluate for body condition score (BCS) on the beginning of the experiment. The females were submitted to fixed time artificial insemination (FTAI), using the same protocol, except for the administration or not of Fosfosal® on Day 0 (D0). Briefly, the FTAI protocol was done with intramuscular administration of 2 mg of EB (Fertilcare Sincronização®, MSD, São Paulo, Brasil) and insertion of an intravaginal device impregnated with progesterone (Fertilcare 1200®, MSD) on D0. On the eighth day, (D8) the intravaginal device was removed and cows received administration of 0.530 mg of PGF2α (Ciosin®, MSD), 300 IU of eCG (Folligon®, MSD), 1 mg of EC (Fertilcare Ovulação®, MSD). The FTAl insemination was done 48 hours after the intravaginal device removal, on the tenth day (D10). The cows were divided in two of both groups: Control Group: received no further treatment and; Treated Group: received the treatment with 15 ml of Fosfosal® on D0 of the FTAI protocol. The animals were homogeneously allocated in groups according to BCS and category. The pregnancy diagnosis was done using transrectal ultrasonography (Mindray DP10VET) thirty days after the FTAI. The results were analyzed using the PROC GLIMMIX (Statistical Analysis System, version 9.3, Institute, Inc.: Cary, NC, USA, 2003). It was observed positive effect of Fosfosal® supplementation on pregnancy rate [Treated = 52% (195/374) vs. Control = 45% (170/374) (P= 0.043)]. There was no interaction between categories*treatment [Treated Multiparous: 52% (140/269) and Treated Primiparous: 50% (55/109) vs. Control Multiparous: 46% (126/273) and Control Primiparous: 44% (44/101) P = 0.98]. It was observed a farm effect (P = 0.007) but not an interaction Treatment*Farm (P=0.67). According to the experimental data, it is possible to conclude that Fosfosal® suplementation improved pregnancy rates at FTAI in suckled multiparous and primiparous Nellore cows.

Acknowledgements: Virbac Animal Health and Firmasa.

A044 TAI/FTET/AI

# Effect of supplementation of long-term progesterone in the conception and in gestational loss of Nellore females submitted to TAI

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The objective of this study was to evaluate the effect of long - acting progesterone supplementation on the conception and gestational loss of Nellore females submitted to TAI. The experiment was carried out at Fazenda Remon located in Porto Real, in the state of Rio de Janeiro, during the period from November 2016 to May 2017. A total of 528 Nellore females (cows and heifers) were used with a minimum 30 days post-partum period and a maximum of 120 days, kept in *Brachiaria decumbes* pasture, with water and mineral salt *ad libitum*. Ovulation synchronization was initiated on a random day of the oestrous cycle (D0) and an intravaginal progesterone device (Primer®, Tecnopec, São Paulo, Brazil) was added to the application of 2.0 mg of estradiol benzoate (Fertilcare®, Vallée SA ), intramuscularly. The device was maintained for 8 days and 500 μg of cloprostenol (Ciosin®, MSD Saúde Animal, São Paulo, Brazil) + 400 IU of equine chorionic gonadotrophin (Folligon®, MSD Saúde Animal, São Paulo , Brazil) were administered both intramuscularly (D8). One day after (D9), 1mg of Estradiol Benzoate (Fertilcare®, Vallée S.A) was given intramuscularly. Two days after the device removal (D10), Fixed Time Artificial Insemination was performed. After the TAI, the animals were divided into three groups: experimental group 1 (EG 1; n = 178), experimental group 2 (EG 2; n = 163) and control group (CG; n = 187). Females were distributed so that each group had the same proportion of nulliparous, primiparous and multiparous females, as well as cyclic and acyclic animals. Experimental group 1 and experimental group 2 were supplemented with 150 mg of long-acting injectable progesterone (Sincrogest LA®, Ourofino, Uberaba / MG, Brazil) in a single dose, and experimental group 1 animals in D15 (5 Days after TAI) and experimental group 2 animals on D21 (11 days after TAI). The animals in the control group did not receive any type of progesterone supplementation. The pregnancy diagnosis (PD) and gestational loss (GL) were evaluated by rectal palpation using transrectal ultrasonography (Mindray D2200 vet). The PD was performed 39 days after insemination and GL evaluated from 39 days to 90 days after TAI. The variable conception rate and loss of gestation were analyzed by the non-parametric chi-square method ($\chi 2$), using the PROC FREQ function of the statistical program SAS® (SAS, 2009). The conception rates were 47% (83/178) 42% (68/163) and 41% (76/187), respectively, for EG1, EG2 and CG. No difference was observed between the treatments ($p = 0.47$). The gestational loss was 6.58% (CG), 2.41% (EG1) and 5.88% (EG2). No statistical difference was observed ($p = 0.19$). It was concluded that long-acting injectable progesterone supplementation, 5 or 11 days after TAI, does not alter the conception rate or gestational loss in Nellore females.

A045 TAI/FTET/AI

# Effect of chute exit velocity on pregnancy rate to FTAI of Nellore females

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The objective of the present study was to evaluate the effect of chute exit velocity at the beginning of the TAI protocol on pregnancy rate of Nelore females. Data were collected from two breeding seasons (2013/2014 and 2014/2015) in a commercial farm located in Piranhas, Goiás, Brazil. The study evaluated 2785 females (2067 suckled cows and 718 heifers) subjected to TAI. On Day 0 (D0) animals received an intravaginal device containing 1.9g of P4 (CIDR®, Zoetis Animal Health, São Paulo, Brazil), followed by administration of 2mg of EB (Gonadiol®, Zoetis); on day 7, animals received 12.5mg of Dinoprost Tromethamine (Lutalyse® Zoetis); on day 9 the P4 device was removed and 1mg of EC (ECP®, Zoetis) and 300 IU of eCG (Novormon®, Zoetis) were administered. TAI occurred 48h after P4 device removal. The animals were evaluated as to the exit velocity, which occurred on D0 of the TAI protocol, in three different classifications: Walking, Trotting and Running. Pregnancy data were analyzed using the PROC GLIMMIX and frequency exit velocity data were analyzed using Qui-Square of PROC FREQ and PROC GENMOD of SAS (Statistical Analysis System, version 9.3 Institute Inc., Cary, NC, USA, 2003). Frequency of exit velocity differed (P< 0.0001) between categories [Cows: Walking = 22%B (455/2067); Trotting = 56%A (1158/2067) and Running = 22%B (454/2067) vs. Heifers: Walking = 17%E (122/718); Trotting = 46%C (332/718) and Running = 37 %D (264/718)]. Interaction category*velocity was observed in relation to the pregnancy rate to FTAI (P<0.0001), thus, categories were analyzed separately. Heifers did not present difference on pregnancy rate to TAI regarding the exit velocity [Walking = 48.4% (59/122); Trotting = 47.6% (158/332) and Running = 48,8% (129/264); P = 0.95]. However, there was effect of the exit velocity on pregnancy rate to TAI for cows [Walking = 66.4%A (302/455); Trotting = 62.7 AB (726/1158) and Running = 57.9%B (263/454); P = 0.04]. Based on these analyses, it is possible to conclude that cows with lower chute exit velocity at the beginning of TAI protocol (D0) presented higher pregnancy rate to TAI. However, no effect was observed of chute exit velocity rate on pregnancy rate to TAI of Nelore heifers.
Acknowledgement: Cria Fértil.

A046 TAI/FTET/AI

# Effect of different reproductive biotechnologies (AI, ET-*in vivo* and ET-*in vitro*) on reproductive performance of Holstein females

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The objective of this study was to evaluate the effect of different artificial reproductive technologies (ART) on indicators of reproductive performance of lactating Holstein cows. The conception rate (CRt) for IA and gestational rate (GRt) for ET and IVF at 30 and 60 days, birth rate (BRt), pregnancy loss rate between 30 and 60 days (PLRt) and between 60 days and birth (PLBRt) were studied in cows receiving AI (8382), ET (6381) and IVF (1503) at Agrindus Farm from 2013 to 2015. The variables gestation length (GL), retained placenta (RP), birth weight (BW), weight at weaning (WW) and age at first conception (AFC) of the calves were analyzed in a subgroup of animals (AI = 471, ET = 429 and IVF = 57). The data were analyzed by the PROC GENMOD and PROC GLIMMIX of SAS. CRt and GRt at 30 days had no effect of year (P = 0.363) and no interaction ART*year (P = 0.393). However, there was effect of ART (AI = 31.7%(2655/8382)B, ET = 39.3%(2510/6381)A and FIV = 27.5%(413/1503)C; P < 0.0001]. A similar result was observed for CRt and GRt at 60 days [AI = 23.9%(2000/8382)B, ET = 29.0%(1851/6381)A and IVF = 19.6%(295/1503)C; P < 0.0001]. The PLRt did not differ in ART (P = 0.175) and there was no ART*year interaction (P = 0.07), but there was effect of year [2013 = 24.4%(426/1746)B, 2014 = 23.7%(438/1849)C and 2015 = 28.6%(568/1983)A; P = 0.0369]. There was ART*year interaction for PLBRt [2013: AI = 18.99%(116/611)C, ET = 20.90%(125/598)BC and IVF = 20.72%(23/111)BC; 2014: AI = 20.94%(151/721)BC, ET = 24.83%(146/588)BC and IVF = 32.35%(33/102)B; 2015: AI = 23.95%(160/668)A, ET = 26.62%(177/665)A and IVF = 21.95%(18/82)C; P < 0.0001]. The GL did not differ between ART (AI = 275.9±0.2; ET = 274.6±0.3 and IVF = 275.9±0.9 days; P = 0.61). Still, there was no effect of year (P = 0.64) and no ART*year interaction (P = 0.96). Also, RP was similar among ART (IA = 17.6%(83/471), ET = 19.1%(82/429) and IVF = 24.6%(14/57); P = 0.91), and there was no effect of year (P = 0.08) and no ART*year interaction (P = 0.35). The BW differed according to ART (AI = 40.1±0.1AB, ET = 39.8±0.1B and IVF = 40.8±0.3A kg; P = 0.0098), however, there wasn't effect of year (P = 0.15) and ART*year interaction (P = 0.71). There was ART*year interaction for WW (P = 0.0016). The AFC differed for ART (AI = 469.2±4.3A, ET = 466.1±4.9B and IVF = 488.8±15.0A days; P < 0.0001). There was difference on results for CRt and GRt at 30 and 60 days between ART, PLRt between years and PLBRt in both. There was not difference for GL and RP, but there was for BW and AFC between ART and for WW between ART and years.

A047 TAI/FTET/AI

# Effect of different progesterone intravaginal devices for estrus synchronization on pregnancy rates of ewes

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The objective of this study was to compare two different intravaginal progestagens devices effect on estrus synchronization, and pregnancy rates on cyclic ewes. The experiment was conducted in january 2017, in a farm in the São Francisco de Assis, Rio Grande do Sul/Brazil. The ewes were non-lactating Australian Merino sheep (n=42), with body condition score of 2,5 (scale 1 to 5: 1-lean, 5-obese). The synchronization protocol started on an unspecific day of estrous (D0). Ewes were randomly assigned into different experimental groups: sponge group (SG – 62.5mg of medroxyprogesterone acetate, Purifarma, São Paulo, SP, Brazil; n=23) and Primer PR® group (PrG– 0.36g of progesterone; PRIMER PR®, Tecnopec, São Paulo/SP, Brazil; n=19). After intravaginal device implantation, ewes received 0.037mg of D-cloprostenol IM (PROLISE®, Tecnopec, São Paulo/SP, Brazil). The devices were removed 12 days (D12) after implantation and 200 UI of equine chorionic gonadotrophin were administered (Folligon®, MSD, São Paulo/SP, Brazil). Right after the end of the protocol ewes had estrus behavior. Four fertile rams (soundness breeding examination) were kept with the females by D19 (1 ram per 12,5 females). The mattings were identified by the presence of paint on their back from the chest of the ram. Forty days following matting, the pregnancy diagnosis was made by transabdominal ultrassonography (5MHz, Medisono P3. Wilmington, USA). Statistical analysis was performed using PROC GLIMMIX of SAS (SAS 9.3). There was no difference on pregnancy rate between treatments [SG = 60.87% (14/23) and PrG = 57.89% (11/19), P=0.40]. Some cases of vaginitis were observed in SG (~40%), while there were observed no signs of vaginitis in the PrG. In conclusion, both devices were effective for estrus synchronization, providing similar pregnancy percentage. However the Primer PR® device showed to be safer and no reactions on vaginal mucosa were noted.
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A048 TAI/FTET/AI

# Effect of ECP on the incidence of estrus in spayed cows and on fertility in timed AI Cows

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Several studies has demonstrated that cows that display estrus before timed artificial insemination (TAI) are more likely to become pregnant. However, the most used ovulation inducer in Brasil are the estradiol esters, that artificially may induce cows to display estrus. In this study, two experiments were performed to evaluate the effects of ECP in cows. The objectives of this study were to evaluate: 1) the incidence of estrus in spayed cows treated with ECP, and 2) the effect TAI protocol without ECP. The Experiment 1 was performed with 14 spayed cows in a cross-over design that were randomly distributed into 2 experimental Groups: ECP Group (n=14) that received a hormonal protocol (2 mL of BE + CIDR insertion on D0 / 2 mL of PGF2 alfa on D7 / 0,5 mL of ECP + CIDR removal on D9), and Group CTL (n=14) that received similar protocol, but instead ECP, cows were injected with saline solution (NaCl 0,9 %). Estrotect devices were used and cows were observed 4 times a day to detect the moment of the estrus. The interval betweeen replicates were 30 d. Spayed cows treated with ECP displayed more estrus (85,7%, 12/14) than cows treated with Saline (0%, 0/14; P < 0,001). Cows displayed estrus 46 h after ECP treatment. In Experiment 2, 94 Nelore cows, between 30 and 60 d postpartum, 2.5 – 3.5 of BCS, were randomly separated into 2 Groups. ECP-GnRH Group (EG Group, n=47) that were given the same protocol as Experiment 1, excepted that all cows were given 300 UI of eCG on Day 9. Moreover, cows that do not display estrus between CIDR removal and TAI, were given 2,5 mL of GnRH (Gonaxal®, Biogénesis Bagó, Buenos Aires, Argentina). The remaining cows were enrolled into GnRH Group (n=47), that received similar treatment as EG Group, however, no ECP was given on Day 9. Stick marker was used to identify cows that display estrus. Cows from EG Group displayed more estrus (57,4%, 27/47) than cows from GnRH group (31,9%, 15/47; P<0,01). However, no difference (P = 0.5) on P/AI was detected between EG (57,4%, 27/47) and GnRH groups (63,8%, 30/47). In conclusion, the injection of ECP at removal of progesterone insert increase the estrus audreydetection, however, the fertility of the TAI protocols was not affected in comparison with cows that did not received ECP.

A049 TAI/FTET/AI

# Effect of time of permanence (7 vs. 8) of different intravaginal progesterone devices (PRIMER® Multidose or Monodose) on conception rate of dairy cows

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The establishment of a systemized reproductive program in dairy farms can enable the use of reproductive biotechnologies on the property. The present study evaluated the use of two different intravaginal P4 devices (PRIMER® Monodose, containing 0.5g of P4 and PRIMER® Multidose, containing 1.0g of P4, Agener União – Saúde Animal, São Paulo, Brazil) and the permanence of the devices (7 or 8 days) on pregnancy rate to TAI. For this study, 505 lactating Holstein cows were distributed into four experimental groups (2 x 2 factorial): Group P8 MONO (8-day protocol with PRIMER® Monodose – n=120); Group P8 MULTI (8-day protocol with PRIMER® Multidose – n=130); Group P7 MONO (7-day protocol with PRIMER® Monodose – n=126); Group P7 MULTI (7-day protocol with PRIMER® Multidose – n=129). Cows from the P8 MONO group received the PRIMER® Monodose and 2mg of EB (RIC-BE®, Agener União – Saúde Animal) on D0, after 8 days (D8) the PRIMER® was removed and cows received 2mL of PGF2α (Estron® 0.2435mg Agener União – Saúde Animal) and 1mg of EC (ECP®, Zoetis, São Paulo, Brasil). Cows from the P8 MULTI group received the PRIMER® Multidose, followed by the same treatment as the P8 MONO group. Cows from the P7 MONO group received the PRIMER® Monodose and 2mg of EB on D1, after 7 days (D8) the PRIMER® was removed and cows received 2mL of PGF and 1mg of EC. Cows from the P7 MULTI group received the PRIMER® Multidose, followed by the same treatment as the P8 MULTI group. All cows were inseminated on the same day (D10), 48h after device removal. Transrectal ultrasonography was used to determine pregnancy rate 30 days after TAI. Data were analyzed by logistic regression (PROC GLIMMIX from SAS). There was no permanence*device interaction (P=0.2966). The pregnancy rate did not differ (P=0.554) between groups [P8 MONO – 27% (33/120); P8 MULTI – 24% (31/130); P7 MONO – 26% (33/126); P7 MULTI – 28% (35/129)]. Analyzing all animals, the permanence of the devices (7 or 8 days) did not influence (P = 0.723) pregnancy rate [27% (68/255) vs 25% (64/250)]. Also, use of different P4 devices (PRIMER® Monodose or Multidose) has no effect (P = 0.792) on pregnancy rate [26% (66/259) and 27% (66/246)]. In conclusion, neither the permanence of the P4 device (7 vs. 8) nor the type of device (PRIMER Monodose® or PRIMER Multidose®) influenced the pregnancy rate of lactating Holstein cows. Therefoe, the 7-day protocol can be used in dairy farms as an alternative to the 8-day protocol, concentrating reproductive managements to two days on the week, instead of three days on the week, simplifying the farms' management and the reproductive schedule.
Acknowledgments: Farm J – Ida Agropecuária Ltda., Farm Tucaninha, Agener União, Tecnopec and CattleVitro.

A050 TAI/FTET/AI

# Effect of treatment with long-acting injectable progesterone on the embryo receptor rate of pregnancy

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The objective of the present study was to verify if the use of long-acting injectable P4 LA (Sincrogest® Ourofino - Saúde Animal, Ribeirão Preto, SP) results in increased pregnancy rate in receivers of in vitro produced embryo. A group of 817 animals was synchronized. At Day -10 all animals received an intravaginal P4 device (0.5g of P4) and administration of 2mg of Estradiol Benzoate (EB); on Day -2 the P4 device was removed and 0.530mg of PGF2α was administered; On Day -1, 1 mg of EB was administered. The animals were randomly assigned to one of three experimental groups: Group 1 (CONTROL; n = 169), Group 2 (P4LAD4; n = 201) and Group 3 (P4LAD7; n = 181). The CONTROL group received an in vitro produced embryo on D7, without any further treatment. The P4LAD4 group received 150mg of P4LA (Sincrogest® Ourofino) on D4 and on D7 the ET. The P4LAD7 group received 150mg of P4LA (Sincrogest® Ourofino) at the same moment as the ET, on D7. Together with the ET, ultrasonography evaluation was performed to evaluate the diameter of the CL (DCL). Pregnancy diagnosis was done using ultrasonography on days 30 and 60 of the synchronization protocol. Data were analyzed using the PROC GLIMMIX procedure of SAS. The utilization rate of the ET protocol was of 69.89% (571/817). Pregnancy rates did not differ between CONTROL, P4LAD4 and P4LAD7 groups at 30 days [41.4% (70/168); 42.3% (89/201); 41.2% (80/181); P = 0.4115], 60 dias [36.3% (61/168); 39.8% (80/201); 38.1% (69/181); P = 0.4859] and did not influence pregnancy loss [5.4% (9/168); 4.5% (9/201); 6.1% (11/181); P = 0.7258]. No interaction between treatment with P4 and DCL on pregnancy rate at 30 days (P = 0.1682), 60 days (P = 0.3543) and pregnancy loss (P = 0.6121) was observed. Despite each treatment, the DCL did not influence the pregnancy rate at 30 days [(≤18mm= 43.9% (72/164); >18mm = 43.3% (167/386); P = 0.8712], 60 days [(≤18mm = 36.0% (59/164); >18mm = 39.1% (151/386); P = 0.5175] and pregnancy loss [(≤18mm = 7.9% (13/164); >18mm = 4.1% (16/386); P = 0.0897]. Therefore, administration of P4LA on days 4 or 7 of the ET synchronization protocol had no effect on conception rate of receivers of in vitro produced embryos.

A051 TAI/FTET/AI

# Effects of progesterone supplementation after the use of fixed time artificial insemination at the pregnancy rate in Nellore cows

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This study aimed to evaluate the effects of using progesterone supplementation in order to increase the pregnancy rate in Nellore cows. The research was developed out of the Center for Reproductive Biotechnology (BIOTEC) and conducted during the months of January-April, 2016 in Maringa-Paraná. Eighty-eight adult Nellore female cows, after 30 and 60 days postpartum, body scale of 3 (scale 1-5). These cows were kept in pasture (Brachiaria brizantha) having access to water and mineral supplementation. The cows were randomized into two groups, A and B. Group A cows did not receive hormonal supplementation, Group B cows received hormonal supplementation using an intravaginal device, dosed at 1.9g of progesterone (CIDR®) from the 14th to the 22nd day after Fixed Time Artificial Insemination (FTAI). The Group B cows were synchronized using the following protocol: Day 0 (D0) the cows received an intravaginal progesterone device (CIDR®) plus 2 mg estradiol benzoate (Gonadiol®, im, Syntex SA). Day 7 (D7) the cows received 2ml of prostaglandin (Sincrocio® Ouro Fino Animal Health) at the day 9 the cows received (ECG®, Novormon, Pfizer / Animal Health) and 0.4ml of Estradiol Cypionate (ECP®, Pfizer Animal Health) where the intravaginal progesterone implant was also withdrawn; and two days after the removal of the intravaginal progesterone device. Day 11 (D11), two days after the removal of the intravaginal progesterone device for FTAI, was performed, using frozen semen from a proven Aberdeen Angus bull. Pregnancy rate (DG) was performed 30 days after the FTAI with the aid of an ALOKA SSD500 ultrasound device and probe of 5.0 MHZ.
The data was analyzed by the PROC GENMOD procedure of the statistical program SAS (2000) version 8.01 and using binomial distribution and identity link function. One can observe that progesterone supplementation from day 14 to day 22 after FTAI, did not change the gestation rate ($P > 0.05$) in Nellore cows. Group 1 showed a gestation rate of 52.27%, not significant difference. Group 2, a gestation rate of 54.54%. Although some authors (KENYON, A. G. Animal Reproduction Science, v.136, n.4, p.223-30, 2013) have observed that there is no difference in the gestation rate, progesterone supplementation has become effective in favor of the expansion and rate of embryo elongation, were shown to be larger and with higher secretion of proteins, making than more mature and functional. With the data obtained in this research project concluded that progesterone supplementation from day 14 to day 22 did not change the gestation rate in Nellore females in Fixed Time Artificial Insemination (FTAI).

A052 TAI/FTET/AI

# Effect of human chorionic gonadotrophin administration 2 days after insemination on progesterone concentration and conception rate in lactating dairy cows

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The aim of this study was to examine the effect of a single administration of human chorionic gonadotrophin (hCG) during the establishment of the corpus luteum (CL) on progesterone (P4) concentration and conception rate in lactating dairy cows. Postpartum spring-calving lactating dairy cows (n=800; mean (± SD) days in milk and parity were 78.5 ± 16.7 and 2.3 ± 0.8, respectively) on three farms were enrolled in the study. All cows underwent the same fixed-time artificial insemination (FTAI) protocol involving a 7-day progesterone-releasing intravaginal device with gonadotrophin-releasing hormone (GnRH) administration at device insertion, prostaglandin at device removal followed by GnRH 56 h later and AI 16 h after the second GnRH injection. Cows were blocked on days postpartum, body condition score (BCS), and parity and randomly assigned to receive either 3000 IU of hCG 2 days after FTAI or no further treatment (Control). Blood samples were collected on Day 7 and Day 14 post estrus by coccygeal venipuncture on a subset of 202 cows to measure serum P4 concentration. Pregnancy was diagnosed by ultrasonography approximately 30 and 70 days after FTAI. The effect of the independent variables on conception rate was determined using a logistic regression model in the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC) with cow treated as a random effect, and the effect of treatment on P4 concentrations was determined using the Mixed procedure of SAS. Administration of hCG lead to an increase in P4 concentrations on Day 7 ($P < 0.05$) and Day 14 ($P < 0.01$) compared to the Control. Conception rate at 30 days after FTAI (CR1) was affected by treatment, farm, BCS, and calving to service interval. Overall, administration of hCG decreased CR1 (46.3% vs. Control 55.1%; $P = 0.02$). Among cows that did not become pregnant following AI (CR1), a higher proportion of Control cows exhibited a short repeat interval (≤ 17 d; 8.6%; $P < 0.05$) than cows treated with hCG (2.8%). In addition, 21-day pregnancy rate (59.6% vs. 52.0%; $P = 0.05$) and 42-day pregnancy rate (78.3% vs. 71.9%; $P = 0.06$) were greater in the Control than in hCG-treated cows. The overall incidence of embryo loss between Day 30 and 70 was 10.7% and was not affected by treatment ($P = 0.4$). In conclusion, administration of hCG two days after FTAI increased circulating P4 concentrations, but did not result in an improvement in reproductive performance.

A053 TAI/FTET/AI

# Efficiency of PRIMER® PR vs. Primer® Monodose in TAI protocols of Nelore heifers

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Willing to compare the efficiency of different intravaginal P4 releaser device, pregnancy per AI (P/AI), and the diameter of the dominant follicle (DF) on the day of P4 device removal were evaluated in 14 (Experiment 1) and 24 months (m) old (Experiment 2) Nelore heifers subjected to TAI. Each age group was distributed in two experimental groups. In Experiment 1, Group 1) 14m old Nelore heifers (n=169) received a conventional intravaginal device, PRIMER® Monodose (Agener União – Saúde Animal, São Paulo-SP) containing 0.5g of progesterone and Group 2) 14m old Nelore heifers (n=153) received a smaller device, made for small ruminant, PRIMER® PR (Agener União) containing 0.36g of progesterone on D0. In Experiment 2, Group 1) 24m old Nelore heifers (n=210) received PRIMER® Monodose device and Group 2) 24m old Nelore heifers (n=196) received PRIMER® PR device on D0. On both experiments, all heifers received the relative P4 device for each group on D0 and 2mg of EB (RIC-BE®, Agener União), on D8 all P4 devices were removed and all animals received 0.5mg of Estradiol Cipionate, 200 IU of eCG and 0.53mg of PGF2α (ESTRON®, Agener União). On D10 all heifers were subjected to TAI. On D8 a subgroup of heifers from Experiment 1 were evaluated by ultrasonography (Mindray M5) to measure the DF [PRIMER® Monodose (n=92) and PRIMER® PR (n=87)]. To obtain the DF measurements, the mean of the diameters obtained by ultrasound images and the standard deviation were calculated. On D30 all heifers were checked for pregnancy using ultrasonography. Data were analyzed by GLIMMIX procedure of SAS. In Experiment 1, P/AI was 43% (72/169) for PRIMER® Monodose and 38% (58/153) for PRIMER® PR (P=0.392). In Experiment 2, P/AI was 42% (89/210) for animals that received the PRIMER® Monodose device, and 52% (101/196) for animals that received PRIMER® PR device (P=0.066). The diameter of DF was 8.3 ± 0.3mm for heifers from the PRIMER® Monodose group and 9.4 ± 0.3mm for heifers from the PRIMER® PR group (P=0.0002). Since heifers treated with PRIMER® PR presented a greater DF diameter on D8, similar P/AI for both 14 and 24 m old heifers, and considering the lower discomfort caused by the small ruminant P4 device, it is possible to conclude that the use of PRIMER® PR is as an alternative to the use of PRIMER® Monodose on Nelore heifers.
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A054 TAI/FTET/AI

# Reproductive efficiency of Nelore cows submitted to three different reproductive strategies in a 64 days breeding season

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This study aimed to evaluate the reproductive efficiency of Nelore cows submitted to three different TAI programs in a 64 days breeding season (BS). The programs were conducted in a commercial farm located in Camapuã-MS, Brazil. A total of 1,505 multiparous Nelore cows were treated for TAI and then homogenously allocated into one of three groups according to the subsequent treatments, resynchronization or bull exposure: 1TAI+NS = one TAI followed by natural service (NS) until the end of the BS (n = 450); 2TAI+NS = two subsequent TAI with 32d interval between AIs followed by NS until the end of the BS (n = 300); and 3TAI = three subsequent TAI with 32d interval between AIs (n = 755). For TAI, cows received an intravaginal device with 1 g progesterone (P4; Cronipres® Mono Dose M-24, Biogénesis Bagó, Curitiba, Brazil) and were treated with 2.0 mg estradiol benzoate (EB; Bioestrogen®, Biogénesis Bagó) IM on Day -10. On Day -2, the device was removed and cows were treated with 300 IU eCG (Novormon®, Zoetis, São Paulo, Brazil), 1.0 mg estradiol cypionate (EC; ECP®, Zoetis) and 112.5 µg D-cloprostenol (PGF; Croniben®, Biogénesis Bagó) IM. All cows were inseminated 48h after the P4 device removal (Day 0). On Day 22, cows from groups 2TAI+NS and 3TAI were resynchronized with the insertion of a P4 device and 1.0 mg EB IM. On Day 30, they were submitted to ultrasonography evaluation (Chison 9300VET, Kylumax, Brazil) for pregnancy diagnosis. Non-pregnant cows went through device removal, received 300 IU eCG, 1.0 mg EC and 112.5 µg PGF IM and were TAI 24h later (48h after P4 device removal; Day 32). Pregnant cows, just have the P4 device removed with no further treatment. On Day 54, cows from group 3TAI were resynchronized with the same protocol to receive a third TAI on day 64. Cows from groups 1TAI+NS and 2TAI+NS were exposed to clean-up bulls (1 bull per 25 cows) 15d after the last TAI. Statistical analysis was performed using GLIMMIX procedure of SAS 9.3. Pregnancy rate after the first TAI was similar between groups [1TAI+NS = 64.0% (288/450); 2TAI+NS = 66.0% (198/300); 3TAI = 65.4% (494/755); P = 0.83]. Pregnancy rate was also similar after the second TAI: [2TAI+NS = 43.1% (44/102); 3TAI = 37.5% (98/261); P = 0.33]. Pregnancy rate for third TAI (group 3TAI) and NS (groups with NS after TAI) until the final pregnancy diagnosis was also similar [1TAI+NS = 36.4% (59/162); 2TAI+NS = 36.2% (21/58) and 3TAI = 43,9% (72/164); P = 0.28] The overall pregnancy rate at end of the BS was different within groups [1TAI+NS = 77.1% (347/450)b; 2TAI+NS = 87.7% (263/300)a; 3TAI= 87.8% (663/755)a; P = 0.0001]. In conclusion, the use of two TAI followed by NS or three consecutive TAI with 32 d interval between AIs were more efficient (greater pregnancy rate) then one TAI followed by NS. Moreover, the program with three TAI enables the establishment of a 64 days BS, without the use of bulls (AI of all cows in all services), what may also allow greater genetic gain.
Credits: Farm Engano and CNPq 152030/2016-6.

A055 TAI/FTET/AI

# Effect of busereline acetate (GNRH) on the fertility in Jersolando (Holstein X Jersey) Heifers submitted to lactation induction protocol

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The present studies' objective was to evaluate the effect of busereline acetate (GNRH) on fertility of Jersolando (Holstein x Jersey) heifers submitted to lactation induction protocol (IL). The effect of the presence of non-ovulatory follicles (NOF), cyclicity rate (RtCyc) and pregnancy rate after three TAIs in heifers submitted to hormonal treatment for IL was verified. The experiment was conducted in Itararé/SP – Brazil, with 106 crossbred heifers (½ Jersey x ½ Holstein), aged 34.7±0.5 months, live weight (LW) of 439.3±5.7kg and BCS=3.05±0.03. On D-20, the hormonal protocol for IL begun on all females (500mg of bSTr (Boostin®, MSD, SP, Brazil)on D-20, -13, -6 and 0; 30mg/heif/d of BE (SincroBE®, Ourofino, Brazil) and 300mg/heif/d of P4 (Sincrogest injetável®, Ourofino, Brazil) IM from D-20 to -13; 20mg/heif/d of BE (SincroBE®) from D-12 to -6; 0.530mg/heif of PGF (SincroCIO®, Ourofino, Brazil) on D-5; 40mg/heif/d of dexametasone (Cortiflan®, Ourofino, Brazil) from D-2 to 0; 5 min daily massage of the teats and udder from D-4 to -1; milking starting on D0; after the onset of lactation, heifers received 500mg of bSTr (Boostin®) every 14d). On D15 US was used in order to evaluate the presence of CL and NOF. On this moment, heifers were divided in two groups: 1) Control (N=53): heifers that did not receive any treatment; 2) GnRH (n=53): treatment with 5mL (0.021mg) of busereline acetate (Sincroforte®). A second US was done 30 days after the first evaluation (D45) and the first TAI was on D80. The same TAI protocol was used for both groups [D0: 2mg of estradiol benzoate (Sincrodiol®); 0.530mg of Cloprostenol sodium (PGF2α; Sincrocio®); 0.01mg of busereline acetate (GNRH; Sincroforte®); and insertion of P4 device (1g of P4; Sincrogest®, Ourofino, Brazil)]. Eight days later the P4 device was removed, associated with administration of 1mg of estradiol cipionate (SincroCP®, Ourofino, Brazil); 0.530mg of Cloprostenol sodium (Sincrocio®) and 400IU of eCG (SincroeCG®, Ourofino, Brazil)]. The TAIs were done 48 hours after the removal of the P4 device, using conventional semen. Two resynchronizations were carried out after early pregnancy diagnosis (30 days after TAI). Data were analyzed using the PROC GLIMMIX of SAS v9.4. The RtCyc 15 days after the first milking was 7.5% (8/106) and the NOF rate was 32.1% (34/106). No interaction between time*GNRH treatment (P=0.20) and GNRH treatment effect (P = 0.12) were observed on the presence of NOF. However, time effect on the decrease of NOF was observed (D15 = 32.1% (34/106); D45 = 10.4% (11/106); P = 0.0003). No interaction was observed between time*GNRH treatment (P=0.80) and GNRH treatment effect (P=0.77) on the RtCyc. Moreover, effect of time on RtCyc was observed (D15 = 7.3% (8/106); D45 = 50.0% (53/106); P = 0.0001). Pregnancy rate to 1st, 2nd and 3rd TAI was 48.9% (45/92) 45.7% (21/46) and 31.3% (5/16), respectively, resulting in na accumulated pregnancy rate of umo out76.1% (70/92). In conclusion, the treatment with GNRH 15 days after the initiation of lactation was not effective to increase the fertility of Jersolanda (Holstein vs. Jersey) heifers submitted to the lactation induction protocol. The induction of lactation protocol can be used as a strategy to boost fertility in crossbred heifers. Also, it can increase milk yield and minimize economic losses referent to reproductive failures.

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A056 TAI/FTET/AI

# Progesterone pre-exposition to ovulation synchronization protocol increases follicular diameter and pregnancy rate in suckled *Bos indicus* cows

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The objective was to evaluate the effect of pre-exposition of injectable progesterone to timed artificial insemination (TAI) protocol on follicular growth and pregnancy rate of suckled-anestrus Bos Indicus cows. Suckled-anestrus Nelore cows (n=681; 325 primiparous and 356 multiparous), at 30-60 days postpartum and body condition score of 2.72±0.01 (scale of 1 – 5) were used. Ten days before TAI protocol (D-10), cows were divided into 3 experimental groups (Control group, P4 group and P4GnRH group). In the Control group, cows received 2 mg of estradiol benzoate (Sincrodiol®, Ouro Fino, Brazil) and a progesterone intravaginal device (Sincrogest®, Ouro Fino, Brazil). On day 8 (D8), the progesterone device was removed and cows received 500µg of Cloprostenol (Sincrocio®, Ouro Fino, Brazil), 300 IU of eCG (SincroeCG®, Ouro Fino, Brazil) and 1 mg of estradiol cypionate (SincroCP, Ouro Fino, Brazil). On the P4 group, cows received 150mg of injectable progesterone (Sincrogest Injetável®, Ouro Fino, Brazil) on D-10 and were submitted to the same synchronization protocol as the Control group. On the P4GnRH group, cows received the same treatment as the P4 group associated to the administration of 10µg of buserelin (Sincroforte®, Ouro Fino, Brazil) on D0. In a subgroup (n=420; 176 primiparous and 244 multiparous), ultrasound exams were performed to evaluate the diameter of the largest follicle (D0, D8 and D10), for evaluation of ovulation rate (presence of CL on D24 ipsilateral to largest follicle on D10) and diameter of the corpus luteum (D24). Pregnancy diagnosis was 30 d after TAI. Statistical analyses were performed by GLIMMIX procedure of SAS and the continuous variables were presented by mean ± standard error. The diameter of the largest follicle (LF) on D10 (P=0.21), follicular growth rate (P=0.34) and ovulation rate [Control 78.2% (104/133), P4 80.3% (110/137) and P4GnRH 75.2% (106/141); P=0,61] were similar among experimental groups. However, there was difference among groups for the LF on D0 [Control (10.9±0.2mm)b, P4 (12.7±0.3mm)a and P4GnRH (12.6±0.3mm)a; P=0.001], LF on D8 [Control 0% (9.7±0.2mm)b, P4 (10.4±0.2mm)a and P4GnRH (9.9±0.2mm)ab; P=0.05], presence of the CL on D8 [Control 0% (0/136)b, P4 0% (0/140)b and P4GnRH 26.4% (38/144)a; P=0.001], diameter of the CL on D24 [Control (19.7±0.4mm)ab, P4 (20.1±0.4mm)a and P4GnRH (18.5±0.4mm)b; P=0.001] and pregnancy rate [Control 35.0% (78/223)b, P4 45.9% (105/229)a and P4GnRH 40.6% (93/229)ab]. In conclusion, the pre-exposition to progesterone on TAI protocol increased diameter of the LF on D0 and D8 without interfering on the ovulation rate. Furthermore, such exposure increases the pregnancy rate in suckled-anestrus Nelore cows.

Support: Ouro Fino Saúde Animal and FAPEMIG.

A057 TAI/FTET/AI

# Factors that affect pregnancy rate to TAI and to natural breeding of 14 months old Nelore heifers

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Two experiments were conducted with the objective of studying the factors that affect the pregnancy rate of 14 months old Nelore heifers submitted to TAI (study 1; E1; n=404) and natural breeding (E2; n = 893; bull:heifer ratio was 1:30). For TAI, heifers received a Norgestomet device (Crestar, MSD, São Paulo, Brasil) and 2mg of EB (Gonadiol, MSD) on D0, followed by device removal and treatment with 0.3mg of EC (ECP, Zoetis, São Paulo, Brasil), 0,530mg of Cloprostenol sodium (Ciosin, MSD) and 300IU of eCG (Novormon, Zoetis) on D9 and TAI on D11. On both studies, gynecological evaluation was performed to determine presence of CL and uterus score (USC; A = uterine horns diameter > 2cm; B = uterine horns with diameter between 1.5 and 2cm; and C = uterine horns with diameter < 1.5cm) on D-10 and D0 of the breeding season (BS), loin eye area (LEA), subcutaneous fat thickness (SCFT), daily average weigh gain (DAWG), withers height (hWIT) and the diameter of the largest follicle (DF). Pregnancy diagnosis was performed by ultrasonography 30 (E1 and E2) and 50 days (E2) after TAI or bull exposure. Data was analyzed by logistic regression (PROC GLIMMIX from SAS). On E1, none of the heifers subjected to TAI was cycling or had an A USC on the beginning of the BS. The pregnancy rate was higher on heifers with USC B [41.1% (122/297)] comparing to USC C heifers [17.8% (19/107); P=0.0005]. Pregnancy probability was higher for animals with greater SCFT (R2 = 0.208; P = 0.005) and DAWG (R2 = 0.168; P = 0.0007). The LEA (R2 = 0.115; P = 0.13) and hWIT (R2 = 0.309; P = 0.28) characteristics did not affect pregnancy rate. On E2, the cyclicity rate of heifers exposed to natural breeding was 5.3% (47/893) at the beginning of the BS. There was no difference (P=0.22) on pregnancy rate according to cyclicity on 30 days of BS [Cycling=27.7% (13/47) and Anestrous=9.9% (84/846)]. However, pregnancy rate on 50 days of BS differed [Cycling=53.2% (25/47) and Anestrous=13.4% (113/846); P>0.0001]. Pregnancy rate on 30 days of BS did not differ according to USC (P=0.2) [A=40.0% (10/25); B=12.4% (80/647) and C=3.2% (7/221)]. However, there was a difference (P<0,0001) on pregnancy rate on 50 days of BS according to USC [A=64,0% (16/25)a; B=17,9% (116/647)b and C=3,2% (7/221)c]. The pregnancy probability was not influenced by SCFT (R2 = 0.096; P = 0.42) and LEA (R2 = 0.061; P = 0.61). DAWG positively influenced the probability of cyclicity (R2 = 0.263; P < 0.0001) and pregnancy (R2 = 0.093; P = 0.005). hWIT negatively influenced pregnancy probability (R2 = -0.082; P = 0.03) and did not have effect on cyclicity probability (R2 = -0.062; P = 0.1). Pregnancy probability increased according to DF (R2 = 0.117; P = 0.0004). Thus, it was possible to verify that heifers with higher SCFT and DAWG had greater pregnancy probability to TAI. Heifers submitted to natural breeding with higher DAWG and DF and smaller hWIT had greater probability to become pregnant. Further, pregnancy rates to TAI and natural breeding were higher on heifers with greater USC.

Credits: Farms Nelore Jandaia, São Geraldo, Terra Boa, Agroandorinha, Martendal, Agropeva, Marca OB and CNPq 152030/2016-6.

A058 TAI/FTET/AI

# Follicular and luteal function of crossbred cows with different circulating concentrations of progesterone during synchronization of ovulation in an TAI protocol

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Due to the lack of information on the impact of serum P4 concentration during ovulation synchronization in crossbred dairy cows, the objective of this work was to study the effect of different circulating concentration of P4 at the time of ovulation synchronization in a TAI protocol under follicular dynamics and characteristics of the corpus luteum (CL). For this, 12 crossbred cows with a mean BCS of 3.07±0.38 were submitted to a pre-synchronization protocol to guarantee that all animals had CL in the beginning of the protocol, ensuring the presence of this structure by transrectal ultrasonography (US). Subsequently on Day 0 (D0), all the animals received a CIDR® device (Zoetis, São Paulo, Brazil) in association with 2mg of GONADIOL® (Zoetis, São Paulo, Brasil) administrated instramuscularly (IM) and half of them were treated with 12.5mg of LUTALYSE® (Zoetis, São Paulo, Brazil) (IM). On D8 the CIDR devices were removed and then 12.5mg of LUTALYSE (IM) and 1mg of ECP® (Zoetis, São Paulo, Brazil) (IM) were administered. At this time, the females were splitted into two groups based on the treatment with PGF2α at D0: group w/CL (n=6) and group W/O CL (n=6). The animals underwent evaluation of the follicular dynamics and vascularization using B-mode and color Doppler US (Mindray Z5, Shenzhen, China), the exams took place every 12 hours from D8 until ovulation. Also, blood samples were collected to determine the serum pre-ovulatory P4 concentration (D0, D8 and D10 of the protocol). On D24 of the protocol, the morphological and functional characteristics of the CL were evaluated through color Doppler and B mode US, and blood samples were collected to measure the serum P4 concentration. The data were analyzed using the ANOVA procedure and the Tukey test in SPSS, $P < 0.05$. The follicular diameters on D10 were 8.78 ± 2.04 and 13.12 ± 3.52 mm for the groups w/ CL and w/o CL respectively, the pre-ovulatory follicle diameter 9.48 ± 2.12 (w/ CL) and 13.66 ± 2.58 mm (w/o CL) and in the vascularized area of the wall of the pre-ovulatory follicle 0.09 ± 0.04 (w/ CL) and 0.18 ± 0.09cm² (w/o CL). The concentrations of P4 were 1.72 ± 1.16ng / mL (w/o CL) and 7.56 ± 3.70ng / mL (w/ CL) on D8. Females of the group w/ CL had a smaller diameter (17.66 ± 1.89 and 23.25 ± 4.46mm) and vascularized area (0.82 ± 0.29 and 1.21 ± 0.27cm²) of CL in D24 compared to females in the group w/o CL group, respectively. The serum P4 concentration on D24 did not differ between groups. In conclusion, high concentrations of P4 at the time of ovulation synchronization negatively impacted on the diameter, and follicular and luteal vascularization of crossbred cows.

A059 TAI/FTET/AI

# Twin pregnancy increases gestational loss in Nelore heifers submitted to FTAI

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The present study aimed to compare the maintenance of single or twin pregnancies between 30 and 60 days, and, between 60 days and calving, in Nelore heifers (*Bos taurus indicus*) submitted to FTAI. A total of 953 pubertal Nelore heifers (CL detection on Day 0) aging from 22 to 26 months received an auricular ear implant containing 3mg of Norgestomet (Crestar, MSD Animal Health, Brazil) associated with 2mg of Estradiol Benzoate IM (Estrogin, Biofarm, Brazil) on Day 0. On Day 8, the device was removed and 0.265mg of Cloprostenol Sodium (Ciosin, MSD, Brazil), 300IU of eCG (Novormon, Zoetis, Brazil) and 0.5mg of Estradiol Cypionate (ECP, Zoetis, Brazil) were administrated intramuscularly. The FTAI was performed by the same inseminator 48 hours after device withdrawal (Day 10). Ultrasonography (Aloka SSD 500, Tokyo, Japan) was performed 30 days after AI (Day 40) to determine pregnancy rate and the frequency of single or twin pregnancies. The animals were divided into 2 groups: Single Gestation Group (SGG) and Twin Gestation Group (TGG). Pregnancy loss between 30 and 60 days was established as the absence of fetus(es) or presence of dead fetus(es) on Day 70-ultrasonography examination on previous pregnant heifers. Moreover, the pregnancy loss between 60 days and parturition was defined as the visual detection of placenta prior to the predicted calving date (292 days after FTAI) and/or no calving until 60 days after this prediction. The results were analyzed by PROC GLIMMIX the SAS® (Statistical Analysis System, version 9.3 Institute Inc., Cary, NC, USA, 2003). The pregnancy rate on Day 40 was 50.2% (478/953). The gestational status verified was: SGG=93.3% (446/478) and TGG=6.7% (32/478). The maintenance of the pregnancy between 30 and 60 days after the FTAI was greater (P=0.03) in the SGG (96.4%; 430/446) than in the TGG (65.6%; 21/32). Pregnancy loss between 30 and 60 days was lower (P <0.001) in SGG (3.6%; 16/446) than in TGG (34.4%; 11/32). Similarly, pregnancy loss between 60 days after FTAI and calving was lower (P<0.0001) in SGG (11.6%; 50/430) than in TGG (71.4%; 15/21). Therefore, we concluded that gestational loss after FTAI was higher in Nelore heifers holding twin pregnancies when compared to heifers presenting single fetus gestation.
Acknowledgments: HoRa Agronegócio e Virbac Animal Health.

A060 TAI/FTET/AI

# Immunization against IBR, BVD, leptospirosis and campilobacteriosis (Bovigen® Repro Total SE) increases conception rate and decreases embryo loss of Nelore cows under TAI program

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This study aimed to evaluate the effect of immunization against IBR, BVD, leptospirosis and campylobacteriosis on the pregnancy rate and embryo loss of Nelore cows submitted to TAI. Nelore cows (n = 323; BCS = 2,78 ± 0,12) were used, from a comercial farm under pasture conditions without history of vaccination against the reffered desease. Females underwent to ultrasound (D-21) assessment to confirm the absence of gestation were divided into two groups based on BCS, as follows: Control (n = 156) - animals that received no vaccination and Vaccinated (n = 167) - 5 mL i.m. of the Bovigen® Repro Total SE vaccine (Virbac Animal Health, Brazil). Vaccinated group received on D0, a second dose of the reproductive vaccine and a TAI protocol was started in all 323 females with an intravaginal P4 device (Sincrogest®, Ourofino, SP, Brazil) introduced and injected i.m. 2 mg EB (Sincrodiol®, Ourofino, SP, Brazil). On D8, P4 device was removed and injected 500µg i.m. sodium cloprostenol (Sincrocio®, Ourofino, SP, Brazil), 1 mg of ECP i.m. (E.C.P.®, Zoetis, SP, Brasil) and 300 IU of eCG (Novormon®, Intervet, SP, Brasil). Females were inseminated 48 h after P4 removal and on D13 was injected 150 mg of P4 i.m. IM (Sincrogest, Ourofino, SP, Brasil). Pregnancy diagnosis and embryo loss exam was performed by ultrasonography (Chison 9300 vet, Kylumax – Indaiatuba/SP) on D40 and D77. Statistical analysis was performed with proc GLIMMIX of SAS, considering significance when $p<0.05$. Pregnant cows on D40 detected without fetus or even fetus with no heart beats were considered with pregnancy loss. However, on D40 there was not difference ($p = 0.1083$) between control and vaccinated [46.15% (72/156) vs. 54.49% (91/167)]. On the other hand, pregnancy was higher for vaccinated on D77 [control = 41.67% (65/156) vs. vaccinated = 53.89% (90/167); $p = 0.0285$)]. Control group showed higher pregnancy loss between 30 and 67 days after AI [control = 9.72% (7/72) vs. Vaccinated = 1.1% (1/91); $p = 0.0372$]. Thus, data suggests that immunization with Bovigen® Repro Total SE (IBR, BVD, leptospirosis and campylobacteriosis) lean Nelore cows under grazing conditions in Central Brazil, never vaccinated for these diseases, provided greater pregnancy at 67 days probably due to the fact that the immunized animals showed less embryonic loss. Complementary studies are underway to evaluate the seroprevalence of these diseases.

A061 TAI/FTET/AI

# Early induction of luteolysis in FTAI protocols increases fertility in beef cows

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Complete luteolysis is essential for pregnancy success in beef cows submitted to FTAI protocols. The objective of this study was to evaluate the effect of PGF administration on Day 7 compared to Day 8 of a FTAI protocol on serum P4 concentration, estrus behavior, ovulatory follicle (OF) diameter, and pregnancy per AI (P/AI). A total of 469 non-lactating Angus cows (with CL, n= 359 or without CL, n= 110), BCS 2.9±0.2 (1 to 5 scale), maintained on pasture were used. Cows received a 1g progesterone-releasing intravaginal device (Reproneo; GlobalGen, Brazil) and 2mg of estradiol benzoate i.m. (Syncrogen; GlobalGen, Brazil) on Day 0. On Day 7, animals were randomly allocated into two groups. Cows were treated with 500mcg sodium cloprostenol, i.m. (Inducio; GlobalGen, Brazil), on Day 7 (PGF7, n= 238) or on Day 8 (PGF8, n= 231). P4 devices were removed and 0.5mg of estradiol cypionate, i.m. (Cipion; GlobalGen, Brazil) was injected in all females on Day 8. FTAI was performed 48 to 52 h after P4 device removal. Females had sacral region painted with appropriate paint (TELL TAIL, GEA, New Zealand) on Day 8 to detect expression of estrus. Transrectal ultrasonography (7.5 MHz linear transducer, MediSono P3V, USA) was performed on Day 0 to detect presence of CL, on Day 10 to measure OF diameter, and on Day 40 to diagnose pregnancy. Blood samples were collected on Days 8 and 10 to measure P4 serum concentration by radioimmunoassay. Estrus behavior and pregnancy rate were analyzed as binary outcomes using logistic regression (Proc GLIMMIX, SAS). OF diameter and P4 concentration were submitted to analysis of variance (Proc GLIMMIX, SAS). The estrus manifestation was greater (Odds ratio= 2.9; P = 0.0002) in females of PGF7 group (91.6%; 218/238) than PGF8 (78.8%; 182/231). The P/AI of cows that exhibited estrus was 60.2% (241/400) vs. 39.1% (27/69), resulting in higher chance (Odds ratio= 2.4) of pregnancy associated with estrus behavior (P = 0.0014). OF diameter did not differ (P = 0.0881) between PGF7 (11.7mm) and PGF8 (11.3mm). The P/AI was higher (P = 0.0034) for PGF7 group (63.9%, 152/238) vs. PGF8 (50.2%, 116/231). In group PGF7, P4 on Day 8 did not differ between pregnant (1.7ng/ml) and non-pregnant (1.9ng/ml) females. However, group PGF8 pregnant females had lower P4 concentration (2.6ng/ml) on Day 8 (P = 0.0005) than non-pregnant (3.4 ng/ml) females. On Day 10, P4 did not differ between treatments (PGF7 = 0.11ng/ml vs. PGF8= 0.09ng/ml) and did not affect fertility (P= 0.2515). According to results, OF diameter and P4 concentration on Day 10 were not influenced by the day of PGF administration, however, earlier PGF injection resulted in higher estrus behavior and increased fertility.

A062 TAI/FTET/AI

# Influence of vaccination against reproductive diseases and use of streptomycin in the pregnancy rate and gestational loss of buffaloes submitted to FTAI in the state of Amapá, Amazon, Brazil

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The objective was to evaluate the effects of vaccination against IBR/BVD/ Leptospirosis and the use of streptomycin in the pregnancy rate and gestational loss of buffaloes submitted to Artificial Insemination at Fixed Time, extensively created in floodplain area in the state of Amapá, Amazonia, Brazil. Eighty pluriparous buffaloes with (ECC) 2.94 ± 0.43 were used. The animals were divided into two groups: Treated Group (GT) with 43 animals and Control Group (CG) with 54 animals. The GT group received the application of streptomycin (Estreptomax®, OuroFino Saúde Animal, Brazil) at D9 of the IATF protocol at a dose of 10 mg / kg intramuscularly, plus a vaccine against IBR/BVD/ Leptospirosis (Poliguard®, Vallée, Montevideo , Uruguay) at the 5 mL dose subcutaneously. A second dose of the IBR/BVD/ Leptospirosis vaccine (Poliguard®, Vallée, Montevideo, Uruguay) was reapplied 30 days after inseminations. The Control- (CG) group received no treatment. The GT and GC were submitted to the same estrus synchrony protocol: {day zero [D0] in the afternoon 2.0mg estradiol Benzoate (BE) by IMP (Sincrodiol®, OuroFino Saúde Animal, São Paulo, Brazil), insertion Of intravaginal progesterone-P4 mono-dose device (Primer®, Tecnopec, São Paulo, Brazil); (D9) and P4 + 0.5 mg of prostaglandin (PGF2α) by IMP (Sincrocio®, OuroFino Saúde Animal, São Paulo, Brazil) + 400 IU of equine chorionic gonadotrophin - eCG by the IMP pathway (SincroeCG®, OuroFino Saúde Animal, Brazil); Day 11 (D11) in the afternoon 25µg of gonadotrophin releasing hormone - GnRH by IMP (Gestran Plus®, Tecnopec, São Paulo, Brazil) and day 12 (D12) morning IATF. The diagnosis of gestation was performed 30 and 90 days after inseminations. The chi-square test was used to evaluate the pregnancy rate. Fisher's exact test was used with 5% significance ($p < 0.05$) for gestational loss. The overall pregnancy rate was 37.11%, the GT pregnancy rate was 41.87% and the GC was 33.33%, and did not differ statistically ($P > 0.05$). The gestational loss rate was 11.11% in GT and 22.22% CG, with no difference ($P > 0.05$). There was no significant difference between the groups, thus, streptomycin and vaccination had no influence on pregnancy rate or embryo losses.

A063 TAI/FTET/AI

# Fixed-time artificial insemination in buffaloes raised extensively in swamp of the state of Amapá

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We evaluated the FTAI in buffaloes raised extensively. Thus, the influence of the body condition score, the insemination period, the semen used and the protocols used in the pregnancy rate of buffaloes was verified. The work was carried out at the Motogeral farm, municipality of Itaubal in the state of Amapá, ground of native pastures in a floodplain region, the FTAI were in the low tide of the Amazon River between September 2016 and January 2017. 138 buffaloes were used, selected for Choice of protocol by ECC and presence of the corpus luteum. The first phase was performed in the months of September / October, 56 females with 3.5 ± 0.6 ECC, received the following protocol. In the D0 (the afternoon) intravaginal Progesterone (Sincrogest®) 3rd use device plus 2.0 ml of estradiol Benzoate IM (Sincrodiol®); D9 (the afternoon) withdrawal from the device + 2.0ml PGF2α (Synchroci®) + 400UI eCG (SincroeCG®); D11 (in the afternoon) 1.0ml of GnRH (Gestran Plus®) and in D12, FTAI in the morning. The second phase was performed in the months of November to January 2017, 34 females with ECC = 3.59 ± 0.7 received the protocol called (ovsynch-plus), in the D0 (the afternoon) single-use progesterone intravaginal device (monodose Primer) plus 1.0 ml of GnRH (Gestran Plus®); D9 (the afternoon) withdrawn from the device and application of 2.0ml PGF2α Sincrocio®; D11 (in the afternoon) 1.0ml GnRH Gestran Plus®; D12 (in the morning) FTAI. Other 48 buffaloes with ECC = 3.09 ± 0.95 received the protocol called (ovsynch-plus) except for the application of 400UI (2.0ml) eCG (SincroeCG®) in D9. After 30 days of the inseminations, a diagnosis of gestation was performed using the Mindray D2200 vet device. The data were evaluated through specialized SAS software (2009) and the chi-square statistical test with significance level of 5%. Of the 138 buffaloes, 57 (41.3%) were pregnant. At the FTAI in September / October, 44.6% (25/56) became pregnant. At the FTAI of November 2016 / January 2017, the pregnancy rate with the protocol called ovsynch-plus was 55.88% (19/34) and the rate in buffaloes with ovsynch-plus plus eCG was 27.08% (13/48). This lower pregnancy rate is explained by the decrease in body condition and the beginning of floods (January) in the region. There was no statistical difference in the body condition score, and the use of national or imported semen. There was a difference ($P < 0.05$) between the protocols used. We conclude that the period from September to January, in this region is a favorable season for FTAI, however, to avoid the onset of floods, and that the ovsynch-plus protocol proved to be satisfactory.

A064 TAI/FTET/AI

# Epidemiological survey based on the prevention of endometritis in mares donating embryos in the state of Pernambuco

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Uterine infections are the most common cause of infertility in mares. In general uterine lesions result from ascending infection infectious agents introduced during manipulations such as artificial insemination, embryo transfer or postpartum. The objective of this work was to do an epidemiological survey from cases sent to our laboratory and guide the veterinarians to minimize these problems. Twenty one mares were examined by rectal palpation and ultrasound, all with a history of infertility or negative embryo transfer flush. Uterine cytology and microbiological samples were collected. For that, sterile swab was used (PROVAR®). Then, a microscope slide smear harvested from the endometrial surface was prepared, and swabs were placed, immediately sealed, identified, chilled and sent to LABRAPE from UFRPE/UAG for further processing. From the 21 mares collect, 6 showed no clinical signs (28.5%) by rectal palpation or ultrasound examination, while the remaining 15 mares (71.4%) presented some type of disfunction such as presence of intrauterine fluid or vaginal discharge. Nineteen (90,4%) of the samples had cytological findings and microbiological tests positive demonstrating a high correlation between these techniques. The microorganism presented most often was Streptococcus spp., being seen in 6 cultures (6/21 - 28.5%), followed by Staphylococcus spp (5/21 - 23.8%), Enterobacter SP and Coccus bacillus (4/21 - 19.0% each) and 2 isolates of Klebsiella sp (9.5%). Fungi were isolated in 9 out of 21 samples analysed (42.8%). Aspergillus spp (4/9 - 44.4%), Candida (2/9-22.2%), and there is still the development of other genres like Curvalaria spp, Cladosporium spp. and Zygomices spp. all with percentage of 11.1% (1/9) each. Mixed fungal and bacterial infections were recorded on 6 samples (28.5%). The rate of fungal infection were considered very high above standards and during epidemiological questionnaires with the veterinarians, were detected 2 important risk factors: history of uterine treatments without achieving bacteriological examination and/or culture, in addition to repeated flushes uninterrupted for embryo collection. The indiscriminate use of antibiotics is associated with reduction of immunity that leads to an increased incidence of these infections. It is important to stress the necessity for agent identification and knowledge of drug susceptibility in order to achieve effectiveness in the therapy employed. The results of this study demonstrate the high rate of fungal infections in embryo transfer donors mares and the need of good practice in the field in order to minimize economic losses and improving reproductive efficiency in equine livestock.

A065 TAI/FTET/AI

# Improved conception results following GnRH treatment on day 2 of progesterone and estradiol-based synchronization protocols in high producing dairy cows

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The objective of this study was to study the possibility of improving conception results (P/AI) with a GnRH treatment given at different times at the beginning of progesterone + estradiol-based protocols in high producing dairy cows. Holstein cows (n = 871) from two commercial herds in Brazil, with daily production of 33.2 ± 4.8 kg at 170.1 ± 11.2 DIM were enrolled at 1st post-partum AI or when found open at pregnancy diagnosis. Cows were randomly allocated one of the following timed AI protocols: 1) EBP4: D0 = 2mg of EB (estradiol benzoate, Biofarm) + progesterone device insertion P4 (PRID®, Ceva), D7 = PGF2a (Veteglan®, Hertape), D9 = 1mg ECP (Cipionato-HC, Hertape) + PGF2a (Veteglan®, Ceva) plus PRID removal, D11 = TAI, approximately 48h after PRID removal; 2) EBG0: similar protocol, with the addition of a GnRH (Cystorelin, Merial) on day 0 (D0); 3) EBG2: similar protocol, with the addition of a GnRH on day 2 (D2). Pregnancy diagnosis by ultrasound was performed 30 days post AI. Statistical analysis were performed with the Glimmix procedure of SAS, 9.4. Cows in EBG2 had greater conception results compared to EBP4 cows (38.2% vs 28.7%; $P<0.05$). Cows in EBG0 had intermediate conception results (34,5%). In addition, there was no interaction between type of protocol and parity ($P=0.11$) or type of protocol and farm ($P=0.79$). These findings suggest a positive effect when associating GnRH 2 days after EB in progesterone-based timed AI protocols, likely related to improved synchrony of follicular emergence in cows responding either to EB or GnRH. Acknowledgements: Ceva.

A066 TAI/FTET/AI

# Morphometry and lutein endocrinology Heifers of Nelore supplemented with sunflower seed

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The influence of nutrition on reproductive performance is a topic that has generated interest in researchers and especially lipid supplementation. Studies using a diet supplemented with polyunsaturated fatty acids (PUFAs), found in seeds such as sunflower and linseed, indicate benefits for reproductive functions, such as: altered follicular development, increased progesterone concentration, improved embryo quality and decreased Luteolytic signals during maternal recognition of gestation. The objective of this study was to evaluate the luteal development and plasma concentration of progesterone in Nelore heifers supplemented with sunflower seeds. Thirty animals aged 2 to 3 years, with a body condition score of 2.5 to 3.5 (1 to 5), were used in Brachiaria decumbens pasture, with water and mineral salt ad libitum. Before initiating ultrasonographic evaluations of the reproductive tract, heifers were divided into two groups, where they received: 1.7 kg / day of food supplement with 40% of soybean meal and 60% of sunflower seed (GT group: N = 15) or 1.7 kg / day feed supplement containing 53% soybean meal and 47% corn (Control Group, GC: n = 15) for 60 days. Immediately after this period the heifers had ovulation synchronized with hormonal protocol of IATF (artificial insemination at fixed time), 3 passages with implant placement at D0 and withdrawal at D8. From the D10, an ultrasound (US) examination of the ovaries and blood collection was performed to monitor the development of the corpus luteum (CL) and plasma progesterone concentration (P4), every 48 hours, for 26 days. Analysis of variance was used followed by the Tukey test when there was difference, using a significance level of 5% ($P < 0.05$). There was no difference ($P > 0.05$) in the CL diameter between the groups studied, with the maximum diameter in the GT 17.61 mm observed on day 10 and the GC 19.51 mm on day 12. The supplementation also did not influence the production of progesterone which reached its maximum concentration in D12 (7.07 ng / mL) in GC and D14 (6.5 ng / mL) in GT. It was concluded that the diet with sunflower seed did not affect the morphology or functionality of CL, since there was no difference in progesterone diameter or plasma concentration. FAPESP (2015/24007-0).

A067 TAI/FTET/AI

# New strategies to improve pregnancy rate at TAI using sex-sorted semen

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Two experiments was conduced to define strategies to optimize pregnancy rate in suckled Nelore cows to TAI with sex-sorted semen. Cows receiveid the same TAI protocol in both experiments (2mg of EB + intravaginal P4 device on D0; device was removed and PGF2α + 300IU of eCG + 1mg of EC were adminstered on D8 and cows were inseminated on D11). Cows' handling was done at end of the day (5:00-7:00pm), and TAI was done on the morning of D11 (5:00-7:00am; 60 hours after P4 device removal). In experiment 1 (n=796), three Nelore bulls had their semen sex-sorted for females. The same ejaculate of each bull was distributed following experimental groups: 1) Conventional, frozen semen without sexing with 20x106 of sptz; 2) Legacy, sex-sorted semen with 2,1x106 of sptz (previous sexing methodology); 3) Sex-Ultra 2, sex-sorted semen with 2,1x106 of sptz (current sexing methodology); 4) Sex-Ultra 4, sex-sorted semen with 2,1x106 of sptz (current sexing methodology with enhanced concentration). The data were analyzed using the PROC GLIMMIX procedure of SAS version 9.3. There was no difference in the pregnancy rate among bulls (P=0.15), among farms (P=0.46) and no interaction bull*group (P=0.84) and farm*group (P=0.95). However, there was difference in pregnancy rate to TAI according to the used method [Conventional=56.2%a (112/199), Legacy 2.1=28.2%c (58/206), Ultra 2.1=37,6%bc (72/191) and Ultra 4.0=43.0%b (86/200); P<0.0001]. In experiment 2 (n=613), three Angus bulls had their semen sex-sorted for males. The same ejaculate of each bull was distributed to the following experimental groups: 1) Conventional, frozen semen without sexing with 20x106 of sptz; 2) Sex-Ultra: 4.0x106 of sptz; 3) Sex-Ultra Pure: 4.0x106 of sptz (with removal of dead sptz). A subgroup of 431 cows had the base of their tail painted for detection of estrus between P4 device removal and TAI. There was no difference in the pregnancy rate between bulls (P=0.12). There was farm effect (P=0.03), however, there as no farm*group interaction (P=0.61) and bull*group interaction (P=0.40). The pregnancy rate was similar between the experimental groups [Convencional=51.2% (107/209), Sex-Ultra: 4.0=42.0% (84/200), Sex-Ultra Pure=43.1% (88/204); P=0.10]. In the subgroup, there was difference in the pregnancy rate according to the estrus manifestation [Estrus=52.6% (161/306) vs No estrus=32.8% (41/125); P<0.0001). Furthermore, there was an interaction method (sex-sorted vs non sex-sorted) and estrus manifestation (P=0.0002). Decreased pregnancy rate to TAI was observed with sex-sorted semen only in cows tqhat did not present estrus. In cows presented estrus, this difference was not observed. It was conclued that sex-sorted semen Ultra with 4.0x x106 of sptz had higher pregnancy rate than Legacy with 2.1x106 of sptz. Cows that presented estrus had the same pregnancy rate at TAI when inseminated with conventional semen or sex-sorted semen. Acknowledgments: Sexing Technologies.

A068 TAI/FTET/AI

# Young Nelore Heifers treated with Fertilcare 600® or Crestar® have similar pregnancy rates following TAI

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The aim of this study was to evaluate the effect of using Fertilcare 600® or Crestar® on the diameter of the dominant follicle (DF), the occurrence of estrus and pregnancy per AI (P/AI) of young beef heifers treated for TAI. A total of 271 Nelore heifers aging 12-16m old (average 14,9±0,07), with average body weight (BW) 273.0±1.3kg, average BCS 2.97±0.01 (1-5 point scale), and average uterine maturity 2,37±0,04 (1-4 scale) from two commercial farms in Rondonópolis and Apiacás, MT State, Brazil were used. Heifers had at least 240kg and were treated with the same protocol of synchronization of follicular wave emergence and ovulation for TAI, except for the use different sources of progesterone (P4) or progestin at the onset of treatment. Briefly, at random days of the estrous cycle (D0) all heifers received 1mg estradiol benzoate (Fertilcare Sincronização®, MSD, São Paulo, Brasil) IM and were homogenously distributed (BCS, BW and age) to receive one intravaginal device containing 0,6g P4 (Fertilcare 600®, MSD) or one progestin ear implant containing 3mg norgestomet (Crestar®, MSD). On D8, the device/implant was removed and 0.265mg Sodium Cloprostenol (Ciosin®, MSD), 200IU eCG (Folligon®, MSD) and 0.5mg estradiol cypionate (Fertilcare Ovulação®, MSD) IM were administered. Also on D8 heifers were painted with chalk on their tailheads, and removal of chalk was used as an indication of estrus. TAI was done by a single veterinary 48h after device removal (sheaths 3W, WTA, Cravinhos, Brazil), concomitant with estrus determination and measurement of the diameter of the DF by ultrasonography on D10. Semen of one Aberdeen Angus and one Senepol bull was homogenously distributed between groups. Diagnosis of P/IA was performed 45d after TAI (Mindray DP2200VET). Data was analyzed by logistic regression (PROC GLIMMIX from SAS). Effect of bull (P=0.02) and farm (P=0.02) was observed. Heifers that showed estrus were older (15.1±0.1 vs 14.8±0.1; P=0.03) and heavier (275.7±1.6 vs 268.0±2.0; P=0.04) than those without estrus demonstration. The proportion of heifers with CL on D0 [3.8% (5/133) and 1.5% (2/138); P=0.45] and the grade of uterine maturity on D0 (2.41±0.07 and 2.32±0.06; P=0.50) were similar in heifers treated with Crestar® and Fertilcare 600®, respectively, evidencing the adequate balance between groups at the onset of treatment. Greater (P=0.02) rate of estrus demonstration on D10 was observed in heifers treated with Crestar® and Fertilcare 600® [71.2% (94/132)] than Fertilcare 600® [56.5% (78/138)]. However, similar diameter of the DF on D10 (10.0±0.1 and 10.0±0.1; P=0,72) was detected. P/IA tended to be greater (P=0.06) in heifers treated with P4-releasing intravaginal device Fertilcare 600® [39.9% (55/138)], compared with those treated with norgestomet ear implant, Crestar® [31.6% (42/133)]. In conclusion, both devices Crestar® and Fertilcare 600® can be used for TAI in young Nelore heifers with similar P/IA.
Credits: Farms São José and São João, CNPq152030/2016-6.

A069 TAI/FTET/AI

# Treatment with GnRH (Gonaxal®) at AI increases pregnancy rate of nelore cyclic heifers that showed or not estrus during the TAI protocol, with greater impact in those without estrus demonstration

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The aim of this study was to evaluate the effect of using GnRH at AI on pregnancy per AI (P/AI) of cyclic beef heifers that showed or not estrus during TAI protocol. A total of 790 Nelore heifers aging 16-20m old, with average body weight (BW) 304.3±1.8kg and average BCS 3.10±0.01 (1-5 point scale) from two commercial farms in Nova Bandeirantes and Apiacás, MT State, Brazil were used. Heifers had uterine maturity and were treated with the same protocol of synchronization of follicular wave emergence and ovulation for TAI, except for the use or not of GnRH at AI. Briefly, at random days of the estrous cycle (D0) heifers received a Cronipres® Mono Dose intravaginal device with 1g P4 (Biogénesis Bagó, Curitiba, Brazil), 1mg estradiol benzoate (Bioestrogen®, Biogénesis Bagó) and 75 µgD-Cloprostenol (PGF2α, Croniben®, Biogénesis Bagó) IM. On D8, device was removed and 75µg D-Cloprostenol, 200IU eCG (Ecegon®, Biogénesis Bagó) and 0.5mg estradiol cypionate (Croni-Cip®, Biogénesis Bagó) IM were given. Also on D8 heifers were painted with chalk on their tailheads, and removal of chalk was used as an indication of estrus. TAI was done by a single veterinary 48h after device removal (sheaths 3W, WTA, Cravinhos, Brazil), concomitant with estrus determination and measurement of the diameter of the dominant follicle (DF) by ultrasonography on D10. At that time, heifers that showed or not estrus were homogenously allocated (BCS, BW and age) to receive or not 10.5µg buserelin acetate (GnRH; Gonaxal®; Biogénesis Bagó), in a 2x2 factorial design. Semen of four Aberdeen Angus bulls was equally distributed between groups. Diagnosis of P/IA was done 45d after TAI (Mindray DP2200VET). Data was analyzed by logistic regression (PROC GLIMMIX from SAS). Effect of bull (P=0.006), estrus (P=0.02), GnRH (P<0.0001) and interaction estrus*GnRH (P=0.02) was observed. The proportion of heifers with CL on D0 [60.6% (206/340) and 64.8% (259/400); P=0.32], showing estrus on D0 [65.6% (240/366) and 66.3% (281/424); P=0.83] and the diameter of the DF on D10 (12.2 ± 0.1 and 12.0 ± 0.1; P=0.17) were similar for heifers treated or not with GnRH, respectively, evidencing the adequate balance between groups before GnRH treatment. Heifers showing estrus had greater BCS (3.13±0.01 vs 3.06±0.02; P=0.002), BW (307.6±1.6 vs 299.6±1.9; P=0.001) and diameter of the DF on D10 (12.6±0.1 vs 11.1±0.1; P=0.001) than those without estrus, regardless of GnRH treatment. P/IA increased when GnRH was given on D10 in heifers with and without estrus demonstration verified on D10 [No estrus No GnRH=29.5% (41/139), No estrus+GnRH=51.2% (63/123), Estrus No GnRH=45.6% (128/281), Estrus+GnRH=51.9% (123/237); P<0.0001]. Thus, the administration of GnRH at AI increases P/IA of Nelore heifers that had showed or not estrus during TAI protocol, with greater impact in those without estrus demonstration. Thus, it can be used as a tool to optimize TAI outcomes. Credits: Farms Beira Rio and São João, CNPq 152030/2016-6.

A070 TAI/FTET/AI

# Treatment with GnRH (Gonaxal®) at AI increases pregnancy rate of Nelore primiparous cows that showed or not estrus during the TAI protocol

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The aim of this study was to evaluate the effect of using GnRH at AI on pregnancy rates of primiparous beef cows that showed or not estrus at the end of the TAI protocol. A total of 703 Nelore primiparous cows with average body weight 341.8 ± 2.6 kg and average BCS 2.79 ± 0.01 (1-5 point scale) from two commercial farms in Nova Bandeirantes, Mato Grosso State, Brazil were used herein. All cows were treated with the same protocol of synchronization of follicular wave emergence and ovulation for TAI, except for the administration or not of GnRH at AI. Briefly, at random days of the estrous cycle (D0) all cows received a Cronipres® Mono Dose intravaginal device containing 1g P4 (Biogénesis Bagó, Curitiba, Brazil) and 2mg estradiol benzoate (Bioestrogen®, Biogénesis Bagó) IM. On D8, the device was removed and 150µg D-Cloprostenol, 300IU eCG (Ecegon®, Biogénesis Bagó) and 1mg estradiol cypionate (Croni-Cip®, Biogénesis Bagó) were administered IM. Also on D8 cows were painted with chalk on their tailheads, and removal of chalk was used as an indicator of estrus. TAI was performed by a single veterinarian 48h after device withdrawal (sheaths 3W, WTA, Cravinhos, Brazil), concomitant with estrous detection by visual analysis of tail-paint score, and measurement of the diameter of the dominant follicle (DF) by ultrasonography on D10. At that time, cows that have or have not showed estrus were randomly blocked by BCS and body weight to receive or not 10.5µg buserelin acetate (GnRH; Gonaxal®; Biogénesis Bagó), following a 2x2 factorial arrangement of treatments. Semen of one Nelore and three Aberdeen Angus bulls were homogenously used among groups. Pregnancy diagnosis was performed by ultrasonography 45d after TAI (Mindray DP2200VET) to determine pregnancy per AI (P/AI). Data was analyzed by logistic regression (PROC GLIMMIX from SAS). Effects of bull (P=0.06), farm (P=0.04), estrus (P=0.06), and GnRH (P=0.03) were observed. There was no interaction estrus*GnRH (P=0.80). The proportion of cows with CL on D0 [10.3% (38/304) and 8.4% (25/299); P=0.08], cows showing estrus on D0 [56.5% (200/354) and 53.9% (188/349); P=0.57] and the diameter of the DF on D10 (12.4±0.1 and 12.2±0.1; P=0.10) were similar for cows treated or not with GnRH, respectively, evidencing the adequate balance between groups before GnRH treatment. Cows that showed estrus had greater cyclicity (presence of CL) on D0 (13.7% vs 6.9%; P=0.02) and greater diameter of the DF on D10 (12.8±0.1 vs 11.8±0.1; P<0.001) than those not detected in estrus, regardless of GnRH treatment. P/IA increased (P=0.03) when GnRH was administered on D10 in both primiparous with estrus [(No GnRH=49.2% (91/185), GnRH=58.3% (116/199)] and without detected estrus [No GnRH=45.9% (73/159) vs GnRH=52.6% (80/152)]. Thus, the administration of GnRH at AI increases P/IA of Nelore primiparous cows that had showed or not estrus during the TAI protocol and can be used as a potential tool to optimize TAI outcomes. Credits: Farms Beira Rio and Vitória, CNPq 152030/2016-6.

A071 TAI/FTET/AI

# Can female body condition influence the "bull effect" at Timed-AI ?

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It is well known that cow body condition score (BCS) affects pregnancy rate (PR). In addition, our group have repeatedly demonstrated the semen influence on reproductive program outcomes ("bull effect"). However, analyzing the present field data, some observations have brought us questions not yet raised. Thus, this study aimed to evaluate if different sires present same reproductive performance regardless cow body condition at Timed-AI. Hence, data of Timed-AI from 596 Nelore multiparous cows, between 45 and 55 days postpartum, were analyzed. All cows received same hormonal protocol with intravaginal progesterone implant for 9 days, being eCG and PGF2α applied on D9 (day of implant removal). Timed-AI was performed on D11. Only animals presenting BCS between 2.75 and 4 (in the evaluation of 1 to 5) were included in the study. The two AI technicians shift between them at each 5 animals entering the chute. Semen of 4 Angus bulls were balanced distributed among cows. Data of PR were compared between groups using Fisher's exact test (P = 0.05) in GraphPad INSTAT program. Overall PR was 45% and no effect of BCS on PR was observed (P>0.05). Cows with BCS between 2.75 and 3.0 (BCS 1 group) presented 44% pregnancy (n = 234), animals with BCS between 3.25 and 3.5 (BCS 2) presented 46% (n = 270) and cows presenting BCS between 3.75 and 4.0 (BCS 3; n = 92) presented PR = 43%. Bulls 2 and 4 presented similar PR (P>0.05), independent of BCS group (Bull2: BCS1=40%, n=55; BCS2=31%, n=71; BCS3=44%, n=25; Bull4: BCS1=32%, n=65; BCS2=42%, n=60; BCS3=36%, n=25). Bull 3 presented higher (P<0,05) PR than other bulls, independent of BCS group (BCS1=62%, n=55; BCS2=58%, n=73; BCS3=59%, n=22; P>0,05). However, bull 1 presented a statistical trend (P=0,0976) for lower PR in fattest cows (BCS1=44%, n=59AB; BCS2=52%, n=66A; BCS3=35%, n=20B). We believe this is the first time that has been addressed the possibility of "bull effect" being influenced by cow's BCS. Thus, we want to share the thought that animals with greater fat deposition may present hormonal and ovulatory alterations (either by delaying or advancing ovulation), or alterations in uterine environment, which may, in turn, favor one or another type of semen.

A072 TAI/FTET/AI

# Pregnancy and embryo mortality of Holstein cows treated with ECG before or after TAI

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The objective of this study was to compare pregnancy per artificial insemination (P/AI) and embryo mortality (EM) of cows treated with eCG 3 days before or after TAI. A total of 963 Holstein dairy cows, primiparous (n = 427) and multiparous (n = 563) (mean±SD 2.0±1.1 lactations, 159.5±122.8 DIM, 3.3±2.6 previous AI, 29.5±9.5 daily milk yield) from 2 dairy herds (Farm 1, n = 414, Águas da Prata-SP, Farm 2, n = 549, São João Batista do Glória-MG ). At random day of the estrous cycle (D-10), cows received two intravaginal devices containing 1.9 g of P4 (iP4, CIDR®, Zoetis, São Paulo, SP, Brazil) and 2 mg of estradiol benzoate (EB, Gonadiol®), im. Seven days later, on D-3, 25 mg of PGF2α (Lutalyse®) were administered and the cows were allocated in one of three treatment: Control (n = 357), cows were not treated; eCG-pre (n = 304), cows received 400 IU of eCG (Novormon®) i.m; and eCG-post (n = 302), cows were not treated on D-3, however, they received 400 IU of eCG, i.m., 3 days after TAI (D+3). On D-2, the iP4 were removed and the cows received 1.0 mg of estradiol cypionate (EC, E.C.P.), i.m. TAI was performed on D0. The P/AI was determined by transrectal ultrasonography (7.5 MHz linear transducer, Mindray DP 2200 vet, Mindray, China), 30 (P/AI 30) and 60 days (P/AI 60) after TAI. Pregnant cows at 30 days but not pregnant at 60 days after TAI were considered to be affected by EM. The data were analyzed using the Glimmix procedure of program SAS 9.3. It was considered statistical difference when $P \leq 0.05$ and tendency when $P > 0.05$ and $\leq 0.10$. Results for groups Control, eCG-pre and eCG-post were, respectively: P/AI 30 - 28.6 (102/357), 31.9 (97/304) and 31.5% (95/302) (P = 0.43); P/AI 60 - 26.1 (93/357), 29.9 (91/304) and 28.5% (86/302) (P = 0.40); EM - 8.8 (9/102), 6.2 (6/97) and 9.5% (9/95) (P = 0.27). The results between Farms 1 and 2 were, respectively: P/AI 30 - 35.3 (146/414) vs. 27.0% (148/549) (P = 0.01); P/AI 60 - 30.7 (127/114) vs. 26.1 (143/549) (P < 0.01); EM - 13.0 (19/146) vs. 3.4% (5/148) (P = 0.25). The only result that tended to present interaction between treatments (Control, eCG-pre and eCG-post) and farms (1 and 2) was P/AI 60: Farm 1 - 29.4 (50/170)ab, 37.1 (46/124)a and 25.8% (31/120)ab; Farm 2 - 23.0 (43/187)b, 25.0 (45/180)b and 30.2% (55/182)ab (P = 0.08). It was concluded that treatment with 400 IU of eCG 3 days before or after TAI does not influence P/AI 30 and EM. In contrast, the eCG-post-treat was the only one able to keep the P/AI close to the highest levels on both farms. Acknowledgments: State of São Paulo Research Support Foundation - FAPESP (processes 2014/00739-9 and 2015/02551-0).

A073 TAI/FTET/AI

# Pre-synchronization by persistent dominant follicle induction using a progesterone device in GnRH-based synchronization of ovulation protocols in lactating dairy cows

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The objective was to evaluate the pre-synchronization by persistent dominant follicle induction using a progesterone device prior to the Ovsynch (Persynch) compared to the Double-Ovsynch (Double-Ov) protocol in lactating dairy cows. Lactating crossbred Holstein x Gyr (n=440) dairy cows were randomly assigned to one of two groups: (I) Double-Ov (n=228), 10 µg of GnRH (Day -17), 7 d later 500 µg of PGF2α (Day -10) and GnRH 3 days later (Day -7) followed by the Ovsynch protocol 7 d later (GnRH on Day 0, PGF on Day 7, GnRH on Day 9); (II) Persynch (n=212), cows received a progesterone (P4) intravaginal device (Day -10), 10d later (day 0) they were started on the Ovsynch protocol, and P4-device withdrawal on Day 7. All cows were inseminated 15 to 20 hours after the second GnRH of the Ovsynch protocol and pregnancy diagnosis was performed 30 and 60 d after AI. In a subgroup (n=102), ultrasound exams were performed on days 0, 7, 9 and 24 of the experimental period and blood samples were collected (n=44) on days 0, 7 and 24 for progesterone assays. Pre-synchronization rates [presence of follicles > 12mm on D0, Double-Ov 94.2% (49/52) and Persynch 92.0% (46/50); P=0.66], follicular diameter at the first GnRH (Double-Ov 17.2 ± 0.7mm and Persynch 18.6 ± 0.9mm; P=0.28), ovulation rate to the first GnRH [Double-Ov 86.3% (44/51) and Persynch 81.2% (39/48); P=0.50], synchronization rate [Double-Ov 84.6% (44/52) and Persynch 86.0% (43/50); P=0.84], follicular diameter at the second GnRH (Double-Ov 17.5 ± 0.6mm and Persynch 18.0 ± 0.5mm; P=0.48), ovulation rate to the second GnRH [Double-Ov 90.9% (40/44) and Persynch 86.0% (37/43); P=0.48] and CL diameter on D24 (Double-Ov 27.9 ± 0.7mm and Persynch 29.4 ± 0.9mm; P=0.19) were similar between treatments. More (P=0.03) Cls were present on D0 in the Double-Ov (57.7%, 30/52) compared to Persynch (36.0%, 18/50). Furthermore, pregnancy rates at 30 (P=0.85) and 60 (P=0.41) days after AI were similar between Double-Ov (39.0%, 89/228 and 34.8%, 79/227, respectively) and Persynch (40.1%, 85/212 and 38.7%, 82/212, respectively). Pregnancy losses from 30 to 60 days after AI were also similar (P=0.13) between Double-Ov (7.9%, 7/88) and Persynch (3.5%, 3/85). Percentage of cows with P4 < 1 ng/ml on D0 [Double-Ov 13.6% (3/22) and Persynch 5.0% (1/20); P=0.37], P4 > 1 ng/ml on D7 [Double-Ov 77.3% (17/22) and Persynch 95.0% (19/20); P=0.14] and concentration of P4 on D24 (Double-Ov 4.7 ± 0.6 and Persynch 5.9 ± 0.9; P=0.84) did not differ between groups. In conclusion, pre-synchronization by persistent dominant follicle induction using progesterone device prior to the Ovsynch protocol yielded patterns of follicular growth and regression, and fertility like those of the Double Ovsynch protocol in lactating crossbred dairy cows.
Support: CNPq and FAPEMIG.

A074 TAI/FTET/AI

# Relation of the serum concentration of the anti-mullerian hormone (AMH) with the pregnancy rate of ovine female

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The objectives of this study were to determine the plasma concentration of the anti-mullerian hormone (AMH) could be detected in the plasma of prepubertal White Dorper ewe lambs and to evaluate their relationship with pregnancy rate at first breeding. The experiment was carried out at the Sheep and Goat Production and Research Center (LAPOC), from the Experimental Farm of Canguiri, Federal University of Paraná (UFPR), from August to December 2015. Twenty nine White Dorper ewes lambs from 120±5 days old, weighing 28±5 kg and body condition score (ECC) 3±0.4 were evaluated. Pre-pubertal ewes were evaluated at 120, 150, and 180 days of age for AMH concentrations and presence of ovulation by videolaparoscopy (Olympus®, Brazil) after IM administration of 600 IU eCG (Novormon®, Zoetis, Brazil). The results were submitted to the Kruskall Wallis test ($P < 0.05$). The correlations between the variables: ovulation occurrence, plasma concentration of AMH and pregnancy rate were evaluated by Spearman correlation (ρ).The results of the present study demonstrated that the plasma concentration of AMH can be detected in 89% of prepubertal ewe lambs. The concentration of AMH was directly related to the presence of ovulation in response to eCG administration at 120 days of age ($p=0.50$; $P<0.50$) and in the total evaluation period ($p=0.42$; $P<0.50$; 120 days+150 days+180 days), but there was no significant correlation for ages 150 and 180 days ($p=0.36$; $P>0.05$, $p=0.17$; $P>0.05$, respectively). The animals that ev The animals that ovulated demonstrated greater than twice the AMH concentrations (133 ± 32 pg / ml) higher than the non-ovulated animals (60 ± 9.2 pg / ml; $P < 0.05$). At 13 months of age, the ewe lambs were synchronized using intravaginal progesterone-releasing devices (CIDR® - Zoetis, Brazil) for seven days, followed by IM administration of cloprostenol sodium (Sincrocio®, Ouro Fino, Brazil) in the day of removal of the device, and then, the ewes were subjected at mating. The animals became pregnant at the first opportunity of mating (n= 9) showed AMH concentration and pregnancy rate corresponding to 99 ± 20 pg / ml and 52.6%, respectively. For the animals that underwent a second attempt (n=8), the AMH concentrations corresponded to 46 ± 12 pg / ml and a 36% pregnancy rate ($P < 0.05$). The pregnancy rate at the first mating was correlated with a concentration of AMH. At 120 and 150 days of age, no significant correlations were found ($P>0.05$), however at 180 days, it was observed that the animals that presented the highest (99±20 pg/ml) concentrations of AMH were also those that became pregnant at first mating ($p=0.66$, $P < 0.05$). It was concluded that the plasma concentration of AMH can be detected in the pre-pubertal period of White Dorper ewe lambs and can be a good parameter to predict fertility.

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A075 TAI/FTET/AI

# Supplementation with protected FAT in the dairy buffalo cows pregnancy rate

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The objective of this study was to evaluate the effect of supplementation with rumen-protected fat source (RPF, palm oil) on the pregnancy rate of dairy buffalo cows submitted to FTAI. The experiment was carried out at the Ouro Negro farm (Bandeirantes-MS) and 80 Murrah buffalo cows were used in the postpartum mean weight of 595 ± 57.6 kg, aged 3 to 8 years and divided in two treatments: CONTR (control (N = 43) - animals received supplementation according to Paul and Lal (2010), to meet the requirement of daily nutrients for maintenance of lactating buffalo cows and GORD (fat; n = 37) - animals received the same diet CONTR, added with 150 g / animal / day rumen-protected fat (ENERFAT® - Kemin). The animals were under rotational grazing of Panicum maximum cv. Mombasa and received supplements in the morning after milking along with the feed (2 kg / animal / day), the supplementation initiated 15 days postpartum and extended until the diagnosis of gestation. The animals were allocated in the experimental treatments according to the date of birth. The females were submitted to the following hormonal protocol: on day 0 (D0) intravaginal progesterone device (P4) was inserted and applied 2 mg of Estradiol Benzoate (BE). On day 9 (D9), the P4 devices were withdrawn concomitantly with the application of Prostaglandin (PGF2α) and Equine Chorionic Gonadotrophin (eCG). On day 10 (D10) Estradiol Benzoate (BE) was applied. On day 12 (D12), FTAI was performed in the morning. After 30 days, the DG was performed with ultrasonography, and the empty were resynchronized and submitted to a new FTAI, totalizing 101 inseminations (80 1st FTAI and 21 Resync). The variables included in the model were treatment, animal category, inseminator, days in lactation and milk production, and when not significant, excluded. For pregnancy rate, a binomial distribution was used (pregnant and empty), using PROC LOGISTIC of the statistical package SAS (SAS Institute Inc., Cary, NC, USA) in a completely randomized design. There was no effect (P> 0.05) of supplementation on the pregnancy rate in the 1st FTAI (53.5% CONTR vs. 62.1% GORD), as well as on resynchronization (66.6% CONTR vs. 55, 5% GORD), as well as in the total pregnancy of FTAI (1st FTAI and Ressinc). It is concluded that supplementation with 150g of rumen-protected fat in the diet does not alter the pregnancy rate of dairy buffalo cows.

A076 TAI/FTET/AI

# Cold semen: An alternative for FTAI protocols

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The use of Insemination Ar in Brazil, although it has grown in the last years, is far behind when compared to developed countries like USA, Canada, New Zealand. It is estimated that only 12% of the Brazilian herd capable of breeding is inseminated (ASBIA, 2014). One of the disadvantages in the use of FTAI is the great variability in the results. An alternative that could be used to improve these indexes would be to use cooled semen in FTAI protocols (VISHWANATH & SHANNON, 2000). Thus, the present study compared the FTAI protocol pregnancy rates using cold and frozen semen. Cows were randomly assigned to 2 groups: cooled (n = 232) and frozen (n = 227). The cows were examined with US and evaluated their cyclicity, using only reproductively healthy and cyclic cows. The FTAI protocol used was the recommended by (BARUSELLI et al., 2002) with the application of eCG 300 IU and ECP 5mg, IM, in the D8. The sperm collection was carried out 24 hours before the AI date. The semen used in this experiment was obtained from bulls of the Aberden Angus breed (Bos taurus taurus; n = 2) healthy (as recommended by CBRA). A semen pool was made from the bulls to remove the individual effect of the animals. The bulls were kept in similar food and sanitary conditions. After collection the semen in botucrio® extender adjusting the volume to the sperm concentration of 60x106 spermatozoa/mL with progressive motility.This procedure was performed in the 2 experimental groups. Progressive motility and vigor were performed by an experienced evaluator under optical microscopy. Soon after the semen was packed in 0.5cc pallets and divided into the experimental groups. The semen for freezing was placed in an SE compact TK freezing machine where the standard freezing curve recommended by the manufacturer was performed. Soon after the pallets were immersed in liquid N2 for later storage. The semen of cooled group was kept in a refrigerator at 5°C/24h. A total of 459 cows were inseminated. The cows presented an average of 4 years old and a body condition score (ECC) of 2.6 ± 0.3 and weighed an average of 352.2 ± 41.5 kg. The data were analyzed statistically through the program GraphPad Prism 5. The comparison between the groups was performed using the t test for data unpaired with the Welch's correction. There was no variation and influence of the variables age, weight and ECC in the experimental groups. Inseminations were randomly performed by the same inseminator. There was a difference ($P < 0.05$) in the pregnancy rate of FTAI with cooled semen (59.91%, 139/227) was to compare FTAI with frozen semen (49.77%, 113/227). Thus IATF with cooled semen increases pregnancy rates and is an alternative to be analyzed in the use of artificial insemination.

A077 TAI/FTET/AI

# Nelore bovine female temperament and its implications in pregnancy rates in FTAI programs

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The Brazilian national beef herd is mainly composed of Nellore cows (Bos taurus indicus), which are characterized by a more reactive and aggressive temperament compared to Bos taurus taurus (CAFE et al., 2011). More reactivity females present a lower reproductive performance (BURDICK et al., 2011). The objective of this study was to correlate the effect of Nellore cow's behavioral reactivity, During the Fixed Timed Artificial Insemination (FTAI) procedure, with pregnancy rates. There were used 403 multiparous Nelore cows from Beef Unit of the Administrative Campus Fernando Costa, University of São Paulo, located in Pirassununga, São Paulo, Brazil. The FTAI protocol was performed as describes: on day zero (D0) an intravaginal progesterone device (Cronipress Monoset®, Biogenesis Bagó, Curitiba, Paraná, Brazil) associated with intramuscular (IM) administration of 2mg of estradiol benzoate (Bioestrogen®, Biogenesis Bagó); After 8 days (D8), the devices were removed and were injected IM 2mL of Cloprostenol Sodium (Croniben®, Biogenesis Bagó), 300 IU of eCG (Ecegon®, Biogenesis Bagó) and 1mL of Estradiol Cypionate (Cronicip®, Biogenesis Bagó); after 48h (D10) the artificial insemination was performed. The cow's Body Condition Score (BCS) was evaluated at the beginning of the FTAI protocol and later at the days of the gestation diagnosis at 30, 60 and 90 days after AI. The first method used to evaluate the animal's reactivity was the Composite Reactivity Score (CRS) by visual observation of the animal restrained in the cattle chute, based on movement, breathing, mooing and kicking, where the animals were classified as calm, normal and reactive. The second method was the Exit Velocity Test (EV), classifying the cows as slow, normal and fast. The statistical analysis used was the chi-square test to determine the pregnancy rate, considering 5% the level of significance. According to CRS, calm animals had a higher conception rate (P = 0.02) compared to normal (58.10% vs. 52.80%) and reactive (58.10% vs. 45.52%) animals, these presented the worst results. For EV, there was no difference (P = 0.47) at the conception rate, which was 53.3%, 51.82% and 50.00% for the slow, normal and fast classification, respectively. Therefore the EV evaluation was not efficient to demonstrate differences on pregnancy rate (P = 0.47). The conclusion of the study was that the use of CRS was efficient to evaluate differences on pregnancy rates of cows in FTAI protocols and to show that more reactivity animals present a lower pregnancy rate compared to normal or calm animals. This methodology can be used for a genetic selection of less reactivity animals, providing improvements in the management and greater fertility on FTAI programs. Consequently, the adoption of this methodology increases efficiency and profitability in the breeding programs in Nellore beef herd farms.

A078 TAI/FTET/AI

# The color Doppler use on monitoring of the equine endometrites

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The objective of this study was to characterize the uterine vascular perfusion by color Doppler ultrasonography in mares undergoing a phytotherapeutic treatment for bacterial endometritis. Mangalarga Marchador and crossbred mares (N=20) presenting endometritis, confirmed through microbiological, cytological and B mode ultrasonography, were used. The vascular perfusion of the uterus was subjectively estimated considering the percentage of color Doppler signals present in the mesometrium, myometrium and endometrium, in longitudinal section of the uterine and transverse body of the uterine horns. The animals were randomly allocated into two groups: c- control group (n = 10) and t - treated group (n = 10) with phytotherapeutic solution Fitoclean® (Organnact Animal Health, Paraná, Brazil). In both groups, uterine culture, antibiogram, endometrial cytology and B mode and color Doppler ultrasonography were performed at T1 (immediately before the treatment), T2 (24h after treatment) and T3 (48h after treatment). For statistical analysis, the Anova test was used to compare the means obtained in the different periods between treated and control group and Chi Square for the evaluation of the Fitoclean® effect on results of the uterine culture, citology and B mode ultrasonography. In the control group, mean values and standard deviation of vascularization at moments T1, T2 and T3 were 75.56 ± 22.28%, 51.67 ± 21.51% and 53.75 ± 14.08%, respectively, while in the treated group were in T1, T2 and T3 69.50 ± 14.99%, 39.00 ± 15.24% and 32.00 ± 16.19%, respectively. No statical difference ($p>0.05$) on vascularization between control and treated groups in the differents moments. The mean values found in T1 in the control and treated groups were significantly higher ($P<0.01$) than those obtained at moments T2 and T3. Regarding the uterine samples, in the control group, at the time T1, 70% (7/10) presented positive culture, cytology and presence of intrauterine fluid. T2, 50% (5/10) identified bacterial growth with intrauterine fluid and 100% (10/10) presented positive cytology; In T3, the results were similar to T2, with one mare showing negative cytology. In the treated group, in T1t, 90% (9/10) presented bacterial growth with positive cytology and presence of intrauterine fluid; In T2, 80% (8/10) of the uterine samples detected bacterial presence, positive cytology and intrauterine fluid; At the time T3, 80% (8/10) identified bacterial growth, 100% (10/10) presented positive cytology and 70% (7/10) with presence of intrauterine fluid. There was no statistical difference ($p = 0.2$). We can conclude that there was no relationship between uterine color Doppler findings and the results of traditional exams used to diagnose endometritis.

A079 TAI/FTET/AI

# Use of estrodial benzoate or cypionate at the time of progesterone device removal on estrus expression, ovulation, and fertility in high producing cows

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Estradiol cypionate (ECP) is commonly used in progesterone-based (P4) synchronization protocols to induce ovulation. However, in P4-based programs with 9d, around 30% of dairy cows anticipate estrus and are inseminated before the scheduled time of AI. Thus, we studied the feasibility of replacing ECP by estradiol benzoate (EB) to induce ovulation in 9d progesterone programs. Holstein cows (n = 556) kept in a freestall in Southern Brazil, producing 39.2 ± 4.1 kg at 160.4 ± 10.2 DIM were synchronized after the voluntary waiting period or when detected open after pregnancy diagnosis. Cows were randomly allocated to receive one of the two timed AI protocols, as follows: 1) 9P4ECP: D0 = 2mg of EB (Estrogin, Biofarm) + P4 device insertion (PRID®, Ceva), D7 = PGF2a (Veteglan®, Hertape), D9 = 1mg ECP (Cipionato-HC, Hertape) + PGF2a (Veteglan®, Ceva) and PRID removal, D11 = TAI, approximately 48h after PRID removal; 2) 9P4EB: D0 = 2mg of EB (Estrogin, Biofarm) + P4 device insertion (PRID®, Ceva), D7 = PGF2a (Veteglan®, Hertape), D9 = 1mg EB + PGF2a (Veteglan®, Ceva) and PRID removal, D10 = TAI, approximately 36h after PRID removal. Estrus behavior was recorded by activity meters (SRC®), and pregnancy diagnosis was performed by ultrasound 30 days after AI. Only cows detected in estrus by the activity meter were inseminated. All statistical analyses were performed with the proc Glimmix of SAS, 9.4. A greater proportion of cows in 9P4BE were detected in estrus and bred (9P4EB = 90.8% vs 9P4ECP = 73.3%; $P<0.05$). In addition, estrus behavior was more concentrated around 12 and 24h after device removal in 9P4EB compared to 9P4ECP (76.3% vs 49.1%; $P<0.05$). Conception results were similar between groups (9P4EB = 44.3% vs 9P4ECP = 42.2%; $P=0.84$). However, because of the greater proportion of cows bred in 9P4BE, the final pregnancy rate was greater in 9P4EB (40.2% vs 30.9%; $P<0.05$). In conclusion, treatment with EB, besides increasing proportion of cows detected in estrus, caused a greater concentration in estrus behavior without affecting conception rate, ultimately increasing pregnancy rate results (% estrus detection x % conception). Acknowledgments: Ceva.

A080 TAI/FTET/AI

# Using pregnancy associated glycoproteins (PAGS) to understand sire effect on pregnancy loss in Nelore beef cows

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Understanding the causes of embryonic mortality (EM) is fundamental to developing management strategies that decrease economic loss in cattle. Though late EM (after day 28 of gestation) represents a smaller proportion of reproductive failure compared to early gestation EM (~10% vs ~35 %), economic consequences are reported to be disproportionately greater due to delayed conception date which limits cow productivity. Placental insufficiency is considered to be a major cause of late EM and bovine pregnancy-associated glycoproteins (PAG) have been used as a marker of placental function. Although the functional role of PAG is unclear, it has been shown that many factors affect PAG concentrations including pregnancy stage, breed, parity, sire and fetal sex. Limited data have been reported on sire effects on PAG concentration, however, based on the influence sire has on placental development, we were interested in this potential relationship. The objectives of this study were to determine how sire used for FTAI influences embryonic loss rate and PAG concentration at day 30. Postpartum Nelore beef cows (n= 736) were artificially inseminated using 6 Angus sires at a fixed time (Day 0) after synchronization of ovulation. Pregnancy diagnosis by ultrasound was performed and serum samples were collected on day 30. Serum concentrations of PAG were quantified using an in house PAG ELISA with antibodies raised against PAGs expressed early in gestation. The SAS PROC MIXED procedure (version 9.4; SAS Institute, Cary, NC, USA) was used for data analysis. Overall pregnancy rate at day 30 was 53.75 % and late EM was 6.21%. Mean concentration of PAG of pregnant cows at day 30 was 8.81±0.24 ng/ml, and cows that maintained a pregnancy from days 30 to 100 of gestations had significantly (p=0.004) higher circulating concentrations of PAG on day 30 compared with cows that did not maintain a pregnancy until day 100 (8.98±0.25 ng/ml vs 5.95±1.02 ng/ml). Although there was variation in sire conception rate to FTAI, there was no linear relationship between sire pregnancy rate and circulating concentrations of PAG (Sire 1 – 51.56%, 7.72 ng/ml; Sire 2 – 49.17%, 8.96 ng/ml; Sire 3 – 55.28%, 8.81 ng/ml; Sire 4 – 55.28%, 10.14 ng/ml; Sire 5 – 55.28%, 8.42 ng/ml and sire 6 – 35.29%, 9.52 ng/ml). Then, sires were classified according to percentage on total embryonic mortality between days 30 and 100 as high embryonic loss (sire 1 - 20%; sire 2 - 28% and sire 3 - 24%) or low embryonic loss (sire 4 – 16%; sire 5 - 4% and sire 6 - 8%). After removing all cows that lost pregnancy after day 30, pregnancies by sires classified as high embryonic loss had lower PAG compared to pregnancies by low embryonic loss sires (8.5±0.35 ng/ml vs 9.48±0.36 ng/ml; p=0.0562). In summary, PAG concentration was driven by the ability of pregnancy maintenance and by sire used at FTAI. Exploring this relationship might be interesting to improve sire fertility in regard to late embryonic loss.

A081 TAI/FTET/AI

# The use of the J-Synch protocol in non-lactating beef cows associated to estrous detection

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The J-Synch protocol was developed aiming to increase the proestrus lenght in beef heifers. The association of this protocol with estrus detection and anticipation of the moment of AI may increase the pregnancy rate by avoiding asynchrony between AI and ovulation. The aim of this study was to evaluate the use of J-Synch protocol in non-lactating beef cows associated to estrus detection and anticipation of the AI. The study was carried out with 116 cows Angus non-lactating beef cows (Bos taurus). On Day 0, all animals received 2 mg of estradiol benzoate (Sincrodiol, Ouro Fino®, Cravinhos, Brazil) and an intravaginal device with 1 g of progesterone (Sincrogest, Ouro Fino®, Cravinhos, Brazil). On Day 6, the progesterone device was removed and animals received 500 µg of cloprostenol (Sincrocio, Ouro Fino®, Cravinhos, Brazil). Estrus detection was performed on Day 8 during an hour. Cows in estrus were inseminated 12 hours later and those not were inseminated in fixed-timed 72 hours after the progesterone device was removed. All cows received 10 µg of GnRH (Sincroforte, Ouro Fino®, Cravinhos, Brazil) at the moment of insemination. The insemination was performed with semen from four sires with known fertility. The pregnancy diagnosis was performed 30 days after AI. The results were analyzed by Chi-square test. The overall pregnancy rate was 57.7% (67/49). The rate of pregnancy did not differ between the cows demonstrating estrous (72.7%) or inseminated in fixed time (54.2%) (P=0.15). However, it must be considered that study involved 116 cows. The use of a higher number of animals could to demonstrate difference between insemination groups. In conclusion, more studies are needed to clarify results of association estrus observation with J-Synch protocol. Nevertheless, the JSynch protocol demonstrated a satisfactory result in non-lactating cows.

A082 TAI/FTET/AI

# Bovigen® Repro Total SE vaccination increases conception rate on FTAI Nelore heifers

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The objective of the present experiment was to evaluate the effect of Bovigen® Repro Total SE vaccine on conception rate of Nelore heifers (*Bos taurus indicus*) on 30 and 60 days after fixed timed artificial insemination (FTAI). The hypothesis was that vaccination, prior to FTAI, would increase conception rate and reduce gestational loss between 30 and 60 days of gestation, when compared to a non-vaccinated control group. A total of 759 Nelore heifers without prior vaccination for IBR, BVD and Leptospirosis underwent ultrasound cyclicity assessment (Aloka SSD 500, Tokyo, Japan) and 459 pubertal heifers were selected for FTAI. These 459 heifers were divided into one of two groups: Control (non-vaccinated) and Vaccinated (two IM doses of Bovigen® Repro Total SE, Virbac Animal Health, São Paulo, Brazil), the animals were randomized using the body condition score as a balancing factor between groups. The animals in the control group received no vaccination at any time, while the Vaccinated Group received the first dose of the reproductive vaccine 25 days before the onset of the FTAI protocol (Day -25; at the time of ultrasonography) and the second dose on the onset of the FTAI protocol (Day 0). All animals received an auricular ear implant with 3mg of Norgestomet (Crestar, MSD, Brazil) and 2mg of Estradiol Benzoate IM (Estrogin, Biofarm, Brazil) on Day 0. After 8 days (Day 8), the device was removed and 0.265mg of Cloprostenol Sodium (Ciosin, MSD, Brazil), 300IU of eCG (Novormon, Zoetis, Brazil) and 0.5mg of Estradiol Cypionate (ECP, Zoetis, Brazil) were administrated IM. The FTAI was performed by the same technician 48 hours after device withdrawal (Day 10) with the same semen batch in all females. Ultrasound evaluations were performed 30 and 60 days after FTAI (Days 40 and 70, respectively) to determine conception rate and gestational loss on each group. Gestational loss was considered as the absence of fetus or the presence of a dead fetus on Day 70, when the heifer was pregnant on Day 40. Data were analyzed using SAS® (Statistical Software Analysis, version 9.3 Institute Inc., Cary, NC, USA, 2003). The 30-day conception rate was lower (P = 0.02) on the Control (42.5%; 96/226) than on the Vaccinated Group (50.2%; 117/233) and remained (P = 0.03) at 60 days after FTAI [Control: 42.0% (95/226) vs Vaccinated Group: 48.5% (113/233)]. The gestational loss between 30 and 60 days did not differ (P = 0.20) among groups [Control (1.0%; 1/96) and Vaccinated (3.4%; 4/117)]. Thus, we concluded that the Bovigen® Repro Total SE vaccine was efficient to increase conception rate at 30 and 60 days post-FTAI in Nelore beef heifers, but no effects upon early pregnancy loss were observed.

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A083 TAI/FTET/AI

# Vaccination against reproductive diseases (IBR and BVD) does not interfere with the pregnancy rate and losses of *Bos indicus* cows submitted to TAI

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The objective of this study was to evaluate the effect of vaccination against bovine herpesvirus-1 (BoHV-1) and bovine viral diarrhea virus (BVDV) on the pregnancy rate and losses of *Bos indicus* cows submitted to ovulation synchronization protocol based on progesterone (P4) and estradiol (E2). In the study, 400 lactanting Nelore cows with average body condition score of 2.83±0.01 (1 to 5 scale) and postpartum between 30 and 60 days were used. On the random day of estrus cycle (D0), cows received 2mg of estradiol benzoate (EB; RIC-BE®, Tecnopec, Brazil) and a P4 intravaginal device (Primer®, Tecnopec, Brazil). On day 8 (D8), the P4 device was removed and the cows received 500mg of Cloprostenol (Estron®, Tecnopec, Brazil), 300 IU of eCG (Folligon®, MSD, Brazil) and 1mg of estradiol cypionate (ECP® , Zoetis, Brazil). After 48 hours, the cows were submitted to TAI. On D0, the cows were randomly allocated to one of two treatments (Control group and Vaccine group). In the Control group, the cows received 2 mL of saline solution by the subcutaneous route and in the Vaccine group, the cows received 2 mL intramuscular of the reproductive vaccine (Cattle Master 4, Zoetis, Brazil) on the D0 and D38. In a subgroup of animals (n = 40), blood samples were collected concomitantly with the administration of the treatments to evaluate the presence of BoHV-1 and BVDV (antibody) by the serum neutralization method. Ultrasonography was performed on D38, D70 and D120 for pregnancy diagnosis and evaluation of pregnancy losses. Statistical analysis was performed using the GLIMMIX SAS procedure. The prevalence of BoHV-1 was 70.0% (14/20) for both groups on D0 and 70.0% (14/20) for the Control group and 85.0% (17/20) for Vaccine group on D38. The prevalence of BVDV antibodies was 80.0% (16/20) for Control group and 75.0% (15/20) for Vaccine group on D0 and 100.0% (20/20) for both groups on D38. In addition, the immunization of the animals did not interfere on the pregnancy rate at 28 days [Control group 64.5% (129/200), Vaccine group 56.6% (112/198); P=0.27], at 60 days [Control group 63.0% (126/200), Vaccine group 55.3% (109/197); P=0.29] and at 110 days of gestation [Control group 61.4% (116/189), Vaccine group 55.3% (104/188); P=0.29]. Also, there was no difference between the groups for pregnancy losses between 28 and 60 days of gestation [Control group 2.3% (3/129) and Vaccine group 2.7% (3/112); P=0.88] and between 28 and 110 days of gestation [Control group 3.3% (4/120) and Vaccine group 2.8% (3/107); P=0.62]. In conclusion, vaccinating cows against bovine herpesvirus-1 (BoHV-1) and bovine viral diarrhea virus (BVDV) does not increase fertility and does not reduce pregnancy losses in *Bos indicus* cows submitted to TAI.

Support: FAPEMIG.

A084 TAI/FTET/AI

# Luteal vascularization as a tool for early pregnancy diagnosis in sheep is more efficient from day 17 post-insemination

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The objective of the present study was to determine the efficiency of luteal vascularization assessment using color Doppler ultrasonography (US) as a tool for predictive pregnancy diagnosis and at which moment post-insemination this evaluation is more efficient. Adult Santa Inês ewes (n=28) with an average age, body weight, and body condition score of 3.0±1.2 years, 44.6±5.4Kg, and 3.0±0.2, respectively, were used. The animals were submitted to a FTAI protocol as previously described by Balaro et al. (Domest Anim Endocrinol, v.54, p.10, 2016). The AI (D0) was performed 56h alter sponge withdrawal using commercial frozen-thawed semen. Luteal vascularization was assessed by color Doppler US (PRF: 1.0 KHz, WF: 75 KHz) from D12 to D20 using a portable device equipped with a 7.5 MHz transducer. Luteal vascularization was classified using a subjective scale ranging from 1 to 4 (Bragança et al. Animal Reproduction, v.13, p.587, 2016). Females bearing a CL with vascularization graded as 2 or superior was presumably considered as pregnant. Pregnancy was confirmed at D30 by visualization of embryonic vesicle using B-Mode US and data was compared with predictive diagnoses performed from D12 to D20. The efficiency of color Doppler US was evaluated by calculating the percentage of false negative (FN) and false positive (FP) results and also sensitivity (SENS), specificity (SPEC), negative (NPV) and positive (PPV) predictive values, and accuracy (AC) of the technique. At D30, 11 females were confirmed as pregnant and 17 as non-pregnant. As expected, use of color Doppler from D12 to D14 was unfeasible to predict non-pregnant animals because all animals still had vascularized CL (pre-luteolysis period), and thus they were all considered as pregnant. From D15 to D17 the number of FP results progressively decreased, increasing the values observed for SPEC, PPV, and AC (SPEC=18%; PPV=44%; AC=50%; FP=50% for D15; SPEC=47%; PPV=55%; AC=68%; FP=32% for D16; SPEC=76%; PPV=73%; AC=86%; FP=14% for D17). Results did not change from D17 to D20. In The present study, four FP results remained until D20. However, CL in these 4 animals kept a vascularization grade equal or superior than 2 until D20, suggesting that failure to predict non-pregnant animals was due to early embryo lost from D20 to D30 and not due to technique limitation. It was not observed FN results, thus SENS and NPV remained constant from D15 to D20 (SENS=1, NPV=1). The present results demonstrated the efficiency of color Doppler US as a tool for early identification of non-pregnant animals from D15. However, due to occurrence of FP results, best results was observed were observed from D17. Acknowledgments: FAPERJ for financially support the project and provide scholarship for EKNA and FZB.

A090 OPU - IVF and ET

# Fertilization rate and developmental kinetics of bovine embryos produced using semen from high and low *in vitro* fertility bulls

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In vitro embryo production is a well-established and commercial technique, being nowadays the best choice for embryo production in some breeds. Therefore, better results can be achieved with the selection of high in vitro fertility bulls. Understanding why a high fertility bull produce more blastocysts than a low fertility one is the key for bull selection. Since embryo kinetics can be linked with embryo viability (Edwards, 2003), it could be a powerful tool to identify differences between these two groups of bulls. The aim of this study was to evaluate early embryo kinetics and fertilization rate from bulls with high (HF) and low (LF) in vitro fertility. For bull selection, a commercial laboratory database was assessed. Bulls were ranked based on blastocyst/cleavage rate (embryo development rate). Ten bulls with high (n=5) and low (n=5) in vitro fertility were used for 5 manipulation of in vitro embryo production, as described by Pontes et al. (2010). Ten oocytes from each manipulation (n=50) were stained with 1 mg/ml Hoechst 33342 (Sigma), washed, mounted on microscope slides and examined with epifluorescence microscopy (Olympus IX80, Olympus Corporation, Tokyo, Japan). The presumptive zygotes were classified in three categories: negative fertilization (1 PN); normal fertilization (2 PN), and polyspermy (>2 PN). Embryos were classified by their specific stage of development (2; 3-4; 6 or 8 cell stage) at 24, 36, 48, 60, 72 hpi (hours post-insemination). Cleavage rate was assessed at day 3 (D3) of embryo culture, viable blastocysts and embryo development rates were assessed at day 7 (D7). Data were analyzed using PROC GLIMMIX of SAS (SAS ® 9.3 Institute Inc., Cary, NC, USA, 2003). Blastocyst rate was higher in the HF group (29.4%) than in the LF (16.0% - $P<0.0001$), similarly to embryo development rate (HF = 34.0%; HL = 189%; $P<0.0001$). There was no significant difference in cleavage rate (HF=86.7%; LF= 84.9%; P= 0.2581), neither in embryo kinetics, in all of the evaluated periods ($P>0.05$). No difference was found in negative fertilization (HF=10.69%; LF= 8.80%; P=0.9925), nor in polyspermy between groups (HF=16.18%; LF=29.20%; P=0.6066). However, normal fertilization was higher in the HF group (72.0%) than in LF group (62.0%) (P=0.0332). In conclusion, early embryo kinetics could not explain the difference in blastocyst rate between high fertility (HF) and low fertility (LF) bulls. Nevertheless, HF bulls had more normal fertilization than LF bulls.

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A091 OPU - IVF and ET

# Effect of insulin: glucose ratio on oocyte and embryo production and pregnancy rate in lactating dairy cows

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Insulin:glucose ratio has been used to determine insulin resistance in dairy cows, which may affect reproductive outcomes in dairy cows. So, the aim of this study was evaluate the insulin:glucose ratio on oocyte and embryo production and pregnancy rate in lactating dairy cows. Follicles of two-hundred-six non-pregnant lactating dairy cows between 80 to 280 days in milk were aspirated in random days of the estrus cycle for embryo production. At the time of follicle aspiration, cows were scored for body condition, and blood samples were collected for determination of plasma concentrations of glucose and insulin. Data was analyzed using a MIXED and GLM procedure of SAS. Insulin:glucose ratio concentrations were classified using its median distribution (0.05 and 0.32 ± 0.01 for I:G below [Be] or above [Ab] or median, respectively). There was no effect of insulin:glucose ratio on oocyte production (13.02 vs. 13.47 ± 1.4 oocyte for Be and Ab, respectively; P = 0.80), embryo production (1.48 vs. 0.98 ± 0.28 embryo for Be and Ab, respectively; P = 0.15) and pregnancy rate (US1 = 53 vs. 47 ± 5% [open vs. pregnant] for Be and Ab, respectively; P = 0.40; and US2 = 57 vs. 43 [open vs. pregnant] for Be and Ab, respectively; P = 0.48). However, there was tended (P = 0.06) for embryo produced per oocyte collected, where cows with insulin:glucose ratio below of median had higher number of embryo produced per oocyte collected compared with cows Ab (0.11 vs. 0.07 ± 0.17, respectively). There was no effect of regression model for insulin:glucose ratio per oocyte production P = 0.20; I:G per embryo production P = 0.96 or I:G per embryo produced per oocyte collected P = 0.55. In conclusion, lower insulin: glucose ratio increase the number of embryo produced per oocyte collected, but they was not efficient to change any others reproductive variables.

A092 OPU - IVF and ET

# Hostein and Gyr gametes contribute differently to the embryonic development morphokinetics

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In the present study we aimed to assess the possible differential contribution of Holstein (Bos taurus taurus) and Gyr (Bos taurus indicus) gametes on morphokinectical embryo development. To do so, we collected oocytes from 16 purebred donors, Holstein (H n=8) and Gyr (G n=8), using the ovum pick up (OPU) technic, and produced in vitro F1 embryos by Holstein and Gyr gametes cross fertilization (HG and GH, in which the first letter represents donor's breed and second bull's breed) and parthenogenetically activated embryos (H and G). Then, assessed the morphokinects development using a Multi-embryo Chamber (MEC), an in vitro culture (IVC) system based in group culture, but allowing individual assessment, in order to compare embryos (HG vs GH and H vs G) cell numbers at 48, 96 and 144 hours post fertilization. The data were expressed as mean ± SD being the cell number means compared using unpaired T-test and Mann-Whitney for non-parametrical data. Analyses were performed using GraphPad Instat software, at 5% significance level. Additionally, only embryos that reach the blastocyst stage of development were compared between groups (HG n=6, GH n=5, H n=8 and G n=9), in order to compare gametes breed contribution, isolated from development potential. The F1 embryos from Holstein oocytes with Gyr spermatozoid cross-fertilization (HG) develop faster than its reverse at 48 h, GH (48 HPI: 5.00 ± 0.63 vs 3.40 ± 0.89 P = 0.0170), however were similar at 96 HPI (6.67 ± 1.21 vs 5.80 ± 0.84 P = 0.2103) and at 144 h HPI (83.45 ± 21.68 vs 60.44 ± 29.08 p = 0.2632), suggesting gametes differential contribution. While embryos produced by Holstein and Gyr oocytes activation presented similar cell number at all time-point (H vs G; 48 HPI: 4.13 ± 0.64 vs 4.11 ± 0.93; 96 HPI: 7.79 ± 2.81 vs 8.99 ± 4.13; 144 HPI: 72.39 ± 27.48 vs 68.70 ± 27.99), which may indicate that Holstein and Gyr oocytes have similar contribution in embryo development. Based on results, we conclude that Holstein and Gyr gametes differently contribute in the kinetics of embryonic development and suggest bull contribution as the main factor for pointed differences. Financial support: CAPES, FAPERJ and FAPEMIG.

A093 OPU - IVF and ET

# Effect of the stage of the estrous cycle of domestic cats on the oocyte maturation competence

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Objective: In vitro fertilization (IVF) can be used in the domestic cat (Felis catus)in order to generate valuable information that can be applied in reproductive physiology of wild feline endangered species (Luvoni, C. Reprod. Nutr. Dev. 40:505-512. 2000). In vitro Maturation (IVM) of Cumulus Oocyte Complexes (COCs) in cats is considered as key in the advancement of IVF in these wild endangered species. The objective of this research study was to evaluate the oocyte competence of COCs obtained at different stages of the estrous cycle of domestic cat females. Materials & Methods: Eighteen clinically healthy 8 months to 2.5 years aged female cats were included in this research. All females were housed under similar conditions (food, water, light) 72 hours prior to surgery (ovaryectomy, OVX). Cats were previously classified according to the stage of the estrous cycle through a vaginal cytology performed hours before the OVX. Three groups resulted according to the previous criteria: a. Follicular Phase (FP), b. Luteal Phase (LP) and Anestrous (A). After OVX, COCs were recovered from follicles using a mixed technique of punction-fragmentation of the ovarian cortex. COCs were thereafter washed in 100 µL droplets (no more tan 25 COCs/droplet) of wash and maturation media supplemented with FSH and LH (Folltropin, Bioniche, Belleville, Ontario, Canada), 17β- Estradiol, BSA, sodium pyruvate and gentamycin. After 30 hours of IVM, COCs were mechanically denuded and fixed in a solution of ethanol:acetic acid (3:1) during 24-72 hours at 4°C. The degree of nuclear maturation was evaluated by staining oocytes with a 1% Aceto-orcein solution (45% of acetic acid) during 30 minutes. Oocyte maturation was characterized as an oocyte in telophase I or metaphase II. Data was analyzed using ANOVA and Chi-square of SAS. Results & Conclusions: The proportion of COCs morphologically fit to initiate IVM did not differ among the different stages of the estrous cycle. In this study 13,7% (41/298) of the oocytes (all groups included) reached nuclear maturation. The maturation rate (%) for oocytes matured in the FP, LP and A were 12,3% (11/89), 17,3% (25/144) y 7,7% (5/65), respectively (P>0,05). Although the difference was not statistically significant, there was a maturation rate 2,5 times greater in the LP than in the A phase (P=0,065). In conclusion, oocytes obtained in different stages of the estrous cycle have similar capacity of reaching maturation under in vitro conditions.

A094 OPU - IVF and ET

# Identification of CD46 and CD59 in Brahman Bull cryopreserved sperm cells

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The main role of the complement system is to protect and defend the host from the attack of pathogenic micro-organisms. Previous studies report the existence of several complement regulatory proteins expressed in different tissues (Harris & Morgan, In: The Complement System: Novel Roles in Health and Disease, pp. 129–166, 2004). These proteins are related with reproduction and are expressed in human and mice sperm cells (Fusi et al. Mol Reprod Dev 29(2):180-188, 1991; Rooney et al. Immunology 75(3):499-506, 1992. The objective of this study was to determine the localization of CD46 and CD59 in frozen-thawed Brahman bull semen, using the Indirect Immunofluorescence Technique (IFI). Materials & Methods: Semen from four ejaculates of five different Brahman bulls was used. Capacitation was induced with heparin while acrosome reaction (AR) was induced with calcium ionophore; and both were evaluated with the chlortetracycline stain (CTC). Afterwards, IFI was used to determine the presence of CD46 and CD59. The statistical analysis was performed using S.A.S for Windows, version 8.2 (S.A.S. Inst. Inc.; Carry, NC, USA). Data were analyzed through a general lineal model ANOVA and results are shown as means ± standard deviation. Results & Conclusions: Data analysis demonstrated that CD46 and CD59 are expressed in 35.93 ± 10.49% and 33.80±12.66%, respectively in bull sperm cells. The expression of both proteins increased significantly after treatment with heparin (CD46: 68.26± 5.29%; CD59: 71.33± 6.24%) and it varied among bulls. It was determined that both proteins are located predominantly in the acrosomal region, which coincides with its location in humans and mice. When bull effect was analyzed, it was observed that the calcium ionophore increased the expression of CD46 and CD59 in all the bulls in comparison to heparin and control groups. However, the effect of heparin was different, and this depended of bull and the antigen. In comparison to control group, heparin only increased the percent of positive sperm to CD46 in two of five bulls, while heparin increased expression of CD59 in all the bulls. In conclusion, the bovine sperm expresses both CD46 and CD59 proteins, mainly in the acrosomal region and this expression was related with the capacitation and AR processes.

A095 OPU - IVF and ET

# Effect of spermatozoa concentration on *in vitro* production of bovine embryos

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The aim of these experiments was to study the effect of different insemination doses on PIV; the semen samples were utilized from two bulls one with a history of low in vitro embryo production (B1) and the other with a good one (B2). The experiment was conducted at the laboratory of animal breeding and reproduction in the experimental station of the Agronomic Institute of Pernambuco (IPA) in the city of Arcoverde. 180 oocytes were aspirated from antral follicles (3-8 mm) of ovaries from recent slaughtered cow and then matured for 24 hours in TCM199 medium. Two Girolando 5/8 bulls (B1,& B2) were the donors of cryopreserved semen; the semen was capacitated by 90% percol gradient. 90 oocytes were divided in 6 groups of 15 oocytes and fertilized as follows, (B1T1), (B1T2), and (B1T3) were fertilized by 2*106, 3*106, and 4*106 respectively, and (B1T1), (B1T2), and (B1T3) the doses and groups were as B1 (Each treatment was repeated 2 times).18 hours after IVF the presumptive zygotes were washed after then cultured in SOF medium. The incubation was in the temperature of 38,5°C, with 5% CO2, and 95% of humidity. The cleavage rate was observed 48 hours after IVF; and 7 days after the IVF the blastocyst rate was observed. The data were analyzed by chi-square test at 5% of signigicance. The cleavage rates in (B1 T1) (B1 T2), and (B1 T3) were 52,17%, 69,23%, and 60% respectively, and in (B2 T1), (B2 T2), and (B2 T3) were 75%, 59,2%, and 82,7% respectively. 7 days after the fertilization the blastocyst rate in B1 groups was 8,7%, 15,3%, and 8 % in (B1 T1), (B1 T2), and (B1 T3) respectively; the blastocyst rate in B2 groups was 18,7%, 25,9%, and 31 % in (B2 T1), (B2T2), and (B2 T3) respectively, as well as 33,3% of the blastocysts of (B2 T1) were hatched. Our results have proved the effect of different concentrations of capacitated bovine spermatozoa and its difference between the two bulls on the blastocyst rate. The increased concentration of spermatozoa of B1, increased the cleavage rate in T2 and T3, however blastocyst rate was increased in T2. In B2T3 the increased spermatic concentration increased the production of blastocysts, as well as the cleavage rate in B2T2 was less than B2T1, and B2T3; however, Heeres et al., 1996 (Theriogenology, v.45, p.266). the best cleavage rate and embryos production were observed when the spermatic concentration of 0.5*106 sperm/1 ml, these observed differences can be related to genetic factors of the bulls.(First and Parrish, J Reprod Fertil, v.34, p.151-165, 1987); The utilization of spermatic concentration which is higher than threshold in IVF can induce increased abnormal fertilization, principally the polyspermy, without changing in the monospermatic fertilization. In this research we have concluded that the individuality of bulls is an important factor to produce bovine embryos in vitro, because we observed that the bull of the history of low IVP increased the production of embryos two times when the spermatozoa concentration was increase from 2*106 to 3 *106, but the spermatic concentration of 4*106 had no effect on IVP, on the other hand the increased spermatic concentration of the bull of history of good IVP, increased the IVP; so more researches are needed to verify the paternal importance in IVP.

A096 OPU - IVF and ET

# Ovum pick up and quality of oocytes in stimulated donors with equine corionic gonadotrofin

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Until recently ovum pick up (OPU) in cattle was generally done at random times of the estrous cycle. However, the increasing need to improve efficiency and increase in demand for OPU at less favorable situations leads to an increase in the use of pre-aspiration protocols. In vitro embryo production (IVP) efficiency has been compromised by poor oocyte quality. The aim of the study was to develop a pre-aspiration protocol to synchronize the follicular wave and stimulate follicular development using equine chorionic gonadotrophin (eCG). Twenty Nelore breed cows were randomly assigned to two treatments in a crossover design with two replicates. T1: CTRL (N = 20): D0 - intravaginal implant of progesterone (DIB™, MSD Animal Health, Brazil) and 2mg of Estradiol Benzoate IM (Gonadiol™, MSD Animal Health, Brazil) and OPU in D5. T2: TRAT (N = 20): Same protocol above with the inclusion of 400UI of eCG (Folligon™, MSD Animal Health, Brazil) IM in D3. In D5 follicular measurement and follicular aspiration were done using an ultrasound equipment (Mindray®-M5). The oocytes classified as viable were sent to IVF in maturation medium, in gassed cryotubes transported in carrier device at 37.5°C. IVF procedures was performed in the same Laboratory using standard protocols. Fertilization was performed 22 to 24 hours after placement of the oocytes in the maturation medium. A semen from the same bull and bath was used. The embryos were cultured for seven days in an atmosphere of 5% CO2 and 38.5°C. After seven days of culture the embryos classified as grade 1 were counted. We evaluated following variables: mean number and size of follicles at D5, quantity and quality of oocytes recovered and embryos. The data were submitted to normality tests and means of treatments were compared by ANOVA considering 5% of probability as significance. No differences were showed in the total follicle mean in D5 (27.1 and 24.9, for T1 and T2). However the diameter was higher in T2 (3.6 + 0.7 and 3.2 + 0.6 - $P < 0.05$). Mean of total oocytes (23.2 + 14.3 and 25.6 + 13.4 for T1 and T2) and viable (14.6 + 11.2 and 16.6 + 9.4 for T1 and T2) did not Differed between treatments. Total embryos (2.6 + 1.7 and 3.5 + 2.8) were higher ($P < 0.05$) in females treated with eCG. The results showed that the amount of oocytes nor their morphological quality was altered by the treatment. However, due to the greater production of T2 embryos, probably the intrinsic developmental capacity of oocytes obtained in animals from the group treated with eCG is better. It was concluded that the stimulation of bovine donors with eCG does not improve the quantity or morphological quality of the aspirated oocytes, but increases the potential of embryo production by the PIVE technique.

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A097 OPU - IVF and ET

# Evaluation of different incubation systems on bovine *in vitro* embryo production

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The use of reproductive biotechniques, such as *in vitro* embryo production (IVP) may contribute to the genetic improvement of the bovine herd. In this context, many efforts have been made to obtain culture systems that are less harmful to embryos. This study aimed to evaluate both the maturation rate and bovine blastocyst production using three different incubation systems. Follicles of 2-8 mm were punctured from bovine ovaries obtained in local slaughterhouses. The obtained *cumulus*-oocyte complexes (COCs) were randomly divided into three groups according to the incubation system: conventional bench incubator - CONV (Thermo, Thermo Fischer, Waltham, USA) with high oxygen tension (5% $CO_2$), mini-bench incubator - MINIb (Eve, WTA, Cravinhos, Brazil) with low oxygen (5% $CO_2$, 5% $O_2$, 90% $N_2$) and mini portable incubator - MINIp (LabMix, WTA) also with low oxygen tension. *In vitro* maturation (IVM) was performed in TCM-199 (Sigma-Aldrich, St. Louis, USA) supplemented for 22 h at 38.5 °C. After this, a sample of oocyte was stained with Hoechst 33342 (Sigma-Aldrich) and analyzed by fluorescence microscopy (Eclipse E400, Nikon, Tokyo, Japan) in order to determine the maturation rate. For *in vitro* fertilization (IVF), COCs were incubated with frozen/thawed semen in Brackett-Oliphant medium (BO) for 6 h at 38,5 °C. *In vitro* culture (IVC) was performed for 8 days in synthetic oviduct fluid (SOF) at 38.5 °C. Cleavage rate was verified on day two (D2) and blastocyst rate on days seven (D7) and eight (D8) of culture. Data were analyzed by the Student *t* test with 5% of significance level. No significant differences were found for the maturation rate, which was 70.4%, 50.8% and 57.6% for CONV, MINIb and MINIp, respectively. A total of 1067 (CONV = 359; MINIb = 356; MINIp = 352) presumable zygotes were submitted to culture. Concerning cleavage rate, CONV (71.0%) produced the better results ($P < 0.05$) when compared to MINIb (44.1%) and MINIp (35.8%). Similarly, significant differences ($P < 0.05$) were verified for blastocyst production in D7 (CONV = 32.0%, MINIb = 21.1%, MINIp = 11.4%) and D8 (CONV = 32.0%, MINIb = 21.1%, MINIp = 12.8%). In conclusion, the incubation system provided by CONV resulted in higher rates for embryo production. However, it is necessary more repetitions for definitive conclusions.

A098 OPU - IVF and ET

# Evaluation of lipid content and gene expression in bovine embryos produced *in vitro* treated with natriuretic peptide C (NPPC)

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The use of natriuretic peptide C (NPPC) has been described to elevate cAMP levels blocking meiosis in oocytes (Zhang, W. Journal of Cellular Physiology, v 230, pp. 71-81, 2015). However, there are no reports on the use of NPPC in the activation of metabolic processes in embryos. Considering the changes in lipid metabolism by increasing cAMP concentrations, possible benefits would happen by activation of protein kinase A (PKA). The present study aimed to evaluate the effect of NPPC supplementation in the culture medium on the development, lipid content and, transcript levels of genes related to the quality and cellular metabolism of bovine embryos produced in vitro. Ovaries (n = 420) from Nelore cows (n = 210) were obtained from the slaughterhouse. On day 5 (D5) of in vitro culture, the embryos of the experimental group were treated with 100 nM of NPPC and the control group did not receive this substance. The evaluation was performed based on blastocyst and hatching rates in D7, D9 and D10. For the evaluation of the lipid concentration, blastocysts (D7; n = 10 / group) were stained with the Sudan Black B. Also, the gene expression analysis of GPX1 (involved in development), POU5F1 (pluripotency), HAND1 (differentiation and implantation), HP1 (homodimerization and chromatin interaction), IFNT2 (maternal fetal interaction), AKR1B1 (glucose metabolism), SREBF1 and SCD (both involved in lipid metabolism) were made by qRT-PCR. The statistical analysis of in vitro culture was performed by ANOVA test with the SAS system; For the lipid content, the ANOVA test was used by Tukey test and, in the analysis of the gene expressions was used the JMP software. The results were considered significant when $P \leq 0.05$. The lipid concentration was similar for both groups, 883 AU (arbitrary unit) in control group and 881 AU in NPPC group ($P > 0.05$). The gene related to lipid metabolism - SREBF1 - showed lower transcript concentrations for embryos in the NPPC group, P=0.06. Another difference was found on IFNT2 gene, with a higher expression for embryos treated with NPPC, P=0.06. There were no differences in blastocyst rates between embryos cultured with NPPC (44.4%) and control group (39.5 %). Interestingly, the rates of hatching at D10 were higher for NPPC group (63.9%) when compared to the control group (54.6%; P=0.09). In conclusion, our data suggested a possible effect of NPPC on embryo development.

A099 OPU - IVF and ET

# Goat incubator: The doe as a life incubator of bovine oocytes - first step

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Despite significant improvements in the in vitro production of cattle embryos, the suboptimal in vitro culture environment still limits the embryo quality and production. Techniques that associate the advantages of in vivo and in vitro systems, such as intrafollicular transfer of immature oocytes, have been proposed mainly to increase the embryo quality. In this context, we tried to use a goat as live incubator and associated nonsurgical embryo transfer techniques in small ruminants to perform ex situ (in vivo) maturation of bovine oocytes. For this, immature bovine cumulus-oocyte complexes (COCs) of grade 1 and 2 were randomly distributed into two groups for in vitro (IVM; n = 38) and ex situ (ESM; n = 40) maturation. The IVM was performed for a period of 24 h in TCM-199 medium (Gibco Life Technologies, Inc., Grand Island, NY, USA) supplemented with 20 mg/mL of FSH (Pluset, Calier, Barcelona, Spain), 0.36 mM sodium pyruvate (Sigma Chemical, St. Louis, MO, USA), 10 mM sodium bicarbonate (Sigma Chemical, St. Louis, MO, USA) and 50 mg/mL streptomycin/penicillin (Sigma Chemical, St. Louis, MO, USA) at 38.8 ºC in an atmosphere of 5% CO2 in air with maximum humidity. For ESM, a pre-synchronized nulliparous goat (12 months old) received 40 immature COCs in the uterine horn apice by transcervical route (Fonseca et al., 2014 Arq. Bras. Med.vet. Zootec) and 24 h after the procedure the structures were retrieved by the uterine flushing (Fonseca et al., 2013 Small Rumin Res). For analysis of the nuclear maturation rate and lipid quantification, the oocytes were denuded (0.1% hyaluronidase), fixed (4% paraformaldehyde) and stained with 10 µg/mL Hoechst 33342 and 10 µg/mL Nile Red (Molecular Probes, Inc., Eugene, OR, USA) dissolved in physiological saline (0.9% NaCl) with 1mg/mL polyvinylpyrrolidone. Oocytes displaying metaphase II plate were considered matured. The lipid amount was inferred by measuring the fluorescence intensity using the ImageJ program and fluorescence intensity were compared by Student's t-test. Forty-seven percent of the structures were recovered after uterine flushing (19/40). The nuclear maturation rate was 94.5% (18/19) and 81.6% (31/38) for ESM and IVM groups, respectively. In vitro-matured oocytes contained more lipid droplets, expressed as a higher ($p < 0.05$) amount of emitted fluorescence light (858 ± 73 arbitrary fluorescence units) than ex situ-matured oocytes (550 ± 64 arbitrary fluorescence units). This is the first report associating nonsurgical embryo transfer techniques with goat as live incubator for maturation of bovine oocytes. We conclude that transcervical transfer of bovine oocytes to uterine goat may be an alternative to in vitro maturation aiming the reduction of lipids without compromising nuclear maturation. Further studies are required to improve the oocyte recovery rate.
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A100 OPU - IVF and ET

# Genetic multiplication center for small farmers

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The small farmers are the ones who could most benefit from embryo technology, but they are the ones who are farthest from the difficulty of genetics, financial resources, opportunity, knowledge and technical ability. The objective of this technological extension project was to eliminate these factors and verify the technical and economic viability of an innovative way of applying the biotechnology. A Biotechnology Centerwas created, where the recipients of the small farmers were taken. Location with adequate infrastructure, trained staff, where all activities were on a larger scale, ensuring good results at relatively lower cost. It was offered to subsidized values: the genetics of donors of the Gir breed and sexed semen of Holtein bulls, the Ovum Pick up (OPU) services, embryo production (IVP), preparation of the recipients, inovulation and ultrasonographic examinations. With the support of the Farmers´ Union and Emater, 13 technical meetings were held to present the Project to small farmers. A veterinary team visited those interested farmers and selected the females with the following characteristics: heifers, weight between 290 and 380 kg, negative to brucellosis and tuberculosis tests and without alteration by rectal palpation and ultrasonography evaluation. Twenty-two small dairy farmers from the southern state of Minas Gerais were selected. The selection criteria were: to have a maximum of 150 animals in the herd; Have the dairy farming as the main source of income of the property; Have conditions for calf creation. When the project completed the 211 recipients, their initial goal, the meetings were discontinued. Each small farmer sent four to fifteen heifers. Twenty-three fixed time protocols for the preparation of recipients were made and respective innovulations. A total of 435 embryos transfer were made and 189 pregnancies were obtained, with an average rate of 43.4% and 90.5% (N=171) of female pregnancies. Each producer paid for the pregnant recipient the fixed value of R$ 300.00 per gestation of female, R$200.00 per gestation of male, added to the heifer weight difference between the entrance and exit of the Central. For those 22 non-pregnant recipients only the difference in weight was paid. The total cost of each pregnant recipient for small producers, including transportation costs was R$384.72. Regardless of the value of donor genetics, donated at no cost by a local breeder, the production cost of each gestation, including fees for the different activities involved, materials for recipient preparation, aspiration, embryo production and semen was R$987.54. The average difference of R$602.87 per gestation was funded by the CNPq Project, Process: 468954/2014-7 and the participating Institutions. We conclude that the methodology used is technically viaible and economic viability depends on cost-benefit analyzes for each situation.
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A101 OPU - IVF and ET

# Nuclear maturation kinetics of immature oocytes into preovulatory dominant follicle

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The objective was to evaluate the effect of maturation time on immature oocytes injected into preovulatory dominant follicle by intrafollicular injection of immature oocytes (IIFOI) on nuclear maturation kinetics. Immature bovines cumulus oocyte complexes (COCs; n=438) of grade 1, 2 e 3 from slaughterhouse were randomly assigned to one of three groups: (I) Control (n = 111), the oocytes were matured *in vitro* for 22 hours; (II) Mat20 (n=172) and (III) Mat30 (n=155), 30 oocytes were injected with the aid of a transvaginal guide with convex probe (7.5MHz) into preovulatory dominant follicle of previously synchronized oocyte recipient cows. In the Mat20 group, oocytes were matured in the dominant follicle for 19.8 ± 0.1 hours and in the Mat30 group for 28.3 ± 0.1 hours. In both experimental groups, cows received 12.5µg LH (Lutropin, Bioniche, Canada) at the time of IIFOI (Mat20 Group) or 10 hours after IIFOI (Mat30 Group). Oocytes from Mat20 and Mat30 groups were aspirated 20 hours after LH administration to evaluate the recovery rate. Oocytes from the experimental groups were denuded, fixed and stained by lacmoid to evaluate maturation kinetics as: germinative vesicle, metaphase I, anaphase I, telophase I, metaphase II, parthenogenetically activated and abnormal [chromosomal aberrations and degenerate (presented diffuse or undefined chromatin)]. Statistical analyses were performed by GLIMMIX procedure of SAS. Oocyte recovery rate after OPU was different between the Mat20 [52.9% (91/172)] and Mat30 [72.9% (113/155); P = 0.001]. The rate of oocytes in germinative vesicle state (P = 0.94), metaphase I (P = 0.98), anaphase I (P = 0.99) and telophase I (P = 0.20) were similar between the experimental groups. However, there was difference between groups for oocyte rates in metaphase II [Control - 81.0% (90/111)a, Mat20 - 74.5% (35/47)a and Mat30 - 41.6% (32/77 )b; P = 0.001], of abnormal [Control - 5.4% (6/111)c, Mat20 - 21.3% (10/47)b and Mat30 - 48.1% (37/77)a; P = 0.001] and parthenogenetically activated [Control - 0.0% (0/111)b, Mat20 - 0.0% (0/47)b and Mat30 - 9.1% (7/77)a; P = 0.001]. In conclusion, oocytes injected and maintained in preovulatory dominant follicle for 20 hours presented nuclear maturation similar to oocytes matured *in vitro*.

A102 OPU - IVF and ET

# Kinetics of embryonic development according to the follicular population in *Bos indicus* donors

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The present study aimed to evaluate the effect of the number of follicles aspirated from Nelore (Bos indicus) donors on the development kinetics of embryos produced in vitro. At random day of the estrous cycle, a total of 30 Nelore cows were synchronized with an intravaginal progesterone device associated with treatment with estradiol benzoate (2.0 mg im). Prostaglandin F2α (2.0 mg im) was also administered for CL removal at the time of ovum pick-up (OPU). Five days later, all donors underwent the OPU procedure. Oocytes were classified and submitted to the in vitro embryo production. Blastocysts production and hatching were verified on days 7, 8 and 9. Data were analyzed by the GLIMMIX procedure of SAS 9.3®. The donors were classified into three categories according to the follicular population: Low (18.1 ± 0.8; n = 10); Medium (30.8 ± 1.5, n = 10) and High (54.3 ± 5.1, n = 9). There was no difference in the rate of viable oocytes (64.3 vs. 65.4 vs. 68.5%, P = 0.59) between the groups of Low, Medium and High follicular population, respectively. However, was verified an increase in the cleavage rate (56.4b vs. 63.5ab vs. 64.6%a; P = 0.05) in the animals of High follicular population, when compared to the Low ones. The blastocysts rates, in relation to recovered oocytes, produced in D7 (25.7 vs. 25.7 vs. 33.3%, P = 0.10), in D8 (29.3 vs. 30.8 vs. 35.7%; P = 0.30) and in D9 (15.7 vs. 21.0 vs. 17.6%, P = 0.41) did not differ between the groups of Low, Medium and High follicular population, respectively. Furthermore, no differences were observed in the rates of hatched blastocysts in D7 (0 vs. 0 vs. 0.1%, P = 0.26), in D8 (52.4 vs. 41.8 vs. 53.4, P = 0.30) and in D9 (20.0 vs. 35.1 vs. 23.6%, P = 0.13), in the respective groups. The rate of total blastocysts production, in relation to the recovered oocytes, (32.1 vs. 34.6 vs. 40.6%, P = 0.15) and the rate of hatched blastocysts (71.1 vs. 74.3 vs. 80.2%, P = 0.36) was similar among the animals of Low, Medium and High follicular population, respectively. It was concluded that the kinetics of embryo development was not influenced by the follicular population of Nelore (Bos indicus) donors submitted to OPU-IVEP.

A103 OPU - IVF and ET

# Comparison of *in vitro* production, lipid content and gene expression of embryos from *Bos indicus* cows cultivated with or without forskolin supplementation

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The aim of this study was to evaluate the effect of supplementation on the culture medium with forskolin on the embryo development, lipid content and transcript levels of genes related to the quality and cellular metabolism of Bos indicus embryos produced in vitro. For IVP, ovaries (n = 420) of 210 Nellore females were collected from local slaughterhouse and transported in saline solution at 35°C to the laboratory. After follicular aspiration, the oocytes were selected (level I and II), MIV, FIV with semen from a single bull previously tested, and CIV. On day 5 of CIV the probable zygotes were randomly divided into the experimental groups: a) control group, cultivated with common medium (n = 447); B) forskolin group (10 µM forskolin supplementation; n = 432). The blastocyst and hatching rates were evaluated in D7, D9 and D10. To evaluate the lipid concentration, blastocysts on D7 (n=10/group) were collected and stained with Black Sudan B stain. The genes expressions of the GPX1, SREBF1, POU5F1, AKR1B1, HAND1, HP1, IFNT2 and SCD were analyzed by RT-PCR. For the statistical analysis, the IVP rates were compared using the ANOVA test from SAS system, for the lipid content the ANOVA test was used followed by Tukey test, and for the analysis of the gene expression data, JMP software was used. The differences were considered significant if $p \leq 0.09$. The blastocyst rate between the groups was 39.5% for the control group and 37.9% for the forskolin group ($p > 0.09$), the hatching rates in D9 and D10 were, respectively, 43.5% and 48.4% for the control group and 55,7% and 62.9% for the forskolin group, showing a significant difference in D10 ($p = 0.09$). The lipid concentration in the treatments was 883 AU (arbitrary unit) in the control group and 772 UA forskolin group, the lipid concentration/ area (AU x 10-10/µm2) was 1.1 ± 0, 8 in the control group and 0.9 ± 0.7 in the forskolin group, and the lipid concentration/ volume (AU x 10-13/µm3) 5,4 ± 4,7 and 4,3 ± 4,0 in the control and forskolin groups ($p > 0.05$). The genes related to pluripotency (POU5F1), development (GPX1), homodimerization and interaction of chromatin (HP1) and differentiation and implantation (HAND1) obtained higher concentrations of transcripts in the embryos from the control group. Genes related to maternal-fetal interaction (IFNT2) and lipid metabolism (AKR1B1 and SCD) presented higher expression in the group supplemented with Forskolin, however for the gene SREBF1, also related to metabolism, we found no difference in expression. In conclusion, the use of Forskolin on the CIV of embryos Bos indicus evidenced differences on genes related to the initial and lipid metabolism, suggesting the beneficial effect of this substance on the IVP of embryos. The lipid concentration in the embryos did not differ with the use of forskolin and the blastocyst rates remained constant, but the D10 hatching rates demonstrated superior results in the forskolin group, thus demonstrating favorable results with supplementation on the in CIV.

A104 OPU - IVF and ET

# Antral follicle count (AFC) and its association with reproductive traits in cows

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This study was conducted to determine if the antral follicle count (AFC) is positively associated with the total AFC, oocyte maturation stage, viable oocytes, total oocytes, and ovarian volume. Materials and Methods: Ovaries were collected in a slaughterhouse from 105 mixed breed cows (predominantly Bos taurus taurus) that were cycling, multiparous, and with body condition 3 and 4 (1-5) from 3 to 10 years of age. The groups were divided into high (≥25 follicles) (H), intermediate (16-24 follicles) (M), and low (≤15 follicles) (L) AFC. The cumulus ooctye complexes (COCs) were aspirated from follicles from 3 to 8 mm diameter. Only oocytes with quality 1 to 3 were used for the experiment. In vitro maturation (IVM) of oocytes of the AFC classes was conducted in an incubator for 24 h at 38.7ºC with 5% CO2 in a humidified atmosphere. The oocytes were examined every 2 days using Nomarski differential interference contrast (200-400 X) in a Nikon Diaphot DTM microscope (Nikon, Tokyo, Japan). Analyses were made by the SAS® statistical program (SAS Institute, Cary, NC, USA). Analysis of variance to test the reproductive variables (total AFC, oocyte maturation stages M1, M2, and M3, viable oocytes, total oocytes, and ovarian volume). The GLM was preceded by the Tukey test for differences between the individual mean values. The mean and the standard error of the mean were used. P was significant when < 0.05. The total AFC, stage 1 ooctyes, viable ooctyes, total oocytes, and ovarian volume were greater in ovaries H (69.69±2.144, 7.86±0.603, 30.97±5.173, 12.60±0.739) ($p<0.001$) compared to M (36.73±2.383, 4.85±0.670, 13.05±1.235, 38.32±5.750, 10.10±0.750) and L (20.65±2.580, 3.27±0.726, 8,07±1,337, 20.81±6.226, 8.09±0.858). In relation to ovarian volume, there was a significant reduction ($P<0.001$) in ovarian weight and size (length and height) in ovaries L (8.09±0.858) compared to ovaries H (12.60±0.739). In relation to the oocyte category, the number of oocytes with degree of maturation 2 and 3 were higher ($p<0.001$) in H ovaries (4.02±0.388 in G2; 2.09±0.389 in G3) compared to M (2.76±0.432 in G2; 2.48±0.439 in G3). For its part, L (1.24±0.467 in G2; 1.27±0.468 in G3) was similar to M ($p=0.009$ for G2 and $p=0.007$ for G3). The AFC is directly associated with oocyte quantity and quality and with ovarian volume, indicating that these traits can be easily used in selection processes of cows.
Acknowledgments: CAPES, FAPEMIG.

A105 OPU - IVF and ET

# Correlation between the concentration of DNA and the call rate of genotyping of bovine embryos biopsied samples

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The use of embryos in genomic selection has been discussed through the application of high density marker genotyping panels. This possibility arose from the evolution of the techniques of genotyping and pre-amplification of small samples that made the use of the whole-genome amplification (WGA). The objective of the present study was to evaluate the DNA quality of the amplified biopsy sample and to associate it with the call rate obtained after genotyping. The call rate is a quality parameter of the genotyping, which indicates the fraction of SNPs found in relation to the total SNPs tested in each sample. For this, biopsies were removed from PIV blastocysts (d7) by the manual section of the trofectoderma fragment (10-20% of the embryo, Bioniche blade). The samples were frozen in nitrogen, and subsequently the DNA was extracted and amplified using the Single Cell GenomiPhi DNA Amplification kit (Illustra, Buckinghamshire, United Kingdom). The quality of the amplified material was analyzed by the 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). Twenty-two biopsy specimens were analyzed. The criteria of total DNA concentration and concentration of DNA fragments greater than 7.000 bp prior to genotyping were adopted. According to the Bioanalyzer, the total DNA concentration reached the minimum value of 3.58ng/ul and the maximum of 726.03ng/ul, average of 25.26 and standard deviation of 155.01. Regarding the concentration of DNA fragments higher than 7,000 bp, the minimum value was 0.98 ng/ul and the maximum was 53.62 ng/ul, the average was 4.98 and the Standard deviation was 12.19. The samples were sent for genotyping using the Bovine SNP50k assay. After the genotyping, the average call rate of each sample was compared with the DNA quantification parameters obtained in the Bioanalyzer. Regarding the call rate, a range of 0.41 to 0.96 (minimum and maximum, respectively) was obtained, an average of 0.79 and a standard deviation of 0.23. The correlation analysis between the total amount of DNA in the Bioanalyzer and the call rate was not significant (R = -0.19 and P = -0.36, Spearman). Similarly, there was no correlation in the parameter concentration of fragments greater than 7,000 bp and the call rate (R = -0.04 and P = 0.85, Pearson). Thus, we can conclude that high total concentration or fragments greater than 7,000 bp of DNA in samples of amplified embryonic biopsies does not suggest that the sample will present a high call rate after genotyping.
thanks to embrapa, faperj and capes for financial support.

A106 OPU - IVF and ET

# Decreased Percoll volume does not influence fertilization rates and early embryonic development of IVP embryos using sexed semen

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Percoll is the most widely density gradient medium used for sperm selection in bovine embryos *in vitro* production. Though, with increased use of sexed sperm, changes in Percoll protocols have been proposed, aiming to enhance the sperm recovery rate and optimize the use of semen. Among these modifications, previous studies have shown that reduction of Percoll volume increases the sperm recovery rate (Missio *et al*., Anim. Reprod., v. 12, p.718, 2015). However, there are no researches evaluating the influence of this reduction in fertilization and early embryo development. This study aimed to evaluate the effect of different Percoll volume in fertilization rate and embryo development kinetics in bovine embryos IVP. Eight replicates were conducted from a pool of sexed semen from two bulls. The sperm selection was performed by discontinuous gradients Percoll (30, 60, 90%; Folchini et al. Rev. Bras. Reprod. Anim., v. 36, p.239-244, 2012), with volumes adjusted as treatments: Control: 300 µL and Treatment 1 (T1):100 µL for each gradient. At the first and second centrifugation a force of 2.200 x *g* was used during 5'and 1', respectively. After the selection process, a dose of $1x10^6$ spermatozoa/mL of each group was utilized for IVF of previously matured oocytes (Day 0). At 18 h post-insemination (hpi), the probable zygotes were divided into two groups for evaluation of the fertilization rate and kinetics of embryonic development. For evaluation of fertilization rate, the presumptive zygotes (93 and 94 for Control and T1, respectively) were stripped and incubated in a Hoechst 33342 solution (10mg/mL), being considered fertilized zygotes that had two pronucleus formation and extrusion of the second polar body or that had extrusion of the second polar body and sperm in decondensation. To evaluate the kinetics of embryonic development, the presumptive zygotes (54/treatment) were individually cultured in SOFaaci in an embryonic monitoring system (Primo Vision, Cryo Mangement Ltd., Hungary) for up to 48 hpi. The embryos were individually evaluated on day 2 for cleavage, moment of first cleavage and number of cells. Data were analyzed by chi-square ($X^2$; $P<0.05$). The fertilization rate after Percoll gradients were similar ($P>0.05$) between the Control and T1 (78.5±4.3 and 71.3±4.7, respectively). No difference ($p>0.05$) in the cleavage rate, the average time of the first cleavage and cell number after 48 hpi between the group Control (81.5±5.3; 32.9±1 and 4.16±0.33) and T1 (68.5±6.4; 35.1±0.99 and 3.77±0.30). The results of this experiment suggest that the Percoll volume decreased to 100 µL does not affect the fertilization rates and early embryonic development. Percoll volume reduction can be used as a routine method for the sperm selection of sexed semen for IVF.

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A107 OPU - IVF and ET

# FSH dose and strategy of administration during ovarian stimulation alter the gene expression profile in ovine cumulus-oocyte complexes

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Ovarian stimulation is an important tool to increase the number of oocytes obtained by laparoscopy for the in vitro production of embryos (IVP). In sheep, different concentrations of FSH administered in single dose (SD) or multiple doses (MD) have been adopted. In parallel, the oocyte quality is fundamental for IVP success, so strategies to produce more competent oocytes have been evaluated. The aim of this study was to evaluate the gene expression profile of BCB+ COC from different hormonal protocols of ovarian stimulation in Santa Inês ewes. To achieve that, a cross-over design was used, where 12 pluriparous ewes had their follicular wave synchronized (Balaro et al., Domest Anim Endocrinol, 54: 10-14, 2016). At 80 h after progestogen implant removal, all ewes received a new vaginal sponge and it started the stimulation by administration of: 80 (Group 1 - 80-SD) or 120 (Group 2 - 120-SD) mg FSH (Folltropin-V®, Bioniche Animal Health, Ontario, Canada) and 300 IU of eCG both in single dose, or 80 (Group 3 - 80-MD) or 120 (Group 4 - 120-MD) in decreasing doses (50/30/20%) every 12 h. The COCs were recovered by laparoscopy and classified morphologically in grade I / II (homogeneous ooplasm and more than 3 cumulus cells layers), III (homogeneous ooplasm and less than 3 cumulus cells layers or partially denuded) and IV (heterogeneous ooplasm or degenerate). For inference of the development competence GI, II and III COCs were stained with bright cresyl blue (BCB) and classified into: BCB+ (competent) and BCB- (non-competent). These variables were compared by ANOVA followed by Tukey test. The abundance of mRNA that encodes proteins associated with steroidogenesis (STAR, FSHr, LHr and ERα), oocyte quality (MATER, BMP15, GDF9 and ZAR1) and apoptosis (BAX and Bcl-2) was assessed by real-time qPCR normalized with GAPDH in BCB+ COCs. The abundance of gene transcripts associated with steroidogenesis was down-regulated ($P < 0.05$) with increasing FSH concentration, when administration was performed in a single dose (80-SD and 120-SD). On the other hand, when the administration was performed in MD, only the LHr was down-regulated (80-MD and 120-MD). In the 80-MD group, FSHr and Erα were down-regulated ($P < 0.05$) in comparison with 80-SD. For genes related with oocyte quality, 80-MD showed up-regulation ($P < 0.05$) to MATER (when compared to 80-SD), ZAR1 and MATER (compared to 120-SD). Nonetheless, apoptosis genes were not affected. These data demonstrate that the FSH dose and strategy of administration affect the gene expression profile in ovine COCs. Subsequent studies are necessary to assess the effect of this change on maturation rate and developmental competence.

A108 OPU - IVF and ET

# eCG stimulation prior to ovum pick-up on expression of oocyte quality markers in immature cumulus-oocyte complexes

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The success of in vitro embryo production is related, at least in part, to the quality of cumulus-oocyte complexes (COCs) harvested by ovum pick-up (OPU). The aim of this study was to evaluate the effect of ovarian stimulation with eCG prior to OPU on the mRNA abundance of TGFβ superfamily components and other markers of oocyte quality in COCs. A total of 30 Nellore (Bos indicus) cows were randomly divided into control (n=15) and stimulated (n=15) groups in a cross-over design. On a random day of the estrous cycle, all cows received an intravaginal P4 device (DIB™, MSD Animal Health, Brazil), 2 mg IM of estradiol benzoate (Gonadiol™, MSD Animal Health, Brazil). On Day 3 morning, the stimulated group received 300 IU of eCG IM (Folligon™, MSD Animal Health, Brazil). On the morning of Day 5, the P4 device was removed and OPU was conducted in both groups. Oocytes and cumulus cells (CC) were mechanically separated from pools of 25 immature COCs of control (n=4 pools) and stimulated (n=4 pools) groups. Total RNA was extracted from pools of 25 oocytes and their respective CC using an RNeasy® kit (Qiagen). Target gene expression in oocytes, including bone morphogenetic protein 15 (BMP15), growth differentiation factor 9 (GDF9), SMAD signal transduction factors (SMAD1, 2, 3, and 5), follistatin (FST), oocyte-derived JY-1, and cathepsins (CTSB and CTSZ), and in CC, including FST, CTSB, CTSK, CTSS, CTSZ, amphiregulin (AREG), epiregulin (EREG), and hyaluronan synthase 2 (HAS2), were assessed by real-time RT-PCR using Power SYBR® green master mix and normalized to the levels of cyclophilin (CYC-A). Relative quantification of mRNA abundance was determined using the ΔΔCt method. Effects of ovarian stimulation with eCG on the expression of target genes in oocytes and CC were analyzed by unpaired t test and $P<0.05$ was considered significant. In oocytes, mRNA encoding BMP15, and SMAD1, 2, and 3 was higher ($P<0.05$) in the stimulated group than in the control group. Moreover, the relative mRNA abundance of CTSZ, a member of the cathepsins family functionally related to reduced oocyte competence, was lower ($P<0.05$) in the stimulated group than in the control group. Similarly, cumulus cell CTSS and CTSK mRNA abundance was lower ($P<0.01$) in the stimulated group compared with control group. However, the relative abundance of AREG and EREG mRNA was higher ($P<0.05$) in CC recovered from stimulated group. No differences on mRNA abundance of GDF9, SMAD5, FST, JY-1, and CTSB in oocytes and FST, CTSB, CTSZ, and HAS2 in CC were demonstrated among different groups. In conclusion, eCG stimulation prior to OPU modifies the profile of mRNA abundance of genes related to oocyte quality in COCs, and it may contribute to the improvement of oocyte competence. Acknowledgements: Fapemig, CAPES, CNPq and Biotran.

A109 OPU - IVF and ET

# Effect of the oocyte competence on the *in vitro* development of embryos of repeat-breeder dairy cows subjected to drying and subsequent induction of lactation

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The objective of the present study was to evaluate the IVEP of Holstein cows (*Bos taurus*) of different categories. A total of 34 cows were used, homogenously distributed in four groups: cows in the beginning of lactation (BL; n=9), repeat-breeder cows (RB; n=7), dry cows (DC; n=9) and cows with induced lactation (IL; n=9). IL cows went through the following drying process: after the last milking, the antisepsis of the teats was done using cotton and 70% alcohol. Next, Ciprolac® (Ciprofloxacin; Ourofino, Ribeirão Preto, Brasil) was administered, the teat was massaged, and, on sequence, Sellat® (Bismuth Subnitrate; Ourofino) was administered on each teat. Also, the drying process included the Mastitis vaccine (5mL of Mastiplus-BR® SC; Vitafort Animal Defense, Ribeirão Preto, Brasil) on the moment of drying and 30 days after. The 60d drying period was concluded for the posterior induction of lactation of the IL cows. The IL group consisted of RB cows that received the induction of lactation protocol 30 days previous to OPU [500mg of bSTr (Boostin®, MSD, São Paulo, Brasil) on D0, 7 and 20; 30mg/cow/d of BE (Sincrodiol®, Ourofino) and 300mg/cow/d of P4 (Sincrogest®, Ourofino) IM from D0 to 7; 20mg/cow/d of BE from D8 to 14; 0,530mg/cow of PGF (Sincrocio®, Ourofino) on D15; 40mg/cow/d of dexametasone (Cortiflan®, Ourofino) from D18 to 20; 5 min daily massage of the teats and the udder from D16 to 19; milking started on D20; after the onset of lactation, cows received 500mg/cow of bSTr every 14d]. The other groups did not receive any treatment. All cows from the four categories went through three OPUs, with a 30d interval. The antral follicle population count (AFC) was done using US in each OPU. Data were analyzed using the Generalized Linear Mixed Models (PROC GLIMMIX) of SAS (*v9.4*). The AFC was lower (P=0.03) in IL cows (9.3±0.9b) when compared to RB (15.9±2.2$^{a}$) and DC (17.7±2.3$^{a}$), not differing from the BL (12.7±1.6$^{ab}$). However, the quantity of recovered oocytes from BL cows (7.15±0.7$^{b}$) differed (P=0.008) only from the DC (14.3±2.0$^{a}$), and was similar to RB (12.9±1.8$^{ab}$) and BL (10.8±1.2$^{b}$). There was difference on the number of viable oocytes (P=0.02) between groups (DC=7.8±1.3$^{a}$; RB=7.3±1.0$^{ab}$; BL=3.38±0.4$^{bc}$ and VL=4.9±0.7$^{c}$). No difference was found (P=0.30) on oocyte recovery rate between groups [IL=77.2% (186/241); BL=78.5% (270/344); RB=80.9% (271/335); DC=81.0% (387/478)]. The cleavage rate (cleaved/total of recovered) was greater (P=0.009) on RB cows [48.7%$^{a}$ (132/271)] in relation to the others [IL=29.6%$^{b}$ (55/186); BL=33.0%$^{b}$ (89/270) and DC=39.3%$^{b}$ (152/387)]. The blastocyst rate (blastocyst/total of recovered) was higher (P=0.02) in RB [24.7%$^{a}$ (67/271)] when compared to IL [9.1%$^{b}$ (17/186)], but similar to the other categories [BL=16.3%$^{ab}$ (44/270); DC=17.6%$^{ab}$ (68/387)]. It is possible to conclude that drying followed by induction of lactation was not efficient to improve IVEP of RB cows.

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A110 OPU - IVF and ET

# Effect of high density lipoprotein during *in vitro* oocyte maturation on initial embryo development in bovine

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High-density lipoprotein (HDL) is the only lipoprotein present in follicular fluid and is responsible for providing cholesterol for steroidogenesis. In addition, it has anti-inflammatory, antioxidant and cytoprotective properties, mainly derived from its lipid composition, apoliprotein-AI and enzymes such as paraoxonase-1. In this sense, intrafollicular levels of HDL-cholesterol have been positively associated with improved embryonic quality in women. The aim of this study was to evaluate the effect of increasing doses of HDL during oocyte maturation *in vitro* on the initial embryonic development in cattle. The IVP was carried out in an incubator with 5% $CO_2$ at 39 °C using commercial media (Progest Biotecnologia em reprodução Animal, Botucatu, SP, Brazil). Cumulus oocyte complex (COCs) were obtained from slaughterhouse bovine ovaries, washed and selected for morphology. COCs of grade I, II and III were randomly distributed into three groups (n=50 COCs/group) according to the addition of HDL protein in the IVM medium (G0: 0 mg/mL; G1: 0.5 mg/mL; G2: 1.5 mg/mL HDL, SIGMA-ALDRICH®, St. Louis, MO, USA). IVM occurred for 22 hours. The IVF (day 0) was performed with a concentration of $1x10^6$ spermatozoa/mL for 20 hours. After this period, the presumptive zygotes were washed and cultured in IVC media covered with mineral oil for 7 days. On day 3 the cleavage rate (cleaved/inseminated) was evaluated and 70% of the culture media was renewed, which was repeated on day 5. On day 7 the rate of embryonic development (blastocysts/inseminated) was evaluated. Therefore, 9 replications were performed, totaling 450 inseminated COCs/group. The effect of HDL on the cleavage rate and embryonic development was analyzed through the repeated measures ANOVA followed by the Tukey post-hoc test. The highest dose of HDL had a negative effect on the cleavage rate ($P=0.0003$) and embryonic development ($P=0.02$). The cleavage rate from G0 (68.8%) and G1 (68.1%) was not different, but G2 cleavage rate (56.3%) was lower in comparison to the other groups ($P<0.05$). Likewise, the embryo development rate was not different between G0 (29.4%) and G1 (29.2%), but G2 (19.5%) had a lower development rate compared to the other groups ($P<0.05$). It is concluded that despite the antioxidant and cytoprotective properties of HDL, when in high concentrations it can negatively affect the initial embryonic development in cattle, since the higher concentration of HDL tested in this study decreased the cleavage and embryonic development rates.

A111 OPU - IVF and ET

# The effect of donor breed on the *in vitro* production of bovine embryos efficiency

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The in vitro embryo production (IVEP) is applied in different breeds of bovine herds in Brazil, seeking greater yield efficiency in each of them. Therefore the purpose of this study was to determine whether the donor breed Gir, Holstein or ½ Holstein-Gir, influences the mean oocytes retrieved per OPU session, blastocyst rate, and blastocyst and expanded blastocyst pregnancy rate. For this, 363 Gir donors (n = 2047 OPU), 109 Holstein donors (n = 265 OPU) and 144 ½ ½ Holstein-Gir (n = 465 OPU) were used. The aspirated oocytes were classified according to the IETS criteria and grade 1, 2 and 3 oocytes were considered viable. After OPU, the oocytes were matured, fertilized (D0) with sexed sperm and cultured in vitro for 7 days (D7). For in vitro fertilization, different previously known fertility bulls (n=71) were used in the laboratory routine. In D7, the embryos produced were transferred to previously synchronized Holstein-Gir cross recipients. The pregnancy status was determinated by transrectal ultrasonography at 28 days and confirmation at 60 days of gestation. Kruskal-Wallis test (P≤0.05) was used to compare the groups. Donor breed effect was observed in the mean of viable oocytes aspirated from ½ Holstein-Gir (19.3 ± 0.63a), Gir (14.8 ± 0.23b), and Holstein (9.00 ± 0.45c) donors. In relation to the blastocyst rate in D7, donors Gir (27.1% ± 0.55a) and ½ Holstein-Gir (24.3% ± 1.08a) presented higher rates than Holstein donors (21.3% ± 1, 46c). However, there was no difference in the pregnancy rate of embryos from Gir (37.8% ± 0.85), ½ Holstein-Gir (35.6% ± 1.67) and Holstein (35.1% ± 3.02) donors. In relation to pregnancy rate according to the stage of embryo development, a higher pregnancy rate was observed when transferring expanded blastocyst (40.2%, n = 6581) in relation to the pregnancy of embryos transferred in the blastocyst stage (29, 32%, n = 1954). The pregnancy rates at 30 and 60 days were similar, and no embryo loss was observed between the two gestation diagnoses. It is concluded that donors of the breed ½ Holstein-Gir provide a greater number of oocytes per OPU than donors Gir, which is superior to the Holstein donors. In addition, Gir and ½ Holstein-Gir donors show a higher blastocyst rate when compared to Holstein donors, without any influence of the donor breed on the pregnancy rate at 30 and 60 days. Furthermore, embryos transferred in the expanded blastocyst stage are more successful in the pregnancy rate than embryos transferred in the blastocyst stage.

A112 OPU - IVF and ET

# Effect of different times of forskolin exposure to induce lipolysis in *in vitro* matured bovine oocytes

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In the present study, the effect of forskolin exposure time to induction oocytes lipolysis was evaluated in order to improve the rate of *in vitro* production of bovines. Oocytes from zebu cows obtained from commercial slaughterhouse were selected (N=708) and in groups of 20-25, they were transferred to drops of 90 µl of IVM medium (TCM 199) with 10% FBS according to the period of 50 µM forskolin (FSK) exposure during IVM: Control (without FSK, N=167), FSK-6h (6h MIV with FSK and 18h without, N=168), FSK-18h (18h MIV with FSK and 6h without, N=178) and FSK-24h (24-hour MIV with FSK, N=194). The IVM was performed in incubator with 5% $CO_2$ in air, temperature of 38.5 °C and humidity for 24 hours. The oocytes from the different groups were fertilized *in vitro* with frozen sperm from the single Nellore bull (*Bos taurus indicus*). Semen was selected by Percoll gradient (Nutricell) and the final concentration was adjusted to 2x106 sperm/mL. The probable zygotes were cultured in SOFaa medium plus 5 mg/mL BSA, 2.5% FBS and 0.11 mg/mL sodium pyruvate. Another part of the oocytes (N=210) was stained with 1 µg/ml of Nile red fluorescent dye to assess lipid content. The pictures obtained from the stained oocytes were taken on an epifluorescence microscope with a magnification of 20X and had the fluorescence intensity measured in the Image J. program. The lipid content of the oocytes was presented by the fluorescence intensity mean per µm2 (IF/µm2). For evaluate embryo quality (N=72), the fluorescent dye of TUNEL kit (*Terminal deoxinucleotil transferase Uracil Nick End Labeling*) was used. Green fluorescent nuclei (fluorescein isothiocyanate (FITC) were considered cells with DNA fragmented and blue fluorescent (Hoechst 333342) indicated the presence of the cell nucleus. To know the rate of apoptosis the number of cells stained in green was divided by the total number of blue cells and multiplied by 100. For statistical analysis, the dependent variables were submitted to ANOVA by the least squares method using the GLIMMIX procedure (SAS Inst. Inc., Cary, NC, USA). If the ANOVA was significant, the data were analyzed using the SEM. The level of significance was 5% ($P<0.05$). The control group ($23.5\pm1.8^{a}$) had a higher accumulation of lipids than the other: FSK-6h ($19.7\pm1.2^{b}$), FSK-18h ($17.2\pm0.7^{b}$) and FSK-24h ($18.8\pm0.9^{b}$) - ($P<0.05$). There was no difference in the cleavage rate: Control (83.8±4.4 - 140/167), FSK-6h (77.3±5.0 - 131/169), FSK-18h (58.1±8.3 - 99/178) and FSK-24h (61.2±7.41 - 18/194). The blastocyst rate of the Control group ($46.4\pm3.9^{a}$ - 77/167) and FSK-6h ($33.0\pm6.6^{ab}$ 54/169) were similar, but FSK-18h ($19.8\pm2.2^{b}$ - 35/178) and 24h-FSK ($16.7\pm2.3^{b}$ - 32/194) were lower than Control - ($P<0.05$). Regarding the total number of intact cells, the Control ($144.7\pm8.3^{a}$), FSK-6h ($145\pm9.4^{a}$) and FSK-8h ($130.5\pm14.9^{ab}$) were similar, but the FSK-24h ($92.5\pm6.3^{b}$) group had a lower number of cells ($P<0.05$). The rate of cellular apoptosis was similar in all groups: Control (8.0±1.4), FSK-6h (6.1±2.1), FSK-18h (3.5±0.9) and FSK-24h (8.5±3.1). Although all times of drug exposure decreased lipid content, without influencing cell apoptosis, the FSK-18h and FSK-24h groups had a decrease in the total production of blastocysts. In conclusion, 6h of oocytes exposure to FSK is sufficient time to reduce lipids without prejudice the later development and quality of the embryos.
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A113 OPU - IVF and ET

# Effect of butafosfan in expression of genes associated oocyte quality

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Butafosfan is an organic phosphorus molecule that has been studied as a metabolic modulator. Phosphorus is fundamental for the growth, differentiation and cellular integrity, it acts in the processes of phosphorylation and dephosphorylation of proteins and cellular signals, as well as in the cycle ADP/ATP. Associated with cyanocobalamin, butafosfan had positive effects on cow folliculogenesis. In view of this, butafosfan becomes a viable alternative to improve oocyte metabolism and thus the acquisition of competence. The aim of this study was to evaluate the effect of butaphosphan addition in the maturation medium in expression of genes associated with apoptosis, cumulus cells expansion, resumption of meiosis and energy metabolism. Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in NaCl 0.9% solution with gentamicin 0.5% at 30 °C. Complex cumulus oocytes (COCs) were aspirated from follicles (3-8 mm in diameter) using a stereo and then, washed three times in washing medium (Animal Biotechnology®, Brasília, DF, Brazil). In total of 809 COCs (n = 809) were randomly assigned to groups of ± 60 COCs/group/routine as supplemented with butafosfan in IVM medium (GC: 0 mg/ml, T1: 0.05 mg/ml; 0.1 mg/ml and T3: 0.2 mg/ml butafosfan, Bayer Animal Health, São Paulo, SP, Brazil). The maturation occurred in 500 µL drops of MIV-TCM medium (Animal Biotechnology®) supplemented with 10% fetal bovine serum at 39 °C in 5% $CO_2$ atmosphere and at maximum humidity for 24 h. After the IVM, 15 COCs from each group were stripped through successive pipings, the rest of COCs continued in the PIVE routine for further analysis. Cumulus cells and oocytes were stored separately in microtubes containing 100 µL TRIzol (Invitrogen, Carlsbad, California, USA) at -70 °C until analysis of gene expression. In this way 3 routines were conducted. Total RNA was extracted from cumulus and oocyte cells using TRIzol and quantified on NanoVue spectrophotometer (General Electric Healthcare Limited, UK). The cDNA synthesis was performed using iScript Reverse Trascription Supermix (Bio-Rad, Hercules, California, USA) according to the manufacturer's instructions. Real-time PCR reactions were conducted on Applied Biosystems 7500 (Applied Biosystems, Foster City, USA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). Expression of the genes: BAX and BCL2 as markers of apoptosis; AREG and EREG as genes related to the expansion of cumulus cells and resumption of meiosis; GDF9 and BMP15 as indicators of oocyte quality and GLUT1 and PFKP related to energy metabolism in oocytes. The results were analyzed using the 2-ΔΔCT method, using the H2A gene as internal control. Statistical analysis was performed in the SAS 9.0 program (SAS, Cary, NC, USA) using the General Linear Model test to determine the linear, quadratic or cubic effect of the supplementation with 0.0, 0.05, 0.1 and 0.2 mg/ml butafosfan in the maturation medium. The relative expression of the genes studied was similar between the groups in both oocytes and cumulus cells (P> 0.05). In conclusion, supplementation of the IVM medium with different doses of butafosfan does not improve oocyte quality.

A114 OPU - IVF and ET

# Effect of the number of recovered oocytes by OPU on *in vitro* embryo production of Holstein (*Bos Taurus*) cows

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Effect of the number of recovered oocytes by OPU on in vitro embryo production of Holstein (Bos taurus) cows
An analysis of 1256 follicular aspirations (OPU) performed by the same veterinarian in 1021 Holstein oocytes donors for in vitro embryo production during the period of 2011 to 2013 in 10 commercial farms was conducted. A total of 16259 oocytes were fertilized using semen from 8 Jersey bulls. For data analysis, donors were divided according to the amount of oocytes on the moment of the OPU into Group 1 (Q1) – lower quartile (n=314); Group 2 (Q2) – intermediate lower quartile (n=314); Group 3 (Q3) – intermediate higher quartile (n=314) and Group 4 (Q4) – higher quartile (n=314). Data were analyzed using the PROC GLIMMIX procedure of SAS (9.4 version) and "interactive data analyses" of SAS was used to calculate probability. There was difference within groups in relation to the number of recovered oocytes (Q1=5.7±0.1; Q2=9.9±0.1; Q3=14.4±0.1 and Q4=26.8±0.5; P<0.0001) and blastocyst production (Q1=1.4±0.6; Q2=1.7±0.7; Q3=2.7±0.9 and Q4=4.5±1.3; P<0.0001) per OPU. The cleavage rate (number of cleaved oocytes/number of viable oocytes) was greater on Q3 (56.3%; 2331/4041) in relation to Q1 (50.3%; 843/1654) and Q2 (53.0%; 1502/2873), P<0.001. The Q4 (53.7%; 4373/7691) did not differ from the other groups. The blastocysts rate [number of blastocyst/number of viable oocytes; (Q1=25.5; 426/1654; Q2=18.7%. 538/2873; Q3=21.2%, 855/4041; Q4=18.3%, 1408/7691; P<0.23)] and the pregnancy rate [number of pregnancies/number of transferred embryos; (Q1=34.0%, 152/447; Q2=31.0%. 220/708; Q3=44.0%, 497/1129; Q4=37.0%, 805/2178; P=0.13)] did not differ within groups. The cleavage rate increased according to the number of recovered oocytes per donor (P=0.0008; R2=0.09436). The blastocysts production rate decreased according to the number of recovered oocytes per donor (P<0.0001; R2=-0.22642). There was no difference on the probability of pregnancy according to the amount of recovered oocytes per donor (P=0.1; R2=0.03608). It is possible to conclude that donors with greater number of oocytes have lower blastocyst production rate. Furthermore, the amount of recovered oocytes per donor does not interfere on pregnancy rate.
Acknowledgments: Sexing Technologies.

A115 OPU - IVF and ET

# Effect of number of oocytes recovered per OPU on *in vitro* embryo production and pregnancy rate of Nelore (*Bos Indicus*) cows

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The aim of the present study was to evaluate the relationship between the number of oocytes recovered per OPU section of Nelore cows with IVEP efficiency, as well as with field fertility (pregnancy after embryo transfer; ET). For that, the analysis of 9,470 follicular aspirations (OPU) performed by nine veterinarians in 1,658 Nelore oocyte donors for in vitro embryo production (IVEP) during the period of 2001 to 2010 in 16 commercial farms was conducted. Semen from 178 Nelore sires was used for the in vitro fertilization. For data analysis, donors were divided according to the amount of oocytes recovered on the moment of OPU into Group 1 (Q1) – lower quartile (n = 13,246); Group 2 (Q2) – intermediate lower quartile (n = 25,376); Group 3 (Q3) – intermediate higher quartile (n = 40,119) and Group 4 (Q4) – higher quartile (n = 75,645). Data were analyzed using PROC GLIMMIX of SAS (9.4 version). "Interactive data analyses" of SAS was used to calculate probabilities. There were differences within groups relative to the number of recovered oocytes per OPU (Q1 = 9.4; Q2 = 18,1; Q3 = 28.6 and Q4 = 53.9), number of viable oocytes per OPU (Q1 = 8.4; Q2 = 15.9; Q3 = 25.4 and Q4 = 47.7) and blastocyst production per OPU (Q1 = 2.6; Q2 = 4.9; Q3 = 7.8 and Q4 = 13.5). However, there was no difference between groups regarding the cleavage rate [number of cleaved oocytes/number of recovered oocytes; Q1 = 68.8% (8,089/11,756); Q2 = 66.7% (14,900/22,337); Q3 = 66.2% (23,582/35,634); Q4 = 63.9% (42,714/66,860; P = 0.08)] and the blastocyst rate [Q1 = 44.9% (3,634/8,089); Q2 = 45.9% (6,841/14,900); Q3 = 46.4% (10,948/23,582); Q4 = 44.5% (19,001/42,714); P = 0.23]. The pregnancy rate was higher for the lower quartile (Q1 = 44.1%, 187/424a) when compared to the other groups [Q2 = 40.3% (302/750)b; Q3 = 39.9% (n/899)b; Q4 = 38.6% (387/1,003)b; P = 0.001). The probability of cleavage (R2 = -0.084; P < 0.001), probability of blastocyst production (R2 = -0.034; P = 0.01), and probability of pregnancy (R2 = -0.072; P < 0.0001) decreased as the number of recovered oocytes per donor increased. In conclusion, both the efficiency of IVEP (cleavage and blastocyst production) and the pregnancy rate are negatively influenced by the increase of the number of recovered oocytes per OPU in Nelore cows.
Credits: Fapesp 2012/50533-2 (GIFT), and CNPq 152030/2016-6.

A116 OPU - IVF and ET

# Efficiency of OPU (Ovum Pick-Up) in females bubalinas (*Bubalus Bubalis*) dairy, in management of pasture

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The objective of this study was to evaluate the efficiency of OPU (Ovum Pick-Up) performed for in vitro embryo production in buffaloes (Bubalusbubalis), through the rate of aspiration follicles, rate of oocytes recovered, rate of oocytes for FIV. The experiment occurred on a farm specialized in the production of milk of buffaloes, located in the municipality of Bujaru 45 km from the capital of Belém. The property presents a herd bubalino consisting of mestizo animals and pure blood (Murrah and Mediterranean) destined for the production of milk to pasture. Used 47 buffaloes multiparous with dairy production above 7 litres in a single milking daily, previously selected through ultrasound examination for determination of ovarian size and follicular population and with body condition score around 3,0 (1 = very lean, 5 = very fat), which originates from producers in the States of Pará, Ceará, Bahia, Rio Grande do Norte and São Paulo. The females were subjected to a total of 43 follicular aspiration sessions (OPU; 18G; 1,7 mm Teflon suction line of internal diameter and 80 cm long; 50 mmHg). Visible follicles were vacuumed (≥ 2mm ≤ 8mm) through ultrasound (DP 4100VET). The aspirations were carried out weekly, each animal being aspired in 14-day intervals, with 7 to 14 buffaloes per session. The data was submitted to ANOVA and the averages compared to the Tukey test ($P < 0,05$). They were vacuumed a total of 5,186 follicles, 2,845 oocytes recovered and 1,800 oocytes for FIV, obtaining a recovery rate of oocytes of 54.86%. The averages obtained by animal and by section of OPU were 12.64 ± 5.87 of aspirated follicles; 6.93 ± 5.55 of oocytes recovered and 4.40 ± 3.89 of oocytes for IVF, higher than that found by other authors, who have achieved averages of 9.1 (aspiration follicles), 3.5 (oocytes recovered) and recovery rate of 38.4% (Baruselli, et al. Revista Brasileira de Reprodução Animal, 31, 285-292, 2005), demonstrating good development of opu in the females bubalinas in this study. However, numerically, the follicular population in buffalo is much lower when compared to the bovine Zebuíno (Gimenes et al., Reproduction in Domestic Ruminants, 1, 357-375, 2010), causing FIV in Bubalino to become much more expensive than in bovine (Ohashi et al., RevBrasReprodAnim, 41, 195-200, 2017). A large individual variation has occurred since donors are from varied backgrounds. Other authors also relara that low results in OPU are coming from individual and race variation (Boni et al. Proceedings of 5th World Buffalo Congress, 5, 787-792, 1997). Therefore, the OPU sessions in the Buffaloes used have shown good efficiency, presenting a good amount of suction follicles, oocytes recovered, oocytes for FIV, in addition to a satisfactory oocitária recovery fee.

A117 OPU - IVF and ET

# Extracts from cerrado plants as antioxidant agents on *in vitro* embryo production

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The present study evaluated the effect of ethanolic extracts of plants from Cerrado- Brazil, containing high levels of polyphenols (antioxidant), on in vitro embryo production (IVEP) in cattle. Ovaries from slaughterhouse were used to collect grade I and II cumulus-oocyte-complexes (COC), which were submitted to in vitro maturation, fertilization (D0) and culture. Different concentrations (0; 1mg/mL; 01mg/mL and 0.01mg/mL) of cagaita (Eugenia dysenterica) and murici (Byirsonima crassifolia) extracts were added to the culture medium during embryo development. The parameters analyzed were: cleavage rate on D3, blastocyst rate in D6, D7 and total and apoptotic cells number by TUNEL method. The ability of those extracts to request free radicals from the culture medium was analyzed by ABTS colorimetric method. To do that an aliquot of the culture media was collected from each treatment drop at two different time points (D0 and D7). Data were analyzed by analysis of variance - ANOVA and the means were compared by TUKEY, with a significance level of 5%.The results of embryo production did not differ between the control group: cleavage 80.5% (136/169), blastocyst rate D6: 30.2% (51/169) and D7: 41% (69/169) and the groups treated with murici 0.1mg: 81.9% (149/182), 23.6% (43/182) and 35.2% (64/182) and 0.01mg: 78% (127/163), 32.% (52/163), 38.7 % (63/163). The total embryonic cells and the proportion of apoptotic cell in expanded blastocyst (BX) in D7 were similar among the groups (P=NS). Except for the 1mg group, that showed high toxicity and death already on cleavage stage evaluation. Regarding the capacity of polyphenols to remove free radical, no differences (P>0.05) between those same groups (control, 0.1 and 0.01mg). The only difference detected ($p < 0.05$) was also for the 1mg/ml group, which showed an increase on the amount of free radicals. However, the cagaita extract showed a similar behavior for cleavage rates in control group: 80.6% (179/222); 1mg: 78.3% (177/226); 0.1mg: 81% (187/231); 0.01mg: 82.5% (184/223). Yet, when evaluating the blastocyst rate at D6, a lower rate ($p<0.05$) was observed for the 1mg group 27/226 (12%) compared to the to control group 57/222 (25.7%), 0.1mg 59/231 (25.5%) and 0.01mg 76/223 (34%) groups. The same profile was observed at D7, with 45.5% of embryos in the control (101/222); 35% in the 1mg group (79/226 $p < 0.05$); 42% in 0.1mg (98/231) and 50% in the 0.01mg (112/223) groups. The number of BX cells was similar among all groups. However, the proportion of apoptotic cells was lower ($p < 0.01$) in the group with 0.01mg cagaita (2.8%) than the others (control: 8.33%, 1mg: 5% and 0.1mg: 5.4%). The ABTS results for cagaita were similar for all groups. The results showed that extracts of the tested plants were toxic at concentration of 1mg/mL in However, when they were diluted thousand times, it was possible to observe a decrease in apoptotic cells using 0.01 mg of cagaita extract (Eugenia dysenterica). This same dilution of the murici extract did not affected any of the evaluated parameters. It can be concluded, that the cagaita extract (0,01mg/mL) is an alternative to be use as a coadjuvant for the reduction of the oxidative stress induced by the adverse conditions of IVP.

A118 OPU - IVF and ET

# Serum FSH, AMH related to number and morphology of oocytes in superovulated 4 to 7 months old Nelore breed females (*Bos Taurus Indicus*)

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The inclusion of prepubertal bovine females in reproductive management can make herd's genetic gain faster by shortening generation interval. However, these female oocytes have lower competence, blastocyst and pregnancy rates, when compared to those obtained from postpubertal animals. So, this study aimed to evaluate serum FSH and AMH concentrations in superovulated (TG-Treated Group) Nelore females of 4 to 7 months old, as in control group (CG) and their respective oocyte retrieval and quality by OPU (Storz Xenon300W Laparoscope, Tuttlingen, Germany). Nine females (cross-over design) were allocated at random to two groups: The CG (n=9), which the greatest follicle ablation was performed on D2 (5 days before OPU) with the aid of transrectal ultrasonography (MyLab 30VetGold, Esaote, 5-7.5MHz transducer, Genova-Italy). And to the TG (n=9), in which D0 represented the protocol beginning with intravaginal device Progesterone insertion (P4, 0.33g. Eazi-Breed-CIDR, Pfizer Animal Health, Brazil) plus 2mg Estradiol Benzoate injection (im Ric-BE, Tecnopec-Brazil). From D4 on, 6 FSH injections were given during 3 days (im, 12/12h: 1x40mg + 5x20mg = 140mg; Folltropin, Bioniche Animal Health, Belleville-Ontario, Canada). At the last FSH injection, LH (2.5 mg) was administered (Lutropin, Bioniche Animal Health, Belleville-Ontario, Canada). Then, the OPU was performed 20-24h after the last FSH injection (D7) and the P4 devices were removed thereafter. The follicles were counted and aspirated COCs were classified. Blood sample collections for FSH measuring were performed 2 days before, at the day and 1 day after the OPU procedure, as for the AMH measuring, it was performed at D5 and at D8. Data were analyzed by Kruskal-Wallis, ANOVA, T-test and Chi-square test. The TG had higher serum FSH concentrations ($p<0.05$) on days 5 (1.16 ± 0.31 ng/ml), 6 (1.21 ± 0.45 ng/ml) and 7 (0.95 ± 0.26 ng/ml) than the CG (0.56 ± 0.17 ng/ml at D5, 0.60 ± 0.25 ng/ml at D6 and, 0.60 ± 0.14 ng/ml at D7). In addition, a greater number of aspirated follicles (152 vs. 95) and higher numbers of oocytes grades I and II (59% vs. 25%) were observed in the TG compared to the CG, respectively ($p <0.05$). However, GC presented more grade III and IV oocytes when compared to TG (53.3% vs 37.1%), whereas the mean AMH concentration (1.48 ± 0.37 ng / ml) was not different between TG and CG nor between the days of collection ($p>0.05$). Thus, this superovulation protocol led to higher serum FSH concentrations, which possibly had a role to a greater quantity and better quality of the retrieved oocytes, without changing the serum AMH levels in the animals.
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A119 OPU - IVF and ET

# Influence of climatological conditions in reproductive variables of Nelore race females

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An extrinsic factor that can interfere in the reproductive variables is the climatic changes occurring throughout the year. This factor has a determinant role in the quality of the pastures that can interfere in the embryo production. This study aimed to evaluate the effect of the seasons of the year and the climatic variables of temperature and precipitation on reproductive variables in Nelore cows. Eighteen cows, with at least 7 aspirations / cows, all from the same genetic lineage were housed 40 km from the city of Maringá, with a Brachiaria grass diet at will and 2 kg of concentrate / day. The reproductive variables evaluated were the production of total aspirated oocytes, viable oocytes and PIV embryos. In vitro embryo production was performed with viable oocytes maturation for 22-24 hours in TCM 199 medium (10% FCS, FSH 0.1µg / mL and LH 50µg / mL); the in vitro fertilization in TALP-FIV medium for 22-24 hours with a dose of 1x106 spermatozoa / mL. The probable zygotes were farmed in SOF (Synthetic Oviduct Fluid) supplemented with 2.5% FCS and BSA (5mg / ml) in an incubator (38.3 °C, 5% CO2 and maximum humidity). On the seventh day of cultivation the viable embryos for transfer were evaluated. The climatological data were obtained at the National Institute of Meteorology (INMET) throughout the year 2012, through the automatic monitoring station located in Maringá region (latitude -23.4º, longitude -51.91º and altitude 542m), and for both the Temperature and precipitation the average of the 10-day period was effectuated. For the data analysis, the logistic regression models (viable oocytes and embryo production) and Poisson regression (total oocytes) were used. Analyzing the climatic data, it was observed that the climate was typical throughout the year (rainy summer and dry winter), without extreme events related to temperature and precipitation. The average winter temperatures were 25 °C in summer, in autumn and in spring 22 °C and 18 °C in winter. The average precipitation of the deciduous was 75mm in the summer and 10mm in the winter. There was no effect of the season of the year with the analyzed reproductive variables having as minimum and maximum mean of: 17 to 28 total oocytes; 10 to 17 viable oocytes; and from 3.5 to 6.5 viable embryos by aspiration. Relating the total oocytes aspirated with precipitation and temperature, no significant difference was found between climatic variations and aspirated oocytes, as did oocytes with viable oocytes and embryo production. The data analyzed show that, in Nelore cows with good nutritional management, there is no influence of precipitation and temperature climatological variables on the reproductive variables of oocyte production and viability, and embryo production in vitro.

A120 OPU - IVF and ET

# Influence of elapsed time from loading to transfer with *in vitro* bovine embryo on pregnancy rates in the State of Mato Grosso do Sul

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Aiming to facilitate the logistics of commercial laboratories regarding the elapsed time from loading to transfer, periods of up to 10 hours and longer periods of up to 24 hours were compared utilizing the "Synthetic Oviduct Fluid" medium with buffered HEPES (HSOF) in order to measure their influence on the pregnancy rates of recipients at 60 days. Selected cumulus-oocyte complexes (COC) were transported in 2 mL cryotubes (1 oocyte/13.3µL of medium), containing 400µL of IVM medium TCM-199 (supplemented with 0.2 mM ofpyruvate, 10% of FCS and gonadotropins) and 300µL of silicone oil, at a controlled temperature of 38.7°C and atmosphere of 5% of $CO_2$, 5% of $O_2$ and 90% of $N_2$. After the transport period, the cryotubes were transferred to incubators with 100% of humidity at 38.7°C, with an atmosphere of 5% of $CO_2$ (≅20% $O_2$), with a total time ranging from 20-25 hours of IVM (24 h on average). The period of fertilization was from 8 to 10 hours, under the same conditions as described for the IVM. Presumptive zygotes were denuded and cultured in SOFaa, supplemented with 5% of FCS for up to 7 days. Cleavage and blastocyst rates were evaluated at 48 and 168 hours post-insemination (hpi), respectively. The procedures were performed at Embriza laboratory, located in Campo Grande, MatoGrosso do Sul, Brazil, and the media produced by the Cenatte Embriões laboratory, located in Pedro Leopoldo, Minas Gerais, Brazil. 1,944 transfers were carried out in the state of Mato Grosso do Sul in the period from July 2016 to January 2017 and were divided into 4 groups: G1 (>10h; n=600), G2 (>10-14 h; n=432), G3 (>14-17 h; n=507) and G4 (>17-24 h; n=405). The analyses of frequency dispersion were performed by a $X^2$ test considering the effects of the technique compared to each other with a level of significance $P<0.05$. The detected pregnancy rates were 44.9%a (G1), 51.16%a (G2), 44.97%a (G3) and 48.28%a (G4). There was no significant difference between the studied groups, keeping for the period from loading to the end of the transfer in the HSOF medium to be of up to 24 hours without any loss in the pregnancy rates.

A121 OPU - IVF and ET

# Lipid content of blastocysts produced *in vitro* from oocytes of small and large follicles – preliminary results

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In vitro produced (IVP) embryos have higher content of cytoplasmic lipid droplets compared with the in vivo counterparts. The reasons for the increased lipid deposit in IVP embryos are still lacking. In previous studies, it was observed that oocyte recovered from large follicles had higher cytoplasmic lipid content when compared with the oocytes from smaller follicle sizes. The aim of this study was to assess cleavage, blastocyst rate and the cytoplasmic lipid content of blastocysts produced in vitro from oocytes of small and large follicles. Slaughterhouse cow ovaries were used for the recovery of oocytes from small (≤5 mm, n= 322) and large (≥6mm, n= 93) follicles. The diameters of follicles were carefully determined with caliper device followed by volume monitoring. Only oocytes with homogeneous cytoplasm and with more than three layers of cumulus cells were submitted to in vitro maturation, fertilization and culture, as previously described. Cleavage and blastocyst production were recorded at day 3 and day 8 after fertilization, respectively. Expanded blastocysts at day seven after fertilization (Day7Ex, n= 22), and embryos that were not expanded blastocyst at day seven but became expanded blastocysts at day eight (Day8Ex, n= 23) were collected for semi-quantitative lipid content evaluation using the Sudan Black B staining. Expanded blastocysts were prepared following a previously established protocol. ImageJ software was used to convert the Sudan Black B-stained blastocysts images to gray scale and to determine the lipid content per embryo expressed as gray intensity. The data were analyzed by ANOVA using the PROC GLIMMIX of SAS. Cleavage (74.8 ± 3.6 vs 51.9 ± 4.4) and blastocyst (26.4 ± 9.0 vs 13.8 ± 1.7) rates (%) were higher ($P<0.05$) on embryos derived from oocytes of large follicles compared with those from small follicles, respectively. Day7Ex from oocytes recovered from large follicles had increased ($P<0.05$) cytoplasmic lipid content compared with the blastocysts derived from oocytes of small follicles (5.3 ± 0.3 vs 4.3 ± 0.4 of gray intensity, respectively). Day8Ex derived from oocytes of large and small follicles had similar ($P>0.05$) cytoplasmic lipid content (7.6 ± 0.5 vs 7.7 ± 0.4 of gray intensity, respectively). However, Day8Ex had increased ($P<0.05$) lipid deposit when compared with Day7Ex. Therefore, the preliminary findings of this study reveal the following: i) Oocytes recovered from large follicles reached increased cleavage and blastocyst rate; ii) Day 7 expanded blastocyst derived from oocytes of large follicles had increased lipid content compared with blastocyst derived from oocyte of small follicle size; iii) Lipid deposit did not vary among follicle sizes at Day 8 blastocysts; iv) Blastocysts with delayed expansion of blastocoel (Day 8) had higher lipid content compared with blastocysts with early expansion (Day 7). Acknowledgements: CNPq, FAPESP, FAPERGS and CAPES.

A122 OPU - IVF and ET

# Nuclear maturation of bovine oocytes submitted to the *in vivo* maturation using intrafollicular transfer of immature oocytes (IFIOT) system

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This study aimed to evaluate the kinetics of nuclear maturation of bovine oocytes submitted to the in vivo maturation using intrafollicular transfer of immature oocytes (IFIOT) system. To do that, ovulatory cows were previously synchronized. On day 0 (D0) the animals received an intravaginal progesterone-releasing device (1 g), and an injection (i.m.) of 2 mg Estradiol Benzoate. On D8, the progesterone implants were removed and an injection of Prostaglandin F2α analog (0.150 mg d-Cloprostenol) was made (i.m.). On D9 a 1 mg of Estradiol Benzoate (i.m.) was administered and on day 10 oocytes were injected into a ≥10 mm diameter follicle together with an injection of a gonadotrophin releasing hormone (GnRH) analogue - Buserelin. A total of 890 grade 1 and 2 oocytes obtained from slaughterhouse ovaries were used, being 417 for TIFOI and the remainder for Control. In the control group the oocytes were placed in IVM and removed at 0, 8, 12 and 16h. For TIFOI, 30 oocytes per ovulatory cow were used, which at 8, 12 and 16h post-injection were recovered by ovum pick up (OPU). Treatments and number of oocytes evaluated by treatment were: Control 0h (n=51); 8h Control (n=60); Control 12h (n=60); Control 16h (n=38); TIFOI 8h (n=79); TIFOI 12h (n=88); TIFOI 16h (n=7). Oocytes from all groups were denuded by and are fixed for further evaluation of nuclear maturation by lacmoid stain. Oocytes were classified according to meiotic stage in: germinal vesicle (GV), germinal vesicle break (GVB), metaphase I (MI), anaphase I (AI), telophase I (TI), metaphase II (MII) and abnormal. Data were analyzed by Chi-square test ($P<0.05$). At 0h, before maturation or injection, 96.1% of the oocytes were found at GV stage. At 8 h, most of the oocytes of the Control group were in MI stage (76.6%), while TIFOI 8h group presented a greater percentage ($P<0.05$) of oocytes in stage (97.5%). The percentage of oocytes in MI at 12h was similar ($P>0.05$) between the Control (81.7%) and TIFOI 12h (73.9%) and both presented oocytes at more advanced stages of meiosis (Control=TI 13.3%, MII 1.7%, TIFOI=AI 4.5%, TI 7.9%). Control group 16h had oocytes abnormal (2.6%), in MI (52.6%), AI (18.4%), TI (21.1%) and MII (5.3%). In the TIFOI 16h group at the time of aspiration the majority of the animals had already ovulated and, therefore, a small number of oocytes were recovered (n=7), being classified as abnormal (14.3%), MI (57.1%), TI (14.3%) and MII (14.3%). The results suggest that the in vivo maturation system using the TIFOI method was adequate, since the oocytes exposed to this system presented kinetics of maturation similar to the in vitro, being even more homogeneous than in vitro with 8 h of maturation.

A123 OPU - IVF and ET

# Glucose metabolism of bovine cumulus oocyte complexes matured *in vitro* with the addition of different suplements

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Cumulus-oocyte complex (COC) is formed by the oocyte and cumulus cells, and between them there exists a paracrine and gap junction bidirectional communication fundamental to determine oocyte viability. COCs preferentially use glucose as substrate, which is converted in pyruvate and lactate through glycolysis. Glucose metabolism during oocyte maturation is involved in meiotic progression regulation, ooplasm maturation, oxidative stress reduction, and it is related to cumulus cells expansion after FSH stimulous. This study aimed to compare the effects of the addition of fetal bovine serum (FBS), polyvinyl alcohol (PVA) or insulin-like growth factor -1 (IGF-I), during in vitro maturation (IVM) over COCs glucose metabolism. Grade I and II COCs (n=20/drop) obtained from ovaries from slaughterhouse were selected in Dulbecco's modified PBS containing 3 mg/mL of PVA and transferred to TCM HEPES. COCs were matured in vitro with TCM199 (supplemented with 0.2 mM pyruvate, 1 µg/mL FSH, 50 µg/mL LH, 100 µg/mL streptomycin, 100 UI/mL penicillin, and 85 µg/mL amikacin) with the respective addition of 10% of FBS (FBS), 3 mg/mL of PVA (PVA) or PVA + 100 ng/mL IGF-1 (IGF). IVM was performed in petri dishes with 90 µL droplets, covered with mineral oil at 38.5ºC and 5% $CO_2$ in humidified air for 22 to 24 hours. Glucose and lactate concentrations were determined in spent maturation media (including media without cells). After IVM, spent media were collected, snap frozen, and stored at -80°C. Samples of five experimental replicates were analyzed by a Hitachi 912 chemical analyzer (F. Hoffmann-La Roche Ltd.). To determine glucose uptake, concentration of glucose of media cultured without cells was taken as reference. Glucose uptake and lactate production were expressed as pmol/COC per h. Data were analyzed by analysis of variance (ANOVA) from PROC GLIMMIX model from SAS software (SAS Inst. Inc., Cary, NC, USA). Tukey test was used to compare the means. PVA (835.53± 7.38) and IGF (824.17± 7.38) groups showed a higher ($p < 0.05$) glucose uptake compared to FBS (769.68 ± 7.38) group. However, it has to be considered that FBS medium had initially lower concentration of glucose than other groups. This may have happened because addition of 10% of serum leads to a dilution of initial concentration of glucose of TCM199 media. IGF group (1908.41± 28.63) had higher lactate synthesis compared to group FBS (1879.77± 28.63), which produced more lactate than PVA (1793.86± 28.63) group. With the presented results it is possible to conclude that the addition of IGF-I in oocyte maturation leads to a higher efficiency in glucose utilization as a substrate and produce higher quantities of metabolites as lactate through the glycolytic pathway.

A124 OPU - IVF and ET

# Live birth of domestic cat by *in vitro* fertilization using oocytes recovered after mild follicular stimulation with equine chorionic gonadotropin

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The domestic cat is a valuable model for the generation of assisted reproductive techniques that might be used in the conservation of endangered wild felids. However, the *in vitro* embryo production systems in the domestic cat still have a low efficiency. The oocyte competence is an important factor that determine the successful in the *in vitro* embryo production systems. In humans, the mild follicular stimulation with gonadotropins (or priming) prior to the *in vitro* maturation (IVM), has been used to increase the oocyte maturation and blastocyst formation rates. The objective of this research was to evaluate the mild follicular stimulation with eCG in the *in vitro* fertilization system (IVF) in the domestic cat. For this purpose, nine domestic cat were treated with a subcutaneous dose of 200 IU of eCG and were subjected to ovariohysterectomy 4 days later for ovaries recovery and cumulus-oocyte complexes (COCs) collection. Additionally, others two cats were synchronized for embryo transfer procedure with 200 IU of eCG and an intramuscular dose of 100 IU of hCG 4 days later. Each cat correspond to an individual biological replicate, for this reason, the COCs recovered from each cat were matured, fertilized and cultured separately. For IVM, only grade I and II COCs were selected and matured in TCM-199 Earle's salts medium supplemented with 4 mg/mL BSA, 0.1 IU FSH-LH (Pluset), 0.36 mM sodium pyruvate, 2 mM glutamine, 2,2 mM calcium lactate, 1 µg/mL 17-β estradiol, 20 µg/mL EGF and 50 µg/mL gentamycin for 28-30 hours in a 5% $CO_2$, 5% $O_2$ and 90% $N_2$ humidified atmosphere to 38.5ºC. The IVF was realized using epididymal cat sperm which was refrigerated to 4ºC for 24 hours, 1.5 – 2.5 x $10^6$ spermatozoa /mL were incubated with 20-30 COCs in TALP medium supplemented with 6 mg/mL BSA, 0.36 mM sodium pyruvate, 1 mM glutamine, 2.2 mM calcium lactate, 1% MEM non essential amino acids (NEAA), 0.01 mg/mL heparin sodium salt and 50 µg/mL de gentamycin for 18 hours in a 5% $CO_2$ humidified atmosphere to 38.5ºC. The presumed zygotes were cultured in SOF medium in a 5% $CO_2$, 5% $O_2$ and 90% $N_2$ humidified atmosphere to 38.5ºC during 7-8 days. The cleavage, morula, blastocyst and hatching blastocyst rates were estimated. Once finished the culture, the blastocysts and hatching blastocysts were fixed and stained with Hoechst for total cell counting. Additionally, a total of 23 blastocysts were transferred into the uterine horn of the two previously synchronized cats (15 and 8 blastocysts per cat, respectively). The descriptive statistic was realized using the statistical software Infostat. Regarding to *in vitro* embryo production, the results of this research demonstrated that the domestic cat oocytes recovered after eCG priming are capable to develop *in vitro* after IVF until the blastocyst stage. Cleavage rate was 155/239 (64.9%), morula rate 115/155 (74.2%), total blastocyst rate 51/155 (32.9%) and hatching blastocyst rate 15/155 (9.7%). Furthermore, the embryo staining revealed the total cell number (mean ± standard deviation) of the blastocysts (182.8 ± 76.9) and hatching blastocysts (420.2 ± 106.1) generated. Finally, one gestational vesicle was detected at the 25 days of gestation in the cat that received 15 blastocysts and a healthy female kitten born after 64 days of gestation. No implantation was detected in the cat that received 8 blastocysts. In conclusion, the mild follicular stimulation might be a useful alternative for the *in vitro* and *in vivo* embryo development in the domestic cat and could be applicable in wild felid species.

A125 OPU - IVF and ET

# Propilenoglycol treatment increases blastocyst production rate on Holstein cows on lactation PEAK

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This study aimed to evaluate the effect of propilenoglycol (PPG) suplementation on IVEP of Holstein (Bos taurus) cows. A total of 84 lactating cows, of those, 45 on lactation peak [(PEAK) DIM < 100 days] and 39 repeat-breeder cows [(RB) DIM > 200 days, not pregnant], were used. On Day 0 all cows were summited to OPU, for follicular ablation on a random phase of the estrous cycle, then distributed in a factorial design 2 x 2 in order to evaluate the treatment and category effect: Control group (CTL; without treatment, n=23 cows PEAK), group RB (without treatment, n=21 cows RB); group PPG-PEAK (treatment with 500mL of intra-ruminal infusion with propilenoglycol twice a day, during 5 days, n=22 cows PEAK) and group PPG-RB (treatment with 500mL of intra-ruminal infusion with propilenoglycol twice a day, during 5 days, n=18 cows RB). On day 5 all cows were submitted to a second OPU. Five replicas were used with animal from both categories and treatments. On each replica, all follicles ≥ 2 mm were picked-up and the amount and quality of the oocytes were registered. Oocytes were submitted to IVP and embryo development (cleavage and blastocyst rate) were evaluated. On day 7of in vitro production, embryos were vitrified for later transfer. Oocytes were fertilized with sexed semen from the same Holstein (Bos taurus) sire and same ejaculate. Data were analyzed using GLIMMIX procedure of SAS®. There was no significant difference (P= 0.52) within categories and treatment for: total number of oocytes, CTL-PEAK (5.09 ± 0.95), PPG-PEAK (3.27 ± 0.67), CTL-RB (6.14 ± 1.11) and PPG-RB (5.38 ±0.76); number of viable oocytes (P=0.847): CTL-PEAK (2.7 ± 0.51), PPG-PEAK (1.82 ± 0.46), CTL-RB (3.33 ± 0.72) and PPG-RB (2.67 ± 0.62); cultivated oocytes number (P= 0.3416): CTL-PEAK (4.39 ± 0.81), PPG-PEAK (2.82 ± 0.65), CTL-RB (5.1 ± 1.03) and PPG-RB (4.89 ± 0.69). The blastocyst number per OPU was similar between the analyzed treatments and categories: CTL-PEAK (0.87 ± 0.28), PPG-PEAK (1.05 ± 0.23), CTL-RBB (2.00±0.51) and PPG-RB (1.56 ± 0.5). There was interaction between treatment and category (P= 0.0031) on cleavage rate of cultivated oocytes, that was superior on group CTL-RB (70%a) when compared to the CTL-PEAK group (36%b) and did not differ from groups PPG-PEAK (58%ab) and PPG-RB (55%ab). There was interaction between treatment and category (P= 0.0118) on blastocyst rate of cultivated oocytes, that was superior (P=0.0118) on cows from group PPG-PEAK (42%a) and CTL-RB (40%a), when compared to CTL-PEAK group (18%b) and did not differ from PPG-RB group (31%ab). The treatment with propilenoglycol for five days increased the cleavage rate and blastocyst rate on lactating Holstein cows on lactation peak.

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A126 OPU - IVF and ET

# *In vitro* embryo production in Canchim primiparous cows (3/8 *Bos indicus and* 5/8 *Bos taurus*) maintained in grazing area with or without shade presence: preliminary results

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The aim was to identify the influence of natural shade in grazing system on in vitro embryo production (IVEP) of suckling primiparous Canchim cows. Were used 18 donors, previously selected for follicular population, with 385.0±10.24 kg and 26.6±3.50 days post-partum at onset of experiment, while grazing pastures with shade provided by in a silvopastoral system (n=10, PRA, presence of eucalyptus trees with 15x2 m spacing) or pastures without shade (n=8, PR), at Embrapa Southeast Livestock. The Temperature and Humidity Index (THI) and Black Globe Humidity Index (BGHI) were measured during all experimental period in both experimental areas. All pastures were intensively managed in a rotational system. To IVEP, 4 OPU sessions were performed 4 OPU sessions, once a month, from January to April 2017, simultaneously, were measured the rectal temperature (oC). The aspirated follicles (AF) was counted to calculate recovery rate (Rr) and then, were performed the counting and morphological evaluation of cumulus oophorus-oocytes complex (COC). To IVF, semen with fertility recognize of the same bull was used. Cleavage rates (Cr) on D3, hatched blastocysts on Day 7 (HrD7), on Day 8 (HrD8) and on Day 9 (HrD9) rates were evaluated. Those classified in Grade I to III were put in maturation medium and carry to Vitrogen Laboratory (Cravinhos, SP, Brazil) to proceed IVF, IVC, and evaluation of cleavage rates (Cr) on D3, hatched blastocysts on D7 (HrD7), on D8 (HrD8) and on D9 (HrD9) rates. The data were analyzed as repeated measures (PROC MIXED, SAS®) and the results showed as least square means±SE. THI (70.4±0.03 and 70.2±0.02, P<0.001) and BGHI (73.3±0.04 and 72.8 ± 0,04, P<0.0001) values were higher in PR than PRA, respectively and were reducing by month during the experimental period in PR and PRA (P<0.0001). There were no interaction between replica and grazing system for any of the variables, as well there were no differences between cows maintained on PRA or PR, respectively, to RT (38.3±0.11 and 38.9±0.12oC, P=0.78), AF (25.6±2.32 and 28.2±2.64, P=0.46), Rr (83.3±8.69 and 75.2±9.89, P=0.54) Cr (90.2±4.68 and 83.8±5.30, P=0.37), HrD7 (37.7±4.64 and 30.8±5.13, P=0.32), HrD8 (30.8±4.81 and 21.2±5.33, P=0.19), or HrD9 (18.8±3.41 and 15.7±3.67, P=0.54). Contrary to expectations, the number of viable oocytes were higher in January (13.0±2.13) and March sessions than in February (9.8±2.05) and April (8.7±2.10) (P=0.03), coincidentally with higher RT in January (39.4±0.17) and March (39.2±0.16), compare to February (38.4±0.16) and April (38.5±0.16) (P<0.0001). The HrD9 was higher in March (2.7±3.40) when compared to February (1.6±0.41) and April (1.2±0.41) (P=0.04). These results allow us to conclude that animals maintained in PR and PRA were not showed body temperatures during morning that determine thermal stress. Therefore, the IVEP was similar between donors maintained in the same for PR or and PRA grazing system.

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A127 OPU - IVF and ET

# *In vitro* embryo production in buffalo: comparison between calves, prepubertal Heifers and lactating cows

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The aim of this study was to compare the production of embryos of calves in relation to prepubertal heifers and lactating cows of bovines species. The experiment was carried out at Paineiras do Ingaí farm, Alambari – São Paulo. For this purpose, 30 bovines females of three animal categories were divided according to the following quantities: 10 calves with 2 to 4 months of age, 10 prepubertal heifers with 13 to 15 months of age and 10 lactating adult cows. On random day, the calves received intravaginal device (Cidr Ovinos, Zoetis) considered as day zero (D0). In the D5 and D6, the calves received 140mg of FSH (Folltropin, Tecnopec, Brazil) divided into 4 decreasing doses at 12-hour intervals and were aspirated, posteriorly, by laparoscopy (LOPU - Laparoscopy Ovum Pick Up) on D7. On random day of estrus cycle, prepubertal heifers and adult lactating cows were submitted to follicular aspiration (OPU). Both LOPU and OPU were performed on the same day. Two of the ten heifers that had been submitted could not be aspirated because they had their bladder filled at the time of laparoscopic intervention. The oocytes produced were selected for morphological appearance, inserted in tubes containing maturation medium, covered with a layer of mineral oil. A gaseous mixture containing 90% N2, 5% CO2 and 5% O2 was placed inside the tube for 15-20 seconds and then maintained on the oocyte carrier (WTA, Cravinhos, Brazil) after capping, at 38°C of temperature until the arrival at the laboratory. All cumulus oocyte complexes recovered (TO - viable + nude + with irregular cytoplasm) were sent to the laboratory. The in vitro fertilization (IVF) occurred between 22 and 26 hours after start in vitro maturation (IVM). Single bull semen was used, keeping the same fertilization match for all oocytes. After 18 hours of the moment fertilization, in vitro culture (IVC) was started and the blastocysts were vitrified six days after IVF. Cleavage and blastocyst rates were done three and six days after of IVM, respectively. The data were analyzed by PROC GLM of SAS 9.3, using Tukey's Test to detect the differences between the groups. Among the animal categories, there was no significant statistical difference in the number of OV (calves = 7.63 ± 2.69, heifers = 6.20 ± 1.55, cows = 3.20 ± 0.90, P=0.1033), cleaved structures (calves = 2.75±0.86; heifers = 3.10±0.67; cows = 2.10±0.43, P = 0.5492) and embryos produced (calves= 1.00±0.57; heifers = 1.50±0.34; cows = 1.10±0.38, P = 0.3621). In contrast, a significant statistical difference was observed in the TO (calves = 10.88±3.25; heifers = 15.50±2.07; cows = 5.80±1.29, P = 0.0129) and in structures conducted for IVC (calves = 10.38±3.06; heifers = 15.30±2.06; cows = 5.70±1.30, P = 0.0110). All the embryos produced were vitrified and only five blastocysts of the calves category were transferred to synchronized recipients at the São Paulo University (Campus Fernando Costa; Pirassununga/SP). Two pregnancies were diagnosed (conception rate = 40%) at the 30 and 60 days of age and two healthy calves were born.

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A128 OPU - IVF and ET

# *In vitro* production of bovine embryos in the different Zebu breeds

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The improvement of the systems involved in the in vitro production (IVP) of embryos in cattle has been pivotal for the study and understanding of several factors and biological mechanisms that may influence the success of the technique, from the collection and maturation of COCs, embryo cultivation, to the development of the embryo for transference. Studies with IVP in different cattle donor breeds reveal different effects in the number of COCs, in the embryo development and in the pregnancy rate. With the purpose of analyzing the effect of different cattle breeds in IVP of embryos, this study analyzed the in vitro production of embryos of Nelore, Brahman and Gir, originated from OPUs performed in two farms by a private company (In Vitro Acre) located in the city of Rio Branco in the state of Acre during 2015 and 2016. Data were submitted to ANOVA and the means were tested by Tukey's test, with a 5% significance rate, using SAS. From a total of 187 OPUs, 39.57% (74/187) were performed in Nelore, 35.29% (66/187) in Brahman and 25.13% (47/187) in Gir donors, resulting in 62.33 ± 13.87 OPUs per each breed. It could be noticed that donors from the Nelore breed presented higher mean results when compared to the Brahman and Gir donors, which presented similar results between themselves ($p>0.05$), in the amount of Grade I COCs and Grade II aspirated and cultivated COCs, cleaved embryos, blastocysts and expanded blastocysts, packaged embryos, embryos lost after packaging, embryos transfered to the recipients and pregnancy total ($p<0.05$). The means of Grade III COCs was higher in Nelore, followed by Gir, which was higher than Brahman ($p<0.05$). The means of denuded oocytes was similar between Nelore and Brahman ($p>0.05$), Nelore and Gir ($p>0.05$), and higher in Brahman donors when compared to Gir ($p<0.05$). The pregnancy rate means by aspirated COCs and by transfered embryos was similar between Nelore and Gir donors ($p>0.05$), between Brahman and Gir donors ($p>0.05$), and higher in Nelore donors when compared to Brahman ($p<0.05$). Brahman donors presented higher morula means than Nelore and Gir ($p<0.05$), which presented similar means ($p>0.05$), and lower related to initial blastocysts than the other breeds studied ($p<0.05$), that presented similar means ($p>0.05$). The means of degenerated COCs, ecloded blastocyst, cleavage rate (% - total cleaved embryos/total aspirated COCs) and the pregnancy rate by cleaved embryo (%) were similar among the breeds ($p>0.05$). In these study conditions, it can be concluded that the donor breed influences most of the parameters of IVF of embryos, without actually interfering on the means of degenerated COCs, ecloded blastocysts, cleaved rate and pregnancy rate by cleaved embryo.

A129 OPU - IVF and ET

# *In vitro* production of Nelore embryos in different farms

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The in vitro production (IVP) of embryos has been studied and used for the genetic improvement of the cattle herd. There is a growing interest for this technique, allowing the recovery of oocytes from donors through follicular aspiration guided by ultrasound (OPU) and the production of embryos with high genetic value, which can be used without major apparent changes in the reproductive tract of females, regardless of their estrous cycle phase. However, this technique is not vastly used in commercial cattle herds due to its operational cost and the variation in results, possibly due to the variation in the technique and in the handling of donors and recipients, which are characteristic of each property, as well as embryo losses and birth of large calves. The objective was to analyze PIVE in Nelore cows in different farms. Within this context, the IVF of embryos in Nelore cows originated from OPUs performed in five farms was analyzed. Three of the farms were located in the state of Acre, one in the state of Rondônia in Brazil and one in Bolivia). The IVF was performed by a private company (In Vitro Acre), which is located in the city of Rio Branco, Acre, in 2015 and 2016. Data were submitted to ANOVA and the means were tested using the Tukey's Test, considering a 5% significance rate, using the R Statistical Program. A total of 465 OPUs were performed, with means of 93 ± 31.63 OPUs per farm, using on average 65.49 ± 16.08% different donors. All variables studied were influenced by the farm ($p<0.05$), and the mean values by OPU ranged from 1.72 ± 3.62 to 0.60 ± 1.43 Grade I COCs; 4.64 ± 4.92 to 2.34 ±3.15 Grade II; 11.53 ± 10.50 to 20.11 ± 13.40 Grade III; 2.12 ± 3.70 to 0.79 ± 1.90 denuded oocytes; 6.86 ± 5.60 to 4.05 ± 3.34 degenerated COCs; 33.20 ± 20.66 to 23.80 ± 14.30 aspired COCs; 29.70 ± 19.33 to 19.82 ± 12.47 cultivated COCs; 22.84 ± 15.55 to 14.24 ± 10.28 cleaved embryos; 1.17 ± 2.45 to 0.14 ± 0.62 morulas; 2.57 ± 3.00 to 0.80 ± 1.51 initial blastocysts; 2.04 ± 3.08 to 1.29 ± 1.92 blastocysts; 7.07 ± 8.70 to 2.50 ± 3.60 expanded blastocysts; 0.15 ± 0.85 ecloded blastocysts; 11.50 ± 10.30 to 6.56 ± 5.61 packaged embryos; 3.32 ± 6.50 to 0.72 ± 2.04 embryos lost after packaging; 8.55 ± 8.75 to 3.94 ± 4.73 transfered embryos; 80.20% ± 55.09 to 61.71% ± 24.49 cleavage rate (total cleaved embryos/total aspired COCs); 3.60 ± 3.84 to 1.64 ± 2.43 pregnancies; 11.80% ± 10.64 to 8.76% ± 9.68 pregnancy rate/aspired COCs; 19.53% ± 17.51 to 9.53% ± 13.75 pregnancy/cleaved embryo rate and 39.14% ± 30.14 to 25.49% to 31.77 pregnancy/transfered embryo rate. In the study conditions, it can be concluded that typical management of each farm influences all parameters of the in vitro production of embryos in Nelore cows.

A130 OPU - IVF and ET

# *In vitro* production of Nelore embryos in different times of the year

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The IVP programs allow an accelerated genetic growth by means of multiplication of animals with differentiated genetics, thus benefiting the entire animal production system. However, it still presents a few hindrances that compromise their results due to aspects inherent to the embryo, the receiving animal and the environment. Among the factors related to the environment, weather variations affect the reproductive performance of the animals and may affect the quality of the oocyst, the fertilizing capability of the spermatozoa, the quality of the embryo and the uterine environment of the receiving animal for the implementation, thus resulting in the compromising of the pregnancy rate. Therefore, the purpose of this study was to assess the performance of Nelore donors in IVP programs in five farms assisted by a private company (In Vitro Acre) located in the city of Rio Branco, in Acre, in 2015 and 2016, in the dry (May to September) and wet (October to April) seasons. Data were submitted to ANOVA, and the means were tested by the Tukey's test, considering a 5% significance rate using the R statistic program. From the 539 OPUs performed, 38.03% (205/539) were performed during the dry season and 61.97% (334/539) during the wet season. It could be observed that, during the dry season, the means of denuded oocytes, morula, blastocysts and ecloded blastocysts were higher than the ones observed in the wet season ($p<0.05$), of 1.70 ± 3.02 and 083 ± 1.84; 0.90 ± 2.00 and 0.17 ± 0.69; 1.99 ± 2.48 and 1.20 ± 2.31; 0.04 ± 0.48 and 0.17 ± 0.82, respectively. The Grade I COCs (1.62 ± 2.98 e 1.36 ± 2.40), Grade II COCs (3.66 ± 3.92 e 3.77 ± 4.64), Grade III COCs (16.00 ± 12.00 e 16.00 ± 12.00), degenerated (5.03 ± 4.27 e 5.41 ± 5.33), aspired (29.20 ± 19.24 e 27.84 ± 18.32), cultivated (25.76 ± 17.90 e 24.64 ± 17.73), cleaved embryos (19.56 ± 14.27 e 18.81 ± 14.80), blastocysts (1.64 ± 2.02 e 1.69 ± 3.04), expanded blastocysts (4.46 ± 5.82 e 5.40 ± 7.02), packaged (9.05 ± 7.82 e 8.62 ± 8.56), lost after packaging (2.09 ± 3.79 e 2.08 ± 5.45), transfered (6.96 ± 7.45 e 6.55 ± 6.48) and pregnancy (3.04 ± 3.57 e 2.80 ± 3.05) means per OPU were similar among dry and wet seasons, respectively ($p>0.05$). In the conditions of this study, it can be concluded that the dry and wet seasons only influence the means of the denuded oocytes, morula, blastocyst and ecloded blastocysts, and does not interfere in the total pregnancy by OPU, as well as other variables, possibly since this study was performed in a location where the seasons of the year are not very evident, only characterized by the amount of rainfall.

A131 OPU - IVF and ET

# Synchronization protocols of oocyte population for OPU/IVM purpose in cattle

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Oocytes isolated post mortem from bovine antral follicles are heterogeneous in chromatin configuration, gap-junction functionality between oocyte and cumulus cells, transcriptional activity and developmental competence. This heterogeneity could account for the low efficiency of current OPU/IVM strategies. We aimed to characterize the oocyte population obtained by OPU at a random day and to develop a protocol to homogenize the oocyte population destined to IVM/IVF. In experiment 1, 10 lactating Holstein cows were used in a crossover design. Treatments were: 1) OPU at a random day (Control); 2) aspiration of all visible follicles at a random day (D0), two IM injections of FSH (Folltropin; 56mg) 12h apart on D2, OPU from follicles >2mm on D4 (ASP-FSH/D4). In Experiment 2, 4 lactating Holstein/Girolanda cows were subjected to ASP-FSH with OPU on D5 (ASP-FSH/D5) and oocytes obtained on D0 were used as Control. Oocytes were fixed in 60% methanol/PBS, stained with Hoechst 33342 and examined by fluorescence microscopy to be classified according with the chromatin configuration in the germinal vesicle as GV0, GV1, GV2, GV3 or as GVBD when resuming meiosis. Data were arcsine transformed and groups compared by paired T test. Control oocytes from experiment 1 (n=90); were in GV0 (8.87%), GV1 (21.94%), GV2 (38.8%), GV3 (19.77%), GVBD (1.25%) and degenerated (9.75%). The protocol ASP-FSH/D4 (n=69 oocytes) abolished GV0, GVBD and degenerated oocytes, increased GV1 to 46.55% (P=0.03), tended to decrease GV3 (13.36%; P<0.1), but did not alter GV2 (40.09%). The number of oocytes recovered was not significantly different but recovery rate was lower in ASP-FSH/D4 (34.5% vs. 54.55%; P=0.01). The protocol ASP-FSH/D5 (n=57 oocytes) increased GV2 (80.76% vs. 47.17%) and decreased GV3 (9.72% vs. 34.5%; P<0.05) in comparison with the Control (n=83). In conclusion, oocytes recovered by OPU at a random day are heterogeneous and strategies combining follicle aspiration with FSH treatment can promote a more homogeneous population of oocytes for OPU/IVM purpose.

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A132 OPU - IVF and ET

# Oocyte quality and quantity differ between primiparous and multiparous *Bos indicus* cows

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The objective was to evaluate quantity and quality of oocyte in primiparous and multiparous *Bos indicus* cows on early post-partum. The study enrolled 48 lactating cows (24 primiparous and 24 multiparous), with post-partum between 30 and 45 days and body condition score of 2.59±0.03 (1 to 5 scale). In a random day of the estrus cycle (D0) all animals received 2 mg of estradiol benzoate (Sincrodiol®, Ourofino, Brazil) and an intravaginal progesterone device (Sincrogest®, Ourofino, Brazil). Five days after hormonal treatments (D5), counting and puncture of all follicles larger than 2 mm were performed. The obtained follicular fluid was transferred to 100 x 200 mm petri dishes containing DMPBS plus 1% PVA for classification and evaluation of cumulus-oophorus complexes (COCs) under stereomicroscope (Nikon®, SMZ645, Japan) according to IETS manual (IETS. Manual of the International Embryo Transfer Society. 4th edition. Illinois: IETS, 2009. 175p 2009). Oocyte quality was evaluated by the Oocyte Quality Index [OQI= (grade I*1+grade II*2+grade III*3+non viables*4)/total of oocytes]. All data were analyzed by GLIMMIX procedure of SAS and continuous variables were presented by mean ± standard error. It was verified that primiparous cows presented a lower number of follicles at D5 (18.0±1.9 for primiparous cows and 20.7±1.5 for multiparous cows; P=0.05). In addition, it was verified that primiparous cows showed a greater number of degenerated oocytes (1.9±0.7 for primiparous cows and 1.2±0.3 for multiparous cows; P=0.05). There was no difference between animal categories (primiparous and multiparous cows) for the number of score 1 oocytes (4.7±0.8 and 5.0±0.8; P=0.83), score 2 oocytes (4.1±1.0 and 3.5±0.4; P=0.23), score 3 oocytes (3.5±0.8 and 3.1±0.6; P=0.51), total number of oocytes recovered (14.2±1.9 and 12.8±1.2; P=0.14), oocyte quality index (2.1±0.1 and 2.1±0.1; P=0.93) and total viable oocytes (12.3±1.9 and 11.5±1.3; P=0.38). In conclusion, primiparous cows presented a lower number of ovarian follicles and a greater number of degenerated oocytes at an early post-partum period.
Support: FAPEMIG.

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# Oocyte quality and *in vitro* embryo production of Taurine and Zebu cattle

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The aim of the present study was to compare the results of Simmental (*Bos taurus taurus*) and Brahman (*Bos indicus indicus*) donors, regarding oocyte recovery and quality and in vitro embryo production. A total of 243 follicular aspirations were realized from 95 Simmental (SIM) and 150 Brahman (BRA) donors cows between November 2015 and March 2017. The ovum pick-up (OPU) aspirations were performed by the same veterinarian using ultrasound equipped with intravaginal microconvex sector transducer (7.5 MHz). Oocytes collection was performed using PBS at 36 °C added heparin. Then oocytes were classified as grade I, II, III (GI, GII, GIII) or naked (N) according to morphological quality, packed in straws with maturation medium and sent to the laboratory on a carrier (atmosphere of 5% CO2; 5% O2 at 36°C). They were cultured in a maturation medium (9.0mL of TCM 199 Earles Salt; 1.0mL of FBS; 20µL of pyruvate; 10µL of FSH; 100µL of LH; 10µL of estradiol; 50µL of amikacin) for 22-24h at 38.7 °C, with 99% humidity and 5% CO2 in air. Following maturation, oocytes were submitted to fertilization (10mL of FERT TALP; 0.06g BSA-FAF; 20µL of pyruvate; 440µL of PHE; 110µL of heparina; 50µL of amikacin) between 18 and 22h. The zygotes were transferred to a culture medium (9.3 mL of CR-2; 0.05 g of BSA-FAF; 500 µL of FBS; 100 µL of alanine; 100 µL of glycine; 40 µL of amikacin), where remained for seven days. The data were processed by Statistical Package for Social Sciences (SPSS) software version 13.0 and evaluated by the Shapiro-Wilk test, not attending normality criteria. The Mann-Whitney test was used to compare the means, considering a level of significance lower than 0.05. Comparing the two breeds, BRA presented a greater oocyte recovery (16.93±1.23) compared to SIM (12.00±0.82) ($P<0.05$). There was a difference ($P<0.05$) in the morphological quality of cumulus oocyte complexes (COCs) (3.91±0.3 and 2.85±0.25 in GI, 4.85±0.49 and 3.06±0.32 in GIII, 0.46±0.08 and 0.21±0.06 in N), with Brahman breed superiority. There was no difference between breeds only for GII oocytes ($P>0.05$). Regarding the embryo production, the total mean of embryos differed ($P<0.05$), being greater in BRA and lower in SIM (6.73±0.55 and 2.38±0.32, respectively). The rate of morulae was higher in SIM than in BRA (0.17±0.59 and 0.01±0.01), although the inverse occured in the number of blastocysts and expanded blastocysts, which is higher in BRA (0.83±0.12 and 5.67±0.49) than in SIM (0.13±0.05 and 1.66±0.26) ($P<0.05$). Brahman cows presented a higher oocyte recovery and a greater number of viable oocytes than Simmental cows, as well as higher production of viable embryos.

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# Results from a commercial *in vitro* embryo production program in Dorper and white Dorper sheep

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In vitro embryo production (IVEP) was tested in a year-round program in a commercial flock of Dorper (DP) and White Dorper (WD) sheep. Donors and recipients were synchronized with intravaginal sponges containing 60 mg medroxyprogesterone acetate for 16 days. Donors received a total of 100 mg FSH (Folltropin®) in 3 injections at 12h intervals starting ~36h before laparoscopic Ovum Pick-Up (LOPU). Recipients received 500 IU of eCG (Novormon®) and 125 mg cloprostenol (Ciosin®) at the time of sponge removal and 50 µg GnRH (Fertagyl®) 36h after that. LOPU, IVM, IVF and IVC procedures were conducted as previously described (Baldassarre et al. 2012. Anim Reprod, 9 (3), 188-194). Briefly, the females were restrained on a laparoscopy table in a 45° angle and then, using a 5 mm laparoscope and an atraumatic grasping forceps to uncover the ovaries, all follicles ≥ 2 mm diameter were aspirated using a 20G needle connected to a vacuum line. IVM were performed in maturation medium under mineral oil, at 38.5°C in humidified atmosphere with 5% CO2 in air for 24 h. Fertilization was conducted in mSOF supplemented with 10% estrus sheep serum with Percoll-enriched frozen semen from 4 males (2 of each breed) at ~50,000 motile sperm per drop. After ~15h in IVF, the presumptive zygotes were cultured in mSOF for 6 days at 38.5°C in humidified atmosphere with 5% O2, 5% CO2 and 90%N2. Blastocyst-staged embryos were transferred into the uterus of synchronized recipients with a morphologically sound corpus luteum. Results were statistically tested for significance by Oneway Anova and t test at 95% confidence level. Overall, 89 LOPU were conducted, which resulted in a total of 1003 oocytes recovered (11.3±6/donor) of which 958 entered IVM (10.7±6/donor). Between breeds, the number of oocytes collected (11.8 vs. 10.8) and % cleavage (61.9 vs. 67.3) were not statistically different, however, the number of transferable embryos/donor was significantly higher in the WD compared with the DP breed (4.9 vs. 3.3, $P<0.05$). Similarly, when comparing results from the seasonal (fall-winter) vs. the non-seasonal (spring-summer) halves of the year, no statistical differences were observed for the number of oocytes recovered/donor (10.7 vs. 11.6) and % cleavage (68.1 vs. 62.9), but the % of transferable embryos was significantly higher during the breeding season (45.8 vs. 32.9, $P<0.05$). Significant differences were found between the 4 males used at the levels of cleavage rate ($31.9^{c}$ vs. $56.4^{b}$ vs. $80.8^{a}$ vs. $81.6\%^{a}$, $P<0.05$) and transferable embryo yields ($19.8^{c}$ vs. $30.9^{bc}$ vs. $42.8^{ab}$ vs. $65.7\%^{a}$, $P<0.05$). Notably, the best two males were one of each breed. In total, 308 embryos were transferred into 273 recipients and the pregnancy rate was 35.5%. Pregnancy rate was not statistically influenced by breed, donor age group, male or season. These results confirm that LOPU-IVEP is commercially ready for application in the propagation of valuable sheep in a year-round scheme.

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# Improvement of bovine *in vitro* embryo production by ovarian follicular wave synchronization prior to ovum pick-up

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This study evaluated the effects of the synchronization of ovarian follicular wave emergence on the efficiency of bovine *in vitro* embryo production (IVP). *Bos indicus* cows (n = 20) with 3- to 10-year-old and weighing an average of 450 kg were divided into two groups (control *vs*. synchronization) to received repeated ovum pick-up (OPU) sessions and subsequent IVP. Cows in the control group (n = 10) were submitted to OPU procedures without any previous hormonal treatment. Animals in the synchronization group (n = 10) received a protocol-based progesterone implant (Crestar®, MSD Saúde Animal, Sao Paulo, Brazil), 2 mg of estradiol benzoate (Bioestrogen®, Biogenesis-Bagó, Garín, Argentina) and 150 µg of D-cloprostenol (Croniben®, Biogenesis-Bagó, Garín, Argentina) on a random day of the estrus cycle (Day 0). In this group the OPU was performed on Day 5. A total of eight aspiration procedures were performed in each group, with an interval of 21 days, and all animals of both groups received the two treatments. After IVP, embryos in blastocyst stage were transferred to recipients synchronized previously by fixed time and the diagnosis was performed 60 days later by transrectal ultrasound (5-MHz linear transducer, Mindray 2200, Shenzhen, China). Data were analyzed by ANOVA or Chi-square Test ($P \leq 0.05$) and are presented as mean ± standard deviation or proportion. The group that received the synchronization of ovarian follicular wave emergence pre-OPU showed a greater ($P < 0,05$) mean of embryo production (5.9 ± 0.5 *vs*. 4.5 ± 0.4), a higher proportion of embryos produced [45.8% (472/1030) *vs*. 38.5% (357/927)) and tendency ($P = 0,07$) to a greater number of conceptions per OPU session (2.2 ± 0.2 *vs*. 1.6 ± 0.2) in relation to the group that did not receive hormonal treatment. The total oocyte mean (17.8 ± 1.2 *vs*. 20.5 ± 1.3), the mean of viable oocytes (11.6 ± 1.0 *vs*. 12.9 ± 1.0), the proportion of viable oocytes [62.4% (927/1424) *vs*. 60.0% (1030/1639)] and the conception rate [37.0% (132/357) *vs*. 37.5% (177/472)] were similar ($P > 0.05$) between the control and synchronization groups, respectively. It was concluded that synchronization of ovarian follicular wave emergence prior to OPU results in a greater mean of embryo production, a higher embryo conversion rate and a tendency to a greater number of conceptions per OPU session, improving the efficiency of the IVP in cattle.

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# Suplementation with forskolin and 2, 4 dinitrophenol in *in vitro* culture improve bovine embryos rates

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Cryopreservation of embryos highlights for making viable the storage of biological material indefinitely, thus enabling the surplus storing and commercialization of these. However, an obstacle to greater dissemination of this technique is the sensitivity, to freezing, of the in vitro produced embryos. Thus, the present study aimed the use of forskolin (lipolytic agent) and 2, 4 dinitrophenol (oxidative phosphorylation uncoupler agent) during the in vitro culture of bovine embryos, with the intent of improving the quality and, consequently, the embryonic cryotolerance. For this, ovaries from slaughterhouse were aspirated to obtain grade I and II oocytes. Subsequently, these oocytes were submitted to maturation, in vitro fertilization and in vitro culture. In the D5 embryo culture, forskolin (treatment 1), 2, 4 dinitrophenol (treatment 2), forskolin associated with dinitrophenol (treatment 3) were added and a group with no adjuvants (control group) was maintained. In D7, the expanded blastocysts were cryopreserved by the conventional freezing technique (TK 1000 BR, Uberaba - Brazil) and 2 hours later, were thawed. We evaluated the effect of these coadjuvants on the blastocysts rates (D7) and the in vitro viability after vitrification/thawing at 24, 48 and 72 h as to survival rates and to hatching rates. Data on blastocyst rates were evaluated by ANOVA and survival and hatching rates were evaluated by Kruskal-Wallis, with a significance level of 5%. In relation to the blastocysts rates in D7, there was a difference ($p < 0.05$) between the control group (40.40%) and the group in which there was addition of forskolin in association with 2, 4 dinitrophenol (50.67%). The other groups did not differ among themselves. Regarding the survival rates at 24 hours (78.24%), 48 hours (64.97%) and 72 hours (65.21%), no differences were observed ($p>0.05$) among the groups. Concerning hatching rates at 24 hours (39.32%), 48 hours (53.28%) and 72 hours (58.40%), there were also no differences ($p>0.05$) among the groups. Thus, supplementation of the in vitro culture media of bovine embryos with forskolin in association with 2, 4 dinitrophenol was efficient in improving the embryonic development, observed through the higher blastocysts rates produced in D7 in this group; however such treatments did not result in embryo benefit after cryopreservation.

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# Pregnancy rate at 60 days of Nelore cattle recipients of embryos frozen through vitrification and ethylene glycol procedures in Mato Grosso do Sul State

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Cryopreservation of bovine embryos provides a series of benefits, such as formation of gene banks, planning of the best time for the transfer and subsequent birth of animals, etc. The ethylene glycol does not only provide all the advantages of cryopreservation, but also brings an even greater ease of application in the field as it allows a direct transfer of embryos without any re-evaluation, eliminating the use of a stereomicroscope, rehydration media and embryo manipulation. Oocytes from 15 Nelore cattle donors were collected in the Arizona center located in the town of Dois Irmaos do Buriti in the state of Mato Grosso do Sul, Brazil, between March 2015 and December 2016. The selected cumulus-oocyte complexes (COC) were transported in cryotubes of 2mL (1 oocyte/13.3µL medium), containing 400µL of IVM medium TVM-199 (supplemented with 0.2 mM pyruvate, 10% FCS and gonadotropins) and 300 µL of silicone oil at 38.7°C and atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$. After the transport period, the cryotubes were transferred to incubators with 100% of humidity at 38.7°C, with an atmosphere of 5% $CO_2$ (≅ 20% $O_2$), with a total time ranging from 20-25 h of IVM (24 h on average). The fertilization period was from 8 to 10 h, under the same conditions described for IVM. Presumptive zygotes were denuded and cultured in SOFaa supplemented with 5% FCS for up to 7 days. The cleavage and blastocyst rates were evaluated at 48 and 168 hours post-insemination (hpi), respectively. The procedures were performed at Embriza Laboratory, Campo Grande, Mato Grosso do Sul, Brazil and the media were produced by the Cenatte Embrioes laboratory, in Pedro Leopoldo, Minas Gerais, Brazil. This study aimed to compare the two cryopreservation techniques (vitrification and ethylene glycol) with a fresh transfer. A total of 671 transfers were carried out at the Recipient Center Arizona, and were divided into 3 groups: G1 (fresh transfers; n=490), G2 (vitrification; n=104) and G3 (ethylene glycol; n=77). The analyses of frequency dispersion were performed by a $X^2$ test considering the effects of the technique compared to each other with a level of significance $P<0.05$. The detected pregnancy rates were 47.86% a (G1), 42.31% ab (G2) and 35.06% b (G3). In regard to the groups, only G1 and G3 showed a significant difference. Both freezing techniques can be used since they do not present any statistical difference, however, application of the ethylene glycol technique brings further benefits due a greater ease of the transfer of the embryo.

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# Assisted reproduction technologies result in different gestation length and birth weights in Dorper and white Dorper sheep

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We analyzed the impact of the type of assisted reproduction technologies on the the gestation length and birth weights, in 68 sheep pregnancies established by AI vs. in vivo (MOET) and in vitro (IVF) produced embryo transfers, at Cravinhos-SP. Protocols for estrus synchronization, AI, superovulation and flushing, in vitro embryo production and embryo transfer are described elsewhere (Baldassarre and Karatzas, Ani. Reprod. Sci. 2004. 82-83: 255–266). Conception day, i.e. gestation day 0, was established as the day of AI (for AI and in vivo embryo groups) or the day of IVF for the in vitro embryo group. Results were statistically tested for significance by Oneway Anova and t test at 95% confidence level. We found that the birth weight was not different between IVF and MOET pregnancies (5.11±1.4 and 4.7±0.9 Kg., respectively) but both were significantly higher than AI pregnancies (3.5±0.5 Kg., $P<0.05$). Interestingly, the gestation length of IVF pregnancies was significantly longer than that of MOET and AI pregnancies (148±3.0 vs. 145±1.6 vs. 146±2.0 days, respectively; $P<0.01$). No statistical differences were observed between breeds when comparing the birth weight (4.83±1.6 vs. 4.58±0.9 Kg) and gestation length (147±3.3 vs. 147±2.2 days) of pregnancies for Dorper and White Dorper. We also looked at the season at the time of conception as a source of variation and found that winter conception resulted in significantly lower birth weights compared with fall, spring and summer (3.7±0.4 vs. 4.9±1.0 vs. 4.9±1.9 vs. 4.9±0.9 Kg., respectively, $P<0.05$). As per gestation length, pregnancies from conception in the spring and summer were longer (149±3.3 and 148±1.9 days) than those from conception in the fall and winter (146±2.0 and 144±1.1, $P<0.05$). In summary, pregnancies from in vitro produced embryos have shown to last for longer and result in heavier lambs at birth which has the potential for increased incidence of dystocia and need for intervention. In that sense, induction of parturition around gestation day 146 may be a recommended management tool to minimize issues at lambing. The gestation length also seemed to be influenced by the season at conception, with longer gestations occurring when conceived out of season (spring-summer), which could very well be associated with seasonal variations in food availability when animals are in grazing conditions.

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# Use of quercetin as antioxidant on *in vitro* maturation of goat oocytes

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The objective of this study was to evaluate the effect of quercetin as an alternative antioxidant to cysteamine during *in vitro* maturation (IVM). Ovary goats were transported from the local slaughterhouse to the laboratory in 0.9% saline at 30 °C until three hours after slaughter. After collection, the oocytes were evaluated and divided into three groups: CIS Group, where the oocytes were immersed in MIV medium: TCM-199, supplemented with EGF (10 µg / mL), FSH / LH (10 µL / mL), Estrus sheep serum (100 µL / mL) and cysteamine (10 µL / mL); In Groups Q4 or Q8, oocytes were immersed in cysteamine-free base medium, supplemented with 4 µM or 8 µM quercetin, respectively. The IVM of the oocytes was performed at 38.5 °C in humidified atmosphere of 5% $CO_2$ in air for 24 hours. After IVM, DNA fragmentation of oocytes was evaluated by TUNNEL assay (Gouveia; Theriogenology, v. 86, p. 1275-1284, 2016) and GSH, ROS and mithocondrial activity levels were quantified as reported previously (Gouveia; Theriogenology, v. 89, p. 263-270, 2016). The data of maturation rate, cumulus cell expansion rate and percentage of DNA fragmentation were expressed as percentages and compared using Chi-Square test. Data from GSH levels, ROS and mitochondrial activity were evaluated by Kruskal–Wallis and Student Newman Keuls tests. The differences were considered significant when $P < 0.05$. The CIS and Q4 groups presented the same percentage of expanded cumulus cells (67.6% and 71.8%, respectively), but the Q8 (46.5%) group was significantly lower than the other groups ($P < 0.05$). The percentage of oocytes in metaphases II was higher in the Q4 group (57.1%) than in the CIS group ($P < 0.05$), but the CIS (25.0%) and Q8 (47.0%) groups were similar. Concerning percentage of oocytes presenting DNA fragmentation, there was a higher ($P < 0.05$) number of TUNEL-positive cells at CIS group (28.2%) than Q4 (0%) or Q8 (0%) group. Oocytes from the CIS and Q4 groups showed the same levels of reactive oxygen species (ROS) and glutathione (GSH). In addition, oocytes matured with 4 µM quercetin showed higher mitochondrial activity than mature oocytes in the CIS and Q8 groups ($P < 0.05$). In conclusion, 4 µM of quercetin can be used as an alternative to cysteamine in the *in vitro* maturation of goat oocytes, as it resulted in rates of oocyte maturation of goats larger than those obtained with cysteamine, maintaining constant levels of cell expansion of the cumulus, glutathione, ROS, in addition to elevating mitochondrial activity. However, the concentration of 8 µM led to the reduction of ROS levels, GSH and oocyte mitochondrial activity, demonstrating lower cell viability.

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# Variation in the number of follicles aspirated according to the quantity of OPU (Ovum Pick-Up) in dairy buffalo, bred to pasture

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The present study aimed to analyze the repeatability of the number of follicles aspirated in OPU sessions in dairy buffalo used as donors of oocytes, as well as checking to classify donors according to their follicular population, aiming to select high-population follicular cows. There were selected 47 buffaloes (Murrah and Mediterranean), of a total of 190 females (24,7%), through the ovarian size, above 2,5cm x 1,5cm (length × width) and the number of follicles, counted by tltrasound exam (DP 4100VET). 14 follicular aspirations (OPU) were carried out using the following protocol, 21G needle; 1,7 mm internal diameter Teflon suction line and 80 cm in length; and pressure 50 mmHg. The OPU were carried out in groups with 7 to 14 animals (aspirated follicles greater than or equal to 3mm) and the suction interval of 14 days by buffalo. The cows have been classified according to the number of follicles aspirated in four groups, G1: Very good cows, with average follicles available over 20; G2: Good cows, ranging from 15 to 19; G3: Intermediate cows, with about 10 to 14 and G4: Bad cows, varying from 5 to 9. The data was submitted to ANOVA and the averages compared by Tukey test ($P < 0,05$). The G1 Group ($n = 2$) presented an average of 20.0±4.2 aspirated follicles, ranging from 38.5±7.7 in the OPU1 and 17.5±0.7 in OPU 14, not occurring statistical difference ($p > 0,05$), between OPU; in the G2 Group ($n = 11$) The average was 16±5,4 follicles, 21,2±8,7 in the OPU1 and 9.7±6.1 in the OPU14 ($p > 0,05$); in the G3 Group ($n = 19$) obtained 10.9±3.5 follicles, ranging from 11.4±5.1 in OPU1 and 8.6±2.8 in OPU14 ($p > 0,05$) and in the G4 group ($n = 15$) presented an average of 8.9±2.1 follicles available 6.0±2.5 in the OPU1 and 9.5±0.7 in the OPU14 ($p > 0,05$). There was a difference ($p < 0,05$) between the number of follicles aspirated among the groups (G1, G2, G3 and G4), demonstrating high variability among the groups and high repeatability within each group, which was probably because the protocol of aspirations did not induce ovarian lesions, maintaining the follicle population constant until the last aspiration in each group. Studies using donor selection by the size of the ovaries and follicle count, above ten per ovary, in buffaloes, has demonstrated that such a process can contribute to increase the number of viable oocytes (Ohashi et al., RevBrasReprodAnim, 41, 195-200, 2017). Although the buffaloes present a low number of follicles and large individual variation, some authors indicate that there are animals that present the population of follicles above the average, indicating that the number of antral follicles have high repeatability in bovine animals, remaining constant 8 to 10 years of age (Burns et al., BiolReprod, 73, 54-62, 2005). Therefore, the selection of donors by ovarian size and number of follicles, presents good efficiency, can contribute to increasing the number of viable oocytes and thereby improving the rates of PIVE, and consequently, decrease the cost of the pregnancy.

A141 OPU - IVF and ET

# Laparoscopic ovum pick-up is a safe procedure for the collection of oocytes for preservation efforts in Pumas (*Puma concolor*)

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Laparoscopic Ovum Pick-Up (LOPU) has been proposed as an ideal method for the collection of quality oocytes for conservation efforts based on *in vitro* embryo production (IVEP) and somatic cell nuclear transfer (SCNT). Furthermore, the procedure is compatible with collecting immature or *in vivo* mature quality oocytes by simply including (or not) hCG in the hormonal stimulation regime. We report herein the results of LOPU conducted in 3 pumas. Follicle development was stimulated by means of 750 IU eCG 4.5 days prior to LOPU. In the first stimulation, 2 females were injected with 500 IU hCG 84h after eCG and 24-30h prior to LOPU to promote in vivo maturation. All injections were conducted using blow darting technique. Animals were deprived from food (24h) and water (12h) in preparation for surgery. The LOPU procedure was conducted as previously described (Baldassarre et al.; Anim. Reprod., v.12, n.3, p717, 2015). Briefly, the females were restrained on a laparoscopy table in a 45° angle and then, using a 5 mm laparoscope and an atraumatic grasping forceps to uncover the ovaries, all follicles ≥ 2 mm diameter were aspirated using a 20G needle mounted in a plastic pipette connected to a collection tube and vacuum line. Interestingly, when hCG was included in the hormonal stimulation, 41 of 42 oocytes recovered showed signs of in vivo maturation (expanded cumulus). Two females were collected twice with a 26-month interval and yielded 55 and 22 (April 2015) vs. 32 and 25 (June 2017) usable oocytes, respectively. No sequels from prior procedure (e.g. adhesions, ovarian scars, etc.) were observed and the results suggested that previous procedure didn't affect the ability of the animals to respond to treatment since they yielded a rather large number of usable oocytes. The third animal that was subjected to LOPU in 2015 and yielded 21 oocytes was released into the environment nine months after LOPU. Prior to release she was equipped with a GPS collar that allowed satellite tracking of movements. Through this monitoring system, it was possible to detect when and where this animal was killed ~80 days after release, an quickly react and conduct a necropsy that showed that she was pregnant with 2 fetuses which reinforces the notion that LOPU is a safe procedure and doesn't impact negatively on the fertility of the animals. We believe this is the first report in which LOPU was repeatedly conducted in pumas and reporting fertility after LOPU. Our results validate LOPU as a safe reproductive procedure for the multiplication of wild felines as part of conservation strategies, specifically pumas and jaguars which are considered vulnerable in Brazil.

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# Xenograft of fresh and vitrified ovarian tissue from agouti (*Dasyprocta leporina*) to scid mice

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The vitrification followed by xenograft of gonadal tissue in the agouti is a useful tool to promote the conservation, multiplication, and formation of germplasm banks, especially when adapted for endangered rodents as the Dasyprocta mexicana. The objective was to evaluate the development of fresh and vitrified agoutis' ovarian tissue after xenograft to C57Bl/6 SCID female mice. Ovaries were obtained from five female agoutis and divided into 16 fragments. Five fragments were immediately transplanted to SCID mice (xeno-control group) and the others were vitrified (xeno-vitrified group) on a solid surface using solution consisting of MEM plus fetal bovine serum, 0.25M sucrose and association of 3 M dimethylsulfoxide with 3 M ethylene glycol, being transplanted only after rewarming. Transplantation was performed under the renal capsule of the recipients. Recipients' ovarian activity return was monitored by observation of external estrus signs and vaginal cytology during 40 days. At the end of this period, the collection of blood destined for estrogen dosage by electrochemiluminescence was conducted. Comparisons between treatments for morphologically normal proportions of ovarian follicles were performed by the Fisher PLSD test ($P < 0.05$). We verified that 80% (4/5) of the mice that received fresh ovarian tissue of agoutis and 16.7% (1/6) who received vitrified tissue returned to ovarian activity at 20.6 ± 8.6 days after xenograft, evidencing proestrus signs such as vulvar edema and presence of mucus, as well as evident increase in the proportion of cornified epithelial cells in the vaginal cytology. At 40 days, the same females showed increase in estrogen levels, related to estrus occurrence. Histological analysis of xenotransplanted tissues showed a predominance of primordial and primary follicles in all treatments. In two individuals (40%) of the xeno-control group, luteal bodies were identified, as well as the presence of a hemorrhagic corporea in one of the females of the same group which was in cytological metaestrus, possibly indicating the occurrence of ovulation. In 80% (4/5 - xeno-control group) and 16.7% (1/6 xeno-vitrified group) of the recipients, there was revascularization of the xenotransplanted tissue through the presence of blood vessels connected to the graft. In conclusion, it has been demonstrated that ovarian tissue xenotransplantation of D. leporina, fresh or vitrified, is able to promote the return of ovarian activity in SCID mice. It should be noted that this is the first study to describe the process of vitrification of agoutis' ovarian tissue followed by in vivo culture.

Acknowledgements: CAPES; CNPq.

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# Characterization of cystic ovarian condition in Nelore (*Bos Indicus*) cows used as oocyte donors

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Repeated ultrasound-guided follicle aspiration (OPU) may alter follicular dynamics in oocyte donors (Viana et al. 2010), probably due to changes in LH/FSH releasing patterns. Moreover, donors cows usually undergo frequent hormonal treatments to synchronize follicular wave emergence and are prone to become obese, both risk factors for the development of endocrine misbalance and, consequently, cystic ovarian disease (COD). Thus, the aim of this study was to characterize COD in Nelore (Bos indicus) cows previously used as oocyte donors. Cows (n=16) were selected based on records indicating recurrent occurrence of COD and lack of response to conventional treatments. The average weight and body condition score were 615.9±11.5 Kg and 4.0±0.1, respectively. Weekly transrectal ultrasonography was performed to evaluate the number of follicles and distribution among size classes, endometrial thickness, and clinical presence of mucometra (scored in a scale of 0 to 3). Non-pregnant, cyclic Nelore cows from the same herd (Controls) were used as a reference of physiological distribution of follicle population among size classes. Results are shown as mean±SEM. The average diameter of the largest follicle in cows with COD was 20.6±0.6 mm, larger than the usual maximum diameter of ovulatory follicles in Nelore (17.0 mm, Sartori et al. 2011), but smaller than the classic reference value to determine follicular cysts in Bos taurus (25 mm). However, 37.5 % (6/16) of donor cows did not have a follicle larger than 17 mm in at last one exam. When compared to controls, donor cows with COD had a greater number (3.6±0.2 vs 0.9±0.1, $P<0.001$) of follicles larger than the maximum diameter reported for the dominant follicle at deviation in Nelore (8 mm) and a lesser number (16.4±1.1 vs 23.5±2.6, $P<0.01$) of small follicles (≤4.9mm). Follicular population was negatively correlated both to the size of the largest follicle and the number of follicles ≥7.9 mm ($R= -0.44$ and $-0.36$, respectively; $P<0.01$). There was no difference in endometrial thickness between groups (4.4±0.2 vs 4.1±0.2 mm; $P>0.05$). In cows with follicular cysts, however, endometrial thickness was negatively correlated to the amount of mucus in the uterine lumen ($R= -0.71$). In summary, our results suggest that: 1) classification of follicular cysts must consider the expected range of follicular diameter in each breed; 2) COD in oocyte donors is characterized not only by ovulation failure and persistent dominant follicles, but also by an abnormal distribution of follicle population of distinct size classes; 3) COD decreases the average number of follicles on the ovaries of oocyte donors and, consequently, may compromise the outcome of OPU/IVEP.

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# Ovarian superstimulation increases oviductal estradiol and upregulates genes involved with gamete interaction

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P-36 protocol for ovarian superstimulation (OVS) using FSH was designed and later adapted for P-36/eCG to increase embryo viability due to the dual action of eCG on FSH and LH receptors (FSHR and LHCGR). Several oocytes are transported into oviduct during OVS, where they are fertilized and take the initial steps towards embryo development until transfer. The present study aimed to analyze the effect of OVS on steroids concentration in the oviduct and on the abundance of mRNA encoding genes related to gamete interaction and hormonal actions. Fifteen Nelore cows (n=5/group) were divided in three experimental groups: non-superstimulated (control) or submitted to two OVS protocols (P-36 or P-36/eCG). At 12h before endogenous LH surge, the ipsilateral oviduct (control cows) and a random oviduct (OVS cows) were collected to quantify the local concentration of E2 and P4. Total RNA was extracted and reverse transcribed from each segment (infundibulum, ampulla and isthmus). Relative RT-qPCR was performed using Power Sybr Green system with bovine-specific primers. The mRNA abundance of genes described as modulators for gamete interaction: Alfa-L-Fucosidase (*FUCA1* and *FUCA2*), Oviductal Glicoprotein 1 (*OVGP1*) and Heat Shock Protein Family A Member 5 (*HSPA5*); and also genes involved with endocrine control (*ESR1*, *ESR2*, *PGR*, *FSHR*, *LHCGR* and *CYP19A1*) was quantified. ANOVA was performed to assess the effect of OVS on mRNA abundance and concentration of hormones in oviduct. Mean values were compared by Tukey-Kramer test using JMP software (SAS Institute Cary, NC). Differences were considered significant when $P \leq 0.05$. In summary, oviductal $E_2$ concentration was higher in cows from P-36/eCG group (464±153 pg/mL) compared with control group (249±32 pg/mL) and showed intermediary values in P-36 group (326±24 pg/mL); however, the oviductal $P_4$ levels were similar among groups. The mRNA abundance of *FUCA1*, *FUCA2*, *OVGP1* and *HSPA5* was higher in infundibulum and ampulla of cows from P-36/eCG group compared to control group; however, no differences were observed in the isthmus. The expression of estrogen receptors (*ESR1* and *ESR2*) and progesterone receptor (*PGR*) were identified in all oviduct and showed higher abundance in ampulla (*ESR1* and *PGR*) and infundibulum (only *PGR*) in P-36/eCG cows compared to control group. The expression of *CYP19A*, *FSHR* and *LHCGR* were not detected in any oviductal segment. In conclusion, OVS increases $E_2$ concentration in the bovine oviduct, which may up-regulate genes involved with gamete interaction through activation of *ESR1* and *ESR2*, maybe setting the oviduct to receive more oocytes and embryos upon OVS treatment. Additionally, the findings presented here suggest no potential interaction of FSH and LH directly into the bovine oviduct during the preovulatory period. Supported by FAPESP #12/09498-9, 13/08629-5, 12/50514-8, 13/11480-3).

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# Prematuration of bovine oocytes with forskolin and IBMX: Cumulus cells transcriptomics and oocyte meiotic status

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Prematuration (Pre-IVM) systems use cyclic adenosine monophosphate (cAMP) modulators such as forskolin (FSK) and 3-Isobutyl-1-methylxanthine (IBMX) for artificially blocking meiotic resumption. To verify Pre-IVM effects in cumulus-oocyte complexes (COCs) we have sequenced cumulus cells transcripts, validated some key genes involved in the regulation of meiotic arrest/resumption and assessed the meiotic status of oocytes. COCs were cultured for 2h in M199 with FSK (100 μM) and IBMX (500 μM) followed by in vitro maturation (IVM) in M199 with rhFSH (0.1 IU/mL) for 24h. Control COCs were only matured for 24h in M199 with rhFSH and were used as the reference group in all comparisons. After 2h (Pre-IVM) and 26h (Pre-IVM + IVM) cumulus cells were recovered and total RNA was extracted. Sequencing was performed on HISEQ2500 Illumina platform and mapped to the Bos t. taurus reference genome UCSC bTau8. Gene expression was estimated with the R/BioConductor and tested with a quasi-likelihood F-test. Enrichment test indicated 80 significant categories of biological processes, 22 for cellular compartment and 25 for molecular function. Also, 7 pathways were significantly enriched: 3 stimulated and 4 suppressed in Pre-IVM. After 2h culture, 727 genes were differentially expressed, 334 upregulated (UR) and 393 downregulated (DR) by Pre-IVM. There were 292 differentially expressed genes after IVM: 185 UR and 107 DR by Pre-IVM. qPCR validated the dowregulation of phosphodiesterases (PDE5A, PDE1B, and PDE4B) and NOS3, which may act in the oocyte with the involvement of gap junctions to maintain higher levels of cGMP and cAMP, sustaining meiotic arrest. Those observations were reinforced by lucifer-yellow staining where gap junctions were substantially more open in Pre-IVM COCs ($P<0.05$) and also by orcein staining indicating higher amount of germinal vesicle oocytes assessed in Pre-IVM (91.0%) when compared to Control (59.3%). Genes related to cumulus expansion (PTX3, HAS2 and TNFAIP6) were also DR by Pre-IVM, however PTX3 became UR after IVM corroborating that Pre-IVM oocytes have the ability to overcome the arresting effects of Pre-IVM. Indeed, Hoechst 33342 staining proved the meiotic progression to be equivalent in both groups after IVM (62.1% of MII oocytes). After 2h we also validated the DR of genes related to lipid metabolism (PLIN2 and LIPE) in cumulus cells, which agrees with the lipolytic action of FSK, although we have observed an increased lipid content in matured oocytes treated with Pre-IVM after Sudan-Black B staining. In conclusion, RNA sequencing data showed that Pre-IVM deeply modulates the transcriptional profile of cumulus cells through the regulation of important genes that might be responsible for arresting meiosis in a reversible manner, since oocytes were able to be fertilized and sustain embryonic development similarly to conventionally matured oocytes. FAPESP grants 12/50533-2, 13/05083-1, 12/10737-8 and 12/23409-9.

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# Effects of different melatonin concentrations on *in vitro* maturation of bovine oocytes: Preliminary results

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Melatonin, a molecule derived from tryptophan and synthesized mainly by the pineal gland, mediates several processes in animal reproduction and has drawn attention for its potent antioxidant, anti-apoptotic, anti-inflammatory action and, more recently, for its benefits on oocyte maturation and embryo development *in vitro*. The aim of this study was to determine the concentration of melatonin that provides greater benefits during the *in vitro* maturation of bovine oocytes. For this, bovine cumulus-oocyte complexes (COCs) were obtained by aspiration of follicles (2-6 mm) from slaughterhouse ovaries, selected (grades I and II) and transferred to 4 well plates (25-30 COCs/well) containing *in vitro* maturation (IVM) medium [TCM199 supplemented with sodium bicarbonate (26 mM), sodium pyruvate (0.25 mM), FSH (0.5 µg/mL), LH (5.0 µg/mL), 0.3% Bovine Serum Albumin (BSA) and gentamicin (50 µg/ml)] with 0, $10^{-5}$, $10^{-7}$, $10^{-9}$ or $10^{-11}$M of melatonin and cultured for 24 hours at 38.5ºC and 5% $CO_2$ in air. At the end of IVM, oocytes were denuded, stained with Hoechst 33342 (10µg/ml) and evaluated for nuclear maturation rate. Cumulus cells (CC) were evaluated for the expression of antioxidant genes (SOD1, SOD2 and GPX4). For transcripts detection in CC, RNA isolation was performed with TRIzol® Reagent (Invitrogen™) and reverse transcription with "High Capacity cDNA Reverse Transcription" kit (Applied Biosystems™; Invitrogen™), following the manufacturer´s recommendations. Relative quantification of SOD1, SOD2 and GPX4 transcripts was performed by Real Time qPCR using Power SybrGreen® PCR Master Mix (Applied Biosystems®) with 3 endogenous controls (β-Actin, GAPDH and PPIA). Nuclear maturation rate and gene expression were tested by ANOVA and means were compared by Tukey's test (4 replicates) ($p<0.05$). In CC, the different concentrations of melatonin used, did not significantly alter the expression of the investigated antioxidant genes ($p>0.05$), however, the concentration of $10^{-7}$M provided a numerical increase in the expression of SOD1, SOD2 and GPX4. In oocytes, the rate of nuclear maturation was not statistically different ($p>0.05$) among the tested treatments, but was numerically higher in the $10^{-7}$M melatonin treated group. In conclusion, under the studied conditions, melatonin was unable to improve maturation rate and expression of antioxidants genes, but was able to improve numerically all the parameters evaluated at an intermediate concentration ($10^{-7}$M), indicating its beneficial effect on the IVM of bovine oocytes. The next step will involve performing more replicates of this experiment, to investigate further the potential cytoprotector role of melatonin and its benefits on oocyte maturation and developmental competence *in vitro*.
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# Changes in MicroRNAs expression in granulosa cells from superstimulated cows

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Ovarian superstimulation (OVS) is widely used in cattle to obtain multiple ovulations and can impact the follicular microenvironment. MicroRNAs (miRNAs) play a key role on ovarian follicle development and are expressed in bovine theca and granulosa cells. Particularly, the mir-222 shows a higher expression in theca and granulosa cells from bovine atretic follicles and mir-122 is described as a negative modulator of progesterone production. On the other hand, mir-144, mir-202 and mir-873 are up regulated in granulosa cells of bovine dominant follicle compared to subordinate follicles and are suggested as markers of steroidogenic capacity. To gain insight into the effects of OVS on regulation of miRNAs abundance in granulosa cells, the present study assessed the expression of mir-222, mir-122, miR-144, mir-202 and mir-873 in granulosa cells of preovulatory follicles from cows submitted to P-36 (only by FSH; n=10) or to P-36/eCG protocol (replacement of FSH by eCG administration on the last day of treatment; n=10). To obtain non-superstimulated preovulatory follicles, cows were only submitted to estrous synchronization (n=10). At 12h before endogenous LH surge, preovulatory follicles were obtained, and granulosa cells were harvested and submitted to miRNA extraction using MirVana™ miRNA Isolation Kit (LifeTechnologies®, São Paulo, Brazil). The relative abundance of miRNAs was measured by real time RT-PCR using TaqMan® Universal PCR Master Mix (Applied Biosystems, São Paulo, Brazil) and RNU43 plus has-miR-191 were used as reference. The effect of OVS on abundance of target mirRNAs was tested by ANOVA and the mean values were compared with orthogonal contrast. Differences were considered significant when $p \leq 0.05$. In summary, the OVS did not alter the relative abundance of mir-144, mir-202 and mir-873 in bovine granulosa cells. On the other hand, the relative abundance of mir-122 was lower in cows submitted to P-36 protocol (0,89±0,36) compared to control group (4,72±2,02). In the same way, the mir-222 showed a higher relative abundance in animals from control group (3,36±1,04) compared to P-36 (0,68±0,29) and P-36/eCG (0,84±0,25) groups. In conclusion, the lower levels of mir-222 and mir-122 in superstimulated granulosa cells reinforce that these miRNAs should be suppressed to allow the antral follicle development. Moreover, the absence on regulation of mir-144, mir-202 and mir-873 suggests that the superstimulatory protocols affect specific pathways to promote the final maturation of preovulatory follicles and corroborates with previous data which indicates that miR-144, mir-202 and mir-873 may exert a special role during follicle deviation in cattle. Financial support: FAPESP (grant #2013/11480-3 and grant #2011/50593-2).

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# Effects of eCG administration 4 versus 2 days prior to timed AI on *Nellore* cows

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The objective of this study was to evaluate the growth of the dominant follicle (DF) in multiparous Nellore cows treated with equine chorionic gonadotropin (eCG) either 4 or 2 d prior to timed AI (TAI). We hypothesized that early eCG administration improves follicular growth and increases DF diameter at TAI. Cows (n = 137) had their estrous cycle synchronized with an intravaginal device containing 1.9 g of progesterone (CIDR) and 2.0 mg of estradiol benzoate (d 0), 12.5 mg of PGF2α (d 7), CIDR withdrawal and 0.3 mg of estradiol cypionate (d 9), and TAI 48 h later (d 11). On d 7, ovaries were evaluated by ultrasonography and cows were blocked based on DF diameter (< 9 mm vs. ≥ 9 mm). Within block, cows were randomly assigned to receive 300 IU of eCG either on d 7 (eCGD7; n = 64) or on d 9 (eCGD9; n = 73). Diameter of the DF was evaluated by transrectal ultrasonography on d 7, 9, and 11. Follicular growth from d 7 to 9, 7 to 11, and 9 to 11 was analyzed by ANOVA using the GLIMMIX procedure of SAS. Follicular diameter was analyzed by ANOVA for repeated measures and cow was considered a random effect. Follicular growth from d 7 to 11 tended to be greater (P = 0.08) for eCGD7 compared with eCGD9 (4.9 ± 0.2 vs. 4.4 ± 0.2 mm). The overall effect of treatment was explained mostly by DF growth from d 7 to 9 (eCGD7 = 2.6 ± 0.2 vs. eCGD9 = 2.0 ± 0.2 mm; P < 0.01), as growth from d 9 to 11 did not differ (P = 0.44) between treatments (eCGD7 = 2.3 ± 0.2 vs. eCGD9 = 2.4 ± 0.1 mm). Follicular diameter was affected (P < 0.01) by the interaction between treatment and day. Although no difference was observed on d 7 (eCGD7 = 9.7 ± 0.2 vs. eCGD9 = 9.6 ± 0.2 mm), DF in eCGD7 cows was larger (P < 0.01) on d 9 (12.3 ± 0.2 vs. 11.5 ± 0.2 mm). In addition, DF on d 11 did not differ (P = 0.12) between treatments (eCGD7 = 14.5 ± 0.3 vs. eCGD9 = 14.0. ± 0.2 mm). Treatment with 300 IU of eCG 4 d prior to TAI improved follicular growth but did not increase overall mean of DF diameter at TAI compared with eCG administration 2 d before TAI in Nellore cows.

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# Glycogen synthase kinase-3 (GSK-3) inhibition during bovine oocytes *in vitro* maturation negatively affects embryo *in vitro* production

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Glycogen synthase kinase-3 (GSK-3) acts on several signaling pathways as cell cycle, protein synthesis and microtubule dynamics. This enzyme was identified in bovine oocytes and cumulus cells at different stages of development. It is know that its unspecific inhibition negatively affects oocytes in vitro maturation (IVM), but there is no research that demonstrate its role on oocyte viability, cytoplasmic maturation and the impact of GSK3 inhibition on embryo development. Therefore, the aim of this study was to evaluate the impact of GSK-3 inhibition using a specific inhibitor, CHIR99021, on bovine oocytes IVM and its effects on embryos in vitro production (IVP). COCs (grade I and II) were in vitro matured in TCM 199 supplemented with 10% FCS, FSH 10 g/mL, LH 5µg/mL and 1% penicillin/streptomycin, supplemented with different concentrations of CHIR99021 [Control group (CG), 1.5 µM(G1), 3.0 µM(G2) e 6.0 µM(G3)]. IVM was performed in 100 µL drop (20 COCs/drop), submerged in mineral oil and maintained in a humidified atmosphere containing 5% CO2 in air, at 38.5°C, for 24 hours. After this period were evaluated the cumulus cells expansion, cellular viability by Calcein AM and Propidium iodide staining, nuclear and cytoplasmic maturation by acetic orcein and Lens culinaris-FITC (LCA) staining, respectively. COCs submitted to same conditions were also fertilized and in vitro cultured to determine cleavage (D3) and blastocysts (D7) rate. The results were analyzed by ANOVA and submitted to Tukey test ($p \leq 0.05$). The highest CHIR99021 concentrations (3 e 6 µM) reduced cumulus cells expansion. The inhibitor use also significantly reduced in a dose-dependent manner the oocyte viability (CG: 81.62±11.15a; G1: 66.67±8.47ab; G2: 52.02±12.60bc; G3: 34.86±10.43c) and nuclear maturation (CG: 92.71±8.58a; G1: 54.67±5.58b; G2: 44.27±7.96b; G3:28.47±1.65c). Cortical granules distribution were also reduced when 3 and 6 µM were used (C: 75.06±5.97a; G1: 60.41±8.46a; G2: 39.11±4.78b; G3:28.31±9.84b). This conditions resulted to lower cleavage (CG: 83.63±9.39a; G1: 80.72±9.06a; G2: 65.44±16.94ab; G3: 39.58±12.39b) and blastocyst rates (CG: 40.50±3.90a; G1: 34.11±5.35ab; G2: 25.34±3.78b; G3: 13.41±4.43c). Thus, it is possible to conclude that GSK-3 is essential for bovine oocyte in vitro maturation and its inhibition negativity effects IVP. To our knowledge, this is the first work that shows the negative effect of GSK3 inhibition during IVM on in vitro embryos production.

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# Analysis of metabolism and hormonal production by canine luteal cells in cyclic and gestational diestrus cultured *in vitro*

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Despite the knowledge about reproductive physiology of the canine species, the similarity between cyclic and gestational luteal phase is still an intriguing and unknown factor. Our objective was to measure glucose and lactate production and P4 and E2 levels in culture medium of canine cyclic and gestational luteal cells. The CLs were collected (n = 40) after OSH on different days after LH preovulatory surge (D10, D20, D40 and D60) during cyclic (n = 20) and gestational (n = 20) diestrus . The CLs were digested enzymatically by collagenase type 1 (1mg / ml, Sigma Aldrich, Saint Louis,USA) plus high glucose DMEN (Sigma Aldrich, Saint Louis, USA) and the contents were filtered and centrifuged (3x, for 10 minutes at 20 ° C). The cell concentration used was 2x105 cells/ml (500 ul / well) and the medium was collected and stored in cryotubes (-80oC) at 3 moments: M1 (36 hours), M2 (48 hours) and M3 (60 hours). A plate containing only culture medium was used as control. For glucose and lactate measurement were used Calibrator 1 VITROS (Ortho Clinicals, Rochester, USA) and for P4 and E2 measurement were used Imunotech and ultra-sensitive DSL-4800 kits (Beckman Counter, Indianapolis, USA). For statistical analysis were used ANOVA and PROC GLIMMIX of SAS, with significance when $p < 0.05$. There was glucose consumption by cyclic ($p = 0.032$) and gestational ($p = 0.0134$) luteal cells in D20 and D60 only by cyclic luteal cells ($p = 0.015$). Probably this higher consumption is due to high energy requirement during this exponential cell growth phase (Zagari F., et al., N Biotechnol., 238-45,2013) and the differentiated consumption in D60 may be associated with a differentiated metabolism during luteolysis. Lactate production was higher in D10 and D40, in M3 ($p < 0.05$) in both cyclic and gestational diestrus, and low in D60, although gestational CL production was higher ($p = 0.02$), suggesting a reduction in metabolic enzymes activity at the time of luteolysis. In relation to P4, there was no difference in production between cyclic and gestational CLs in all days studied. Only in the cyclic C.L, the concentration in D10 was similar to D40, both higher than D20 and D60 in M3 ($p < 0.05$). In relation to E2, there was no variation between the cyclic and gestational luteal cells in D10, D20 and D60, but in D40, in cyclic diestrus, the estradiol production was high (342 pg / ml), 9 times more than gestational phase (51.51 pg / ml, $p = 0.042$). In bitches, the concentrations of P4 in vitro culture showed the same pattern observed in vivo. E2 values were high in D40 cyclic diestrous, a fact also evidenced by Concannon et al, 2002 (Reprod Domest Anim, page 3-15), but never reported in cell culture when compared to gestational diestrus. This differentiated pattern may indicate a distinct role for the action of E2 on the cyclic CL Luteal cells from cyclic and gestational diestrus, cultured in vitro, presented metabolic and hormonal differences, mainly in phases close to luteolysis moment.

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# Evaluation of oocyte meiotic arrest after the combined use of natriuretic peptide precursor C (NPPC) and rhFSH during *in vitro* prematuration of bovine cumulus-oocyte complexes

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Studies have shown that natriuretic peptide C (NPPC) and its type 2-receptor (NPR2) are essential for the maintenance of oocyte meiotic arrest. Further details are still lacking, especially in the bovine species, regarding the NPPC-NPR2 signaling and the possible effects of their association with other factors used during in vitro maturation (IVM). Among those, FSH is widely used to stimulate cumulus cells expansion and meiosis progression. Since the active NPPC-NPR2 system can delay the spontaneous resumption of meiosis in vitro and FSH has an antagonistic action, the combined use of these two drugs is avoided when designing systems for IVM or pre-IVM. The objective of this study was to evaluate the effect of NPPC, associated or not to recombinant human (rh)-FSH, in a 6h pre-IVM system on the progression of meiosis in bovine oocytes. Cumulus-oocyte complexes (COCs) were matured according to the following groups: I) Laboratory control: 24h IVM with control medium (M199, pFSH, pLH, E2, FCS, pyruvate and amikacin); II) Experimental control: 6h pre-IVM with basic medium (M199, BSA, pyruvate and amikacin); III) Pre-IVM-NPPC: 6h pre-MIV with basic medium plus NPPC (100 nM); IV) Pre-IVM-FSH: 6h pre-IVM with basic medium plus rhFSH (0.1 μg/ml); V) Pre-IVM-NPPC/FSH: 6h pre-IVM with basic medium plus NPPC and rhFSH (at same concentrations of groups III or IV). In all groups, except I, pre-IVM was followed by 24h IVM with the control medium. COCs were collected at 9h and 15h of culture time, i.e., COCs from groups II, III, IV and V were collected after 3h and 9h of IVM. Collected COCs were stained with orcein to assessed the percentage of oocytes still arrested in meiosis at the germinal vesicle stage (GV). Five replicates were performed with 15 to 20 COCs per group. The effect of treatments was tested by one way-ANOVA after data normalization and the means were compared with the post-hoc test by Fisher LSD method. Significance was considered with $P \leq 0.05$. After 9h IVM, the percentage of GV oocytes was higher in groups III and V (79,1% and 51,7%, respectively) when compared to groups I, II and IV (38,1%, 40,3% and 33,2%, respectively). After 15h IVM, the percentage of GV oocytes of group III (28,8%) was increased when compared to groups IV and V (12,4% and 14,1%, respectively), as well as of group IV with group II. We can conclude that after 9h there was a partial inhibition of the effect of NPPC due to the combined use of rhFSH, indicating that the use of drugs with opposed effects was not fully detrimental. However, this inhibition was complete after 15h IVM.

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# Morphological and morphometric evaluation of preantral multi-oocyte ovarian follicles of *Bos indicus* fetuses

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The objective of this study was to evaluate the morphology and morphometry of preantral multi-oocyte follicles of bovine fetuses in initial, middle and final third of gestational development. Thirty two ovaries (16 pairs) of Bos indicus fetuses in three distinct gestational phases: initial (6 pairs), middle (3 pairs) and final third (7 pairs) were removed after the slaughter of females. The pregnant uterus were packed in boxes and sent to the lab, where the foetuses were dissected for the obtaining of the ovaries. These were immediately fixed in Bouin for 18 hours, subsequently placed under running water for 12 hours and packed in 70% alcohol to histological processing. To make the blades, the paraffin blocks were cut with a rotary microtome (Leika ®, Germany) in cuts 5 µm thick serials and interval of 6 histological sections. The fragments were stained with periodic acid Schiff (PAS) and were analyzed under an optical microscope (Axio, Scope. A1-Zeiss, Switzerland). The total number of multi-oocyte follicles was evaluated and these were classified morphologically into primordial, primary and secondary. Morphometry was performed with the aid of the Image Pro Plus Version 4.5 Program by image capture of the slides, calculating the mean area of each follicle. The results were submitted to analysis of variance ($P<0.05$). Seven and forty six preantral multi-oocyte follicles were found from 16 pairs of ovaries. There was a higher proportion of primordial follicles in thirds and end multi-oocyte [70.3% (154/219) and 69.4% (245/353)] respectively, compared to the initial third [28.7% (50/174)]. The primary follicles showed similar proportion ($P> 0.05$) in the initial third [50.5% (88/174)], middle third [28.3% (62/219)] and final third [22.6% (80/353)]. The secondary follicles presented a higher proportion ($P<0.05$) in initial thirds [20.6% (36/174)] and end [7.9% (28/353)] in relation to the middle third [1.3% (3/219)]. The morphometry resulted in different sizes for the primordial follicles multioócitos in three-thirds of pregnancy (third: 246.8 µm; middle third: 388.0 µm; final third: 409.0 µm) in relation to primary follicles (third: 797.2 µm; middle third: 851.1 µm; final third: 569.1 µm) and secondary (third: 713.3 µm; middle third: 1141.6 µm; final third: 699.6 µm). The primordial follicles were lower in the initial third of gestation (246.8 µm) in relation to the middle third (388.0 µm) and final (409.0 µm). Only the primordial follicles, initial third pregnancy showed lower size ($P<0.05$) compared to medium and final thirds. The secondary follicles were similar ($P > 0.05$) in three-thirds of pregnancy (third: 713.3 µm; middle third: 1141.6 µm; final third: 699.6 µm). We conclude that primordial multioocyte follicles were predominantly found in ovaries of Bos indicus fetuses in the middle and late thirds, and this follicular class presented a smaller diameter in the fetuses in the initial third of gestation by morphometric evaluation.

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# Varian morphological characteristics influencing population of anthropological foliculos in femesas *Bos indicus* of different ages - preliminary results

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The antral follicular population has been associated with fertility traits in Bos taurus (Ireland et al., Reprod Fertil Develop, v.23, p.1-14, 2011; Jimenez-Krassel et al., Biol Reprod, v.80, p.1272-81, 2009), but not in Bos indicus (Rodrigues et al., Pesq agropec bras, v.48, p.801-804, 2013). However, in both species there is positive relation of the antral follicular population (PFA) with the number of embryos produced in vitro (Ireland et al., Reprod Fertil Develop, v.23, p.1-14, 2011; Rodrigues et al., Pesq agropec bras, v.48, p.801-804, 2013), which has made this feature interesting for selection (Oliveira Jr. et al., Semina, v.36, p. 3741-50, 2015). The best understanding regarding the relation of ovary morphology and PFA can optimize the selection method based on this characteristic. The objective of the present study was to evaluate the relationship between ovary weight and diameter and the number of antral follicles ≥1 mm in bovine females of different ages. The ovaries of 398 Bos indicus females were individually collected at a local slaughterhouse. The age of the animals was determined by analysis of the dentition as recommended by McManus et al. (Serie técnica: Genética. UnB- DF, 2010). After that, the ovaries were sent to the laboratory and the weight, diameter and count of the antral follicles were measured. The obtained data were submitted to analysis of variance and Pearson correlation by the program R. Large variations in ovarian weight and PFA were observed among the animals. Significant positive correlations were found between ovarian diameter and weight ($r \geq 0.66$) and between ovary diameter and PFA ($r \geq 0.30$). There was a difference ($P<0.05$) in PFA of the animals presenting CL in one of the ovaries, when compared to those who did not have CL (32.8±2.04; 27.1±0.95 respectively). There was no difference ($P>0.05$) in PFA between null females of 15 (n=41), 24 (n=38), 36 (n=31), 48 (n=45) and >60 (n=43) months of age. There was a correlation between age and ovarian weight ($r=0.33$, $P<0.05$) and corpus luteum diameter ($r=0.18$, $P<0.05$). The same did not occur when the age was correlated with the weight and diameter of the FD ($P>0.05$). It was concluded, therefore, that PFA varied according to the diameter, ovary weight and presence of CL, but no influence of FD was observed on Bos indicus females. And although ovary weight increased with age, PFA did not vary in females between 15 and 60 months of age.

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# Comparison among different systems of *in vitro* culture of preantral follicles in cattle

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The aim of this study was to evaluate the efficiency of four methods for *in vitro* culture method of preantral follicles in the bovine species. Ovaries (n = 10) were collected from local slaughterhouse, from cyclic Nellore cows (n = 5). After collection, the ovaries were washed in 70% ethanol and PBS. The surrounding ovarian tissue was removed and the ovary cortex was dissected into fragments of approximately 3x3x1 mm. One fragment per animal was immediately fixed in Bouin and the remaining fragments (n = 8) were individually randomly distributed into four groups: i) standard culture in plastic plate, ii) culture on the agarose gel; iii), agarose gel culture on the plate; and iv) Millicell culture. Fragments were grown with supplemented minimal essential medium (MEM, Gibco) with ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin, and 6.25 ng/ml selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxantina, 1.25 mg/ml bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20 IU/ml penicillin and 200 mg/ml streptomycin. Culture methods were tested for six (D6) or fourteen (D14) days. The culture media were replaced with fresh aliquots every two days. For the analysis of the integrity and degree of development of the follicles, the classical histology with Shiff Periodic Acid (PAS) and Hematoxylin staining, and the morphometric evaluation of the follicles and oocytes was performed. Classification was based on evaluation of the stage of follicular development (primordial, primary or secondary) and morphological integrity in intact or degenerate. For the analysis of morphometry, 5 healthy follicles were observed per treatment. For the morphometric analysis, images were used to measure oocyte and follicle diameter, calculating the arithmetic mean of two perpendicular measurements, using the program Motic Plus 2.0. Data were analyzed by ANOVA tests ($p \leq 0.05$). We evaluated 1,330 preantral follicles, of which 326 were primordial follicles and 1,004 developing follicles. After six days of culture, the fragments grown on the agarose gel support presented a higher proportion of intact follicles (75.3%; 113/150) compared to the other methodologies tested: 58.7% (88/150) for the standard culture; 62.6% (94/150) for culture with agarose gel on the plate; 54% (81/150) for the Millicell culture ($p<0.05$). As for the percentage of developing follicles in D6, there were no differences between the culture methods tested ($p<0.05$). After fourteen days of culture, the standard cultures on the agarose gel and agarose gel on the plate allowed a higher percentage of morphologically intact follicles: 54.7% (82/150), 58% (87/150) and 54.7% (82/150), respectively, when compared to the millicell culture (35.3%, 53/150). For the rate of development in D14 the treatments also did not differ among themselves ($p> 0.05$). Morphometric evaluation at six days of culture presented similar results among the four groups. However, after fourteen days, the follicles cultured on the agarose gel presented a greater average diameter in comparison to the other treatments. We concluded that the culture method on the agarose gel was more effective in maintaining the morphological integrity and allowing bovine preantral follicles to reach greater diameters.

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# Progesterone dosage with Sincrogest Injetável® on the induction of ciclity

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The induction of cyclicity (IC) with intravaginal progesterone device associated with estradiol IM treatment increases cyclicity and conception rate at TAI in Bos indicus heifers (Sá Filho, et al. Anim.Reprod. Sci., v.160, 2015). Furthermore, similar results are obtained when 150mg of long-action injectable P4 was used (P4LA; Sincrogest Injetável®, Ourofino Saúde Animal; Neto, et al., Anual da SBTE, Foz do Iguaçú, 2016). However, the period between the increase of blood P4 concentration after treatment with P4LA in prepubertal Nelore heifers remains unknown. Therefore, the objective of the present study was to evaluate plasma concentration of P4, the diameter of the dominant follicle (Ø FolDom) and ovulation rate (RtOv) in prepubertal Nelore heifers treated with 150mg of P4LA. The experiment was conducted in a commercial farm in Mato Grosso state in Brazil, where 21 prepubertal heifers were selected [absence of CL in two US with a 6-day interval (D-18 and D-12)] aged 27.0±0.3 months and BCS = 3.01±0.03. On D-12, concomitantly with the US, blood collection was performed and heifers were separated in four experimental groups: 1) Control Group: did not receive hormonal treatment; 2) InjD10 Group: received 1.0ml (150mg) of P4LA on D-10; 3) InjD12: received 1.0ml (150mg) of P4LA on D-12; 4) DispD12: received an intravaginal progesterone device previously used for 24 days. Blood collection and US was performed promptly on D-12 and daily from D-2 to D2. The P4 device was removed on D0, at the same time the heifers of the InjD10, InjD12 and DispD12 groups received 0.5ml of estradiol cipionate (SincroCP®, Ourofino Saúde Animal). Data were analyzed using SAS (v9.4), the "Fisher's Exact Test" was used for binomial variables and the PROC GLIMIX for continuous variables. Treatment effect was observed for RtOv [Control = 20%b (1/5); InjD10 = 20%b (1/5); DispD12 = 50%b (3/6) and InjD12 = 100%a (5/5); P = 0.02]. Treatment effect was observed (P = 0.07) for Ø FolDom (Control = 10.4±0.83b; InjD10 = 13.2±1.1a; DispD12 = 9.5±2.5a and InjD12 = 12.2±0.85a). Time*treatment interaction was observed (P = 0.08) for [P4]. Time effect was observed (P < 0.001). Treatment effect for [P4] was observed (Control = 0.25±0.04b ng/mL; InjD10 = 0.59±0.1a ng/mL; DispD12 = 0.71±0.14a and InjD12 = 0.71±0.12a; P = 0.03). Punctually evaluating the D-1 time, Sincrogest Injetável®, presented [P4] near 1ng / ml, not observing difference when compared to the P4 device (DispD12 = 1.728 ± 0.38a ng/mL; InjD10 = 1.160 ± 0.28a ng/mL; InjD12 = 1.207 ± 0.5a ng/mL) as well as remaining superior to the Control Group (0.56 ± 0.2b ng/mL). However, Sincrogest Injetavel® showed intermediate values to DispD12 and the control group observed in other times. Therefore, it was concluded that Sincrogest njetável® promoted intermediate levels of [P4] compared to the Sincrogest Dispositivo®, mantaining [P4] above 1ng/ml for 11 days. Injectable Sincrogest® may be an alternative to induction of cyclicity in zebu heifers.

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# Effect of overfeeding with rehydrated grain corn silage on ovulation of crossbred Santa Inês Ewes

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Overfeeding (flushing) during the breeding season is an usual practice to increase the number of ovulations, once the greater energy intake stimulates folliculogenesis. Regarding the better ruminal digestibility of the starch on the rehydrated grain corn silage (RGCS), the objective of this study was to evaluate the effect of flushing with RGCS on ovulation of ewes. Fifteen crossbred Santa Inês ewes were randomized allocated to treatments: diet with ground corn (GC, n=7) or with RGCS (RGCS, n=8). Diets contained corn silage (40% of the dry matter of the diet), soybean meal, ground or rehydrated corn, and mineral salt were offered for 30 days before the breeding season. Estrus behavior was synchronized with progestagen impregnated implant (Progespon®, Zoetis, Campinas, Brazil) for 11 days, i.m. administration of 37.5 mg of d-cloprostenol (Prolise®, Arsa, Buenos Aires, Argentina) and 300 UI of eCG (Novormon®, Zoetis, São Paulo, Brazil) two days before the implant removal (Day 9). Estrus behavior was observed twice a day. Daily from the day 9, the number and diameter of all follicles ≥ 3 mm were observed through ultrasonography (Aloka SSD 500, linear transducer of 7.5 MHz) until the ovulation or until 8 days after the implant removal. The ovulation time was estimated as the mean interval between the last follicle ultrasound image and its disappearance on the following examination. Daily dry matter intake (DMI) was evaluated. Blood samples were collected every six days for glucose dosage. Percentages of estrus and ovulation, estrus length, interval from implant removal to the beginning of estrus and interval from estrus to the ovulation were analyzed by generalized linear models, using SAS® software (v 9.3, SAS Institute, Cary, USA). Variables such as DMI, serum glucose, number, growth rate and diameter of the ovulatory follicles were analyzed by variance analysis. Initial body weight was used as covariate in all analyses. The DMI (1.2 ± 0.1 and 1.1 ± 0.0 kg/day) and the serum glucose concentration (73.0 ± 8.7 and 82.2 ± 8.0 mg/dL) did not differ (P> 0.05) between the treatments GC and RGCS, respectively. The percentage of estrus behavior (57 vs 63%) and the estrus length (43.6 ± 10.6 vs 37.1 ± 9.5 h) did not differ (P>0.05) between treatments, but the implant removal-estrus interval was shorter (P<0.05) in the treatment GC (26.5 ± 8.5 vs 62.8 ± 8.3 h). The estrus-ovulation interval (41.2 ± 11.2 vs 36.6 ± 10.0 h), the percentage of ewes that ovulated (86 vs 75%), the number (2.7 ± 0.5 vs 1.7 ± 0.2 follicles) and growth rate of the ovulatory follicles (0.5 ± 0.1 vs 0.7 ± 0.1 mm/day) did not differ (P>0.05) between treatments. However, the ovulatory follicles diameter was greater (P<0.05) in the RGCS treatment (6.2 ± 0.5 mm vs 8.2 ± 0.7 mm). Flushing with RGCS did not promoted increase in the ovulation number, but delayed the estrus beginning and resulted in the ovulation of greater follicles. Acknowledgments: FAPEMIG.

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# Effect of different melatonin concentration on meiosis resumption and lipid content of bovine oocytes matured *in vitro*: Preliminary results

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Melatonin (MLT) is synthesized in pineal gland and can act on reproductive competence, has antioxidant and antiapoptotic activities and influences different cell signaling pathways. The objective of this study was to evaluate the effect of the addition of different concentrations of MLT during the *in vitro* maturation (IVM) on the meiosis resumption rate and lipid content of bovine oocytes. Pools of 25 to 30 cumulus-oocyte complexes (COCs) were randomly selected and submitted to IVM in TCM 199 medium supplemented with 11 µg/mL sodium pyruvate, 10 µg/mL gentamicin and 3 mg/mL BSA (negative control; NC) or added with hormones: 1 µg/mL FSH (positive control; PC) or different concentrations of MLT ($10^{-11}$, $10^{-9}$ and $10^{-7}$ M), in order to evaluate the individual action of each treatment on IVM and lipid content. Only melatonin was used and not its association with FSH in order to evaluate its individual action on IVM and lipid content. After 9 hours, the oocytes were denuded and stained with Hoechst 33342 and Nile Red, and evaluated by epifluorescence microscope to determine the nuclear maturation stage and lipid content, respectively (emission 445-450nm and 475-490nm excitation and emission 590nm and 516-560nm excitation, respectively). The fluorescence intensity (FI) was measured by ImageJ software. The statistical analyses were realized by ANOVA, followed by Tukey (3 replicates/group), with 5% of significance (GraphPrism software). There was an increase ($P<0.05$) on the meiosis resumption rate (metaphase I) for MLT $10^{-9}$ M group (39.0%, 32/82) when compared to NC group (5.6%, 5/89). For the other treatments, there was no difference in the meiosis resumption (13.3%, 10/75; 8.3%, 6/72 and 35.8%, 29/81 to MLT $10^{-11}$, $10^{-7}$ M and FSH, respectively) in relation to NC group. For MLT $10^{-11}$ M group was observed an FI of 8.43 ± 7.61, characterized by a higher lipid content ($P<0.0001$) in relation to the others treatments with MLT $10^{-9}$ M (5.6 ± 4.13), MLT $10^{-7}$ M (4.8 ± 3.21), FSH (5.34 ± 3.66) and NC (4.27 ± 3.28). In conclusion, under the conditions studied, MLT $10^{-9}$ M was able to influence meiosis resumption rate during the initial 9 hours of IVM. In the same way, MLT $10^{-11}$ M stimulated the increasing intraoocyte lipid content. Further studies are necessary to improve the knowledge of MLT role on the lipid synthesis and if it is associated with meiosis resumption in bovine oocyte. Acknowledgment: FAPESP (HF-2016/24884-3, LS, FCC, DMP, scholarships; CLVL, financial support 2015/20379-0).

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# Effect of epidermal growth factor on the development of preantral follicles of *Bos taurus indicus* females cultured *in vitro*

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Epidermal Growth Factor (EGF) is considered a potent mitogenic factor for follicular cells. Produced by the oocyte and granulosa cells, EGF plays an important role in the modulation of ovarian folliculogenesis. The objective of the present work was to test the addition of different concentrations of EGF to the in vitro culture medium for 5 or 10 days on the development of preantral follicles included in bovine ovarian fragments. Ovaries (n=10) of Bos taurus indicus females (n=5) were collected at a local slaughterhouse. Ovarian tissue fragments (n=9) with approximately 3x3x1 mm were obtained from each animal. One fragment was immediately fixed (Bouin's fixative) and processed for histological analysis (control group: Day 0), the remainder was placed in MEM® (Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/L, pH 7,2) supplemented with penicillin (200 IU/mL) and streptomycin (200 mg/mL) at 20°C, remaining for 1 hour (period of transport to the laboratory). The other fragments were in vitro cultured for 5 (D5) or 10 days (D10) in MEM+ (cultured control) or MEM+ plus different concentrations of EGF (50, 100 or 200 ng/mL), as the following groups: control (D0); MEM+ (D5); MEM+ + 50 ng/mL de EGF (D5); MEM+ + 100 ng/mL de EGF (D5); MEM+ + 200 ng/mL de EGF (D5); MEM+ (D10); MEM+ + 50 ng/mL de EGF (D10); MEM+ + 100 ng/mL de EGF (D10); MEM+ + 200 ng/mL de EGF (D10). The preantral follicles were evaluated by light microscopy and classified according to the stage of developmental (primordial or developing follicles) and morphology (intact or degenerate). The averages were compared by the Tukey's test ($P \leq 0.05$). In this experiment, 2,203 follicles in 720 histological sections were evaluated, totalizing 240 slides and 45 ovarian fragments. The non-cultivated control treatment (D0) predominantly presented preantral follicles at the primordial stage (82.8%), some primary follicles and rarely secondary follicles (17.2%). There was an increase in the number of developing follicles when 100 ng/mL EGF was added to the culture medium for 10 days (48.4%), compared to the control treatment (17.2%). In this way, we conclude that the addition of 100 ng/mL of EGF for 10 days to the in vitro culture medium of bovine preantral follicles was efficient in promoting follicles development and maintaining follicular integrity.

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# Study on nuclear maturation kinetics of bovine ovocyts with different degrees of competence

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Oocyte nuclear maturation is a complex process involving modification of chromatin from the germinal vesicle stage to metaphase II. Studies have reported that oocytes from large follicles have greater developmental capacity than oocytes derived from small follicles, resulting in higher rates of in vitro embryo production. The aim of this study was to evaluate the chromatin condensation and nuclear maturation kinetics in oocytes with different degrees of competence. For this, small follicles were used ( SF= 1.0-2.9 mm diameter follicles with less competent oocytes; n=120) or large (LF= 6.0-8.0 mm with more competent oocytes; n=130) dissected from the ovarian cortex were (Caixeta ES et al., Reprod. Fertil. 21,p.655–664, 2009).The control group (Con; n=151) was recovered by aspiration from 3 to 8 mm follicles. The COCs obtained from each treatment were fixed at 0 and 24 hours of maturation and then stained with lacmoid to identify meiotic stage: GV (GV0, GV1, GV2, GV3; Lodde et al., Mol. Reprod. Dev. 74, p.740–749, 2007), GVBD, MI, AI, TI, MII and abnormal. The data were analyzed by Chi-square (P<0,05). The results showed that at 0 hour of maturation, a higher percentage (P<0.05) of SF(98.33%; 59/60) were at GV stage than the Con (89.77%; 79/88). At the 24 h of culture, the Con and LF groups did not present any other ovocyte in GV, and the LF group presented 93.4% (57/61) of MII. The SF group still had 10% (6/60) oocytes in GV (P <0.05) and only 81.7% (49/60) in MII. Regarding to the degrees of chromatin condensation at GV stage, the SF (20%;12/60) showed a higher percentage of GV0 than the Con (2.27%;2/88) group. However, the more competent and the less competent groups did not differ (P <0.05) for the percentage of oocytes in GV1, GV2 and GV3. It can be concluded that SF have the lowest capacity to reach metaphase II, and that the degree of chromatin condensation in GV, when evaluated by lacmoid stain, is not a good parameter to estimate oocytes competence in cattle.

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# Fibroblast growth factor 2 regulates apoptosis and viscosity of the extracellular matrix in bovine cumulus-oocyte complexes undergoing *in vitro* maturation

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In vitro maturation (IVM) is one of the main obstacles to improve IVF outcomes. There are evidence that cumulus expansion and apoptosis are influenced by in vitro conditions. Fibroblast growth factor 2 (FGF2) expression is upregulated by the LH ovulatory peak in bovine cumulus cells (CC). In bovine granulosa cells, FGF2 activates ERK1-2, a pathway known to regulate cumulus expansion and cell survival. Extracellular matrix (ECM) chemical and physical properties affect adhesive and invasive capacity of the COC and thus fertilization rates. In this study, we assessed whether activation of the ovulatory cascade with FSH increases FGF2 mRNA abundance in CC, and tested the effects of FGF2 on apoptosis, degree of expansion and viscosity of the ECM in bovine COCs undergoing IVM. Groups of 20 COCs (grades 1 and 2) from 3-8 mm follicles of abattoir ovaries were cultured in TCM 199 (0.4% BSA, 1µl/mL FSH, 22µg/ml sodium pyruvate, 75µg/mL amicacin) supplemented with graded doses of FSH (0, 0.1, 1, 10 and 100 ng/mL; IVM for 12h; n=4/dose) or FGF2 (0, 1, 10 and 100 ng/mL; IVM for 22h; n=4/dose) at 38,5°C in humid atmosphere. FGF2 mRNA was assessed by real time RT-PCR using SybrGreen (LifeTech) and CYC-A as the reference gene, after RNA extraction from cumulus cells with RNEasy (Qiagen). Expansion degree was visually assessed and classified in grades 1 to 3 (1 poor expansion and 3 complete expansion). ECM viscosity was estimated by the number of pipetting movements necessary to completely denude the oocyte. To assess apoptosis, CC were tested for annexin V and caspase 3/7 staining in a flow cytometer (kits APC Annexin V, BD Pharmigen; CellEvent™ caspase 3/7, Thermo Scientific). Effects of treatments were tested by ANOVA and means were compared by the Fisher protected test. Treatment with FSH increased FGF2 mRNA levels in a dose dependent manner (mRNA relative values: 0.92±0.12a, 0.82±0,09a, 1.44±0.64a, 4.44±0.52b, 19.92±5.74c). FGF2 did not alter expansion degree, but increased ECM viscosity at all concentrations tested and there are empirical evidence indicating that cumulus ECM is far more viscous when maturation occurs in vivo in comparison to in vitro. FGF2 increased non-apoptotic cells (annexin V and caspase 3/7 negative; 80.48±2.43%a, 82.11±2.36%ab, 85.14±1.09%ab, 86.08±0.83%b) and reduced caspase 3/7 positive cells (17.64±2.06%a, 15.60±2.66%ab, 12.52±1.39%b, 12.25±1.02%b), suggesting an inhibitory action on the intrinsic pathway of apoptosis. In conclusion, the present data suggest that activation of the ovulatory cascade increases transcription of FGF2 in bovine cumulus cells, which appears important for the control of ECM viscosity and apoptosis in the bovine COC. These data have potential practical implications for IVM and suggest novel actions for FGF2 as a mediator of the ovulatory cascade favoring cumulus cells survival and fertilization.

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# Lactation influence on antral follicles count (AFC) in Holstein cows reared in the semiarid

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This study was conducted at the Experimental Station of São Bento do Una (ESSBU/IPA) Pernambuco, Brazil (Latitude 08 31' 35'' S and Longitude 036 27' 34.8" W), with the objective of evaluating the influence of lactation on the diameter and count of ovarian antral follicles (AFC) in Holstein cows submitted to hormonal protocol. Eighty-seven lactating cows aged 36 to 96 months, body condition score 2-3 and between 50 and 120 days in milk (DIM) or non-lactating were used. The cows were kept in a semi-intensive system, receiving a diet composed of cactus pear (Opuntia ficus-indica Mill), sorghum silage (Sorghum bicolor (L) Moench) and protein concentrate with 28% crude protein along with mineral supplement and water ad libitum. All cows after clinical and gynecological evaluation were distributed in six experimental groups (G1 to G6). In groups G1, G2 and G3 cows were not submitted to hormonal protocol, being G1 (n = 15) composed of non-lactating cows, G2 (n = 15) by lactating cows with average production (15-20 kg / milk / day) and G3 (n = 15) lactating cows with high production (21-35 kg / milk / day).The G4, G5 and G6 groups were submitted to hormonal protocol, with G4 (n = 14) being non-lactating cows, G5 (n = 14) being cows with average production, and G6 cows with high production. The hormonal protocol consisted of an intravaginal device containing 1g of P4 and 2mg of Estradiol Benzoate in D0, 0.530mg of Cloprostenol along with 200 IU of FSH in D4. All females were submitted to ultrasound examination for AFC and measurement of follicular diameter in D6. An ultrasound equipped with a 7.5 MHz microconvex transducer coupled to a transvaginal guidewire was used. The antral follicles of both ovaries were measured obtaining the diameter of the largest and the smallest follicle in each ovary. The data were submitted to ANOVA and to the Tukey test considering the level of 5%. There was no difference ($P > 0.05$) in the diameter of the large follicles, being observed variations from 9.6 ± 0.75 to 11.9 ± 0.87 mm. As for the smaller diameter follicles varied from 3.3 ± 0.99 to 4.7 ± 1.02 mm ($P<0.05$). In the AFC cows from G4 had an average of 25.8 ± 1.72 follicles, being superior ($P<0.05$) to the other groups. However, G1 cows had 18.7 ± 1.54 follicles and were also superior ($P <0.05$) to G2 cows (8.7 ± 1.04), G3 (12.4 ± 1.16), G5 (11.3 ± 1.12) and G6 (10.3 ± 0.93). Therefore, it was concluded that lactation exerted influence on AFC in Holstein cows, even with the use of hormonal protocol.

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# Lipids of bovine oocytes recovered from different follicle sizes

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Oocyte development is a complex process involving sequential molecular, biochemical and ultrastructural changes. The aim of the present study was, therefore, to access the amount of cytoplasmic lipid droplets and lipid profile fluctuation in oocytes derived from different follicle sizes and to associate with the oocyte mRNA levels of key lipid metabolism-related genes, and follicular fluid metabolic molecules. Cow ovaries collected from a slaughterhouse were used for the recovery of oocytes and follicular fluid from follicles with ≤2 mm, 3-5 mm, 6-8 mm and > 8 mm diameter. The diameters of follicles were carefully determined with caliper device followed by volume monitoring. Only oocytes with homogeneous cytoplasm and with more than three layers of cumulus cells were selected and denuded after trypsin and acidic tyrode's solution treatment. Oocyte lipid content and profiles were accessed by Sudan Black B staining (n=20 to 30 oocytes per group) and MALDI-MS (35 to 65 oocytes per follicle size, n=7 to 13 per group), respectively. Relative abundance of oocytes mRNA transcripts for ACSL3, ELOVL5, ELOVL6, SREBP and LXRα genes were determined by quantitative realtime PCR (n=4, total of 60 oocytes per follicle size). The patterns of follicular fluid molecules were investigated trough commercial biochemical assays. The data were analyzed by ANOVA using the PROC GLIMMIX of SAS and MetaboAnalyst 3.0. Oocytes recovered from follicles >8 mm had higher cytoplasmic lipid content when compared with the oocytes from other follicle sizes (6.0 ± 0.4; $P<0.05$). The other groups showed no difference in lipid content (≤2 mm: 4.8 ± 0.4; 3-5 mm: 4.1 ± 0.3 e 6-8 mm: 4.2 ± 0.4; $P>0.05$). The mRNA abundance of ELOVL6 was reduced ($P=0.02$) in the oocytes recovered from 6-8 mm (5.3x;) and > 8mm (5.6x) follicles compared with the ≤ 2mm group. The mRNA levels of ACSL3, ELOVL5, SREBP and LXRα were similar ($P>0.05$) among groups. Follicular fluid of large follicles (6-8 mm and/or >8mm) presented higher ($P<0.05$) levels of glucose, cholesterol, reactive oxygen species, glutathione and superoxide dismutase activity compared with small follicles (≤2 mm and/or 3-5 mm). Triglyceride concentration has reduced ($P<0.05$) in large follicles (6-8 mm and >8mm) compared with small follicles (≤2 mm and 3-5 mm). Additionally, oocytes recovered from different follicle sizes have presented fluctuations of membrane lipids such as phosphatidylcholines and sphyngomielins. Therefore, the findings of this study reveal the following: i) oocytes recovered from follicles greater than 8 mm have higher lipid content; ii) the oocyte phospholipid membrane profiles varies among different follicle sizes; iii) the mRNA level of ELOVL6 in oocytes recovered from large follicles suggests lower elongation reaction rate of fatty acids containing 12 to 16 carbons; iv) follicular fluid triglyceride, cholesterol, and glucose concentrations may be influencing oocyte lipid content and profiles. Acknowledgements: CNPq and FAPESP.

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# Increased expression of Mitofusin 2 in relation to Mitofusin 1 in oocytes impairs folliculogenesis in mice

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Mitochondria are highly dynamic organelles that change their morphology by undergoing fusion and fission processes. We previously addressed the role of mitochondrial fusion in oocytes by conditional knockout of Mfn1 (M1), Mfn2 (M2) and Mfn1+Mfn2 (DM) based on Zp3-driven expression of Cre recombinase. As a result, M1 and DM females were infertile, whereas M2 females were subfertile. Compared to the M1 group where infertility was caused by defective folliculogenesis, DM females were infertile due to ovulation of oocytes that arrested meiotic progression. These results suggested that ablation of Mfn2 in DM oocytes partially rescued the effect of Mfn1 deficiency. To further investigate this, using 5-week-old mice injected with eCG, we evaluated oocyte ultrastructure, ATP and $FADH_2$ level as well as gene expression in oocytes and ovaries. The number of follicular cells was determined using ovaries from 8-week-old females injected with BrdU. Data were evaluated by ANOVA followed by Duncan test ($P<0.05$), with at least biological triplicates. DM ovaries expressed normal levels of follicular markers that were downregulated (*Inhba*, *Inhbb*, *Fst* and *Fshr*) or upregulated (*Kitl* and *Lhcgr*) in M1 ovaries. In addition, these genes were not altered in M2 ovaries, except for *Fst* and *Fshr* that were increased. A similar pattern was found in oocytes since *Bmp15* and *Fgf8b* were downregulated in the M1 group, but remained unchanged in the WT, M2 and DM. These findings are in accordance with our previous results, suggesting the lack of Mfn2 enabled folliculogenesis in the DM group. In keeping with this, the number of follicular cells did not differ between the WT and DM groups, but it was decreased in the M1 and increased in the M2. The number of replicating cells was also decreased in M1 follicles, but unchanged among WT, M2 and DM. Ultrastructural analyses revealed that DM and M2 oocytes contained fewer mitochondria which were swollen in comparison to WT and M1 oocytes. Moreover, ultrastructure characteristics of mitochondria (i.e. increased number of inner membrane vesicles) were more often altered in the M1 and DM groups. Mitochondrial dysfunction in the DM group became more apparent after ovulation, as indicated by enhanced mitochondrial aggregation, increased amounts of $FADH_2$ and decreased levels of ATP and ATP/ADP. These defects possibly led to defective meiotic progression of DM oocytes. Therefore, ablation of Mfn2 in DM oocytes could have enabled follicular development through inducing replication of follicular cells. As a result, this might have compensated for the mitochondrial defect in DM oocytes until ovulation when the oocyte loses interaction with cumulus cells. This suggests an important role of Mfn1/Mfn2 ratio to oocyte development. Considering that Mfn1, but not Mfn2, were downregulated in oocytes from aged females, Mfn1/Mfn2 imbalance might underpin part of the effects of aging on oocyte competence.
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# Mutant mitochondrial DNA is selectively eliminated in the mouse oocyte by mitophagy

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Several lethal and cureless diseases are caused by mutations in the mitochondrial DNA (mtDNA). However, since wild-type mtDNA molecules are preferentially transmitted to the next generation, understanding the underlying mechanism might enable manipulation of mitochondrial inheritance. Recently, we conditionally (driven by Zp3-Cre) knocked out mitofusins 1 (Mfn1) and 2 (Mfn2) in heteroplasmic oocytes (C57BL/6 (B6) and NZB/BINJ (NZB) mtDNA) to evaluate the effect of mitochondrial fusion on mtDNA inheritance. NZB mtDNA differs from B6 mtDNA in 91 nucleotides, which are known to affect mitochondrial function. We found that Mfn1-null oocytes did not differ (-10.3±1.58) from wild-type (WT) oocytes (-10.1±1.25) regarding the ΔNZB (difference between the percentage of NZB mtDNA in the oocyte or progeny and that present in the mother). However, the ΔNZB was mitigated in Mfn2-null (-3.0±1.45) and Mfn1-null+Mfn2-null (DM-null) (-4.4±1.21) oocytes. This indicates that during oocyte development B6 mtDNA is preferentially inherited by a mechanism relying on Mfn2 expression. Considering that Mfn2 seems to play a key role during mitophagy, this work aimed at associating the higher levels of NZB mtDNA in Mfn2-null oocytes with a possible defect on the autophagic flux. In this regard, we used heteroplasmic B6/NZB mice and estimated the effect on mitochondrial inheritance by quantitating the ΔNZB and the occurrence of autophagosomes in the oocyte. Data were analyzed by ANOVA followed by T test or comparison of means by Duncan. Differences with $P<0.05$ were considered significant. First, we confirmed that the conditional knockout of Mfn2 in the oocyte resulted at weaning in pups with higher levels of NZB mtDNA (ΔNZB – WT=-8.1±1.07 vs. Mfn2-null=2.4±1.77). In addition, both the level of NZB mtDNA in spleen (ΔNZB – WT=-22.4±2.47 vs. Mfn2-null=-13.4±3.51) and liver (ΔNZB – WT=9.3±3.06 vs. Mfn2-null=19.9±2.77) were increased in pups that were 100 days old. These tissues, respectively, eliminate and accumulate NZB mtDNA with aging, giving evidence that the lack of Mfn2 in the oocyte impacted on mitochondrial inheritance. Next, we investigated whether the underlying mechanism by which NZB mtDNA is eliminated was dependent on autophagy. To this end, heteroplasmic oocytes were *in vitro* matured in the presence (CQ) or absence (CT) of 100 μM of chloroquine, an autophagic inhibitor. The higher amounts of autophagosomes in CQ oocytes, as indicated by anti-Lc3b immunofluorescence (CQ=52.0±2.51 vs. CT=42.1±5.11), suggested the treatment interfered with the autophagic flux. As a result, the treatment with chloroquine reduced NZB mtDNA elimination by ~70%, agreeing with our results from Mfn2-null oocytes. In conclusion, these results provide evidence that mitochondrial inheritance is regulated in the oocyte by mitophagic elimination of mutant mtDNA.

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# Ovarian response to hormonal stimulation treatment in collared Peccary (*Pecari tajacu* linnaeus, 1758)

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The *Pecari tajacu* species, commonly known as collared peccary, has arouse commercial interest. The study of the animal reproductive physiology is necessary in order to assist captive management and biodiversity conservation. This study aimed to compare two different hormonal treatments in collared peccaries females. The animals (n=4) were provided by Wild Animals Multiplication Center (CEMAS) located at Federal Rural University of Semi-Arid (UFERSA) and were subjected to two ovarian stimulation protocols with PG600® (eCG and hCG, MSD Saúde Animal, São Paulo, Brasil). The hormone was administered by single intramuscular injection and the treatments consisted of 5mL as recommended for swine ovarian stimulation (SD, Swine Doses) or 1.2mL as determined by allometric calculation (AD, Allometric Doses). Five days after hormone administration, ovaries were recovered and visualized follicles were classified as (S) small (<0.3cm), (M) medium (0.3-0.5cm) or (L) large (>0.5cm). The number of corpora lutea (CL) in each ovary was registered. Biopsies of follicles were achieved with Castroviejo scissors, weighted and stored in liquid nitrogen for downstream applications. Data were presented as mean ± s.e.m and compared by Student t test at $P<0.05$. The follicle numbers for each group (SD and AD, respectively) were 6.00 ± 0.71 and 12.00 ± 2.83 for S, 8.00 ± 0.71 and 8.00 ± 0.00 for M and 10.00 ± 1.41 and 4.50 ± 1.77 for L follicles. Despite numerically different, the number of S, M and L follicles were statistically similar ($P>0.05$) between SD and AD groups. The biopsy weights of SD animals were 5.44 ± 1.71 mg for S, 11.92 ± 2.67 mg for M and 17.53 ± 2.92 mg for L follicles. While for AD group, the follicular biopsies weighted 2.09 ± 0.28mg for S, 2.41 ± 0.36mg for M and 2.69 ± 0.59mg for L follicles. Thus, the mass of follicular wall increased significantly ($P<0.05$) from S to M and from S to L follicles in DS, but not in AD group. Consequently, DS treatment yielded a greater amount ($p<0.05$) of follicular tissue sample from L follicles than AD (378 ± 53.49 *vs* 28.4 ± 3.81 mg, respectively). Finally, both treatment produced similar ($P>0.05$) ovulation rates (accessed by CL number) in females. Thus DS produced 2.50 ± 0.35 CL/animal and AD produced 3.00 ± 0.71 CL/animal. Additionally, a female of AD treatment presented two structures macroscopically classified as luteinized follicles. In conclusion, both SD and AD treatments produced similar ovarian stimulations (denoted by the number of follicles) and ovulation rates in collared peccary females. On the other hand, AD treatment generated luteinized follicles, which might indicate that the dosage is not completely optimal to induce ovulation. Finally, the follicular wall mass seems to increase with follicular size after SD, but not after AD treatment. Additional investigations about oocyte quality or molecular profiles of ovarian follicles can elucidate reproductive physiology of collared peccary.

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# Embryonary recovery rates after the induction of ovulation using human chorionic gonadotropin (HCG) in mares with different follicular diameters

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Human chorionic gonadotropin is used as an ovulation inducer (OI) in mares and does not affect the embryo recovery rate. This hormone is important in embryo transfer programs in order to obtain an effective synchronization degree of ovulations between the donor and the recipient to obtain improved gestation rates. (JACOB, et al., 2011. A Hora Veterinária, v. 180, p. 9-13). However, it is unknown whether there is an influence in the embryo recovery rate after the induction of ovulation in follicles (FL) with diameters < 35mm. Observing this fact, the aim of this study was to evaluate the embryo recovery rate after the induction of ovulation with hCG in mares with follicles with different diameters. Estrous cycles (n=101) from embryo donor mares (n=40; with ages ranging from 3 to 20 years) were used in this study, between the 2015/2017 season in the State of Rio de Janeiro, Brazil. The estrous cycles were divided into two groups where: GI: control group (35 cycles), with spontaneous ovulation; and GII: hCG group (64 cycles), with ovulation induced using 1000 UI, iv., of hCG (Chorulon® - MSD Saúde Animal, Brasil), subdivided by follicular class: Follicles with diameter ≥ 32mm < 35mm; FL ≥35mm ≤37mm; FL >37mm to 41.5mm. Only mares that had single ovulations were included in the study. The follicular diameters were accompanied in intercalated days using transrectal ultrassonography until the observation of a follicle ≥ 28 mm of diameter, when the ultrasonography was realized daily until the ovulation. The mares were inseminated with fresh semen as close as possible of the ovulation for the GI. For the GII the insemination occurred 24h after the induction of the ovulation with fresh semen. The embryos were collected 8-10 days (D8-D10) after the ovulation, using the transcervical method. The exact Fischer test was used for the statistical comparison of all data. The positive (CP) and negative (CN) embryo recovery rate of the GI was: CP 48.57% (17⁄35) and CN 51.42%(18⁄35) to follicles which had a variation in diameter of 37.5 - 52mm. The obtained results from GII was: CP= 46.96% (31/66) and CN= 51.42% (35/66). The GII was divided by class related to the follicle diameter: FL ≥ 32 < 35mm = CP 40% (10⁄25) and CN 60% (15⁄25); FL ≥ 35 ≤ 37 = CP 54.16% (13/24) and CN 45.83% (11/24); FL >37mm = CP 47.05% (8⁄17) and CN; 52.94% (9⁄17). There was no statistical difference between the embryo recovery rate in follicles induced with hCG and the control group (P=0.6), and also with the different diameters in follicles induced with hCG (P=0.09). We conclude that: 1) the embryo recovery rates did not differ between mares that spontaneously ovulated or had the ovulation induced with 1000UI of hCG; 2) there is no difference between the embryo recovery rates after ovulation induction of follicles with different diameters.

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# Software efficiency for counting sheep spermatic cells: Preliminary results

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This study aimed to develop a software for sheep sperm cell count, from images taken under a microscopy. This program should be compatible with notebooks and PCs for home use, easy to use, in the Portuguese language and free. It is intended, with this software, standardize the sperm concentration analysis carried out by veterinarians during andrological examinations at field, cheapening the cost of acquisition of specific equipment. Ten images of sheep semen were made by microscopy from thawed semen doses. The 0.5 mL reed were thawed for 30 seconds in a water bath at 37°C. Semen drops were analyzed by CASA (Hamilton Thorne Research) and deposited on slides and Neubauer chamber both covered with cover slip for microscopy. The images were obtained from phase contrast microscope (Jenamed2) with 1.3 MP camera attached (Coleman). The software was developed from resources already available in an open source Java solution called ImageJ. The approximate count of the sperm contained in the image was possible through particle analysis capabilities. Initially, the video images were converted into frames and subjected to some treatments, using only 8-bit color and segmenting grayscale so that the software could do the analysis of the image particles. The 10 semen samples were analyzed by CASA, by the technician in Neubauer chamber and by the software for spermatozoa count. For statistical analysis, the results of the counts were subjected to analysis of variance (SAS, 2012) at a significance level of 5%. The average values of the sperm cell counts did not differ and were 186.00 ± 78.63, 171.00 ± 50.63 and 282.00 ± 155.81 by CASA, by the technician and by the software, respectively ($P > 0.05$). The software was highly efficient for sperm cell count, being a convenient and easy to use solution. The CASA instruments have shown high levels of accuracy and reliability using different methodologies of classification that provide a great tool to improve our knowledge and ability to analyze sperm, making it essential to research, personnel training and standardization between laboratories. Regardless of the manufacturer, the different instruments are based on similar principles, but differ in terms of optics and software used to identify the sperm and the construction of the track, respectively. Our differential is the gratuity and ease of use, since it is a specific software for sperm analysis and available in Portuguese. In conclusion, the developed software showed the same efficiency as the count carried out by CASA e by the technician.

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# Evaluation of spermatic characteristics of small and medium-sized dogs: Partial results

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The present study has the objective of evaluate the spermatic parameters of small and medium size dog breeds. Nine adults dogs were used, with good reproductive histories, from private breeders, five (n=5) of medium size Breeds (one American Pit Bull Terrier, one Basset Hound and three Sharpei) and four animals of small Breeds (Poodles), the age was ranging from three to six years, clinically healthy and trained for the semen manual retrieve procedure. The testicles were evaluated in length and width. The semen was obtained by digital manipulation of the genitalia and the parameters volume, color, odor, motility, vigor, concentration and spermatic morphology have undergone descriptive analysis. The mean values±standard deviation (SD) of length and width of the small dogs testicles were 2.17±0.70 and 1.53±0.50 (left testicle) and 2.17±0.47 and 1.50±0.30 (right testicle), respectively, and of the medium sized dogs were 3.64±0.42 and 2.14±0.27 (left testicle) and 3.64±0.54 and 2.08±0.31 (right testicle), respectively, all testicles were considered symmetric after the evaluations. In relation to the spermatic parameters, the mean±SD to the small Breed dogs were volume = 3.75±0.66, color = 50% (2/4) yellowish and 50% (2/4) white, aspect = 100% (4/4) aqueous, odor = 100% (4/4) suis generis, motility = 83.33±5.77%, vigor = 3.33±0.58, spermatic concentration = 380±62.41x$10^6$Sptz/mL, major defects = 15.67±5.13% and minor defects = 31.33±4.51%, while the medium-sized dog parameters were volume = 4.20±0.57, color = 60% (3/5) yellowish and 40% (2/5) white, aspect = 80% (4/5) milky and 20% (1/5) aqueous, odor = 100% (4/4) suis generis, motility = 70±4.18%, vigor = 3.50±0.55, spermatic concentration = 439±18.72x$10^6$Sptz/mL, only one animal was bellow 200x $10^6$Sptz/mL concentration, major defects = 21.40±5.18% and minor defects = 27.20±13.22%. The morphologic alterations presented in large incidence were strongly folded tail, absence of acrosome, medium piece defects, proximal and distal citoplasmatic droplet, besides the folded tail. The pathology of dog semen limits were not regulated by the MAPA (Brazilian Agricultural Government Ministry) as Bulls and Stallions were, however the Brazilian Animal Reproduction College has the recommendation of total defects maximum in 20%, and the major defects not exceeding 10%. Despite the high incidence of spermatic defects, all animals showed a good history of fertility, with breeding followed by positive pregnancies. It could be concluded that are some variances among the spermatic parameters between small and medium size breeds, some alterations could be explained by the testicle size, which is related to the animal size, therefore more animals should be analyzed in order to obtain an effective comparison among the reproductive parameters of different size dog Breeds.

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# Angiotensin-converting enzyme characterization before and after the cryopreservation of Gyr bulls semen

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The aim of this study was to characterize the angiotensin-converting enzyme (ACE) before and after the cryopreservation of Gyr bulls semen. Ejaculates from five sexually mature bulls were used. After semen collection, 1mL aliquot was removed for fresh semen analysis, the rest was submitted to cryopreservation process. Frozen semen sample was maintained in liquid nitrogen until thawing at 37ºC for 30 seconds. Both fresh and thawed semen were twice centrifuged with TALP for plasma and diluent withdrawal, respectively. The samples were submitted to western blot, immunocytochemistry and enzymatic activity. The means of the stained areas in immunocytochemistry, mean pixels of the protein bands and the enzymatic activity were submitted to analysis of variance (F test) and the differences between the groups were compared by Student test, considering 5% of significance. After western blot execution with anti-ACE monoclonal antibody it was possible to observe 100 kDa band in the semen extract protein of analyzed bulls. All the observed bands intensity decreased by 70% order ($p<0.05$) after cryopreservation. ACE periacrossomal location was demonstrated by immunocytochemistry and the stained area by fluorescent antibody decreased significantly ($P <0.05$) after cryopreservation. The enzymatic activity evaluated by hydrolysis the furanylacryloyl-L-phenylalanylglycylglycine (FAPGG) substrate was significantly lower ($p <0.05$) in cryopreserved semen in relation to the fresh also. It was concluded that cryopreservation process leads to intensity decreases in ACE bands, in the stained area by immunocytochemistry and enzymatic activity of ACE in semen from Gyr bulls.

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# Comparison of different surgical procedures to prepare teaser boars

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Besides the production, the swine industry is now facing an important pressure from the consumers to develop better systems to address the animal welfare issues and the use of antibiotic. The use of collective gestational pens by the European Union, have forced producers from different parts of the world, to adapt and to implement to their system. The heat detection procedure also needs some adaptations, since females are moving from individual crates to collective pens during the detection procedure. Therefore, the use of teaser boars could be an alternative to avoid the undesired pregnancy, and facilitating the heat detection procedure in collective pens. Therefore, the objective of this study was to evaluate different surgical procedures to produce boar teasers. For that, 39 male pigs (30-35kg) were used, (9-10 per technique).Animals were kept for 12 hours prior the surgery without food and water. Animals were sedated with tiletamine hypochloride and zolazepam hypochloride (Zoletil 50® - 5mg/Kg/IM); azaperone (Strenil® - 2mg/ kg/ IM) and local anesthesia was conducted at the incision line with lidocaine without vasoconstrictor (1,5 mg/Kg). The surgical procedures were: Vasectomized animal via the inguinal access (VI): Removing 2cm of the deferent duct between the last pair of teats. Tail-epididymectomy (CE): removal or the bilateral part of the epididymis tail, using the ventral access of scrotal sac. Vasectomized animal via scrotal access (VE): removal of 2 cm in the funicular portion of the deferent duct using a caudal access of the scrotal sac. To evaluate the surgical procedures, cortisol levels were measured 48hrs afer the procedures using radioimmunoassay, and at seven months of age a complete Breeding Soundness Examination test was conducted, including (testicular volume, and testosterone levels measurements). Data were analyzed using PROC MIXED (SAS®) with comparisons using a Tukey test, significance was considered if ($p<0.05$). During the Breeding Soundness Examination, no changes or reduction of libido was identified due to the surgical procedures. Only one animal from the VE group had spermiation. No differences were observed among the procedures and the control group. The cortisol concentrations were 3.29 ± 0.47 µg/dl (VI); 3.23 ± 0.47 µg/dl (CE); 3.48 ± 0.27 µg/dl (VE) and 3.29 ± 0.51 µg/dl (cont.) ($p=0.98$), testosterone levels 6.25 ± 1.92 ng/dl (VI), 5.92 ± 1.74 ng/dl (CE); 6.14 ± 1.82 ng/dl (VE) and 5.29 ± 2.02 ng/dl (cont.) ($p= 0.98$) and testicular volume 343.52 ± 35.29 $cm^3$ (VI); 463.05 ± 33.65 $cm^3$ (CE); 422.76 ± 35.29 $cm^3$ (VE) and 423.70 ± 37.20 $cm^3$ (cont.) ($p=0.12$). All the surgical procedures could be used to produce a teaser boar, since they have produced low levels of stress and have not caused any negative effect on the libido and the testosterone levels to the animals.

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# Effect of cyclophosphamide associated or not with propolis on the thickness of muscle stroma and height of the prostate secretory epithelium in pubescent guinea pigs

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The prostate in guinea pigs is an odd gland, with flanged edges and presence of a shallow groove (isthmus) on its dorsal surface (Gradela et al. 2013, *Biotemas* 26: 221-31). Prostatic secretions play an important role in semen production (Cepeda et al. 2006, *Int J Morphol* 24:89-97) and ovum fertilization (Carvalho 2005, Tese em Biologia Celular e Estrutural[1], UNICAMP[2]) and the gland is often affected by diseases associated with old age, such as benign prostatic hyperplasia (BPH) (Brianezi et al. 2006, *Braz J Vet Res Anim Sci* 43:65-73; Averbeck et al. 2010, *Rev AMRIGS* 54:471-7) and cancer (Srougi et al. 2008, *Rev Med* 87:166-77). Cyclophosphamide (CF) causes infertility due to overproduction of reactive oxygen species (Abd-Elmoaty et al. 2010, *Fertil Steril* 94:1531–34), while propolis has been recognized as a powerful antioxidant (Russo et al. 2006, *Life Sci* 78:1401–6). However, the effect of CF and propolis on the prostate remains unknown. This study aimed to evaluate the effect of CF alone or associated with ethanolic extract of propolis (EEP) on the thickness of muscle stroma (ST) and height of the secretory epithelium (EH) of prostate in pubescent guinea pigs. This study was approved by Comitê de Ética Experimental em Humanos e Animais[3] of UNIVASF (Protocol nr. 0001/160315). The guinea pigs were divided into six groups: CONT (0.10 ml/10 g of saline PV, N=6); CF100 (100 mg/Kg of CF, N=6); CF200 (200 mg/Kg of CF, N=5); EEP (50 mg/Kg of EEP, N=5); CF100+EEP (100 mg/Kg of CF + 50 mg/Kg of EEP, N=4); CF200+EEP (200 mg/Kg of CF + 50 mg/Kg of EEP, N=5). Treatments were performed once a week/5 weeks. IP administration route was used to saline and CF and gavage to EEP. In the sixth week, animals were anesthetized and euthanized, and the prostate was collected and submitted to routine histological processing with HE staining. ST (μm) was evaluated with 10x augmentation at four points corresponding to the maximum heights of the wall, not including the mucosa, and EH (μm) with 40x augmentation in the same areas using an Olympus BH-2 microscope and the Image Pro Plus 2.0 software (Media Cybernetics, Brazil). Data (mean + SEM) were evaluate by ANOVA with test hoc Student´s t-test (α=5%) (ASSISTAT 7,6 Beta). Results showed decreased ($P < 0.05$) ST and EH in groups CF100 (514.54 + 38.47 and 202.,51 + 9.,47, ST and EH, respectively), CF200 (563.57 + 33.29 and 217.34 + 3.36); CF100+EEP (490.32 + 24.40 and 192.75 + 12.93) and CF200+EEP (540.85 + 31.80 and 179.06 + 16.94) compared to CONT (660.64 + 34.05 and 252.35 + 22.41). In conclusion, cyclophosphamide alone or associated with propolis decreases the thickness of the stroma and the height of the epithelium in the prostate and it may be beneficial for the treatment of diseases that cause the increase of these parameters such as benign prostatic hyperplasia and prostate cancer.

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[1] Cell and Structural Biology Thesis; [2] Universidade Estadual de Campinas – University of Campinas; [3] Committee on Experimental Ethics in Humans and Animals; [4] Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazilian Council of Scientific and Technological Development.

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# Effect of cyclophosphamide associated or not with propolis on the projection of the folds of the tunica mucosa and height of epithelial cells of vesicular gland in pubescent guinea pigs

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Guinea pigs have even, tubular, craniocaudally elongated vesicular glands, with smooth surface and consistent appearance located at the origin of the pelvic urethra, being the largest accessory glands (Gradela et al. 2013, *Pesq Vet Bras* 33:942-8), whose secretions are essential for reproduction (Wong et al. 2001, *Reprod Toxico*l 15:131-6). Cyclophosphamide (CF) causes gonadal failure with oligozoospermia or azoospermia and even irreversible infertility (Freire et al. 2006, *Rev Bras Reumatol* 46:12-20), however, its effect on the vesicular glands has not been studied, as well as the effect of natural antioxidants such as propolis, which improved the function and integrity of sperm DNA (Safarinejad; Safarinejad 2009, *J Urol* 181:741–51). This study evaluated the effect of CF alone or associated with ethanolic extract of propolis (EEP) on the projection of the folds of the tunica mucosa (FP) and height of epithelial cells (EH) of vesicular gland in pubescent guinea pigs. This study was approved by Comitê de Ética Experimental em Humanos e Animais[1] of UNIVASF (Protocol nr. 0001/160315). Guinea pigs were divided into six groups: CONT (0.10 ml/10 g of saline PV, N=6); CF100 (100 mg/Kg of CF, N=6); CF200 (200 mg/Kg of CF, N=5); EEP (50 mg/Kg of EEP, N=5); CF100+EEP (100 mg/Kg of CF + 50 mg/Kg of EEP, N=4); CF200+EEP (200 mg/Kg of CF + 50 mg/Kg of EEP, N=5). Treatments were performed once a week/5 weeks. IP administration route was used to saline and CF and gavage to EEP. In the sixth week, animals were anesthetized and euthanized, and the prostate collected and submitted to routine histological processing with HE staining. FP (μm) was evaluated at six fields per animal using 10x augmentation, while EH was determined in 40x augmentation at the same points using an Olympus BH-2 microscope and the Image Pro Plus 2.0 software (Media Cybernetics, Brazil). Data (mean + SEM) were evaluated by ANOVA with test hoc Student´s t-test (α=5%) (ASSISTAT 7,6 Beta). Results showed marked decrease ($P < 0.05$) of FP in animals treated with CF200+EEP (954.05 + 153.11) compared to CONT (1628.83 + 211.58) and EH in animals treated with CF200 (149.81 + 4.59); CF100+EEP (128.64 + 18.70) and CF200+EEP (108.90 + 14.25) compared to CONT (212.00 + 13.08). In conclusion, cyclophosphamide decreases the projection of the folds of the tunica mucosa and height of the secretory epithelium at the 200 mg dose and height of the epithelium in both doses when associated with propolis, confirming its toxic effect on vesicular cells.

Acknowledgement: CNPq[2], UNIVASF.

[1] Committee on Experimental Ethics in Humans and Animals; [2] Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazilian Council of Scientific and Technological Development.

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# Effect of diet containing gossypol on testicular histology in Santa Inês rams

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The gossypol is a Polyphenolic pigment present in all parts of the cotton seed plant and its derivatives. Earlier experiments showed side effects in caused by feed containing high percentage of cottonseed and claim that gossypol might affect reproductive system, causing abnormalities in sperm and subsequent infertility. The objective of this work was to evaluate the effects caused by a diet rich in cottonseed gossypol on testicular histology and seminal parameters of hair sheep gossypol Santa Inês Breed. For that, 22 rams, same age, which were divided into 2 groups, being the animals confined in individual pens. The gossypol Group (Gg = 15), supplemented with 500 g of cottonseed and the control group (Gc = 7), supplemented with 500 g of corn for 104 days and subjected to semen evaluation before (7 days), during (at intervals of 15 days) and after (10 days) the period of supplementation. The cottonseed used was analyzed to the levels of free gossypol by CBO laboratory (Campinas – SP), founding 3,39 g/Kg of gossypol of cottonseed. No statistical difference was observed in sperm parameters between Gg and Gc during all the experiment. After the end of the supplementation period, 5 animals (2 from Gc and 3 from Gg group) were submitted to unilateral orchiectomy. The material was used for conducting comparative Histopathological examination in historesin, No difference was seen in the diameter of seminiferous tubule, between control and gossypol group, however it showed a statistical difference in height of the seminiferous epithelium, having a larger epithelium in the Gg compared with control group. The height of the seminiferous epithelium is an effective feature for assessment of sperm production in mammalians, however in this study there was no significant difference in sperm parameters between control and treatment group. Our results showed that supplementation with cottonseed did not influence sperm quality, while the testicular morphology despite having demonstrated a significant difference in height of the seminiferous epithelium, did not affect sperm quality in vitro. Further studies may be conducted by increasing the duration of treatment to see whether there may be a cumulative effect.

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# Effect of the selenium supplementation on the ovine spermatic DNA integrity

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The present study aimed to evaluate the effects of different concentrations of selenium (Se), at Ovine nutritional supplementation, on its Spermatozoa DNA integrity. Thirty male ovine were used, aging from 18 to 24 months, housed in an intensive system of creation, divided in five experimental groups, control group (CG; n=6), with supplementation at mineral salt without Se added, G1 (n=6), same mineral salt mixed with 5mg of Se (Sodium selenite)/Kg, G2 (n=6), control mineral salt mixed with 10mg of Se/Kg, G3 (n=6), control mineral salt mixed with 15mg of Se/Kg, and, G4 (n=6), control mineral salt mixed with 20mg of Se/Kg. In every group was respected an adaptation period of 14 days, following a treatment time of 56 days. The samples were obtained by electroejaculation, before starting treatment and after the end of the treatment, totaling 30 ejaculates per experimental group. The DNA fragmentation detection of the semen samples were performed using the Comet Assay, adapted for ram semen (Martins et al. Ani. Reprod., v.10, 697-703, 2013). The experimental were implemented using a Latin Square 5x5, i.e., five treatments and five experimental times. The dates were evaluated using GLM process (SAS, 2009). The mean differences were compared using Tukey`s test at 5% of significance. The mean percentage of DNA integrity of CG were 12.34±2.21%a, G1 = 6.60±0.59%b, G2 = 6.38±0.71%b, G3 = 6.74±0.64%b, and, G4 = 6.52±0.51%b ($P < 0.05$). The CG had shown a higher percentage of DNA fragmentation when compared to the other groups, but no statistical difference were observed among the Se supplemented groups. According the results presented in this study, the Selenium supplementation (at the presented experimental conditions) had shown a benefic effect on the DNA integrity preservation at ovine spermatozoa.
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# Effect of different doses of cyclophosphamide associated or not with propolis on the tubular diameter and height of cells of the epididymal duct in pubescent guinea pigs

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Sperm maturation occurs in the body of the epididymis (Hermo; Robaire 2002, *The epididymis*: from molecules to clinical practice, p.81-102), thus, changes in its function cause infertility (Jarvi 2012, *Fertil Steril* 97:1298). Cancer in young adults increases every year and the success of treatments has made studies on the impact on future fertility relevant (Anders; Souza 2009, *Cienc Cuid Saude* 8(1):131-7). Cyclophosphamide (CF) causes oligozoospermia and even irreversible infertility (Freire et al. 2006, *Rev Bras Reumatol* 46:12-20), however, its effect on the epididymis is still unknown, as propolis, which has a protective effect on human sperm (Russo et al. 2006, *Life Sci* 78:1401–6). This study evaluated the effect of CF alone or associated with ethanolic extract of propolis (EEP) on the tubular diameter and height of the cells of the initial segment (distal portion of head and body) and tail of the epididymis in pubescent guinea pigs. This study was approved by Comitê de Ética Experimental em Humanos e Animais[1] of UNIVASF (Protocol nr. 0001/160315). The animals were divided into six groups: CONT (0.10 ml/10 g of saline PV, N=6); CF100 (100 mg/Kg of CF, N=6); CF200 (200 mg/Kg of CF, N=5); EEP (50 mg/Kg of EEP, N=5); CF100+EEP (100 mg/Kg of CF + 50 mg/Kg of EEP, N=4); CF200+EEP (200 mg/Kg of CF + 50 mg/Kg of EEP, N=5). Treatments were performed once a week/5 weeks, using IP administration route to saline and CF and gavage to EEP. In the sixth week, guinea pigs were anesthetized and euthanized, and the right epididymis was collected and submitted to histological processing with HE staining. The tubular diameter of the epididymal duct (TD, μm) was evaluated on 10x augmentation and the height of the epithelium (EH, μm) on 40x augmentation using an Olympus BH-2 microscope and the Image Pro Plus 2.0 software (Media Cybernetics, Brazil). A total of 24 measurements per segment, 72 measurements per animal and 216 measurements per group were performed. Data (mean + SEM) were evaluated by ANOVA with test hoc Student´s t-test (α=5%) (ASSISTAT 7,6 Beta). Results showed decreased ($P < 0.05$) TD in the initial segment of the epididymis in groups CF200 (1085.48 + 84.51), CF100+EEP (919.65 + 131.31) and CF200+EEP (969.50 + 72.33) and in the tail only in CF200 (1244.94 + 69.79) compared to CONT (1491.64 + 119.81 and 1619.15 + 129.16, initial and tail, respectively). There was EH decrease ($P < 0.05$) in the initial segment in groups CF100 (480.46 + 16.68), CF200 (618.54 + 74.07), EEP (675.45 + 62.47), CF100+EEP (554.68 + 92.24) and CF200+EEP (467.73 + 4.00) and in the tail in EEP (486.79 + 26.91) and CF200+EEP (233.59 + 24.52) compared to CONT (913.31 + 83.01 and 651.80 + 57.10, respectively). In conclusion, cyclophosphamide was toxic specially for the initial segment of the epididymis, and propolis, in the dose tested, had no protective effect against this action, increasing this effect in some cases.

Acknowledgement: CNPq[2], UNIVASF.

[1] Committee on Experimental Ethics in Humans and Animals; [2] Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazilian Council of Scientific and Technological Development.

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# Heat shock effect on redox balance and acrosomal reaction in Holstein bulls spermatozoa

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Heat stress affects spermatogenesis and compromises sperm quality. However, the direct effect of elevated temperature on spermatozoa is not fully understood. The natural or artificial insemination of animals under heat stress condition, exposes the spermatozoa to temperatures above of physiological in the female reproductive tract, which can compromise the spermatozoa quality and fertilization capacity. Therefore, the present work objective was determine the effect of heat shock (in vitro heat stress) on the acrosomal reaction and the production of reactive oxygen species (ROS) in bovine spermatozoa. It has already been shown that protein kinase C (PKC), involved in the regulation of acrosomal reaction, is also activated by oxidative stress. Thus, PKC role was evaluated in this process. Straws from five Holstein bulls (pool of 2 bulls/replica) were thawed at 37°C and subjected to Percoll gradient. The pellet was diluted in SP-TALP at 2.5x106sptz/mL. After dilution, spermatozoa were incubated for 4 hours according to the following treatments: control 0hr (non-incubated), 35°C (testicular temperature control), 38.5°C (body temperature control) and 41°C (heat shock). The acrosomal membrane integrity (FITC-PSA 100 µg/mL for 10 min) and ROS (CellROX® Green 5 µM for 30 min) were analyzed by flow cytometry (BD FACSCALIBUR) and the data obtained were analyzed by the FlowJoLLC program V.10. Phosphorylated PKC was assessed by immunofluorescence using the rabbit antibody anti-phosphoPKC (1:100) and the secondary antibody Alexa Fluor-555 anti-rabbit (1:200). DNA stained with Hoechst 33342 (5 µg/mL). The PKC (15 µM bisindolylmaleimideII: BIMII) inhibitor was used to determine the PKC role on spermatozoa acrosomal reaction subjected to heat shock. Data were analyzed by ANOVA (parametric data) and Wilcoxon test (non-parametric data) using SAS JMP Statistical Discovery 11.0. The incubation of spermatozoa at 35°C (67.9 + 12.3 %), 38°C (81.7 + 12.3 %) and 41°C (86.9 + 12.3 %) increased ($p<0.05$) ROS production when compared to control group 0h (8.9 + 12.3 %). However, the magnitude of this increase on ROS production was higher ($p<0.01$) in spermatozoa incubated at 41ºC when compared to 38°C. Similarly, incubation of spermatozoa at 38°C (30.5 + 1.45 %) and 41ºC (51 + 1.45 %) induced ($p<0.001$) acrosomal reaction when compared to 35°C (22.2 + 1.45 %) and 0hr (7.4 + 1.45 %). Inhibition of PKC with BIMII did not affect acrosomal reaction at any temperature evaluated, suggesting that PKC is not involved in this process. To confirm this result, the phosphorylated form of PKC was evaluated. Immunofluorescence results showed that sperm incubation at 35°C (24232,838 + 926,10883), 38.5°C (21479,378 + 926,10883) and 41°C (21452,572 + 926,10883) increased ($p<0.01$) phosphorylation of PKC when compared to group 0h (18249,092 + 926,10883). However, phosphorylation of PKC did not differ between spermatozoa incubated at 38.5°C and 41°C. In conclusion, the increase in temperature results in a higher amount of ROS and acrosomal reaction by a pathway independent of PKC.

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# Effect of bovine oviductal fluid obtained in the follicular or luteal phase of the estrous cycle on the ram sperm function and capacitation

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Despites advances in the use of *in vitro* capacitating substances in other species, in small ruminants the presence of estrous sheep serum is essential to achieve ideal IVF rates. In attempt to mimic the sperm capacitation process that occurs *in vivo*, this study aimed to evaluate the effect of oviductal fluid on ram sperm function and *in vitro* capacitation. Oviducts from cows were collected in regional slaughterhouses and classified as follicular or luteal phase. Then the oviductal fluid obtained was stored at -20 ºC until its use. Subsequently, semen was collected from three rams simultaneously forming a pool. After sperm selection (swim up), sperm were incubated in different media, using Fert-TALP (positive control; commonly medium used for IVF) as the base medium (1) or with the following changes: Fert-TALP (2) without capacitating substance (negative control), (3) without capacitating substance and supplemented with 10% of oviductal fluid in the follicular phase and (4) without capacitating substance and supplemented with 10% of oviductal fluid in the luteal phase. The sperm were incubated at 38 ºC in 5% CO2 and the parameters of sperm kinetics, plasma membrane (PM) integrity and capacitation status were evaluated after 0, 2, 4, 6, 18 and 24 h. The variables were subjected to ANOVA and Tukey analysis ($P < 0.05$). There was no difference ($P > 0.05$) among all treatments during incubation for PM integrity. At 2 h and 4 h of incubation, the negative control showed lower ($P < 0.05$) values in the velocimetric parameters (VSL, VAP, LIN, STR, WOB, ALH e BCF) and progressive motility. Media supplemented with oviductal fluid (both phases) were greater in these parameters, compared to the negative control. At 6 h, there was no difference ($P > 0.05$) among all treatments. At 4 h and 24 h, the negative control showed higher ($P < 0.05$) capacitation rate compared to other groups. At 18 h, the positive control showed higher ($P < 0.05$) values in the velocimetric parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH e BCF) compared to other groups. At 18 h and 24 h, sperm incubated with oviductal fluid in the follicular phase presented higher ($P < 0.05$) acrosome-reacted cells compared to other groups. In conclusion, supplementation with 10% of oviductal fluid regardless the phase of estrous cycle to the IVF medium, promotes an improvement in the velocimetric parameters and sperm kinetics for up to 4 h of incubation. This strategy can be considered as a possible alternative because it presents lower cost compared to defined and synthetic additives and, due the simplicity of the technique in relation to the extraction and purification process, necessary to obtain estrous sheep serum.

Financial support: FAPERJ, CNPq.

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# Effects of nutritional supplementation with polyunsaturated fatty acids on seminal quality and fecundity of Nelore males classified for residual feed intake

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Nutrition has great influence on sperm production and quality. Thus, diets with polyunsaturated fatty acids (PUFAs) may exert a positive effect on the seminal quality of bulls. In addition to diet, food efficiency is another important factor for genetic breeding programs, and the indicator "residual feed intake" (RFI) may have effects on the reproductive performance of bulls. Within this context, the objective of the present study was to evaluate the effects of long-term dietary supplementation with PUFAs (200g/animal/day Megalac®) in Nelore bulls classified for RFI on the quality of frozen semen and fertility (evaluated by *in vitro* production of embryos - IVP). Twenty-four young bulls from the Animal Science Institute (IZ) of Sertãozinho, SP, were used. The animals remained in performance test from 7 months to 12 months of age for determination of RFI, being classified as low RFI (< average - 0.5 standard deviation of the mean (SD)) and high RFI (> average + 0.5 SD). The animals were then divided into 4 treatments (n=6 bulls per treatment): 12 low RFI animals receiving control diet or supplemented with AGPs, and 12 high RFI animals receiving control diet or supplemented with AGPs. Experimental diets were isoproteic and were formulated to meet the nutritional requirements of young bulls growing on pasture (*Brachiaria brizantha* cv. Marandu pasture in continuous stocking). The animals were 14.3 ± 0.13 months old at the beginning of the experiment and initial weight was 389.5 ± 5.43 kg. The semen of all 24 animals was frozen at the end of work (1 single ejaculate of each bull) when they reached 24.6 ± 0.13 months of age. Samples of thawed semen were submitted to computerized analysis of sperm kinetics (CASA, Hamilton Thorne Research, IVOS-14, USA) and IVP. The parameters evaluated were: total motility (TM%), progressive motility (PM%), rapid cells (RAP%), lateral displacement of sperm head (ALH, μm), beat frequency (BCF, Hz), cleavage rates (%) and rates of embryonic development to the blastocyst stage (%). The data were submitted to analysis of variance by SAS proc MIXED with significance of $P < 0.05$. No differences were observed between the treatments for the post-thawed semen analysis by CASA, and the means between the 4 groups were 75.5% ± 6.5 MT, 57.2% ± 5.8 MP, 71.5% ± 6.6 RAP, 5.8 ± 0.2 μm ALH and 26.2 ± 1.47 Hz. There were also no differences between treatments for cleavage rates and blastocyst rates, and the means between the 4 groups were 78.2% ± 2.1 (3922 cleaved oocytes, out of a total of 5014 matured in 12 replicates) and 35.8% ± 2.1 (1799 embryos produced out of a total of 5014 matured oocytes), respectively. It is concluded that the supplementation with sources of AGPs for bulls classified as high and low RFI did not influence the post-thawing sperm characteristics and the fecundity of the semen evaluated by the *in vitro* production of embryos. Thus, further studies are needed to determine the real influence of AGPs and RFI on sperm quality and on IVP.

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## *In vitro* effects of bovine semen cooling and cryopreservation

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The FTAI protocols, when well implemented, result on high percentages of ovulations (70 to 90%), occurring in a window of approximately 48 hours. However, the pregnancy results show a large amplitude (30 to 65%), part of these results may also be in response of the semen quality used. With the hypothesis that refrigeration causes less damage to sperm than cryopreservation, an in vitro experiment was carried out to compare the effects of refrigeration and cryopreservation of bovine semen. Eighteen ejaculates of 10 Nelore bulls were used. After the semen collection, each ejaculate was fractionated in two equal aliquots on 50 mL conical tubes, pre-diluted in Botubov® medium (BB, Botupharma/Brazil). One sample was subjected to refrigeration (5ºC) for 48 hours and the other was cryopreserved. Both semen samples were diluted (40x106 sperm/straw), filled in 0.5 mL French straws (IMV® Technologies/France) and placed into Botuflex® (Botupharma/Brazil) refrigeration boxes for evaluations at the 24 , 36 and 48 hours of refrigeration. Another 10 straws were submitted to the cryopreservation process on an automated freezing system (TK 3000®/Brazil). Post-refrigeration and cryopreservation semen samples were evaluated in duplicate by computerized system of sperm movement (SCA program - Sperm Class Analyzer, Spain), sperm abnormalities by differential interference contrast chamber (DIC), membrane integrity evaluation and acrossomal, mitochondrial membrane potential by epifluorescence microscopy with the fluorescence probes Hoescht 33342, Propidium iodide, FITC-PSA and JC-1. The fixed effects of the cooling times were evaluated, besides the effects of the cryopreservation vs refrigeration evaluated, using PROC GLIMMIX of the SAS. After 24 hours of refrigeration at 5ºC, the semen presented a decrease on progressive motility (from 69.59 ± 3% pre-refrigeration to 50.36 ± 5%) and on percentage of spermatozoa with intact plasma and acrossomal membranes and high mitochondrial potential (PIAIA from 55.7 ± 5% to 33.7 ± 5%). Any other alterations were found up to 48 hours at 5°C. When comparing the sperm characteristics of cooled semen for 48 hours at 5ºC (REF) with cryopreserved semen (CRIO), it was observed that the refrigeration presented higher values only for VCL (CRIO = 106.04 ± 6 and REF = 152.5 ± 5 μm/s); (CRIO = 69.3 ± 4 and REF = 50.4 ± 1%), WOB (CRIO = 79.4 ± 4.8 and REF = 60.6 ± 1%), and cryopreserved semen showed higher values for BCF (CRIO = 23.6 ± 0.48 and REF = 13.46 ± 4.42). However, the values of total motility, progressive motility, percentage of fast cells, VSL, VAP and percentage of cells with plasma membranes integrity, acrossomal and high mitochondrial potential were similar between refrigerated and cryopreserved samples.

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# Influence of seasonality in the physiological parameters and buffalo seminars in the West Region of Pará

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The presente study was undertaken with the objective of evaluating the influence of climatic variables on parameters of ejaculates of Murrah buffalo bulls raised in humid tropical Amazonian climate, evaluate some physiological parameters such as heart frequency (HF), respiratory frequency (RF), rectal temperature (RT) and superficial temperature (ST) during the rainy and non-rainy periods in Amazon region. Five buffaloes (n=5), average of age 2.5 ± 0.5 years and body weight 450.0 ± 35.5 kg were selected from a group of 12 adult bulls. In order to characterize the study area, a mini-station was installed at the experiment site to measure ambient temperature, relative humidity, rainfall, Solar radiation and wind speed. The experiment was carried out in the municipality of Santarém, Meso-region of Lower Amazonas, West of Pará State, with experimental animals kept in lairage regime. The animals had constant access to fresh and clean water, as well as mineral salt ad libitum in a trough. The sanitary control was carried out according to pre-established criteria for the species. The experimental period was divided into two phases: rainy season (RS), from February to May, and non rainy season (nRS), from August to November 2016. <span style="font-family:times new roman,serif; font-size:12.0pt; line-height:150%; mso-bidi-font-family:" times="" new="" roman";="" mso-bidi-theme-font:minor-bidi;="" mso-fareast-font-family:"times="" mso-fareast-language:pt-br"="">The evaluations physical and morphological of the semen samples were performed with fresh semen, immediately after each collection. The immediate analyzes pertinent to the physical and morphological characteristics of the ejaculates were carried out and corresponded in the RS volume of 3.4 ± 2.0 mL; mass activity of 4.4 ± 0.5; motility of 80.4 ± 5.6%; vigour of 4.4 ± 0.4; concentration of 657.300 ± 237.865,1 x$10^6$sptz / mL; major defects of 9.0 ± 2.6%; minor defects of 11.2 ± 3.9%; total defects 2of 0.2 ± 5, 3% and sperm plasma membrane integrity (SPMI) 84.8 ± 5.6%, where as in the nRS, the results were 4.0 ± 2.1 mL; mass activity of 3.0 ± 1.0; motility of 56.2 ± 13.4%; vigour of 3.0 ± 1.0; concentration of 586,000 ± 291,925.9 x$10^6$sptz / mL; major defects of 20.8 ± 9.9%; minor defects of 27.5 ± 6.3%; total defects 48.3 ± 9.3% and SPMI of 57. 9 ± 12.4%. Furthermore, it was observed a statistical difference (P<0,01) for the parameters mass activity, motility, vigour, major defects, minor defects, total defects and sperm plasma membrane integrity between the both periods. The data on heart frequency, surperficial temperature (head, back, groin and scrotal pouch) showed statistical difference between both periods (P<0,01). However, the relative data, for RF and TR did not show any difference between both periods (P>0,01). The HF data were 54 ± 10.9 bpm and 48 ± 11.8 bpm; RF 22 ± 6.8 mpm and 23 ± 6.7; RT, 38.3 ± 0.8 $^oC$ and 38.2 ± 0.8 $^oC$; ST, in the head region (STH) were 33.3 ± 2.5 °C and 36.2 ± 2.4 °C; in the back region (STB) 33.6 ± 2.1 $^oC$ and 35.1 ± 2.1 $^oC$; in the groin (STG) 32.3 ± 2.2 $^oC$ and 34.6 ± 2.2 $^oC$; and scrotal pouch (STS) 30.3 ± 2.0 $^oC$ and 32.3 ± 2.0 $^oC$, RS and nRS, respectively. The physiological parameters that were influenced by the non-rainy period, compared to the rainy season were heart rate and surface temperature, of head, back, groin and scrotal pouch (p<0,01).

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# Embryo production using epididymal sperm submitted to different selection methods and their influence on the embryo sex

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Epididymal spermatozoa and their use in assisted reproductive technologies (RT), such as IVP, have an important role in the multiplication of genetic material from sires that die suddenly and/or have acquired reproductive failure. However, in order to establish an appropriate procedure to use those sperm in embryos IVP, a better knowledge about their physiological behavior facing events involved IVP, such as different methods of sperm selection, is needed. The aims of this study were evaluated different methods of sperm selection for IVP procedures and their influence on the embryo sex. A pool of epididymal (EP) and ejaculated (EJ) cryopreserved spermatozoa, recovered from seven Gir bulls through eletroejaculation followed by bilateral orchiectomy were used. The pool of the two groups were selected by three different methods: Percoll gradient 45%90% (GE Healthcare Bio Science, Uppsala, Sweden), PureSperm gradient 40%80% (Nidacon Laboratories AB, Gothenborg, Sweden) and wash in Tyrode's Albumin Lactate and Pyruvate (SpTALP). Four groups were formed: ejaculated on Percoll (EJ-P), control group; epididymal on Percoll (EP-P); epididymal on PureSperm (EP-PS) and epididymal on SpTALP (EP-T). After selection, sperm samples were co-incubated with a total of 759 cumulus-oocyte-complexes (COC's) in fertilization medium in 7 replicates experiment. Embryos were evaluated two days (D2), six days (D6), seven days (D7) and eight days (D8) after fertilization and then, embryos were storage for sex evaluation. Embryo sexing was performed according to Sousa et al (Theriogenology, 90, p.25, 2017), by PCR technique. Embryo rates data were analyzed using Chi-square (mean±SD; $P<0.05$) and sexing date by Wilcoxon using Prophet 5.0 (mean±SD; $P<0.05$). Cleavage rates (D2) and blastocyst rates on D6 were higher for EP-PS group (80% and 48%, respectively) than the other groups. At the D7 and D8, blastocyst rates were similar ($P>0.05$) between EP-P (D7 54%; D8 55%) and EP-PS (D7 37%; D8 37%) groups. EP-T and EJ-P groups showed similar blastocyst rate in D6 (27%; 32%), D7 (37%; 44%) and D8 (37%; 45%), which was lower than the others groups that used EP sperm. Male and female embryos showed differences only in EP-P group (38% and 62%, respectively). For others methods of sperm selection differences were not significant ($P>0.05$). These results suggest that PureSperm and Percoll were the better methods of sperm selection, for embryos production using EP. Furthermore, the relation between male × female embryos only showed differences when Percoll gradient was used, resulting in more female embryos. Financial support: CNPq and Embrapa.

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# How much sperm can we collect from the epididymides of Crioulo stallion??

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Stallion sperm spermatozoa capable of fertilization can be harvested from the cauda epididymis and be used for artificial insemination (Barker & Gandier, 1957) of a limited number of mares or for cryopreservation, being the last chance to obtain viable doses from a valuable stallion. The expansion of Crioulo breed is growing fast over the years and still little research is done regarding reproductive parameters, especially with epididymal stallion sperm. The aim of this experiment was to evaluate number of total spermatozoa collected during harvesting Crioulo stallions epididymides. In order to perform the evaluation, 15 Crioulo stallions, aging 4-18 years, were submitted to elective bilateral orchiectomy. The epididymis (n=30) were washed wish ringer lactate and the cauda of epididymis was isolated. The connective tissue was carefully dissected and the cauda was straightened, as long as possible. The sperm harvesting was performed by a retrograde flushing technique and the smaller parts of the cauda by a flotation technique, with skim-milk based extender. Sperm concentration was measured by computer-assisted semen analyses (CASA - AndroVision®, Minitub, Tiefenbach, Germany) and total sperm count was calculated from volume (mL) of spermatozoa with extender x sperm concentration (per mL). Descriptive stastistic analyses was performed with the program Statistix 9® (Statistix. Statistix 9 for Windows. Analytical Software, Tallahassee, FL, USA, 2008).
Similar with what happens with ejaculates, the total sperm count presented a large variability among stallions. Minimal sperm count was 1.225x106; maximal was 12.500x106 and medium was 4.122x106 spermatozoa per epididymis. Minimal sperm count was 2.450x106; maximal was 22.500x106 and medium was 8.245x106 spermatozoa per pair epididymis of the same stallion. The epididymides evaluated from the same stallion had similar size, even so, it was observed a variation of up to 45% in the total sperm count, when compared spermatozoa collected from each epididymides. According to Amann et al. (1979), the two cauda epididymides of a normal, sexually rested, adult stallion can contain 54x109 sperm or approximately 61% of the sperm in the excurrent duct system. Bruemmer (2006) reported that 5 to 25 breeding doses, consisting of 800x106 sperm per dose, are typically obtained from a given stallion, resulting in 4.000-20.000x106 spermatozoa harvested from the two epididymides and 2.000-10.000x106 per epididymides. In the present study with only Crioulo breed horses, large variations in the total spem recovery were also reported. According to the literature and the present findings, it is possible to affirm that the variability between individuals is very evident. If you considered the variation of total sperm count between 4.000 to 20.000x106 (Bruemmer, 2006) it is observed up to 5x times difference, if considered the variation between 2.450x106 and 22.500x106 it is observed almost 10x times the difference.In conclusion, the number of spermatozoa that can be collected from adult Crioulo stallions had a huge individual variation, from minimal 2.450x106 up to maximal 22.500x106 spermatozoa per stallion, including both epididymides.

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# Sperm subpopulations defined by sperm head morphometry and the relationship with fertility *in vivo*

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The presence of subpopulations of a sample semen, defined by the sperm head morphometry to reduce the in vitro fertility. (Santos, et al. 2016. VI ISABR), But no studies have evaluated this effect on fertility in vivo. The objective of the present study was to identify and compare sperm subpopulations (SBP) distributed according to the sperm morphometry in the semen of bulls with high and low fertility. Seven batches of 3 bulls Aberdeen Angus of high fertility (FA) determined by the fertility score (0,536; 0,495 e 0,284) and pregnancy rate (PR: 54.32 ± 1.60%) and 6 batches of low fertility bulls 3 (FB) with negative fertility score (-1.579, -1.758 and -2.447) with lower PR (41.25 ± 3.47%). Data from 29,939 IATF for the group of FA and 3,259 IATF for the bulls of BF. The semen batches were thawed (37 ° C / 30 sec) and a sample was diluted and fixed in formal saline 4% (37 ° C), one drop (2.5 µl) being placed between slide and cover slip. Images of at least 200 spermatozoids were obtained from each batches and analyzed individually 2,841 spermatozoa using a phase contrast microscope (1000x, Eclipse E200, Nikon®) and a digital video camera (Eurekam 5,0). Sperm head morphometry was automatically analyzed using Image J. software. All parameters have been activated in the measurement set. Data were analyzed by multivariate statistics using the SAS FATSCLUS tool, using the k-means method. PROC MIXED was used to make inferences of each parameter evaluated, considering the effects of fertility, SBP and fertility interaction x SBP, in addition to the random effects of animal and residue. In case of significant effects, the Tukey Test (5%) was adopted. Four SBPs were defined by the area (pixels2), perimeter (pixels), height (pixels) and circumference (pixels) of the spermatic head. The area was the main variable that explained the variance of SBP (R2 = 0.83). SBP1, 2, 3 and 4 varied in the head area in both groups (P <0.05). The spermatic head area for SBP1, SBP2 and SBP3 was similar among the AF groups (SBP1 = 27092 ± 606.87, SBP2 = 26199 ± 611.72, SBP3 = 29719 ± 606.08 pixels2) and BF (SBP1 = 27373 ± SBP2 = 27410 ± 606.07, SBP3 = 29629 ± 623.22 pixels2, however, SBP4 was lower (P <0.05) for the AF group (21564 ± 728.93 pixels2) than for the BF (27000 ± 616.71 pixels2). It was also noticed a higher frequency (P <0.05) of SBP4 for BF semen (6.5%) than for AF (3.32%). SBP4 is characterized by being the population of cells that have the smallest area and the largest perimeter, height and circularity of the other SBP (P = 0.05). Despite the smaller area for AF, the algorithm that forms the SBPs included other parameters of the morphometry to characterize the SBP4 that should be considered. The data suggest that there are changes in sperm DNA that lead to changes in sperm head morphometry. It is concluded that the higher SBP4 frequency influences on fertility.

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# Prolonged use of supplementation with fatty acids in Nelore bulls raised in pasture system improves the frozen semen?

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The production and quality of semen are influenced by several factors, especially nutrition. Thus, diets with polyunsaturated fatty acids (PUFA) have an effect on the reproductive performance of males. Within the context, the objective was to study the effects of prolonged use of supplementation with PUFA (200g/animal/day Megalac®) on the quality of frozen semen of Nellore bulls in pasture. Twenty-four young bulls from the traditional herd of the Animal Science Institute (IZ) of Sertãozinho, SP, were used. The animals were divided into 2 groups: with (n=12) and without supplementation with PUFA (n=12). The animals were kept in Brachiaria brizantha cv. Marandu pasture in continuous stocking during the experimental period. The animals were 14.3 ± 0.13 months of age at the start of the experiment and reached 24.6 ± 0.13 months of age at the end and initial weight of 389.5 ± 5.43 kg. Animals from the group supplemented with PUFA received 1 kg/animal/day, the control group received supplementation without PUFA of 1.25 kg/animal/day. Supplements were initially formulated to meet the nutritional requirements of growing young bulls (NRC, 2000; level 2), according to the nutrient content of the pasture, and for them to be isoprotic. For this, the animals in the control treatment received 0.25 kg supplement / animal / day more than the animals treated with PUFA. Samples of thawed semen were submitted to computerized analysis of sperm kinetics (CASA, Hamilton Thorne Research, IVOS-14, USA). The material was previously homogenized and evaluated in a Makler chamber preheated at 38°C, five random fields were observed for each sample and a minimum number of 150 cells per field. The parameters evaluated were: total motility (TM%), progressive motility (PM%), rapid cells (RAP%), path velocity (VAP, μm/s), straight velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), lateral displacement of sperm head (ALH, μm), beat frequency (BCF, Hz), straightness (STR%) and linearity (LIN%). The data were submitted to analysis of variance by SAS proc MIXED with significance of $P < 0.05$. The post-thawed semen evaluation, the sperm velocity analyzed by CASA was higher for bulls supplemented with PUFA (82.0±1.8, 68.6±1.2 and 132.0±3.9 for VAP, VSL, and VCL, respectively) in relation for bulls without AGPs (76.2±1.8, 64.2±1.2 and 122.3±3.9, respectively) ($P<0.05$). Despite these differences in sperm velocity, evaluations are needed in conjunction with other seminal tests, especially in natural service and artificial insemination breeding programs, to determine the actual influence of PUFA on sperm quality.

Acknowledgment: CNPq (process 447036/2014-9) and Fapesp (process 2015/06733-5).

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# Resistance Doppler index of stallions treated with human chorionic gonadotrofin (hCG)

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The use of hCG may stimulate the steroidogenic response of Leydig cells and it is used in insufficient testosterone production and cryptorchidism (LIMA et al., Brazilian Journal of Vet and Animal Science, v.37, p.52-55, 2000). Analysing these factors, this research aimed to evaluate possible changes in the resistance index (RI) of the testicular artery and in parameters of the reproductive behavior in the semen collect from fertile stallions after hCG administration. The study was developed during two cycles using four Mangalarga Marchador stallions at the four seasons (January, April, July and October 2016), in Rio de Janeiro, Brazil. The animals were separated in two groups: GI (n = 4), administering 5ml of saline and GII (n = 4), administering 5000 IU of hCG (5ml) (Chorulon® - MSD Animal Health, Brazil), endovenous (ev), in bollus. In each month, the procedures were subdivided into two cycles (C1 and C2) during six days (D) each and with a three days off between cycles. On odd days two stallions were evaluated (one from each group) and on even days the others two remaining stallions from each group. For C2, the crossover experimental rotational scheme was applied, where: CI = animal 1 (GI) and 2 (GII) evaluated on days D1, D3 and D5 and animal 3 (GI) and 4 (GII) on D2, D4 and D6; CII = animal 1 (GII) and 2 (GI) evaluated on days D1, D3, D5 and animal 3 (GII) and 4 (GI) on D2, D4 and D6. The treatment was conducted only on the first day of each cycle (D1 and D2). Following the treatments and with the animals contained and relaxed using Xylazine Chloridrate 2% (0.00028 mg/kg), ultrasonography (US) was performed in spectral Doppler, measuring the RI in the testicular artery one hour before and immediately after semen collect. During the semen collect, Flehmen's reflex frequency and number of mating without erection, reaction time to erection and duration of mating were evaluated. All data were submitted to chi-square and ANOVA tests with significance of 5%. No statistical differences (P> 0.05) was observed for the following variables: testicular artery RI, Flehmen reflex frequency, number of mating without erection, reaction time to erection, duration of mating between hCG or saline group, as well among the four seasons. We concluded that administration of a single dose of 5000 IU of hCG in stallions was not enough to change the blood flow in testicular artery, measured by the resistance index, and also did not cause behavior changed in the experimental conditions studied.

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# Injectable long-acting P4 supplementation stimulates IFNt-signaling, but not pregnancy success after transfer of multiple embryos in beef cattle

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In cattle, the P4 supplementation at early diestrus stimulates conceptus growth increasing IFNt-signaling, however also anticipates luteolysis occurrence. These paradoxically effects are associated with inconsistency on pregnancy rates. Using a multiple embryo transfer model, we aimed measure the luteolytic and embryotropic effects triggered by the P4 supplementation in beef cattle. This model allows to eliminate pregnancy failures related to the unique embryo transfer (Therio.73,250-260,2010). The hypothesis were (1) the embryo presence inhibits the early luteolysis induced by P4 supplementation and (2) P4 supplementation stimulates embryos retention. Experiment was conducted in two replicates; estrous cycles of non-lactating multiparous cows were synchronized by an estradiol/P4-based protocol followed by estrus detection twice a day (day 0 = estrus; D0). Cows detected in estrus (n=70) were split to receive an IM administration of 150 mg of long-acting P4 (iP4, Sincrogest, Ourofino Saúde Animal) or vehicle (no-iP4) on D4 and transcervical transference of none or 5 embryos produced in vitro on D7 on a 2x2 factorial arrangement. The CL development and function was evaluated daily by B-mode and Color Doppler ultrasonography from D3 to 21. Criteria for determining the day of luteolysis was when the CL area was <2.0 cm2 and colored blood flow signals covered ≤ 25% of total CL area. Pregnancy was confirmed by ultrasonography on D30. Abundance of transcripts for the interferon-stimulated gene ISG15 was measured by qPCR in peripheral blood mononuclear cells (PBMCs) isolated on D14, 16, 18 and 20. Transfer of multiple embryos (5 embryos: 16.60 ± 0.47 days vs. None embryo: 17.91 ± 0.26 days; $P < 0.05$), but not iP4 ($P > 0.10$), reduced luteal lifespan of animals. Regarding the embryotropic effect of iP4, there was no difference in the proportion of cows presenting functional CL on D20 between cows underwent to embryo transfer treated with iP4 (72.2% [13/18]) or not (77.8% [14/18]). Similarly, there was no difference on conception rate between iP4 treated cows (44.4% [8/18]) compared with no-iP4 treated cows (55.6% [10/18]). Despite of similar conception rate, the pregnant cows treated with iP4 presented greater ($P=0.05$) abundance of ISG15 mRNA on D18 (2.69-folds) and D20 (2.05-folds), than no-iP4 treated cows. In conclusion, our results demonstrated that luteolysis occurrence was not anticipated by iP4 supplementation, but it was by multiple embryo transfer. The iP4 supplementation at early diestrus stimulates the abundance of ISG15 mRNA in cows that underwent multiple embryo transfer, although not increase the embryo retention.
Acknowledgements: FAPESP, CNPq, CAPES, In Vitro Brasil.

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# Spatial differences in energy substrates across the bovine uterus

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The bovine endometrium is a dynamic tissue that undergoes spatio-temporal functional changes directed by the ovarian hormones, estradiol and progesterone. The arrangement of vessels that irrigate the uterus allows a greater input of ovarian steroids to the cranial portion of the uterine horn ipsilateral to the CL. In cattle, the morula-stage embryo enters in the apical uterine horn on days 4–6 post-mating and then develops into a blastocyst. Development of the preimplantation embryo/conceptus depends on uterine secretions to supply nutrients and growth factors. The uterine luminal fluid (ULF) is composed of molecules synthesized and secreted by endometrial cells as well as selectively transported from blood. Transport of molecules to the uterine lumen is a spatially programmed process, but knowledge of the biochemical composition of the ULF along the uterine horns is lacking. The aim was measure the abundance of energy substrates in spatially defined regions of the uterine environment. Estrous cycles of multiparous, non-lactating Nelore cows (n=7) were synchronized and uterine horns ipsi- and contralateral to the CL were isolated and divided in anterior, middle and posterior thirds, starting from the uterotubaric junction 7 days after estrus. Each uterine third was washed individually with D-PBS to obtain regional ULFs. Subsequently, intercaruncular endometrial samples were collected from each uterine third in the mesometrial side. Glucose and pyruvate were quantified in the ULF using fluorometric assays and concentrations were adjusted to units of endometrial area. Relative abundance of transcripts for Glucose transporters (SLC2A1 and SLC2A4) and Pyruvate transporter (SLC16A7) were measured on endometrial samples by qPCR. Protein quantification of SLC2A1 on endometrium was performed by Western Blot. Metabolite concentration on ULF and relative abundance of transcripts and protein was analyzed by split-plot ANOVA (SAS 9.3) and included the effects of horn (ipsi vs. contra) and third (anterior vs. middle vs. posterior) and their interaction. Concentration of pyruvate was similar across uterine horns and thirds. In contrast, concentration of glucose was 48% greater ($P<0.05$) in the anterior ULF of the ipsi- compared to the contralateral horn and was similar between the remaining thirds of both horns. Abundance of transcripts for SLC2A1, SLC2A4 and SLC16A7 was not affected by horn or third, neither the protein abundance of SLC2A1. The regional pattern of the glucose composition across the bovine uterus was not consistent with transcripts and protein abundances of main transporters in the endometrium, indicating a complex-regulation of the energy substrates-transport to the uterine lumen. In conclusion, glucose, but not pyruvate ULF abundance in the bovine uterine environment is associated with physical proximity to the CL-containing ovary.
Acknowledgments: FAPESP, CNPq e CAPES.

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# Presence of a pre-hatching embryo influences the metabolite composition of the uterine environment in beef cattle

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The oviductal and uterine environments play critical roles in the success of early embryonic development. *In vitro*, the bovine pre-implantation embryo secretes bioactive molecules (embryotropins) from early developmental stages. We reported that the endometrial transcriptome is altered by the presence of an embryo 7 days after estrus. However, potential effects of embryotropins in the uterine environment *in vivo* are unknown. We hypothesize that exposure to an embryo changes the biochemical composition of the uterine fluid in the cranial region of the pregnant uterine horn. The present study aimed to assess a spatially defined region of the uterine environment for responses to a day 7 embryo *in vivo*. Uterine luminal fluid (ULF) and endometrium were collected from the cranial region of the uterine horn ipsilateral to the CL 7 days after estrus from sham-inseminated (Con; n=8) or artificially inseminated and confirmed pregnant (Preg; n=10) cows. We performed absolute quantification of 205 metabolites in the ULF, including amino acids and biogenic amines, acylcarnitines, phophatidylcolines, lysophophatidylcolines, sphingolipids, hexoses and eicosanoids using electrospray ionization and tandem mass spectrometry. Metabolite concentrations were normalized by the endometrial area. Relative abundance of endometrial transcripts was determined by Real-Time PCR. Statistical analysis was carried out by one-way ANOVA with FDR correction for multiple comparisons. Of the 205 metabolites quantified, 166 were detected in 50% or more samples. Twenty two (13%; $P<0.05$) metabolites showed different concentration between Con and Preg ULF samples. Concentration of two metabolites (12-S-HETE and 15-S-HETE), associated with the Lipoxygenases pathway, were significantly greater (2.5 and 2.8-folds, respectively) in the Preg group. While the remaining 20 metabolites were less abundant in the Preg ULF, including Glycine (0.7-fold) and Sarcosine (0.6-fold). A Quantitative Enrichment Analysis revealed that Arachidonic Acid Metabolism and Glycine, Serine and Threonine Metabolism pathways were positively enriched and decreased in the Preg group, respectively. Relative abundances of transcripts for Lipoxygenases (*ALOX5*, *ALOX5AP*, *ALOX15B* and *ALOX12*) were evaluated by qPCR in endometrial samples and were similar between groups. However, a downregulation of *SLC6A9* (a Glycine transporter; 0.76-fold; $P=0.01$) transcripts was found in the Preg endometrial tissue, suggesting an endometrial origin of regulation that was consistent with the lower Glycine concentration in the ULF. Although the endometrial or embryonic side of modulatory biochemical processes can only be speculated, it is clear that regulation is complex. We conclude that uterine environment changes in response to embryo presence and believe that differences in ULF metabolite composition are important for both uterine receptivity and embryo development. Acknowledgments: FAPESP, CNPq e CAPES.

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# Changes in the profile of PGFM following an oxytocin challenge during the first 60 days of pregnancy in dairy cows

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It has been shown that pregnant (P) cows on d18 have a higher concentration of prostaglandin F2α (PGF) and its metabolite 13,14-dihydro-15-keto-PGF2α (PGFM) in the uterine lumen than non-pregnant (NP) after challenge with oxytocin (Parkinson et al., J. Reprod. Fert. 90, 337-345, 1990). However, little is known about PGF release throughout other stages of pregnancy. The aim of this study was to evaluate and characterize the profile of PGFM, before and during a challenge with oxytocin, throughout the first 2 months of pregnancy in lactating Holstein cows (n = 121) inseminated by FTAI (GnRH–7d–PGF–1d–PGF–3h–GnRH–16h–AI). On d11 (n = 23), 18 (n = 23), and 25 (n = 12) after AI, and on d 32 (n = 13), 39 (n = 13), 46 (n = 12), 53 (n = 13), and 60 (n = 12) of pregnancy, cows were challenged with 50 IU of oxytocin, i.m. Blood was collected before (0 min), 30, 60, 90, and 120 min after challenge for plasma concentration of PGFM (pg/mL) by ELISA. Ultrasound evaluations were performed for pregnancy diagnosis on d32, 39, and 60 post-AI. In addition, cows on d11 and 18 after AI had whole blood collected for concentrations of interferon-stimulated genes (ISGs) using RT-PCR, as a marker of an elongating embryo. Samples from cows on d11 were used to establish a confidence interval, to identify a cut off value of maximum expression of ISGs for NP cows. The analysis of PGFM used data only from NP cows on d18, based on ISGs, or cows diagnosed pregnant on d32, using ultrasound. Data were analyzed by PROC MIXED of SAS 9.2 comparing P vs. NP on d11 and 18 in one analysis and comparing the effect of day of pregnancy from d11 to 60 in a second analysis. On d11, there was no difference between P and NP with low PGFM before and after oxytocin challenge and no effect of oxytocin on PGFM. On d18, NP tended to have greater basal PGFM than P (16.3 vs. 9.5; $P = 0.08$) and had 3-fold greater PGFM after oxytocin (72.9 vs. 24.4; $P < 0.05$). Comparing only P cows from d11 to 60, the basal PGFM concentrations increased ($P < 0.0001$) as pregnancy progressed with d11 and 18 of P, lower than d25 and later days of pregnancy. After oxytocin, PGFM increased throughout gestation, and there was an interaction between gestation day and time after challenge ($P < 0.001$). Pregnant cows on d18 had little increase in PGFM following oxytocin but it tended ($P = 0.06$) to be greater compared to the negligible PGFM after oxytocin in P cows on d11. The oxytocin-induced PGFM in P cows on d25 (48.5 ± 9.1) was greater than P cows on d18 (22.0 ± 3.3; $P = 0.006$), especially 60 min after challenge ($P = 0.01$) when values were 2.8-fold higher on d25 However, there was no difference between d25 and 32 (68.9 ± 12.2). The oxytocin-induced PGFM had maximum values 60 min after challenge in P cows for d25 to 60 (d25 = 48.3 ± 8.6; d32 = 72.1 ± 12.0; d39 = 89.4 ± 18.7; d46 = 93.3 ± 14.5; d53 = 136.5 ± 19.3 and d60 = 106.1 ± 29.4). Basal and oxytocin-induced PGFM was greater on d53 (26.9 ± 4.6; 122.4 ± 18.4) and d60 (28.0 ± 4.2; 106.4 ± 19.5) of P than on d25 (22.6 ± 3.7; 48.5 ± 9.1), with intermediate values on d32 (30.5 ± 4.8; 68.9 ± 12.1) and d39 (25.8 ± 3.2, 80.7 ± 16.5). Thus, consistent with previous reports, the CL of early pregnancy is protected due to suppression of PGF secretion probably due to actions of interferon-tau. However, during the second month of pregnancy, PGF secretion is not suppressed since basal PGFM and oxytocin-induced PGFM secretion are greatly elevated (equal or greater than in d18 NP cows) indicating alternative mechanisms for protection of the CL during the second month of pregnancy.

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# Sex steroids drive the remodeling of oviductal extracellular matrix and regulate embryo receptivity in cattle

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The extracellular matrix (ECM) is a group of molecules that offers structural and biochemical support to cells and interacts with them to regulate their functioning. Also, growth factors (GF) can be stored in the ECM and locally released during the ECM remodeling process. Here, we hypothesize that the abundance of ECM components and remodelers is different in the oviduct of cows treated to ovulate larger or smaller follicles. It was employing an animal model that, using synchronization protocols, produces two groups: cows that ovulate small follicles (SF-SCL; n= 20) or large follicles (LF-LCL; n=21) and therefore have differences in their luteal development and E2 and P4 plasmatic concentrations. In preliminary studios, these animals had significant differences in their fertility. Ampulla and isthmus samples were collected after slaughter in day four (Day 0 = ovulation induction by GnRH) and immediately frozen or fixed in 4% buffered formalin. After RNA extraction, transcriptome (n = 3 / group) were evaluated by RNA sequencing. Human MMP Antibody Array (Abcam: ab134004) was used to detect and quantify several ECM remodelers proteins in parallel. While the paraffin embedded samples were used to localize and quantify the immunostaining for type I Collagen. Digital analysis was performed using Image J and the immunostaining signal was analyzed separately for each structural layer (i.e., tunica mucosa, t. muscularis, and t. serosa). Data analyses were performed using the GLIMMIX procedure of SAS. The model included the fixed effects of group, region, and their interaction. The transcriptome analysis revealed clusters with overrepresented ontology terms and activation of pathways associated with ECM organization, and remodeling in the LF-LCL group, especially in the isthmus region. Molecules up-regulated in LF-LCL cows could be further classified as ECM components (Collagens), ECM remodelers (ADAMs and MMPs), and ECM-related growth factors. Protein-intensities for MMP3, MMP8, MMP9, MMP13, and TIMP4 were 65.84, 43.50, 44.30, 76.08, and 65.23% greater for the LF-LCL compared to the SF-SCL group, respectively ($P < 0.05$). Additionally, the protein expression of MMP1 and TIMP2 tended to differ between groups, and was greater in the LF-LCL group (71.48 and 63.39% greater intensity in comparison to SF-SCL; $P < 0.10$). When type I collagen content was quantified in the t. mucosa, even though no region effect was observed, it was detected a stronger signal in SF-SCL in comparison to LF-LCL samples on both regions (LF-LCL ampulla: 14.48 ± 5.21% and isthmus: 17.05 ± 2.52%; SF-SCL ampulla: 27.77 ± 1.70% and isthmus: 29.55 ± 3.58%; $P < 0.01$). We concluded that ECM remodeling process takes place in the oviduct of high fertility cows (LF-LCL) when the embryo is being transported from the oviductal lumen into the uterine environment. This remodeling process is more intense and probably occurs earlier in these cows, when compared with low fertility cows (SF-SCL).

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# Pregnancy-associated plasma protein-A (PAPP-A) added during in *vitro* maturation: effects on meiosis progression, oocyte DNA fragmentation and gene expression in bovine cumulus-oocyte complexes

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The insulin-like growth factor (IGF) is a stimulatory factor for oocyte maturation and embryonic development *in vivo* or *in vitro*. The bioavailability of free IGF is restricted by IGF-binding proteins (IGFBPs) and increased by pregnancy-associated plasma protein-A (PAPP-A), which cleaves the bond between IGF and IGFBPs. Thus, we added PAPP-A to the *in vitro* maturation (IVM) of *cumulus*-oocyte complexes (COCs) and evaluated effects on oocyte meiosis progression, DNA fragmentation and transcriptional profile of cumulus cells and oocytes. For this, COCs from a local abattoir were submitted to IVM for 24h with TCM199 medium supplemented with PAPP-A (P1:1ng/mL, P10: 10ng/mL e P100: 100ng/mL) or not (Control). After IVM, oocytes were submitted to evaluation of DNA fragmentation (TUNEL assay) and meiosis progression (stained with Hoechst 33342; n=5 replicates with approximately 88 oocyte/group). Further, after the same IVM treatments groups, oocytes (n=4 replicates with 20 oocytes/group) were separated from their respective cumulus cells (n=6 replicates with *cumulus* cells from 20 COCs/group) and each cell type was analyzed for the transcriptional profile of 96 genes (3 reference genes and 93 target genes) by RT-qPCR using Taqman® assays in the HD-Biomark System®. The mRNA abundance of target genes was normalized with the geometric mean of reference genes (ACTB, GAPDH and PPIA), and the effects of PAPP-A supplementation on meiosis progression (%), apoptosis rate (%) and mRNA abundance (fold-change) were tested by ANOVA, using JMP software (SAS Institute Cary, NC). Means were compared with Tukey-kramer or Wilcoxon tests and differences were considered significant when $P \leq 0.05$. The addition of PAPP-A did not alter the percentage of oocytes in metaphase II (Control: 57.8±12.7, P1: 49.9±10.4, P10: 45.1±12.9, P100: 54.8±10.3) or oocyte DNA fragmentation (Control: 42.2±2.7, P1: 50.1±10.4, P10: 55.0±12.9, P100: 45.2±10.3). Regarding the mRNA abundance of transcripts in cumulus cells, FOXO3 was lower in P100 group (Control: 0.005±0.0002; P1: 0.004±0.0001; P10: 0.004±0.0004; P100: 0.003±0.0006). In oocyte, the mRNA abundance of DNMT1 was lower in P100 group when compared with P10 group (P10: 1.035±0.065 and P100: 0.841±0.026); H1FOO was higher in P100 group when compared with P1 group (P1: 2.211±0.078 and P100: 3.031±0.229), TXNRD1 was higher in P100 group when compared with control group (Control: 0.099±0.005 and P100: 0.140±0.006) and CPT1B was higher in P100 group when compared with P1 and P10 groups (P1: 0.004±0.0004; P10: 0.005±0.001 and P100: 0.009±0.002). In conclusion, the addition of PAPP-A, particularly at 100ng/mL, could modify the oocyte competence and maybe results in further developmental changes in blastocysts due to the down-regulation of FOXO3 (involved on premature primordial follicle activation) and DNMT1 (related in methylation pattern) and up regulation of TXNRD1, H1FOO, CPT1B (involved in oxidative stress processes, control of gene expression during oogenesis and fatty acid beta-oxidation) in matured bovine COCs.

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# Pregnancy-associated plasma protein-A (PAPP-A) increases free IGF-1 on *in vitro* maturation medium and modulates important genes related to embryonic development in bovine *in vitro*-produced blastocysts

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The insulin-like growth factor (IGF) acts as an important modulator for oocyte maturation and early embryonic development and its bioavailability is modulated by action of pregnancy-associated serum protein A, which breaks down the binding of IGF-1 to IGF binding proteins (IGFBPs). So, the aim was to verify the effect of PAPP-A on the modulation of free IGF-1 during bovine oocyte in vitro maturation (IVM) and its impacts on embryo yield and transcriptional profile of blastocysts. For this, ovaries were obtained from a local abattoir and COCs (5 replicates; 20 COCs/replicate per group) were in vitro matured in humidified atmosphere with 5% CO2 in 90 ul drops, into serum free medium on the absence (control group) or presence of PAPP-A (100 ng/mL; P100 group). After 24 h IVM, the medium was collected to measure levels of free IGF-1 by in house ELISA and the matured oocytes were submitted to IVF (5% CO2) with semen from Nelore bull and thereafter, presumptive zygotes were cultured in controlled atmosphere of 5% CO2, 5% O2 and 95% N2 until blastocyst stage. Medium was exchanged (45 ul – 50%) on the third and fifth day of culture. On day 3 and 7, the cleavage and blastocyst rates were verified, respectively. On day 7, blastocysts were collected to analyze the embryonic gene expression (n=4 pools for control group and n=5 pools for P100 group; 3 blastocysts/pool). The transcriptional patterns of 91 genes were analyzed by RT-qPCR using Taqman® assays in the HD-Biomark System®. The in vitro performance (cleavage and blastocyst rates) was calculated as percentage and transformed to arcsine. The mRNA abundance of target genes was normalized by geometric mean of four reference genes (ACTB, GAPDH, PPIA and SDHA) and data were transformed to fold change. The levels of free IGF-1 was calculated and transformed to fold change. The statistical analysis was performed with t-test, using JMP software (SAS Institute Cary, NC) and differences were considered significant when $P \leq 0.05$. In summary, the addition of PAPP-A (100 ng/mL) increases 1.27-fold change the levels of free IGF-1 (P=0.03) on IVM medium. There was no difference in embryo yield. The rates of cleavage and blastocysts were, respectively, 83% and 12% for control group and 79% and 17% for P100 group. The mRNA abundance of genes related to cellular stress (ATF4, GPX4 and H1F1A) and lipid metabolism (FASN and SREBF1) was lower in embryos of P100 group. On the other hand, genes related to cellular proliferation/differentiation (MAPK1) and pluripotency (POU5F1) were up-regulated in embryos of P100 group. In conclusion, the addition of PAPP-A during oocyte IVM increases the bioavailability of free IGF-1 and modulates the expression of important, genes related to cellular stress ,lipid metabolism, embryo development and pluripotency genes in in vitro-produced bovine embryos.
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# Follicular viability and tissue damages are influenced by time and temperature of ovarian transportation

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Despite the great prospect of preantral follicles, their application in studies or in assisted reproductive techniques comes up against the fact that these ovaries are generally at some distance from the manipulation centers. Thus, the effects of ovarian transportation should be evaluated in order to minimize the injuries to the follicular population. Thus, the aim of this study was to establish the best time interval and the ideal temperature of the holding medium to transport the bovine fetuses' ovaries from the slaughterhouse to the laboratory. The preantral follicles morphology and tissue viability by confocal microscopy were the evaluated parameters. For this, the work was divided into two experiments. In experiment 1, ovaries of bovine fetuses were collected in test tubes and stored in thermal bottles with temperatures of 4°C, 22°C, and 33°C to test in which temperature range would maintain the follicular morphology and tissue viability. Experiment 2 was carried out, evaluating the parameters by the time the ovaries were manipulated after their collection., and each group was manipulated with intervals of 4-, 10- and 16 hrs. The control group was immediately fragmented and fixed at the slaughterhouse (H0). Experiment 1 demonstrated that the highest proportion ($P < 0.05$) of viable preantral follicles was observed at 4°C. Furthermore, the group of 33 °C showed the highest fluorescence intensity relative to tissue degeneration emitted by propidium iodide. The proportion of normal primordial follicles was lower ($P < 0.05$) in all groups after in vitro culture compared to control and D0. Experiment 2 demonstrated that the follicles morphology and tissue viability were better during the 4 hs-interval compared to the 10 and 16-hour intervals. The group transported for 4 hrs presented the lower fluorescence emission relative to apoptotic cell index ($P < 0.05$) compared to the control (H0) and to the other treatments. In conclusion, the follicular morphology and tissue viability were influenced by time and temperature of ovarian transportation. Also, the results showed that the best temperature for the transport of bovine fetuses ovaries is 4 °C within the time interval of 4 hours.

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# Effect of increased circulating insulin with propylene glycol on ovarian dynamics in Holstein cows

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Based on the assumption that cows with high feed intake have lower circulating steroid concentrations due to increased catabolism by the liver, it is necessary to identify new strategies to alleviate these problems as alternatives to hormonal supplementation. Several studies have suggested that increasing circulating insulin would stimulate steroidogenesis in ruminants. Therefore, we tested the hypothesis that augmented circulating insulin during pre and post follicle deviation periods would increase follicle and corpus luteum (CL) development and function. Estrous cycles of 16 non-lactating and non-pregnant Holstein cows were synchronized using an intravaginal progesterone (P4) device of 1.9 g (CIDR; Zoetis, SP, Brazil) and i.m. treatment with 100 µg gonadorelin diacetate (GnRH ; Cystorelin, Merial, Canada) on day -12 of the protocol. On day -5, 25 mg were administered i.m. of dinoprost tromethamine (PFG2α; Lutalyse, Zoetis). On day -3, the P4 device was removed and cows received 25 mg of PGF2α i.m. On days 10 and 11 ± 1 of the estrous cycle all follicles > 5 mm were aspirated to synchronize the emergence of a new follicle wave. The second day of aspiration was considered D1 of the experiment. On D1, treatments were initiated and cows were divided into two groups: water (control; C) or propylene glycol (P) provided orally in four daily doses of 300 mL every 6 h for 3 consecutive days (D1 to D3, pre follicular deviation period), and another 3 consecutive days (D5 to D7; after follicular deviation period). The experimental design was a Latin square in a 2x2 factorial arrangement. Thus, four groups of 16 cows each were formed: 1) CC = water pre and post follicle deviation; 2) CP = water pre and propylene glycol post follicle deviation, respectively; 3) PC = propylene glycol pre and water post follicle deviation, respectively; and 4) PP = propylene pre and post follicle deviation. Blood samples were taken 0 (immediately before), 15, 30, 60 and 120 min after propylene glycol for circulating insulin and glucose, and daily for P4 measurement. Ovarian ultrasound examinations were performed daily for evaluation of follicular and luteal dynamics until ovulation, as well as during the following 9 d of CL development post ovulation. Statistical analysis was performed by the MIXED procedures of SAS. Plasma insulin concentrations (µIU/mL) were greater for groups receiving P compared to controls (0, 15, 30, 60 and 120 min: 17.5 ± 1.4, 26.3 ± 1.4, 31.2 ± 1.4, 21.8 ± 1.4, 16.9 ± 1.5 vs. 12.1 ± 1.5, 11.6 ± 1.5, 11.2 ± 1.5, 10.8 ± 1.5, 11.1 ± 1.5; $P \leq 0.05$). Glucose concentrations were also greater in the groups receiving P. Despite increased circulating insulin and glucose due to treatments with propylene glycol, there was no difference ($P > 0.05$) among groups for rate of pre deviation follicle growth (1.5 ± 0.14, 1.3 ± 0.15, 1.5 ± 0.14, 1.4 ± 0.15 mm/d), or post deviation follicle growth (1.2 ± 0.13, 1.4 ± 0.14, 1.4 ± 0.13, 1.4 ± 0.13 mm/d), ovulatory follicle diameter (15.5 ± 0.56, 16.1 ± 0.55, 15.6 ± 0.52, 15.6 ± 0.54 mm), CL volume, or circulating concentrations of P4 for the groups CC, CP, PC and PP, respectively. Thus, increasing circulating insulin using treatment with propylene glycol four times per day, either before or after the expected time of deviation, did not alter growth rate of the dominant follicle, luteal volume, or circulating P4.
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# 17-beta estradiol action on the synthesis of endometrial PGF2alpha in cows

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17β-E2 stimulates expression of the endometrial ER and OXTR receptors. The activation of OXTR induces the PGF2α cascade synthesis. The hypothesis was that the signaling enzymes and PGF2α synthesis are regulated by 17β-E2. The objective of this study was to determine the effects of 17β-E2 on expression of key transcripts and proteins in PGF2α synthesis. Cyclic and non-lactating Nelore cows were synchronized by application of 3mg BE (Ourofino, Cravinhos, Brazil) and insertion of P4 device (1g; Ourofino, Cravinhos, Brazil). After 8 days, the P4 device was removed and PGF2α (0,5mg; Ourofino, Cravinhos, Brazil) was applied, followed by 4 days of estrus observation (D0). On D15 cows were randomly assigned into three groups: Control (C, untreated), Placebo (P; 6mL of ethanol 50%, IV) and Estradiol (E; 3mg 17β-E2 plus 6mL of ethanol 50%, IV).Uterine biopsies were collected at the times 0h (C; N=10), 4h (E4h; N=11 and P4h; N=10) or 7h (E7h; N=10 and P7h; N=11) for PCR, WB e ICQ assay. Plasma concentration of PGFM concentrations were measured at D15. Group E presented decrease in CL area (cm2) and blood flow (%) and P4 concentration ($P<0.05$), compared to group P. Continuous dependent variables were analyzed by factorial 2 X 2 (PROC MIXED; SAS 9.3 program). PCR and WB were analyzed separately at times 4 and 7 hours. The Kruskal-Wallis test was performed for IHC. Compared to group P, functional and structural luteolysis of group E was anticipated 2 and 3 days, respectively. Group E presented greater concentration of PGFM at 4h, 6h, and 7h (38%, 119% and 150%; $P<0.05$), compared to the group P. At 4 hours, the abundance of the genes ESR1(fold change; FC=0.3), ESR2(FC=0.1), PRKCα(FC=0.5), PRKCβ(FC=0.3), PLA2G4(FC=0.2), AKR1B1(FC=0.2), and AKR1C4(FC.=0.3) was lower in the E4h, while OXTR was greater in the same samples compared to the P4h (FC=4.7; $P<0.05$). The gene expression of PTGS2 was not different between groups E4h and P4h ($P>0.05$). At 7 hours, E7h also showed lower abundance of ESR1(FC=0.8), PRKCα (FC=0.5), PRKCβ (FC=0.5), AKR1B1 (FC=0,2), and AKR1C4 (FC=0,2; $P<0.05$) and there was a tendency for lower ESR2 expression, compared to P7h (FC=0.3; $P=0.08$). However, there was no difference in the abundance of OXTR, PLA2G4, and PTGS2 between E7h and P7h ($P>0.05$). The abundance of the enzyme PKCα was decreased in both, E4h and E7h, relative to the samples P4h and P7h, respectively. E4h showed greater PGR immunostaining in the glandular epithelium (GE; $P<0.05$) and there was a tendency for greater PKCγ immunostaining in the luminal epithelium, compared to the P4h ($P=0.08$) e for reduced ERα immunostaining in the GE of the E4h compared to the E7h ($P=0.1$). 17β-E2 stimulates the plasmatic concentration PGFM and trancription of OXTR, but inhibits the transcription of molecules of the PGF2α-synthesis cascade. The increased concentrations of PGFM have led to speculate that the enzymes of the PGF2α synthesis cascade were activated by 17β-E2.

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# Cyclic GMP modulators (NPPC and Sildenafil) effects during *in vitro* maturation on cytoplasmic lipid content and transcripts abundance in bovine cumulus-oocyte complexes and embryos

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Recent studies have indicated the influence of C-type natriuretic peptide (NPPC) on meiosis resumption in mammalian oocytes. NPPC is synthetized by granulosa cells and binds to the natriuretic peptide receptor 2 (NPR2) present mostly in cumulus cells. NPR2 activation induces the synthesis of cyclic guanosine monophosphate (cGMP), which is transferred via gap junctions from cumulus cells to the oocyte, where the cGMP inhibitory action over PDE3A maintains high concentration of cAMP in the oocyte, sustaining meiosis arrest. The concentration of cGMP is controlled by the balance between its synthesis and degradation, which is carried out by the PDEs themselves. Sildenafil is specific inhibitor of PDE5 (inhibiting cGMP hydrolysis), utilized during IVM to delay spontaneous meiosis resumption. The signaling pathway activated by cGMP/PKG may trigger a lipolytic action through phosphorylation of perilipin and lipases. The objective of this study was to evaluate the effects of natriuretic peptide type C (NPPC) and/or sildenafil during in vitro maturation (IVM) on lipid content (LC) by Nile Red staining and gene expression in cumulus-oocyte complexes (COCs) and embryos by Fluidigm Biomark™ HD system and on embryo production. In experiment I, cumulus-oocyte complexes (COCs) were cultured in maturation medium with a NPPC (100 nM) associated or not with a Sildenafil (10 μM PDE5 inhibitor) for 24h and after this period, the samples were collected to lipid content (oocytes) and gene expression (COCs). In experiment II, COCs were cultured in the same conditions as experiment I and the COCs were submitted to IVF and IVC. The developmental rates, embryo LC and gene expression were evaluated on D7. Statistical analyses were performed using the JMP Software, the effects of the treatments on lipid content, gene expression and embryonic development were analyzed by one-way ANOVA followed by Tukey parametric post hoc testing or non-parametric Kruskal-Wallis test. Differences with probabilities of $P < 0.05$ were considered significant. NPPC during IVM decreased the LC in oocytes compared with control group (16.10 and 20.60 FI, respectively, $P < 0.05$), but genes involved in lipid metabolism and glucose transport were not affected in COCs ($P > 0.05$). NPPC increased one gene involved in cumulus expansion (PTX3) compared with control group ($P < 0.05$). Embryo development rates and their LCs were not affected in d7 ($P > 0.05$). Analysis of embryos transcripts showed that when COCs were matured with NPPC, transcript abundance was not different from the control group ($P > 0.05$). Only FOXO3 was increased relative to sildenafil or NPPC+sildenafil ($P > 0.05$). Sildenafil treatment during IVM increased HSF1 and PAF1 ($P < 0.05$) and decreased REST transcripts abundance relative to controls ($P < 0.05$). When both NPPC and sildenafil were used during IVM, only REST was lower than control ($P < 0.05$). PAF1 and HSPA1A were reduced relative to sildenafil alone and FOXO3 relative to NPPC alone ($P < 0.05$). This study reports, for the first time, the effect of NPPC during IVM on cytoplasmatic LC in bovine oocytes and effects on genes involved in cumulus cell expansion. However, the reduce LC in oocytes and the an increase in the expression of cumulus expansion related genes had not affected embryo production, still, these embryos recover the LC after IVC. We acknowledge São Paulo Research Foundation (FAPESP) for funding (Grant 2012/50533-2, 2013/05083-1 and 2015/20379-0).

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# Molecular and endocrine factors involved in future dominant follicle dynamics during the induction of luteolysis in *Bos indicus* cows

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The growth profiles of the future dominant (DF) and subordinate (FS) follicles and the gene expression of the granulosa cells during luteolysis induction in Bos indicus cows were evaluated. Forty non-suckled multiparous Nelore (*Bos indicus*) cows scoring 3.0±0.2 body contition score (1-thin to 5-obese) and between 5 and 7 years-old were synchronized with a progesterone and estradiol based protocol. After synchronization, cows with a CL were evaluated by ultrasonography every 12 hours, beginning at eight days post ovulation. Cows identified with a follicle of at least 6.0 mm in diameter in the second wave were split into two groups (BD– before deviation and AD- after deviation. In the BD group cows received 500µg of cloprostenol when the DF reached a mean diameter of 7.0mm (6.5 to 7.5mm). In the AD group, cows received 500µg of cloprostenol when the DF follicle reached a mean diameter of 8.0 mm (7.5 to 8.5 mm). Cows in both groups were submitted to the aspiration of the DF at 96 and 72 hours after was given cloprostenol. Follicular aspirations were performed to quantify IGF1r, LHr and PAPPA transcripts in the granulosa cells. Statistical analyses were performed using the Statistical Analysis System for Windows (Statistical Analysis Software 9.3, SAS Institute Inc., Cary, NC, USA). The GLIMMIX procedure was used to determine significant differences between groups for the variables related to follicular dynamics. Relative gene expression analysis was performed by the REST software Relative gene expression using the Pair-Wise Fixed Reallocation Randomization Test. The relative expression values are expressed as the mean ± SEM. Transcripts abundance were compared between BD and AD groups. The diameter of the DF at the moment of prostaglandin administration (BD=6.76 ±0.13 mm and AD=8.17±0.13 mm; P=0.001) and growth rate of the SF (BD=-0.04±0.09 mm/day and AD=0.07±0.14 mm/day; P=0.05) were greater in the AD group. There was greater abundance of LHr transcripts in BD cows (P=0.04). The remaining variables tested were similar between the experimental groups (P>0.05). In conclusion, the induction of luteolysis before follicular divergence does not interfere with dominant follicle dynamics. However, it causes granulosa cell LHr down regulation.
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# Endometrial cytological and microbiological evaluation of postpartum dairy cows from properties in the Caparaó, ES

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Dairy farms have experienced a contrast between increasing milk production and reducing the fertility of high-yielding cows. Among the factors involved in the impairment of fertility, uterine conditions, such as different types of endometrites, stand out. Thus, the aim of this study was to diagnose and classify the occurrence of uterine infections through endometrial cytological and microbiological examinations of dairy cows. Fifty-five cows (20 primiparous and 35 multiparous) lactating cows belonging to breeders from the Caparaó, ES region were used. Subclinical endometritis was diagnosed by endometrial cytology at 30 days postpartum when more than 3% of neutrophils were identified from 200 cell. However, animals in estrogenic phase, 18% neutrophils was considered (Salasel et al., Theriogenology, 74: 271-1278, 2010). Clinical endometritis was identified by the presence of cloudy, catarrhal or purulent uterine secretions. The culture, isolation and identification of microorganisms from the endometrial microbiological examination were performed according to a technique described by Koneman et al. (Microbiological Diagnosis, 5th ed. 2001, p.720-785). Results were submitted to descriptive statistics. In the microbiological examination, 52.7% (29/55) of the samples presented positive growth in plates containing blood agar media in aerobiosis. Anaerobic growth was observed in 47.2% (26/55), and in 27.2% (15/55) of the samples there was no microbiological growth. All positive samples were evaluated by catalase test, obtaining 100% of catalase positive samples. Thirteen (23.6%) animals were diagnosed with clinical endometritis (9 multiparous and 4 primiparous) by observation of mucopurulent, purulent, catarrhal or sanguineo-purulent secretion. According to endometrial cytology, 49 animals (89.1%) had no uterine infection, while 6 animals (10.9%) presented subclinical uterine infection. It was observed that only 46.1% of clinical uterine infection, diagnosed by secretion observations, also demonstrated a diagnosis of uterine infection in the cytology, suggesting that it may have be originated from cervicitis or vaginitis. Gram staining showed predominance of positive cocci (09/29) in aerobiosis and anaerobiosis (07/26). Despite several treatments described for endometritis, the early diagnosis of this pathology is essential for its success. Thus, vaginoscopy, cytology and endometrium microbiological examinations are important alternatives for diagnosing these conditions.

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# *In vitro* evaluation of progesterone production of luteal cells on pregnant domestic cats

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Feline species represents an important experimental model for wild felids with physiological similarities (Pope, C.E., Theriogenology, 163-174, 2000). Several factors are involved in steroidogenesis process of luteal cells and in domestic cats this knowledge is unrecognized. Our objective was performed standardization of luteal cell culture in vitro of domestical cats and measurement of P4 production in culture medium during pregnancy. Nine pairs of ovaries were obtained through OSH tecnic performed in three gestational phases: initial, intermediate and final. The ovaries were isolated and maintained in phosphate-saline buffer solution (DMPBS-FLUSH, Saint Louis, USA) containing 1% antibiotic and antimycotic solution (10,000 IU penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml; Sigma Aldrich, Saint Louis, USA) and amikacin (4µL/40mL). After ovary scarification, then were submitted to collagenase type 1 action (1mg / ml; C0130, Sigma Aldrich, Saint Louis, USA) plus DMEN high glucose (cod.41965-039, Sigma Aldrich, Saint Louis, USA) to obtain the luteal cells. The resulting contents were filtered (70um) and centrifuged serially (3 times) for 10 minutes at 20oC. The final pellet was resuspended and cell viability and concentration determined (10x10 5 cells/ ml for plating, 500 ul / well). The culture medium was collected in 2 stages: M1 (36 hours after beginning of cell culture) and M2 (60 hours after after beginning of cell culture) and stored in cryotubes (1mL) at - 80 ° C. The P4 assays were accomplished at PROVET laboratory by radioimmunoassay (RIE). The results were analyzed by ANOVA, two-way and average were compared in pairs by Student-Newman-Keuls method, with significance when $p \leq 0.05$. In initial phase of pregnancy there was a higher production of P4 at M1 when compared to M2 ($p = 0.043$); however, in intermediate and final phases there was no difference between analyzed moments ($p = 0.71$ and 0.27, respectively) . At M1, the lowest production of P4 occurred in intermediate phase of gestation, ($0.74 \pm 0.25$ ng/m), and at M2 there was no difference between gestational phases. According to results, P4 secretion in culture medium was higher in initial gestation after 36 hours of cellular culture (M1), which was expected, since P4 exercises an important role on endometrium, modulating the regulation of important genes for uterine receptivity and concept growth (Ayad, A. et al., Theriogenology, 1503-1511, 2007). In intermediate and final stages of pregnancy, P4 concentrations differs from those observed in serum profile, suggesting a possible interference of placental progesterone, a fact emphasized by Siemieniuch et al. (Reprod Biol Endocrinol., 89, 2012). According to results obtained a standardized method of cellular culture for feline species was established although P4 mensurements in culture medium during pregnancy did not follow seric pattern known.

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# Evaluation of ultrasound parameters in the diagnosis of the functionality of corpus luteum after ovulation induction in prepubertal Nellore heifers

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Due to the common occurrence of premature regression (PR) of the corpus luteum (CL) in heifers after first ovulation (OV), the aim of the present study was to determine differences in luteal dynamics in relation to the functionality of these ovarian structures. Fifty-seven prepubertal Nellore heifers (BW 289.61±32.28 kg, BCS 5.66±0.65 and 17.47±0.81 months old) were divided into two treatment groups for OV induction: GP4+GnRH and GGnRH. In the first, an intravaginal progesterone (P4) device (CIDR®, Zoetis, São Paulo, Brazil) of 3rd use was used for 10 days, and 48 hours after its removal 0.02 mg of buserelin acetate (GnRH hormone analogue) (Sincroforte®, Ouro Fino, São Paulo, Brazil) was applied intramuscularly, and in the second group only GnRH was used. Formed CLs were monitored by B-mode and color Doppler ultrasonography every two days until their functional regression (determined by the decrease in Doppler vascular signal and serum P4 concentrations below 1 ng/mL). For each day of evaluation was determined the area and percentage (%) of vascularization of CL and serum concentration of P4. The CLs with luteal phase greater than 16 days were classified as normal duration (ND), and those with duration less than 16 days as prematurely regressed. The characteristics were compared between treatments, CLs functions, evaluation days and their interactions, using the MIXED procedure of SAS program ($p \leq 0.05$). Six animals (6/57 = 10.5%) did not respond to treatment, due to the absence of CL within 96 hours after GnRH administration. Based on the results found for the serum P4 concentration, another category of CLs was established in the study, the nonfunctional (NF), which were those that presented at all days of evaluation values less than 1 ng/mL. In GP4+GnRH was observed 80.7% (21/26) of CL with ND and 19.2% (5/26) with PR; and in GGnRH, 4.0% (1/25) of the CLs were of ND, 44.0% (11/25) RP and 52.0% (13/25) NF. The area and % of vascularization of CL presented interaction between the treatments and the CLs functions ($P<0.0001$ and $p=0.05$ respectively). The NF CLs presented the lowest area ($31.9\pm11.4^{c}$mm2) in comparison to the other categories (GP4+GnRH: ND - $112.1\pm8.2^{a}$ and PR - $78.4\pm13.8^{b}$; GGnRH: ND - $154.4\pm22.9^{a}$ and PR - $58.3\pm10.7^{b}$), and the ND of the highest % of vascularization (GP4+GnRH: ND - $20.8\pm1.2^{a}$ and PR - $15.7\pm2.1^{b}$; GGnRH: ND - $18.1\pm3.3^{ab}$, PR - $13,6\pm2.3^{b}$ and NF - $10.9\pm2.6^{b}$). The concentration of P4 presented interaction with the treatments, CLs functions and evaluation days ($p=0.03$), with higher values for ND CLs in both treatments, intermediate values for prematurely regressed and values always lower than 1 ng/mL for NF. In conclusion, formed CL after OV induction of prepubertal heifers presented differences in the functionality, being able to be differentiated by ultrasound parameters area and % of vascularization of CL and serum concentration of P4. Acknowledgment: FAPESP (2015/13079-0), Ouro Fino and Centro APTA Bovinos de Corte (Instituto de Zootecnia/Sertãozinho – SP).

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# Changes in the expression of epigenetics related genes during the embryonic genome activation in cattle

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Genome activation is an important event during early embryonic development. The transition from maternal to embryonic genome is gradual and in bovines its peak is evident in embryos at the 8-6 cells stage. During this period the depletion of maternal transcripts by degradation or translation and the replacement by new transcripts of embryonic origin occurs. Epigenetic control is an important component of transcriptional regulation and can be modulated by non-coding RNAs, mainly involved with post-transcriptional mechanisms such as gene silencing and modulation, as well as epigenetic changes related to histone modification and DNA methylation. Considering that epigenetic changes are essential to control gene transcription, the aim of this study was to evaluate genes related to these alterations before and after the major genome activation in bovine embryos. For this, IVF embryos were produced and collected with 4 and 8-16 cells for the analysis of genes related to epigenetic alterations. Samples were pooled into 5 polls of 10 embryos each. Total RNA was extracted by combining the QIAzol reagent (Qiagem) with the miRNeasy kit. Reverse transcription was performed with the high-capacity cDNA reverse transcription kit (Applied Biosystems) and the analysis of gene expression through the Biomark HD System (Fluidigm). Relative levels were analyzed by Student's T-Test. Six differentially expressed genes related to epigenetic changes were identified. Four genes (*DNMT1*, *DNMT3A*, *DNMT3B* and *H1FOO*) are increased in 4-cell embryos and two genes (*H2AFZ* and *HDAC2*) in embryos of 8-16 cells stage. The 4-cell embryos showed higher levels of three DNA methyltransferases (DNMTs), enzymes involved in the maintenance of gene methylation patterns, and could be related to maintenance of the inactive embryonic genome. For this same reason, higher expression of *H1FOO* in 4-cell embryos, a key histone H1-encoding gene, was expected based on chromatin remodeling occurring during early embryonic development. The genes *H2AFZ* and *HDAC2*, higher in the group of embryos of 8 to 16 cells, are related to histone coding. The *HDAC2* is associated with embryonic development and cell differentiation and the *H2AFZ* controls gene expression through regulation and chromatin silencing. As we know, embryonic genome activation is a critical period of early embryonic development, and in vitro culture may alter the pattern of gene expression. Thus, is important to understand the pattern of expression of epigenetic modifiers during critical embryonic developmental periods in order to improve the production of in vitro bovine embryos.
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# Cumulus cell gene expression as a biomarker of blastocyst´s development in dairy cows involuntarily culled

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Intensive genetic selection and management in Holstein breed has resulted in modern dairy cow with very high milk yields. Thus, a high metabolic activity to maintain the level of production, which increases the metabolism of steroids, affecting reproductive physiology and reduce oocyte competence, even more in dairy cows involuntarily culled. Consequently, selecting oocyte with the highest development potential is of great importance in assisted reproductive technology. The aim of our study was to identify the cumulus cell (CC) gene expression associated with oocyte the high development potential to blastocyst in involuntarily culled dairy cows (referred as dairy cow). COCs from crossbred cattle were used as control. The donors were identified at the moment of ovary collection and grouped in dairy cows (Holstein–Friesian breed) or crossbred cattle (mostly Angus). CC biopsy from cumulus oocyte complex (COCs) was performed in 184 COCs from dairy and 188 COCs from crossbred cattle, divided in 7 replicates per group. Eight genes were analyzed in CCs biopsies (LUM, KRT18, KRT8, AGPAT9, CLIC3, BMPR1B, GATM and SLC38A3. In vitro maturation (IVM) and fertilization (IVF) as well embryo culture (IVC) was performed individually. IVM was performed in TCM199 for 22 h; oocytes were fertilized using frozen–thawed semen during 18 h and both at 39˚C under 5% CO2 atmosphere. IVC was performed using SOFaa culture medium at 39˚C under 5% CO2, 5% O2 and 90% N2 during 7 days. At day 7, embryos were classified; after scoring the developmental fate of embryos derived from the COCs that were biopsied, two groups were created: G1 (n=10); CCs from COCs that developed to blastocysts and G2 (n=10); CCs from COCs that did not develop to blastocysts stage. Also, the expression level of 12 genes (OCT4, SOX2, NANOG, CDX2, GATA6, TP1, BCL2L1, BAX, CASP3, LPIN1, LPIN2 and ELOVL5) were evaluated in individual blastocysts derived from dairy cows COCs (n=10) and controls (n=10). Statistical analysis were conducted using chi square test for embryo development and Wilcoxon non-parametric test for gene expression analysis using the software InfoStat (Buenos Aires, Argentina). Less than 10 % (7.4 ± 2.1%; mean±SEM) of COCs derived from dairy cows were able to develop to blastocyst stage (In control: 20.7 ± 2.7%;). Also, 68.4 % of blastocysts (day 7) from crossbred COCs were at expanded or hatching stage while only 20 % of blastocysts from dairy cows were at these stages (p=0.004). Two genes, LPIN1 and CASP3, were differentially expressed (P<0.05); LPIN1 was downregulated while CASP3 was upregulated in blastocysts derived from dairy cows COCs. The gene expression analysis of cumulus cells showed that more competent oocytes (G1) had a higher expression of six genes: LUM, KRT18, KRT8, CLIC3, BMPR1B and SLC38A3 (P<0.05). A positive correlation of gene expression in cumulus cell with embryonic developmental ability (r=0.7, P<0.05), can be used as biomarkers of blastocyst´s development in involuntarily culled dairy cows.

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# The effect of using estradiol cipionate on antecipated luteolysis control in *Bos indicus* (Nelore) cows suplemented with a long acting injectable progesterone at the beginning of diestrus

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In cattle, lower estradiol concentration in the preovulatory period have been associated with premature luteolysis. The objective of this study was to evaluate the effect of estradiol cypionate (ECP) on proestrus associated with a treatment of a long acting injectable P4 (iP4) at beginning of the diestrus on the expression of genes that control the release of PGF2α in the endometrium [progesterone receptor PGR], Oxytocin receptor (OXTR), estradiol receptor 1 (ESR1)] in Nelore cows. The ovulation of 80 cyclic cows was synchronized with a conventional E2 plus P4 protocol, which started on day -10 (D-10). After the P4 device removal (Sincrogest®, OuroFino Saúde Animal) in the D-2, animals received 0 or 1 mg of ECP (SincroCP®, OuroFino Saúde Animal). After 48 h following P4 device removal, all animals received 1 μg of buserelin acetate [(GnRH), Sincroforte®, OuroFino Saúde Animal). Therefore, the groups were defined as: GnRH + ECP (n = 12), GnRH + P4 (GnRH + ECP), GnRH + ECP (n = 12) and GnRH + P4 (N = 13) and GnRH + ECP + P4 (n = 13). Endometrial tissue samples were collected transcervically on D1, 3, 5, 7, 13 and 16 after ovulation using cytobrush. Color Doppler ultrasonographic evaluations were performed from day D3 to 25 to evaluate the development and regression of CL. Data analysis was performed with PROC GLIMMIX (SAS, 9.4), considering statistical difference $P \leq 0{,}10$. Structural luteolysis occurred earlier ($16.9 \pm 0.3$ vs. $18.4 \pm 0.4$; $P = 0.10$) in animals receiving iP4 compared to groups not receiving iP4. The GnRH + ECP + P4 group presented a greater ($P < 0.05$) rate (53.9%) of early luteolysis (≤ D16) compared to GnRH group (0%). Regardless of use of ECP, animals that received iP4 and presented early luteolysis demonstrated: lower CL area at D6, 7, 15 and 16, lower blood flow at CL at D14, 15 and 16 after ovulation ($P = 0.01$), greater P4 concentration in D4, 5 and 6 and lower concentration in D14, 15 and 16 ($P < 0.0001$) compared to those not having early luteolysis. In general, there was a greater abundance of transcripts for PGR in D1, which decreased over time ($P < 0.0001$). Cows receiving ECP presented lower ESR1 expression over time compared to animals that did not receive ECP ($P = 0.03$). Cows that received iP4 and presented early luteolysis there was greater OXTR expression in D16 compared to animals that did not have early luteolysis ($P < 0.0001$). In conclusion, the early luteolysis induced by iP4 is associated with an increased transcript for the endometrial OXTR gene. It is speculated that this molecular event is associated with early release of PGF2α pulses that will lead to CL regression. Furthermore, the increase in P4 concentration in the initial diestrus followng iP4 use may have damaged the development of the CL, which may have influenced the early luteolysis of these animals. However, estradiol (through of the ECP use) was not able to reverse this luteolysis process. Furthermore, iP4 treatment had no effect on the modulation of the PGR and ESR1 receptors in the initial diestrus, so the modulation of these receptors at this time had no influence on early luteolysis response. Acknowledgments: FAPESP, CNPq, CAPES, Ourofino Animal Health.

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# Eight-cell embryo developmental rates after been expose to gaseous sublethal stress

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High gaseous pressure (HGP) has been reported as a sublethal stressor able to induce embryo stress response, providing cell protection to subsequent stress, such as cryopreservation, although the involved cellular mechanisms are little understood. This principle was investigated in this report aiming to evaluate the effect of 16 MPa high gaseous pressure (HGP) during 2 (P1 group) and 4 hours (P2 group) on the development and viability of 8-cells stage murine embryos. From 60 superovulated 6 weeks old Mus musculus domesticus females, it was recovered 1303 8-cells stage embryos that were aleatory segregated in control (C group) and experimental groups (P1 and P2). HGP was applied on embryos of experimental groups while control embryos were immediately after recovery cultured in vitro in mKSOM media + 0.4% BSA during HGP treatments at 37ºC under atmosphere of 5% CO2, 5% O2, 90% N2 with satured humidity. After, embryos from experimental groups were also cultured at the same in vitro conditions. Blastocysts rates were observed after 48h in vitro culture and compared by Chi-square test ($P < 0.05$). No differences were observed in blastocysts rates between control and experimental groups: (C) 94.2% (419/445); (P1) 95.4% (395/414), (P2) 94.1% (418/444). We concluded that murine 8-cells embryos could be exposed to16 MPa HGP during 2 and 4 h without loss of in vitro embryo viability to reach blastocyst stage. This approach will be studied as subletlhal stressor in order to induce embryo cell response aiming to improve survival rates in a subsequent stress such as cryopreservation.

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# Study of the relationship of transcription and metilation profiles of genes related to lipid metabolism, cell differentiation and cell stress/death in bovine blastocysts with different kinetics of development

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Blastocysts with distinct kinetics in early cleavages present differences in global methylation DNA, which potentially lead to the activation/suppression of genes from various biological pathways, such as lipid metabolism, cell death/stress and cell differentiation (Ispada, et al. Anim. Reprod.13(3): 708, 2016). In this work, genes previously identified as differentially methylated were investigated aiming to stablish the relation between these two parameters and its impact on essential pathways for embryo viability. For this, bovine embryos were in vitro produced by conventional protocols, using sexed semen (female). After 40 hours of insemination the embryos were classified as Fast-cleavage (FBL - 4 or more cells) or Slow-cleavage (SBL - 2 or 3 cells), remaining in culture until the blastocyst stage (12 blastocysts per group obtained in 4 replicates). These embryos were analyzed through BioMark™HD for the characterization of gene transcripts related with lipid metabolism (9), stress (10) and cell differentiation (4). For statistical analysis, PPIA gene was used as endogenous control for ΔCt calculation, later submitted to Student's t-test. All results will be presented in SBL in relation to FBL. Upregulation of ACSL3, ELOVL6, PPARA and FADS was observed in the transcripts of genes related to lipid metabolism and previously identified as hypomethylated genes, whereas PPARG and PTGS2 showed no statistical difference. SCD and FASN genes, although hypermethylated, showed upregulation or non-difference, respectively. ACSL6, which did not show difference in DNA methylation, showed upregulation. Gene transcripts related with cell survival/death that were hypomethylated, BAX, PA2G4, HSPA1A and NOS2 were dowregulated and BID was upregulated, whereas NFE2L2 had no statistical difference. Furthermore, the CASP9 transcripts were observed as downregulated and TXNRD1 and FOXO3 showed no difference, although these genes were hypermethylated, and DDIT3 was upregulated, although it did not had DNA methylation difference. Regarding the control of cell differentiation, it was observed that of the genes POU5F1 and SALL4 (hypomethylated) only the POU5F1 presented statistical difference, being dowregulated. However, the NANOG gene that showed no DNA methylation difference was upregulated. Herewith, we can conclude that differentially methylated pathways identified previously between blastocysts with distinct development kinetics during the first cleavages are also altered in transcripts and could lead to metabolic differences with impact in embryo viability. In addition, the lack of correlation between DNA methylation status and some transcripts may be a result of other epigenetic mechanisms influence.
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# Genome wide association test with scrotal hernias in pigs

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Hernia is an abnormal protrusion of an organ or tissue through a defect or natural opening in the covering skin or muscle. In the swine industry, it is considered the most common congenital defect involved with high morbidity and mortality in the herds. The scrotal hernias are found in high frequencies ranging from 1.7 to 6.7%, and they have been linked to several boar lines and breeds, with moderated to high heritability (0.2- 0.86). However, even avoiding the use of those boar lines, hernias have not being completely eliminated from herds. Therefore, new approaches are needed to elucidate the genetic mechanisms involved with this condition to better select animals. Therefore, the objective of this study was to conduct a genome-wide association test (GWAS) in a cross-bred swine population (Landrace, Large-White and Pietrain) for the identification of genetic markers associated with the appearance of scrotal hernias. Animals were all from the same commercial swine herd located in the Northwest region of the RS. Piglets had similar age and were kept with the sow until 28 days of age and then weaned; castration was conducted during the first week of age. After weaning, piglets were moved to a group-housing with 100 piglets per pen with mixed sex. The phenotype classification was based on visual appearance of scrotal hernias. Each affected pig was matched to a healthy control from the same pen. In the total, 68 animals were genotyped using the Porcine SNP60 Beadchip, out of those, 41 animals had the presence of hernias and 27 were healthy animals. Markers and animals were submitted to a quality control process to remove individuals with difference in their genetic background and SNPs with a Minor Allele Frequency< 1% or if they failed in more than 10% of the samples. After after quality control, 50,797 SNPs from 18 healthy animals and 35 piglets with scrotal hernia were tested using an allelic $X^2$ test. From this test, we have identified two markers (MARC0114274, $P = 1.6 \times 10^{-7}$ and CASI0004285, $P = 1.6 \times 10^{-5}$) located on SSCX at 50,001,848 bp and 55,903,957 bp, respectively, and one with unknown location (MARC0063079, $P < 1.6 \times 10^{-5}$) associated with appearance of scrotal hernias in this population. In this study, we were able to refine the region linked with the appearance of hernias previously identified by Grindflek, E., BMC Genet. V. 7, P.1-12, 2006. We have identified that the segregation of these predisposing alleles for hernias in this population is via maternal inheritance. Therefore new approaches will be needed to eliminate those predisposing alleles to hernias out of this population, by removing the carriers females and replacing the boars being used.

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# Hematological profile and reproductive tract lactic acid bacteria during the estrous cycle in beef cattle

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The interactive role of the immune system and the microbiota in regulating the physiological outcome of specific organs has been increasingly explored across different species and body systems. Considering the marked fluctuations of ovarian steroids throughout the estrous cycle, the aim of the this work was to assess effect of the phase of the estrous cycle on the response of systemic blood variables and on the isolation of lactic acid bacteria (LAB). Thirteen cyclic cows received on Day -3 (D-3) an injection of 25 mg of dinoprost. Observation of estrus behavior was performed twice daily, beginning on D-2. A cross over design was setup, where in the first round cows were randomly selected to be collected either on D-3 (diestrus; n=5) or on D0 (estrus; n=8). Subsequently, cows collected on D-3 and D0 on the first round were collected on D0 (n=5) and D-3 (n=8), respectively, on the second round. The interval from sampling to estrus (SEI) was calculated in hours. Uterine sampling was performed by washing the uterine horn ipsilateral to the dominant follicle with 60 ml of saline. Vaginal secretion was obtained from the vaginal fornix with a Metricheck-like apparatus. For the assessment of LAB, uterine washing and vaginal secretion were seeded in Man Rogosa Sharpe agar and broth and incubated in anaerobic chamber at 37ºC for 48 hours. Blood samples were collected by jugular venipuncture on D-3 and D0 to determine hematological profile. Total leukocyte count was performed on Neubauer chamber, and differential leucocyte count was obtained by Quick Panoptic-stained blood smear. Packed cell volume (PCV) and total plasma protein (TPP) were determined by microhematocrit method and refractometry, respectively. Microsoft Excel was used to compare group means by Student´s T-Test and run regression analyses. Fibrinogen, PCV, eosinophil, neutrophil and lymphocyte counts did not vary between D-3 and D0 ($P>0.05$). TPP concentration and leukocyte count were observed at greater values, whereas monocytes decreased on D0 ($P<0.05$). Furthermore, both TPP ($r=0,66$; $r2=0,44$; $P<0.01$) and leukocytes ($r=0,63$; $r2=0,4$; $P<0.01$) were positively, whereas monocytes ($r=-0,58$; $r2=0,33$; $P<0.05$) was negatively correlated with SEI. No LAB growth was detected on any uterine or vaginal samples, except for a single animal, whose sample collection was the only one carried out 24 hours post-estrus. It is suggested that the major endocrine events taking place around estrus interfere with the population of circulating leukocytes as well as total plasma protein. Such changes may impact the regulation of local reproductive tract immune cell population and the regulation of the colloid osmotic pressure, which in turn may influence the uterine influx of plasma-derived substances. We thank the State Foundation for Agricultural Research (FEPAGRO; Uruguaiana, RS) for providing the animals.

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# *In Vitro* viability of murine 2-cells embryos after high gaseous pressure exposure

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Successfully genome activation is the first critical checkpoint in embryo development and it is an essential step for correct cell metabolism and differentiation. In mice it occurs at 2-cells stage and the further embryo development can be used as quality indicator for in vitro culture conditions. Many studies were made to evaluate high pressure effects on embryonic developmental capacity and its transcriptional response. Sublethal stress caused by high pressure activates different genes as embryo cell response that confers protection to a subsequent challenge such as cryopreservation (Pribenszky, Biol Reprod, 83, 690, 2010; Rodrigues, Reprod Fertil Dev, 25, 282, 2012). The present report aimed to evaluate the effect of 28 MPa high gaseous pressure (HGP) during 2 hours on the development and viability of 2-cells stage murine embryos to reach hatched blastocyst stage. A total of 148 2-cells embryos recovered from 10 six weeks old superovulated Mus musculus domesticus females were aleatory segregated into control (C) and experimental (P) groups. HGP was applied on embryos of P group while control embryos were immediately after recovery cultured in vitro in mKSOM media + 0.4% BSA for 120 h at 37ºC under atmosphere of 5% CO2, 5% O2, 90% N2 and satured humidity. After HGP treatment, embryos from P group were also cultured at the same in vitro conditions. Blastocysts hacthing rates were observed after 120 h in vitro culture and compared by Chi-square test ($P < 0.05$). No differences were observed in hatching rates between control and experimental groups: (C) 74.2% (43/60); (P) 74.0% (65/88). Therefore, we concluded that 28 MPa HGP can be used as sublethal stressor on 2-cells embryos without compromising in vitro embryo development, viability and genome activation overcoming. Transcriptional response analysis will be conducted to identify if modifications at molecular level could affect later organism development.

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# Maternal breed influence in Holstein-Gyr reciprocal F1 embryos

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Since several reproductive particularities exist between Bos taurus and Bos indicus, a common question is whether maternal contribution affects embryo developmental capacity. We compared reciprocal embryo development, in order to detect the most efficient maternal breed for F1 production in tropical conditions, and understand possible origins for any disparities. Crossbred embryos were produced by fertilization of Gyr oocytes with Holstein semen (HGyr) or by fertilization of Holstein oocytes with Gyr semen (GHol). Blastocysts were transferred to recipients and post-transfer development was assessed until birth. Spermatozoids bearing X-chromosome were used in all replicates of the experiment for IVF, and only viable oocytes (grade I, II and III). Mean number of viable oocytes and blastocysts were compared between groups using Mann Whitney Test. Rates of viable oocytes (percentage of viable oocytes in regards to total oocytes), cleavage, blastocyts, and pregnancy 30 and 60 days, calving, and embryonic loss were compared between groups using Fisher's Exact Test. Gestation length was compared between groups using T Test. We performed 88 OPU sessions in Gyr and 90 OPU sessions in Holstein donors, and retrieved 1040 Gyr oocytes and 609 Holstein oocytes. Oocyte quality, assessed by mean viable oocyte number (Gyr=8.94±0.69, Holstein=4.20±0.33; P<0.0001) and viable oocyte rate (Gyr=74.73%; Holstein=62.07%; P<0.0001), were decreased in Holstein oocytes. Embryo cleavage (HGyr, 562/694 – 80.97%; GHol, 255/343 – 74.34%; p=0.015) was decreased in GHol group. Overall, we observed a reduction of 2.39 fold in the total number of F1 blastocysts obtained in GHol group in this experiment (GHol 106 vs HGyr 254), and comparison of mean blastocyst number per OPU revealed decreased numbers for GHol (GHol 1.36±0.15, HGyr 3.34±0.35; P<0.0001). Blastocyst rate (per cleaved embryos) was similar between groups (GHol 41.56, HGyr 45.19; p=0.36). Post implantation development revealed similar pregnancy rates 30 days after IVF (GHol 38.57, HGyr 47.92; p=0.24) and 60 days after IVF (GHol 31.43, HGyr 44.44; p=0.08), but decreased calving rates for GHol group (GHol 22.86, HGyr 40.97; p=0.01). Embryonic loss was increased for GH group (GHol 40.74, HGyr 14.49; p=0.01). Gestation length of F1 embryos was affected by reciprocal cross (GHol 281.23±0.71, HGyr 286.72±0.96; P<0.0001). We conclude despite similar genetic background, maternal/paternal breed has deep influence on embryo development in bovine. We acknowledge CNPq (Grant 309271/2009-6), Embrapa (Grant 01.13.06.001.05.01.003), Faperj (Grant 111.466/2014) and Fapemig (Grant PPM 00167/15) for financial support.

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# Influence of endometrites on ovarian structures and oocyte quality of cows

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The objective of this study was to diagnose clinical endometritis (CE) and subclinical (SE) by means of macroscopic, cytological and histological evaluations of bovine uterus, its influence on ovarian structures and alterations, number of oocytes recovered and oocyte quality. A total of 171 reproductive tracts of females of different ages, from unknown races and / or crosses, slaughtered in a slaughterhouse were collected. ES were diagnosed by endometrial cytology and identified more than 3% of neutrophils from 200 cells. Females in the estrogenic phase, with follicle between (12,5 and 15,5 mm) in diameter was considered the percentage more than 18% of neutrophils. The CE were identified by the presence of uterine secretion. The diagnosis of CE and ES was confirmed in the histopathology by the observation of neutrophil infiltrates in the endometrium. The ovaries were evaluated macroscopically for ovarian structure and alterations, being: dominant follicle (DF), corpus luteum (CL), ovarian cysts (OC) and oophorites (OP). The oocyte quality was performed according to the number of cumulus cell layers and cytoplasmic aspect, being: grade I (GI), grade II (GII), grade III (GIII) and grade IV (GIV). The results were submitted to descriptive statistics, parametric ANOVA and Tukey's test. Non-parametric data were analyzed by the Kruskall Wallis test and Dunn test at 5% significance. CE were present in 8,2% (n = 14) of the animals, while SE in 4,1% (n = 7), both confirmed by histopathological evaluation. DF were observed in 32,7% of the animals (n = 56), of these, 7,1% (n = 4) presented CE and 5,3% (n = 3) SE. CL were found in 42,1% (n = 72) of the animals, of these 11,1% (n = 8) were diagnosed with CE, and 5,5% (n = 4) with SE. CF and OF were found in 1,2% (n = 2) of the animals with CE. In the oocyte retrieval analysis, animals with CE, ES and healthy presented mean recovery of 10,3 ± 7,1, 15,0 ± 9,6 and 10,6 ± 8,3 oocytes / animal, respectively. In the assessment of oocyte quality, healthy animals presented GI oocytes in 12,4% (n = 188), GII in 19,8% (n = 300), GIII in 42,4% (n = 643) and GIV in 25,3% (n = 384). Animals with CE showed 11,7% (n = 17) of GI oocytes, 13,1% (n = 19) GII, 33,1% (n = 48) GIII and 42,1% (n = 61) GIV. In those diagnosed with SE, GI oocytes were obtained in 15,2% (n = 16) of the animals, GII in 9,5% (n = 10), GIII in 29,5% (n = 31) and GIV in 47,7% (n = 48). In the comparison of the quality degree between the groups, no difference was observed between the rates of GI, GII, GIII and GIV oocytes. The presence of endometrites did not influence ovarian structures and alterations and number of oocytes recovered per animal. The endometrites altered the oocyte quality within each group, however, it did not affect the oocyte quality when evaluated between the groups.

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# Does lipopolysaccharide affect the rate of cleavage and embryonic development *in vitro*?

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Cows with postpartum uterine infection reduce the growth of dominant follicles and the production of estradiol, which results in postpartum delay in ovulation (Sheldon, Biology Reproduction, 81, 1025, 2009). Gram-negative bacteria, such as Escherichia coli, contain on its outer membrane lipopolysaccharide (LPS) that produces an acute inflammatory response at systemic level, which causes an increase in body temperature and serum levels of pro-inflammatory cytokines (Carroll , Innate Immunity, 15, 81, 2009). Thus, the objective of this work was to evaluate the effect of the supplementation of MIV medium with LPS (0.1, 1.0 and 5.0 µg / mL) in the cleavage rate and initial embryo development in vitro in cattle. In vitro embryo production was performed in a 5% CO2 incubator at 39°C using commercial media (Progest - Biotechnology in animal reproduction, Botucatu, SP). Oocytes (COCs) were obtained from slaughterhouse, washed and selected by morphology. COCs of grade I, II and III were randomly distributed into four groups (n = 50 COCs / group) according to the addition of LPS in the IVM medium (G1: 0 µg / mL, G2: 0.1 µg / mL, G3: 1.0 µg / mL and G4: 5.0 µg / mL LPS, SIGMA-ALDRICH®, St. Louis, MO, USA). IVM occurred for 22 hours. IVF was performed with a concentration of 1x106 sperm/mL during 20 hours. After this period, the probable zygotes were cultured in CIV medium covered with mineral oil for 7 days. At day 3, the cleavage rate (cleaved / inseminated) was assessed and on the same day, and also at day 5, 70% of the culture medium was renewed. On day 7 the embryonic development rate (blastocysts / inseminated) was evaluated. Thus, 4 replicates were performed with approximately 150 oocytes / group. The effect of LPS on the cleavage and embryo development rate was analyzed by repeated measurements of ANOVA after data transformation and the Tukey post-hoc test. There was no difference for cleavage rate, G1: 21.5 ± 3.3 (58.9%); G2: 22.5 ± 8.1 (55.6%); G3: 23.7 ± 5.3 (60.1%); G4: 30.2 ± 10.6 (72.9%) (P> 0.05); or embryonic development: G1: 6.7 ± 2.1 (13.7%); G2: 8.0 ± 3.5 (15%); G3: 9.3 ± 5.1 (17.7%); G4: 10.7 ± 8.1 (19.3%) (P> 0.05). These data suggest that exposure of oocytes to LPS does not affect the initial embryonic development in vitro in cattle.

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# Modulation of cholesterol biosynthesis pathway in bovine embryos produced *in vitro*

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Embryos with high amounts of lipids have their impaired development, with increased embryo death and lower survival after cryopreservation. A component which is present in both cytoplasmic lipid droplets as the cell membranes is cholesterol, which has been suggested by recent studies as a major lipid to be investigated, having demonstrated differences in the amounts of related genes transcribed this pathway in embryos cultured in the presence and absence of serum, as well as embryos with different kinetics as compared to those produced in vivo. Therefore, the aim of this study is to test if different concentrations of a inhibitor (I) of cholesterol biosynthesis affects the blastocysts rate and the lipid content of IVP bovine embryos. In such a purpose, slaughterhouse cows oocytes (n=250 oocytes/group; 3 replicates) where submitted to IVM, IVF (5% of CO2, 38.5°C and saturated humidity) and in the IVC (5% of CO2, 5% of O2, 38.5°C and saturated humidity) were divided in six treatment groups (inhibitor dose): I (20μM), 100x I, 10x I, 0,1x I, 0,01x I, and control (C). After 40hpi the cleavage and additional rate was evaluated, in the embryos droplets, the inhibitor doses corresponding to each treatment. Expanded blastocysts (n=15 blastocysts/group) were collected and submitted to lipid quantification by Sudan Black B prepared following previously stablished. The ImageJ software was used to convert the images in gray scales and determinate, in gray intensity by area, the mean of lipid content by embryo. The test t Student was used for the comparison between kinetics and ANOVA to the else analysis involving comparison between the three treatments. The cleavage rate was the same to all groups, once the inhibitor wasn't added yet ($P>0.05$). In the 100x I treatment, no embryo survived after the addition of this inhibitor dose. There was no difference between the else groups ($P>0.05$). Embryos form the treatments 0,01x I, 0,1x I and C did not presented differences in the lipid content (0,01x I: 11,67 AU±1.47; 0,1x I: 11.73 AU±1.32; C: 11.23 AU±1.14. $P>0.05$), just as the treatments 10x I e I (10x I: 15.51 AU±1.36; I: 15,16 AU±1.29. $P=0.82$). But the embryos from the treatments 10x I (15.51 AU±1.36) and I (15,16 AU±1.29) presented higher ($P< 0.05$) lipid content when compared to the group C. Concluding, i) the most concentrate dose of the inhibitor blocked the embryo development, demonstrating the importance of the cholesterol biosynthesis in the early development; ii) the dose of 10x I, 0,1x I, 0,01x I and I did not affected in the blastocyst rate; iii) 10x I and I embryos presented higher lipids amount, possibly, by a deviation in the substrate from the cholesterol biosynthesis pathway to the triglycerides pathway and PL reflecting in a higher amount of lipid cytoplasmic droplets when compared to C.

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# TIMP1 levels in follicular cells and extracellular vesicles pattern in follicular fluid from ovarian follicles of different stages of the bovine estrous cycle

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During the bovine estrous cycle ovarian follicles are exposed to different physiological profiles, which can influence oocyte quality and atresia contents. Extracellular vesicles (EVs) are nanovesicles carrying bioactive molecules (mRNA, miRNA and proteins) with size between 50-150 nm found in follicular fluid (FF). It has been recently shown that transcript levels of metallopeptidase inhibitor 1 (TIMP1) are increased in follicular cells (FCs) in atresia. Our hypothesis is that *TIMP1* levels are associated with the pattern of EVs originated from follicles at different stages of the estrous cycle. To test this hypothesis, slaughterhouse ovaries were collected in pairs and classified according to the appearance of the corpus luteum in stage 1 (hemorrhagic corpus luteum, characteristic of the post ovulatory period) and stage 3 (developed corpus luteum, orange, characteristic of the diestrus period). Next, we evaluated the transcript levels of *TIMP1* in FCs of the different stages, as well as the EVs pattern, and the IVP rates from the oocytes of each stage. In order to do that, follicles between 3-6 mm exposed to different moments of the luteal phase were punctured for collection of FCs, FF, and cumulus oocyte complexes (COCs). FCs were placed at -80 ° C for subsequent mRNA extraction and evaluation of *TIMP1* levels by RT-PCR. The EVs were isolated from FF by two ultracentrifugations at 100,000xg for 70 minutes and were analyzed for concentration and particle size. The COCs were selected and kept in an incubator at 38.5 ° C, 5% CO2. After 26 hours of maturation the oocytes were parthenogenetically activated and cultured in SOFaa at 38.5 ° C, 5% CO2. Data analysis were performed using Student's T-test with significance level of 5%. The results showed an increase in *TIMP1* levels in stage 3 ($p = 0.051$) compared to stage 1. There was no difference between size (113.8 ± 9.40; 123.62 ± 7.62 nm) and concentration ($5.2x10^{11} \pm 2.06x10^{11}$; $6.52x10^{11} \pm 1.28x10^{11}$ particles/mL) of the EVs at the analyzed stages. As for IVP, there was no difference between cleavage rates at day 3 (68.89 ± 15.54; 67.28 ± 10.55%) and blastocysts rates at day 7 (30.09 ± 9.71; 33.80 ± 13.32%) for stage 1 and 3 respectively. Our results demonstrate that *TIMP1* levels are increased in FCs of stage 3 follicles of the estrous cycle. However, it was not possible to observe differences in size and concentration of EVs found in FF as well as in IVP, probably due to the exclusion of poorer quality oocytes prior to IVP. In this way, we believe that although we cannot find differences in the EVs size and concentration, the vesicles contents in the different stages of the estrous cycle can influence the PIVE results. These results will help to understand the molecular events involved in follicular maturation and oocyte quality in cattle. Funding: FAPESP (2014/22887-0; 2015/21674-5; 2015/21829-9; 2017/02037-0).

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# The ketone body acid β–hydroxybutyric does not affect H3K9ac levels in bovine oocytes maturated *in vitro*

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Recently the ketone body acid β–hydroxybutyric (BOHB) was demonstrated to be a potent inhibitor of histones deacetylases (HDACs) both in vitro and in vivo. It makes this molecule a potential candidate to connect the animal nutrition with the regulation of gene expression via chromatin modifications. The modern high-producing dairy cows present reduced fertility, which might be caused by altered levels of metabolites (e.g. non-esterified fatty acids and ketone bodies) originated from metabolic disorders affecting the maturing oocytes. The process of oocyte maturation is characterized by changes in chromosome morphology and dynamic changes on histone modifications. HDACs are chromatin-remodeling proteins that participate in this process catalyzing histone deacetylation, which is essential for normal chromosome condensation and segregation. To gain mechanistic insights about the epigenetic effects of BOHB on maturing oocytes, we decided to investigate whether exposure of immature cumulus-oocyte complexes (COCs) with BOHB levels normally found circulating in blood of ketotic cows and paralleled in follicular fluid affect the oocyte in vitro maturation and histone acetylation. We supplemented the in vitro maturation (IVM – TCM199 +10 % FCS + 50 mg/mL hCG + 1 mg/mL FSH) medium and treated the COCs with 2 mM BOHB during the IVM (~ 21-23h). We denuded the oocytes and observed the maturation rate based on the presence of the first polar body. We carried out 10 independent replicates, culturing a total of 994 oocytes in the control group (0 mM BOHB) and 1013 oocytes in the treated group (2 mM BOHB). We did not observe difference between the groups, with the control group presenting maturation rate of 74.27 ± 2.22% and the treated group 69.74 ± 2.01%, (P=0.15; Student's t test). Since BOHB is a HDAC inhibitor, we decided to investigate whether exposure of COCs to BOHB affect histone acetylation levels as well the relative nuclear area in oocytes, an indirect measure of chromatin condensation. After confocal measurement of 61 oocytes from each group, we did not observed difference on the H3K9ac levels in the control oocytes (52.76 ± 2.23; N=61) compared with the treated with BOHB (56.95 ± 2.74; N=61; P=0.24; Student's t test). Regarding the relative nuclear area, there is no difference in the diameter of the metaphase plate, with the control group presenting 0.06058 ± 0.003816 (arbitrary units), and the treated group 0.06039 ± 0.003852, (P= 0.97; Student's t test). Our data suggest that BOHB levels normally found in cows with ketosis does not compromise the in vitro maturation neither the H3K9ac levels in bovine oocytes.
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# Prostaglandin E2 in ovulation of prepubertal female mice

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The objective of this study was to evaluate the effect of prostaglandin E2 (PGE2) on ovulation of prepubertal mice. In Experiment 1, 96 prepubertal female BALB/C mice were given 5 IU of eCG intraperitoneal (i.p.) on Day 0. On Day 2, females were randomly distributed into 3 Groups to receive: 1) 0.5 mL of PBS (n=31), 2) 5µg of GnRH (Gonadotropin Releasing Hormone; Gonaxal®, Biogénesis-Bagó, Curitiba, Brasil; n=32), and 3) 25 µg of PGE2 (Prostaglanin E2; Sigma-Aldrich, MO, USA; n=33). On Day 3, mice were killed and the oviducts were collected for cumulus oocyte complex (COC) detection and counting according to Bogle et al. Reproduction, v. 142, p. 277-283, 2011. Number of oocytes was analyzed by one-way analyses of variance and Tukey's test was used to determine differences among treatments. The proportion of ovulated mice was compared among groups by chi-square test or Fischer, when appropriate. The proportion of mice that ovulated was higher ($P<0.001$) in the GnRH group (93.7%, 30/32) than PBS (9.7%, 3/31) and PG E2 (3%, 1/33) groups. Similarly, the mean number of oocytes observed per treatment group was higher ($P<0.001$) in the GnRH-treated group (10.2 ± 1.4) compared with PGE2 (0.1 ± 0.1) and PBS (0.1 ± 0.1) groups. The proportion of mice that ovulated was similar in the PBS and PGE2 groups. In the Experiment 2, 50 prepubertal female BALB/c mice were randomly distributed into PBS (n=18), GnRH (n=16) and PGE2 (n=16) groups. The mice were treated similarly to the experiment 1, except mice from PGE2 group that received 250 µg of PGE2 i.p. There were no difference in the proportion of mice that ovulated in females treated with GnRH (93.7%, 15/16) and PGE2 (93.7%, 15/16), and both were higher ($P<0.001$) than PBS group (0%, 0/18). Similarly, the mean number of oocytes observed per treatment group was higher ($P<0.001$) in the GnRH-treated group (8.9 ± 1.3) and PGE2 (6.4 ± 0.8) compared with PBS (0.0 ± 0.0) groups. The results demonstrated that the PGE2 dose of 250 µg successfully induces ovulation in prepubertal female mice.

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# Redox potential profile of the reproductive tract environment at diestrus and estrus in beef cattle

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Drastic fluctuations of ovarian steroid hormones trigger a number of molecular and cellular mechanisms that drive relevant reproductive tract functional outcomes. The aim of the present work was to assess the redox potential of the uterine and vaginal compartments under the regulation of diestrus and estrus endocrine environment. Thirteen cyclic cows were assigned in a cross over design, where in the first round cows were randomly selected to have vaginal and uterine samples collected either on D-3 (diestrus; n=5) or on D0 (estrus; n=8). Observation of estrus behavior was performed twice daily, beginning on D-2. Cows received on D-3 an injection of 25 mg of dinoprost. Subsequently, cows collected on D-3 and on D0 on the first round were collected on D0 (n=5) and on D-3 (n=8), respectively, on the second round. Uterine sampling was performed by washing the uterine horn ipsilateral to the dominant follicle with 60 ml of saline, and vaginal secretion was obtained by scooping up the vaginal fornix with a Metricheck-like apparatus. Reactive oxygen species (ROS) were assessed by spectrofluorimetric method, using the 2',7'-dihydrodichlorofluorescein diacetate (DCHF-DA) probe. Antioxidant capacity was determined by using "ferric reducing antioxidant potential" (FRAP) assay. Microsoft Excel was used to compare group means by Student´s T-Test and run regression analyses. Uterine FRAP and ROS content did not differ between D-3 and D0 ($P>0.05$). Vaginal secretion from D-3 was not processed for redox potential. Uterine washings from D-3, obtained within 108 and 36 hours pre-estrus, showed a linear increase of ROS ($r=-0,84$; $r2=0,71$; $P<0.05$) and FRAP ($r=-0,76$; $r2=0,57$; $P<0.05$) content as the sampling to estrus interval (SEI) decreased. On D0, for washings obtained within 36 hours prior and 24 hours post-estrus, longer SEI were positively correlated with higher ROS ($r=0,67$; $r2=0,45$; $P<0.05$) and FRAP ($r=0,68$; $r2=0,47$; $P<0.05$) counts. On vaginal secretions, ROS ($r=-0,69$; $r2=0,47$; $P<0.05$) and FRAP ($r=-0,7$; $r2=0,48$; $P<0.05$) were negatively correlated with SEI. At estrus, the lowest FRAP and ROS counts in uterine washing and the highest counts in vaginal secretion were expressed by the only cow that was sampled 24 hours after estrus detection. It is suggested that the uterine and vaginal redox environments are regulated by the endocrine events that take place around estrus. We speculate that estradiol concentrations play a major role in the proposed regulation. As ROS and FRAP concentrations were highly positively correlated with each other, it is possible that antioxidant agents are stimulated as the oxidant activity rises; however, further investigation is warranted to determine what the precise mechanism is. It is hypothesized that major endocrine changes happening around estrus trigger equally relevant changes in the uterine and vaginal environment. We thank the State Foundation for Agricultural Research (FEPAGRO; Uruguaiana, RS) for providing the animals.

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# Requirement of follicular estradiol at the time of luteolysis in Nelore cows supplemented with progesterone at early diestrus

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In beef cows, long-acting injectable progesterone (iP4) supplementation in the early diestrus has paradoxical effects, as both it increases conception growth and fertility as it induces early luteolysis in a part of treated animals. Estradiol (E2) from the ovarian follicles during the luteal phase plays a central role in the induction of PGF2α release and lysis of the corpus luteum (CL). We tested the hypothesis that follicular E2 is necessary for the occurrence of luteolysis in cows treated with iP4 at the beginning of diestrus. Cyclic Nellore cows (n = 35) were synchronized and three days after ovulation (day 3; D3), the animals were randomly assigned to receive 300 mg (im) of iP4 (Sincrogest®, Ourofino Saúde Animal) and also, to be submitted or not to daily follicular aspiration (FA) from D9 to 18, thereby composing 4 groups: -iP4-FA (n=6), +iP4-FA (n=8), -iP4+FA (n=6), e +iP4+FA (n=7). Ovarian ultrasonography was performed from D6 to subsequent ovulation using B-mode for evaluation of the largest follicle in D6, development and regression of CL, and Doppler color for evaluation of CL blood perfusion area (blood perfusion x CL total area). The day of structural luteolysis was considered to be the day when there was a 25% reduction in the largest CL area measured between D8 and 10, and 50% of the luteal blood perfusion in D8. The data were analyzed by PROC MIXED of SAS (9.3 version) for the main effects of treatment with iP4, FA, day, and their interactions. The groups treated with iP4 had a smaller follicular diameter in D6 (9.6 ± 0.4 vs. 11.8 ± 0.5 mm; P = 0.03) and lower mean value for CL area between D8 and 10 (2.9 ± 0.3 vs. 2.4 ± 0.2 cm2; P = 0.02) compared to the placebo groups. Cows from the +iP4+FA group presented earlier luteolysis compared to the +iP4-FA group (D16.4 ± 0.7 vs. 18.2 ± 0.6 d; P = 0.05). In the moments prior to luteolysis (D12, 13 and 14), cows from the +iP4+FA group with luteolysis ≤ D16 had CL with a lower total and blood perfusion areas compared to cows of the same group with luteolysis > D16 (2.1 ± 0.2 versus 2.67 ± 0.2, P = 0.08; 1 ± 0.2 vs. 1.35 ± 0.2 cm2; P = 0.07, respectively). Luteolysis occurred in the presence of smaller follicles in the groups submitted to FA (2.8 ± 0.6 vs. 10.75 ± 0.7 mm; P < 0.0001) compared to groups in which there was no FA. Cows submitted to FA had a longer cycle compared to non-aspirated cows (24.9 ± 0.8 vs. 22.1 ± 0.9d; P < 0.01). In summary, iP4 supplementation 3 days post ovulation affected the development of the dominant follicle of the first wave and CL. In addition, a deficiency in size and blood perfusion of CL from animals that entered luteolysis up to D16 in the +iP4+FA group was noted. It was concluded that despite extending the duration of the cycle, follicular aspiration was not able to postpone the timing of luteolysis in cows supplemented with iP4. Thus, we reject the hypothesis that follicular E2 is necessary in this process.

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# Variation of seroprevalence of bovine viral diarrhea (BVD), infectious bovine rhinotracheitis (IBR), leptospirosis and neosporosis in dairy herds from different regions of Rio Grande do Sul

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Reproductive disorders, such as embryonic death, abortion, repeat breeder cows lead to decreased reproductive efficiency and consequent milk production. Frequently, the etiology of reproductive losses is related to the occurrence of infectious diseases, such as leptospirosis, infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD) and neosporosis, causing great economic losses. Strategies for diseases prevention may vary from region to region according to disease prevalence as well as their respective risk factors. The objective of the study was to estimate seroprevalence among individuals for leptospirosis, IBR, BVD and neosporosis in dairy cattle in different meso regions of the state of Rio Grande do Sul. Simple random sampling was performed considering an expected seroprevalence of 50% for IBR, BVD and leptospirosis and 15% for neosporosis. The 95% confidence level and 5% sample error were the other parameters used in the calculation of the sample size. Blood samples were collected in different mesoregions: MR1 (north-west and northeast, n=459), MR2 (north-west, n= 259), MR3 (south-west and southeast, n=373) for laboratory diagnosis by enzyme-linked immunosorbent assay (ELISA) Seroprevalence for IBR was was 61% (MR1), 54.8% (MR2) and 59.7% (MR3), with no difference (P> 0.05) among mesoregions. For BVD no difference (P> 0.05) was found between the seroprevalences according to the mesoregions (45.5% MR1, 30.1% MR2 and 39.9% MR3). For the neosporosis, higher serum prevalence (34.6%; P <0.05) was observed in the north-west mesoregion (MR2) compared to other mesoregions (24.5% MR1 and 21.7% MR3). In the case of leptospirosis, the highest serum prevalence was 27.5% in the southeast-southwest mesoregion (MR3), which differed (P <0.05) from other mesoregions (15.2% MR2, 17.8% MR1). The results indicate that IBR and BVD were homogeneously distributed according to the mesoregions studied. However, mesoregions with higher sero-prevalence were identified for leptospirosis and neosporosis, indicating a spatial variation in the health problems of these diseases.

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# Effect of *in vitro* co-culture of buffalo embryos with bovine cumulus cells on the potential for early embryonic development

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In vitro embryo production (IVEP) in buffaloes is a promising technique for the multiplication of genetic material from maternal origin, however, embryo production rates are still lower than those observed in cattle, probably due to the lower quality of buffalo oocytes (fragility of the zona pellucida and the cumulus cells). Thus, cell co-cultures are widely used during IVC of buffalo embryos, especially with cumulus cells from the IVM stage. The present study aimed to evaluate the possible benefits provided by the co-culture of buffalos embryos with bovine cumulus cells newly obtained in the IVM, believing in the contribution of these cells to the production of growth factors that, in turn, stimulate the initial embryonic development. After IVM for 22 h in TCM199 medium supplemented with 10% FBS, hormones, sodium pyruvate and antioxidants, buffalo oocytes were fertilized in Talp-IVF medium supplemented with 0.6% BSA for 24 h. Then the development culture was performed in modified SOF medium supplemented with 2.5% FBS and 6 mg/mL BSA, and the structures maintained at 38.5 °C and 5% $CO_2$ atmosphere in air during 7 days, when the blastocyst production rate was evaluated. Three replicates of IVEP were performed, totalling approximately 100 oocytes per group. Analyses were performed in the program GraphPad Prism 7, and the proportions of blastocysts were evaluated by Fisher's exact test. Although there was a higher average blastocyst production in the group co-cultured with bovine cells, there was no difference ($P>0.05$) between the groups (co-culture with buffalo cells - 17/106 - 20.0% , Co-culture with bovine cells - 22/110-16.0%). Attanasio et al. (Theriogenology, v.74, p.1504-1508, 2010) performed a study in which buffalo oocytes were cultured with bovine somatic cells after the vitrification process and observed that only those exposed to intact bovine cumulus oocytes (CCO) complexes presented the restoration of initial developmental capacity. It is possible that co-cultivation in these systems fails to mimic the association between oocytes and radiate corona cells provided by gap junctions. Thus, it is concluded that the use of buffalo embryo co-culture with bovine cumulus cells originated from IVM does not provide preimplantation development increments, and we recommend that the strategy of co-culture of buffalo embryos with intact bovine CCOs also be investigated.

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# Comparison of cytokines concentration in conditioned medium of non-immune stimulated mesenchymal stem cells (MSCS) derived from equine amnion (AM), allantois (AL), adipose tissue (AT) and bone marrow (BM)

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Studies have shown a bidirectional interaction between MSCs and immune cells, also different sources of MSCs may release divergent paracrine factors amounts. Further, some reports suggested MSCs are not spontaneously immunosuppressive, needing to be immune-challenged to exert their immunomodulatory potential. The aim of this study was to compare the presence and concentration of pro-inflammatory (PI) INFγ, IL1α, IL2, IL4, IL08, IL15, anti-inflammatory (AI) IL1RA, IL10, MCP-1(CCL-2) cytokines and VEGF in the conditioned medium derived from AM, AL, AT and BM-MSCs, non-challenged by immune stimulus factors (LPS, IL-1α and β, TNFα ot INFγ). Cells in P3 (~5*103), previously characterized, were obtained from our cell bank, thawed, plated and cultured in medium composed of 80% DMEM/F12, 20% FBS, supplemented with antibiotics and antimycotic, at 37,5°C, 5% CO2 (Gibco-USA) and controlled humidity incubator. After reaching at least 70% of confluence, samples were washed six times with HBSS, and cultured in deprivation of FBS for 4 days. The conditioned media (CM) was then collected, centrifuged 10 min/900G, filtered in 0.22µm syringe filter and frozen at -80°C. The concentrations of cytokines were measured by quantibody array analysis, in quadruplicates, using a microarray scanner (Innopsys, model InnoScan 710). Images were analyzed by Mapix 7.0 software (Innopsys). Statistics were performed by T-Student test and one-way ANOVA, followed by Tukey's test when differences between groups were found, using Past3 Software (PAST3, 2017). Significant differences were considered when $P<0.05$. No concentrations of PI cytokines INFγ, IL2, IL4 and IL10 or AI cytokine ILRA were detected (bellow Limit of Detection) in all groups (AT, BM, AM and AL). However, IL1α low concentrations were detected, with no significant differences, for the groups AM (9,75pg/mL), AL (1,92pg/mL) and AT (1,0 pg/mL) but not in MO-CM. All groups presented IL-8 concentrations, but it was significantly higher in AL (118,02 pg/mLa) than AT and MO (21.93b and 1.53 pg/mLb), with no significant difference with AM (87,02 pg/mLab). IL15 was present only in AM (81.35 pg/mLa) and AT (70.1 pg/mLa) CM, with no differences. Also, MCP-1 (AM: 333.2 pg/mLa, AL: 341.2 pg/mLa, MO: 397.2 pg/mLa, AT: 301.8 pg/mLa) and VEGF (AM: 548.52 pg/mLa, AL: 466.6 pg/mLa, MO: 484.7pg/mLa, AT: 248.6 pg/mLa) were highly found in all MSCs-CM groups. IL8, MCP-1 and VEGF are known to be produced by MSCs in response to inflammatory stimuli and, in this study, they were found in CM of non-immune challenged MSCs from different sources, indicating their spontaneous immunomodulatory potential. Still, presence and concentrations of cytokines may vary between MSCs sources.
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# Mesenchymal stem cells (MSCS) derived from equine allantois (AL): Characterization and immunomodulatory potential of an alternative source

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Perinatal tissues such as umbilical cord and amniotic membrane (AM) have caught the attention of the scientific community as an advantageous source of MSCs for regenerative medicine due to their plasticity, proliferative and immunomodulatory potential, in addition to their non-invasive sampling. To obtain equine AM-MSCs, the AM and AL are detached from each other and, even though the membranes are juxtaposed by their connective tissues, ALs are often discarded. The aim of this study was to isolate, characterize and evaluate AL-MSCs proliferative, differentiation and immunomodulatory potential. Cells were obtained from 5 allanto-amniotic membranes harvested during term delivery. Membranes were manually detached, and AL portion was fragmented in smaller pieces (3cm2), excluding vessels, for enzymatic digestion with 0,1% collagenase type 1 (Sigma-Aldrich, USA) in culture medium (80% DMEM/F12, 20% FBS, antibiotics and antimycotic - Gibco, USA). Cells were cultured in the medium described, at 37°C, 5% of CO2 and controlled humidity. Immunophenotype was characterized by flow cytometry (P3) for mesenchymal and pluripotency markers. Proliferative potential was evaluated by growth curve (plated in P3, resuspended and counted every 48 hs/8 days), fibroblastic colony forming unities assay (CFU-F) and efficiency of CFC-U (EFCU-F). For that 200cells/cm2 were plated and cultured for five days and then fixed and stained with violet crystal (1%). Colonies with 20 or more cells were considered. In vitro adipogenesesis and osteogenesis differentiations were perfomed and immunomodulatory potential were analyzed by quantibody array, in quadruplicate, for IFNγ, IL1a, IL1ra, IL2, IL4, IL8, IL10, IL.15, MCP-1 (CCL2) and VEGF concentration in the conditioned medium (CM) obtained after 4 days culture in FBS deprivation. Samples displayed plastic adherence, fibroblastic morphology, good proliferation (cells/cm2 - D0: 2000; D2:3820.26; D4:14660.13; D6:25525.47; D8:40433.56), high CFU-F (189,7) and ECFU-F (10.53). High expression of mesenchymal markers (CD44: 95.6%, CD29: 97.88%), low expression of hematopoietic marker (CD34: 8.4%), low expression of MHCII: 8.26%, and low expression of epithelial cells (Cytokeratin: 9.88%) were observed. High expression of pluripotent markers SOX2: 88.76, OCT4:75.9% and NANOG: 68.3% corroborates with another fetal sources' expressions, indicating high plasticity and differentiation potential. Further, in vitro osteogenic and adipogenic differentiations were positive. No relevant concentrations of IFNγ, IL1ra, IL2, IL4, IL10 and IL15 were shown and low concentration of IL1α (1.8pg/mL) were found. However, relevant concentrations of IL8 (118pg/mL), MCP-1 (341pg/mL) and VEGF (591.6pg/mL) were found. IL8 and VEGF are known to be released by MSCs after inflammatory stimuli. These results showed AL is, indeed, a good alternative source of MSCs, releasing cytokines involved in immune response and must be further studied.

Acknowledgments: FAPESP.

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# Influence of OCT4 and SOX2 exogenous expression on imprinting maintenance at H19/IGF2 locus in cattle

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OCT4 and SOX2 are fundamental genes for the acquisition and maintenance process of cellular pluripotency and therefore for induced pluripotent cells (iPSCs) generation. Recently, it has been reported that both factors may have a huge influence on the regulation of some imprinted genes, especially at locus H19/IGF2, known to be important for the normal development of embryo and placenta. Herein we aimed to understand the possible influence of their expression, together or separately, on H19/IGF2 imprinting regulation. For that, bovine fetal fibroblasts cell lines were transduced with lentiviral vectors containing human OCT4 or SOX2 cDNAs. The fibroblasts were analyzed trough cell cytometry and positive cells were sorted. Fibroblasts expressing OCT4, SOX2, both (OCT4+SOX2), none (control) together with a non-sorted and non-transgenic control (five treatments) were investigated regarding pluripotency and imprinted gene expression as well maintenance of DNA methylation patterns at H19/IGF2 locus. Expression of OCT4, SOX2, H19 and IGF2R genes were analyzed after RT-qPCR in triplicate for each experimental group using ACTB e C2ORF29 as constitutive genes. Sodium bisulfite treatment and sequencing of the differentially methylated region (DMR) were used for imprinting analysis. The amplification of fragments from the imprinting control region (ICR, H19-ICR) and sequencing for a single nucleotide polymorphism (SNP) at IGF2/H19 locus between Bos indicus and Bos taurus allowed for the allele-specific analysis of DNA methylation. The transcript quantification by qRT-PCR showed that OCT4 and SOX2 expression increased in the respective groups, the expression of H19 gene increased in the control sorted group and IGF2R expression was not different between groups. Imprinting pattern methylation at H19/IGF2 locus showed that OCT4+SOX2 group was slightly different from others whereas the control sorted group presented a demethylation on the maternal allele: Control non-sorted (21, 42% for DMR and CTCF, respectively), control sorted (0%), OCT4+ (21, 24%), SOX2+ (2, 4%), OCT4+SOX2 (5, 95%). In conclusion, this study shows that the production of cells expressing exogenous pluripotent factors was successful, the H19 imprinted gene expression pattern was influenced by cytometry and sorting and imprinting maintenance at H19/IGF2 locus may be influenced by both cytometer/sorting procedure and pluripotency related genes overexpression. Such results are unprecedented and may greatly contribute to the understanding of the role of these pluripotency factors in acquiring and maintenance of the epigenetic patterns in reprogrammed cells in cattle. We acknowledge the São Paulo Research Foundation (FAPESP) for the funding (Grant number: 15/01407-2).

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# Sirna-mediated silencing of HMTs in nuclear donor cells decreases H3K9me in cloned embryos at the 8-16 cells stage, but not at the blastocyst

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Epigenetic mechanisms are responsible for many cellular functions. Amongst them, is the epigenetic memory maintained during cell division. Consolidation of this information is made by stable chemical marks as DNA methylation and histone modification. Understanding epigenetic mechanism allows the development of several studies, such as nuclear reprogramming, which is directly influenced by those marks. Thus, H3K9 methylation has been shown as the main barrier on the rodents nuclear remodeling, it modulation showed significantly improves in both iPS generation and Somatic Cell Nuclear Transfer (SCNT). Hence, the main goal of this project was to knockdown HMTs responsible for H3K9 methylation in bovine fibroblasts preceding SCNT, and to investigate it consequences on embryo development. To achieve these modifications, histone methyltransferases enzymes EHMT2 (G9a), SUV39H1, SUV39H2, and SETDB1 were silenced by small interference RNA technique (siRNA). The H3K9me2 and H3K9me3 levels were analyzed by immunostaining. Those cells were also used as nuclear donor in SCNT to understanding their role during nuclear reprogramming. For this, cells combining siRNAs for each gene (siRNA) or not (Control) were used as nuclear donor on SCNT (N=5). IVF embryos were produced at the same oocyte collection and used as biological control. Embryos at the blastocyst stage were accounted and compared among the groups. After analyzed, our blastocyst rates were 34% (2.4), 60% (4.8), and 28% (6.3) for IVF, SCNT-Control and SCNT-siRNA, respectively. Embryos at both 8-16 cells stage and blastocysts (N=10) were collected to evaluate by immunostaining the effect of HMTs knockdown upon H3K9me2 and H3K9me3 levels. The analysis showed that, in SCNT-SIRNA group, levels of H3K9me2 were considerably lower than control and IVF at the 8-16 cells stage, but not in blastocyst. In H3K9me3 levels, this decrease at the 8-16 cells stage was less evident, but did not change on blastocysts. Changes at this stage are noteworthy since it is the same period that occur the genome activation. The recovery of H3K9 methylation levels displayed at the blastocyst stage may explain why blastocyst rates did not increase as described in mice. Studies describing the kinetics of epigenetic modifications during embryo development in bovine are needed to elucidate the divergence between species.
Financial support: FAPESP-CEPID 2013/08135-2; FAPESP-BEPE 2015/25111-5; FAPESP 2015/08807-6.

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# Comparison of cytokines concetrations in conditioned medium derived from canine amniotic membrane and adipose tissue mesenchymal stem cells non-immune stimulated

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It is well known that the presence of cytokines is crucial for an appropriate cell communication and the stimulation of the immune system in order to restore homeostasis against an aggression. The conditioned medium (CM) is an outcome of paracrine factors secreted by the mesenchymal stem cells (MSCs) and has an essential role in inhibiting apoptosis, enhancing angiogenesis, reducing inflammation and stimulating the immune system. The purpose of this study was to compare the presence and concentration of IL2, IL6, IL8, IL10, GM-CSF, MCP-1, RAGE, SCF, TNFα e VEGF cytokines in CM derived from canine amniotic membrane (AM) and adipose tissue (AT) MSCs non immune-stimulated. The previously characterized samples, in P3, were obtained from the laboratory cell bank, thawed, plated and cultured in 80% DMEM/F12 culture medium supplemented with 20% FBS, antibiotics and antimycotic (Gibco, USA), at 37.5°C, 5% of CO2 and controlled humidity incubation. After reaching confluence of approximately 70%, the cells were washed six times with HBSS and cultured in FBS deprivation for 4 days in order to obtain the CM from both sources. Subsequently, the medium was harvested, centrifuged 10 min/900G, filtered in 0.22μm syringe filter and frozen at -80oC for future analysis. The cytokines concentrations were analyzed by quantibody array of four AM and three AT samples, in quadruplicate, using a microarray scanner (Innoppsys, model InnoScan 710). Images were analyzed by Mapix 7.0 software (Innopsys). Statistics were performed by unpaired T student test with PAST3 Software 20017. Significant differences were considered when $P<0.05$. The pro-inflammatory cytokines IL2, IL6, RAGE and TNFα were not detected in either MSC sources. IL10 (AM: 808.72 and AT: 722.02 pg/ml), GM-CSF (AM: 10.83 and AT: 9.28 pg/ml), MCP-1 (AM: 2632.89 and AT: 3914.58 pg/ml) and SCF (AM: 18.65 and AT: 33.79 pg/ml) concentrations were detected, but with no significant differences between the studied groups. IL8 concentration in the AM-CM was significantly higher than AT-CM (2049.52 pg/mla and 42.90 pg/mlb). The cytokines detected in these samples are known to be released by the MSCs after an inflammatory stimulus with LPS, IL1α and β, INFγ or TNFα. The relevant concentrations of the cytokines found in CM derived from non-immune stimulated MSCs of this experiment encourage further studies regarding its spontaneous immunomodulatory potential. Acknowledgments: CAPES e FAPESP.

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# Development of an *in vitro* model to study cell-to-cell communication utilizing transgenic fibroblasts capable of secrete extracellular vesicles tagged with GFP

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Extracellular membrane vesicles, also called as extracellular vesicles (EVs), are produced and secreted from different cell types. They recently emerged as a new and important component of cellular communication. Thus, EVs can transfer different kinds of signals between the cells that produce them and the recipient cells. However, the role and tracking of the VEs during the cellular communication process is still not clear. Therefore, the development of transgenic cell lines capable of produce EVs tagged with GFP can facilitate these studies. Herein, our aim was to develop a transgenic fibroblast cell line that secretes GFP-EVs. For this purpose, bovine fibroblasts were transfected with the DNA constructs using a stable transducer with lentivirus vectors (MGH Vector Core, Boston, MA, USA). Briefly, lentiviral particles were produced by transfection of 293T cells (Invitrogen) with Lipofectamine 2000 (Invitrogen) for 12-16h. After this, the medium was recovered and centrifuged to harvest the viral particles. Fibroblasts were plated at the density of 105 cells and 50 µl of the viral concentrate plus 8ng/mL polybrene (hexamethrin bromide, Sigma) were added to the culture. The culture medium was replaced every 12h for five days. Afterwards, the cells were sorted by flow cytometry. The size, complexity and the percentage of GFP-positive cells (GFP+) were analyzed. For this, 10.000 fibroblasts from the both groups, Control (non-transfected) and GFP+, were selected by sorting of cells with GFP protein-fluorescence presence. In the control group, there were no GFP+ cells and 87.4% (n = 8.741) of the cells were retrieved. Regarding the GFP+ group, we retrieved 94.2% (n = 8.043) and all of them were expressing the fluorescent-protein. The recovered cells from both groups were re-plated at the same concentration (105 cells/mL) and after three days of culture, the medium was recovered for evaluation of the size and concentration of the produced EVs. In relation to the EVs production, the Control and GFP+ groups presented concentration of 2.86x108 and 5.95x108 particles/mL and the size average were 165nm ± 2.5 and 141nm ± 5.2, respectively. Based on the preliminary results presented here, we were able to establish and produce a transgenic cell line capable to release EVs tagged with the GFP protein. However, others evaluations are still necessary for complete characterization of these vesicles. In addition, through this in vitro model, future studies will be able to investigate the content, tracking and action of EVs in several biological models.
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# Effect of stem cells application on the follicular population of bovine females (preliminary results)

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The objective of the present study was to evaluate the ovarian follicular population in bovine females after the application of allogenic (from another individual of the same species) mesenchymal stem cells (MSCs) in the ovaries. For this, 15 Nelore cows (Bos indicus) were submitted to synchronizations of the follicular growth wave to measure the amount of follicles at the beginning of the emergence of the follicular growth wave [Day-5 (D-5): insertion of an intravaginal P4 device (1.0 g) for 5 days and administrations of EB (2.0 mg im) and PGF2α (0.53 mg im, cloprostenol sodium)]. On D0, cows were divided to one of three experimental groups: CONT (n=5), MSC1 (n=5) and MSC2 (n=5), according to the amount of follicles verified in the ovaries. MSCs of adipogenic origin were isolated and cultured with IMDM culture medium with 10% SFB and 1% P/S at 37°C in 5% CO2 for cell expansion till third passage. Cells were frozen in DMSO and kept in liquid nitrogen until the day of application in the ovaries (D1). On this day, the cells were thawed and the DMSO removed. They were then maintained in IMDM culture medium until the time of application. In the CONT females were not applied MSCs. In MSC1, MSCs (3x106 cells) were applied to the cortical layer of one of the ovaries. In MSC2, MSCs (3x106 cells per ovary) were applied to the cortical layer of both ovaries. The cows were submitted to ovarian ultrasonographic (US) evaluations to quantify the follicular population on D0, D7, D14 and D21. The data were analyzed as time-repeated measure using the GLIMMIX procedure of SAS. The ovarian follicular population on days 0, 7, 14 and 21 were respectively: CONT (42.0±9.8, 44.5±9.4, 37.0±8.0 and 41.5±8.8), MSC1 (42.8±8.6, 41.8±4.3, 49.2±10.3 and 49.8±8.4) and MSC2 (40.2±6.4, 42.6±8.3, 59.2±15.3 and 48.0±8.1; Ptreatment=0.87, Ptime=0.33 and Pinteraction=0.21). Despite the numerical increase, in the present study no statistical difference was verified in the ovarian follicular population of bovine females submitted to the application of allogeneic mesenchymal stem cells in the ovaries. Further studies are needed to evaluate the long-term effect of MSCs treatment on the ovarian follicular population.

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# Effect of the passage number on the viability of brown brocket deer (Mazama Gouazoubira) fibroblasts

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The brown brocket deer (Mazama gouazoubira), one of the ten recognized deer of the Neotropical region, has recently been placed in a critical position due to current threats, mainly hunting and habitat loss. Several studies have shown that some endangered species may benefit from interspecific Somatic Cell Nuclear Transfer (iSCNT) due to the possibility of using fibroblasts as karyoplasts. Thus, the objective of this study was to verify the viability of M. gouazoubira fibroblasts submitted to several cell culture passages. Cells were obtained by auricular biopsy and then cultured and cryopreserved at passage 1. Cell culture was performed from this stock until the passages of interest were achieved (4, 7 and 10). Cells were cryopreserved in cryotubes (frozen/warmed group) and there was a cultured control group for each cryopreserved passage. Population Doubling Time (PDT) of each passage was determined using cultured control groups. Trypan Blue test, MTT assays and flow cytometry analysis (apoptosis and necrosis level) were performed using frozen/warmed groups with their respective cultured control groups for comparison. Statistical analysis was performed by means of ANOVA. Student's t-test and Fisher's exact test were applied when convenient. In order to detect statistical differences, a p-value of 0.05 was specified as the significance level. Fibroblastic cells were observed to grow between 12 and 14 days after the tissue explants had adhered to the culture plates. The PDT was higher ($P < 0.05$) in passage 10 (229.7 ± 3.3 h) when compared to passage 4 (89.3 ± 0.95 h) and 7 (90.8 ± 1.21 h), which were not different between them ($P > 0.05$). Cell proliferation rate of passage 10 was significantly lower ($P < 0.05$) when compared to passages 4 and 7. A high cell survival rate of more than 80% was observed in all groups, except for passage 10 in the frozen/warmed group. Regarding the MTT test, no significant difference ($P > 0.05$) was observed for the metabolic activity in the cells of passage 4 compared to passage 7. However, passage 10 exhibited low metabolic activity (62%) with respect to their control in culture. This value was significantly lower ($P < 0.05$) when compared to the passage 4 (80.5 %). Moreover, concerning the percentage of viable cells (in frozen/warmed group), it was observe that cells from passages 4 (88.6 %) and 7 (87.8 %) were statistically superior ($P < 0.05$) to passage 10 (59.1 %). Therefore, our findings clearly show the viability of M. gouazoubira fibroblasts derived from passages 4 and 7, and these cells could be used as a useful tool in conservation programs for this species.

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# Effects of buffer medium and cytochalasin B on survival, development and reporter gene expression following embryo cytoplasmic microinjection with DNA

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The aim of this study was to compare the in vitro efficiency of distinct buffer media to deliver nucleic acids into bovine embryos by cytoplasmic microinjection (MI). Tris-EDTA (TE) and KCl-based (PS) buffers were compared for DNA dilution (circular GFP vector) at 30 ng/µL for the MI of bovine embryos after 5-min incubation or not in 5 µg/mL cytochalasin B (CCB) in the medium prior to MI. Bovine in vitro-matured oocytes were chemically activated in 5 µM ionomycin for 5 min, followed by incubation in 2 mM 6-DMAP for 4 h. Embryos were allocated to one of five groups: non-manipulated controls, and TE, PS, CCB+TE, and CCB+PS microinjected groups. The MI of 15 pL (1.5% of total embryo volume) was performed using a microinjector apparatus (Femtojet 4i, Eppendorf, Germany). Then, embryos from all groups were in vitro-cultured for seven days. Survival, cleavage and blastocyst rates, and proportion of GFP+ blastocysts were compared by the Chi-square test ($P<0.05$). After five replicates, 1,432 matured oocytes were chemically activated, and 1,053 microinjected. Overall, CCB-treated embryos had higher survival (82.3%, 427/519) and blastocyst (28.3%, 121/427) rates than non-treated counterparts (67.6%, 361/534; and 21.9%, 79/361, respectively), irrespective of the buffer medium, with no effect on cleavage rates (61.8%, 264/427 vs. 63.2%, 228/361, respectively), with cleavage and blastocyst rates similar to controls (70.6%, 190/269; and 26.4%, 71/269). The use of TE or PS buffers, regardless the CCB treatment, did not affect survival (73.1%, 384/525 vs. 76.5%, 404/528) or blastocyst rates between groups (28.1%, 108/384 vs. 22.8%, 92/404) and controls (26.4%, 71/269), but cleavage rate was lower using PS buffer than controls (61.1%, 247/404 vs. 70.6%, 190/269, respectively), with both being similar to the TE group (63.8%, 245/384). The TE and CCB+PS groups had similar (61.3%, 106/173; and 57.9%, 125/216) cleavage rates, but lower than controls (70.6%, 190/269), whereas the PS (64.9%, 122/188) and CCB+TE (65.9%, 139/211) groups were similar to all groups. The CCB+TE group had better blastocyst development (30.3%, 64/211) than PS (18.6%, 35/188), with both being similar to the Control (26.4%, 71/269), TE (25.4%, 44/173) and CCB+PS (26.4%, 57/216) groups. The CCB incubation did not affect GFP expressivity (50.4%, 61/121) in blastocysts when compared to no CCB exposure (51.9%, 41/79). However, DNA dilution in TE buffer improved the number of GFP+ blastocysts (62.0%, 67/108) than the PS buffer (38.0%, 35/92). A higher proportion of GFP+ embryos was seen in the CCB+TE group (63.5%, 40/64) than the PS (40.0%, 14/35) and CCB+PS (36.8%, 21/57) groups, with the TE group being similar (61.4%, 27/44) to the CCB+TE and PS groups. In summary, the embryo pre-exposure to CCB and the use of TE buffer for DNA dilution improved survival after microinjection, cleavage and blastocyst rates after culture, and proportion of GFP+ blastocysts on Day 7 of development.

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# Evaluation of the production of cytokines by bovine endometrial mesenchymal stem cells

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The aim of this work was to evaluate with a comercial kit the production of cytokines by bovine endometrial mesenchymal stem cells (eMSCs) challenged (treated – T) or not (control – C) with bacterial lipopolysaccharide (LPS- 1µg/ml : E. coli serotype 0111: B4, Sigma, St Louis, USA). Bovine eMSCs were collected in two phases of the estrous cycle (Phase, II N=6; Phase III N=6), and characterized. Cells on thrid passage were cultured with medium composed of DMEM high glucose/F12 (1:2), 20% fetal bovine serum (FBS), 100IU/mL penicillin, 100µg/mL streptomycin, 3µg/mL amphotericin B (Thermo Fisher Scientific®, (Walthan, USA)) and 11µg/mL amikacin (Teuto®, (Anápolis, BRA)) until 60% confluence. After confluence, cells were divided into studied groups and cultured with FBS free medium (C) or with 1µg/mL LPS (T) diluted in medium. Conditioned medium was collected after 2, 6, 12 and 24 hours of exposition, filtered through 22 µm filter and centrifuged at 2000 x g for 5 minutes. Samples were analyzed by Quantibody® Bovine Cytokine Array 1 kit (QAB-CYT-1, Raybiotech®, Norcross, USA), according to manufacturer's specifications and concentration of INFα, INFγ, IL-13, IL-1α, IL-1α, IL-F5, IL-21, MIP-1β, TNF-α on conditioned medium was analysed. The slides were scanned using a microarray scanner (Innopsys, model InnoScan 710) and the images were analyzed at Mapix 7.0 software (Innopsys). Each sample was evaluated in quadruplicate, and their data were normalized according to intra-slides positive controls. Results were achieved by eliminating the background. Sample concentration (pg/mL) was calculated based on linear regression of standard curves. Data were analyzed using the software GraphPad Prisma, version 6.01. Normality test (Shapiro Wilk) was done and groups were compared and analyzed using the non-parametric Wilcoxon test and moments at the same group were analyzed using the non-parametric Kruskal-Wallis test. For the analysis, P values < 0.05 was considered to represent a significant difference. The quantication of cytokines reveled production of all cytokines by all samples. When studied groups were compared, it was observed significant difference ($P < 0.05$) only for the cytokine TNF-α on moment 6, with greater concentration on samples of treated group (74.3 pg/mL±40.3 x 32.4 pg/mL±27.3). No difference ($P>0.05$) was observed when moments from same group were evaluated. In both experimental groups we observed the presence of pro and anti-inflammatory cytokines in the conditioned medium at all moments. The elevation of TNF- α evidences and confirms the action of LPS on the stimulated cells. We believe that future studies should be carried out with higher concentrations of LPS, mimicking pathological uterine processes, and with shorter times of collection of conditioned medium, enabling the earlier analysis of cytokine release.

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# Exposure *in vitro* of endometrial bovine endometrial mesenchymal stem cells to prostaglandin E2

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The objective of this research was to evaluate the transcriptomic changes of bovine endometrial mesenchymal stem cells (MSCs) in response to different PGE2 concentrations. Primary cultures of MSCs from healthy cycling cows in late luteal phase (LLP1 and LLP4)1 were seeded at 4 x 104 cells/cm2 and maintained in DMEM-F12 with 10% FCS, supplemented with 1% AAM solution, 1 mM sodium pyruvate and 2 mM L-glutamine, and cultured in 5% CO2, 39°C and 100% humidity. The PGE2 (Caymann Chemical, Ann Arbor, Michigan, USA), was diluted in DMSO and added in concentrations of 1, 3 and 10 uM in triplicates. After 28 hours the cells were scrapped, subjected to RNA extraction using EZNA Total RNA Kit I (Omega Biotek, Santiago, Chile) and synthesis of complementary RNA with Agilent Low Input Quick Amp Labeling kit (Agilent Technologies©, Santa Clara, CA, USA). The bovine (V2) Gene Expression Microarray 4x44 (Agilent Technologies, USA) was used for differential gene expression and data obtained were analyzed using GeneSpring 12.5 extraction software (Agilent Technologies, USA). Significantly differentially expressed genes with a fold change greater than or equal 2.0 were selected and analyzed Gene Ontology (GO) using Panther software 11.1 (University of Southern California, USA), and gene interaction network was created with the GeneMania Prediction Server. To validate microarray data, the expression profile of 13 genes were selected and evaluated using qRT-PCR, and statistical analysis was conducted employing Pearson's correlation test. We found 1127 genes differentially expressed between the control group, (PGE2 concentration=0) and the rest of the doses of PGE2 considered together at p value of 0.05, and 2X fold change. The FDR was of 0.05%. The top 40 most deregulated genes demonstrated through GO that the biological processes affected the must were: cellular component organization or biogenesis and cellular and metabolic processes. Among other represented processes found were: biological regulation, development, growth and immune system. While the most represented molecular functions were: binding, catalytic, receptor, transport and structural molecule activity. GeneMania Prediction Server determined the predominant interaction was a coexpression (70.5%). A qPCR was an absolute coincidence between the microarray and qPCR data, with an average value of R = 0.89. Our findings indicate that the exposure in vitro of bovine endometrial MSCs to a mediator of inflammation such as PGE2, modifies their transcriptomic profile. So PGE2 could have a potential role in the fate of stem cell activation, migration, and, homing processes during pathological, uterine inflammation like as in endometritis and also in healthy puerperal endometrium.

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1. Cabezas, J. et al. Reproduction in Domestic Animals, 49(4): 550–559, 2014.

A289 Cloning, transgenesis, and stem cells

# Generation of transgenic cattle for human proinsulin and human blood coagulation factor IX

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The generation of transgenic animals as bioreactors for expression of recombinant proteins is an important advance in biotechnology. Cows can produce large quantities of proteins in the milk and mammary glands are able to perform post-translation modifications in proteins, which become therefore active biologically. In this way, the mammary gland is considered one of the best tissues to produce recombinant proteins in transgenic animals. Few transgenic cows have been generated in comparison to other species due to the 1) low efficiencies of the technologies in this species, 2) to the low number of offspring generated per gestation and 3) to the long period of gestation. Mammary glands have been used for the production of biopharmaceuticals in milk and currently, only two products from the milk of transgenic animals have been approved and are being used for human health. Herein, we have generated two transgenic cows to produce recombinant proteins, one for human proinsulin and other for the human blood coagulation factor IX. Previously, two lentiviral vectors containing the genes of were constructed, with the tissue specific expression driven by β-casein promoter. The lentiviral vectors produced were used to transduce bovine fibroblasts culture. The modified cells were selected by blasticidin-resistence and used to produce transgenic embryos via somatic cell nuclear transfer methodology. Embryos at seven days of age post-fusion were transferred to recipient cows previously synchronized, the pregnancy was monitored and live born animals were obtained. The transgenic animals generated are being subjected to hormonal induction of lactation to evaluate recombinant protein expression in the milk.

A290 Cloning, transgenesis, and stem cells

# Isolation and culture of stromal cells from buffalo Wharton's jelly

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Stem cells are undifferentiated cells, which can be used for the treatment of various conditions due to their immunomodulatory and therapeutic properties. The aim of the present study is to describe the isolation and culture of stromal cells (presumably mesenchymal stem cells) from buffalo Wharton´s jelly. For this purpose, approximately 30 cm of umbilical cord (n = 4) was collected in a slaughterhouse and transported to the laboratory in a solution composed of DMEM + F12, penicillin (100 IU/mL), streptomycin (100mg/mL), amphotericin B (3mg/mL) (Thermo Fisher Scientific, USA), and amikacin (11 mg/mL) (Teuto, BRA). In the laboratory, the umbilical cord was placed in 70% alcohol solution for 1 minute, followed by dissection of the umbilical vessels and collection of the surrounding tissue corresponding to Warthon´s jelly. The Wharton´s jelly was then sectioned into smaller pieces and enzymatic digestion was performed in 0.1% collagenase solution in PBS for 1.5 hours at 37.5°C. Every 10 minutes the samples were homogenized in a vortex to improve the digestion process. After digestion, the samples were filtered on 70 micron filters and centrifuged in culture medium composed of DMEM + F12 penicillin (100 IU/mL), streptomycin (100mg/mL), amphotericin B (3mg/mL) (Thermo Fisher Scientific, USA), and amikacin (11 mg/mL) (Teuto, BRA). The pellet was then resuspended and the cells cultured at 37.5°C in a humidified atmosphere, containing 5% CO2 in air. The samples showed adherence to the plastic within 24 hours and the presence of colonies with fibroblastoid morphology was observed within 72 hours. The first medium exchange was performed at 96 hours. The first passage was performed one week after the isolation and the samples were cryopreserved for later characterization. According to the morphological characteristics and adhesion to plastics, we can assume that we have isolated buffalo mesenchymal stem cells, as previously performed in other species. The characterization process is in progress to confirm this affirmation. Acknowledgment to FAPESP: Process 2015/01057-1.

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# Isolation and culture of stromal cells from adipose tissue of sea turtle Caretta caretta

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The loggerhead sea turtle (Caretta caretta) is an endangered animal, according to the IUCN Red list of threatened species. Sea turtle populations have been seriously depleted worldwide due to human impacts. The TAMAR Project monitors approximately 1,100 km of beaches located on the mainland coast and oceanic islands. The project works with rehabilitation, aiming to nurse these animals back to health and release them into the sea. Isolation and culture of mesenchymal stem cells (MSC) may allows the treatment of various diseases by the use of cellular therapy and permits storing valuable genetic material. The objective of this study was to isolate and culture adipose tissue stem cells from the sea turtle Caretta caretta, comparing the efficiency of 5 protocols. The subcutaneous adipose tissue was collected from a female donor, with the authorization of the Tamar Project. The animal was submitted to general and local anesthesia and antisepsis of the inguinal region, followed by a skin incision and collection of the subcutaneous adipose tissue. The material collected was sent to OMICS Biotechnology Animal Laboratory. For transportation (6 hours), the adipose tissue fragments were placed in a solution containing penicillin, streptomycin, amphotericin and amikacin (AAS). In Group A, the fragments were placed in a solution with 0.04% of collagenase and the enzymatic digestion started during transportation. The digestion protocols were: A) Transport in collagenase solution followed by incubation in 0.04% collagenase solution in a water bath at 37.5°C for 30 minutes B) 0.04% collagenase solution for 30 min passing through the vortex every 10 min. C) 0.04% collagenase overnight. D) Collagenase 0.25% 15 min in incubator at 37°C. E) Trypsin (TrypLE Express ®) overnight. After enzymatic digestion, the solution was filtered, diluted 1: 1 in DMEM F12 with 20% fetal bovine serum (FBS), antibiotic and antifungal and centrifuged at 1500 rpm for 10 min. The cell pellet was re-suspended in two 25 cm² culture bottles/group. Culture was performed in DMEM F12 with 20% FBS, antibiotic and antifungal at 28°C and 5% CO² in air. Culture flasks were observed every 4 days to assess cell growth. After 15 days of culture fibroblastoid-like cells adherent to the plastic were observed in one bottle of Group A and one of Group B, as well as in the two bottles of Group E. After 30 days of culture the cells of group E reached confluence and were submitted to first passage with 97.5% of cell viability according to Trypan Blue staining. While those in the groups A and B, are still growing. We concluded that both, the use of trypsin overnight and 0.04% collagenase solution were efficient in isolating stromal cells in sea turtles, though the results with the use of trypsin appear to be superior. To our knowledge this is the first report of isolation and culture of adipose tissue stromal cells obtained from Caretta caretta.

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# Isolation and *in vitro* culture of somatic cells derived from Jaguar (*Panthera onca*) ear tissue

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The establishment of *in vitro* culture of cells derived from skin fragments has been proposed as a useful technique for the conservation of endangered species, especially when associated with nuclear transfer (cloning). Among the species vulnerable to extinction, the jaguar (*Panthera onca*) requires strategies for the conservation of its genetic diversity. Therefore, the aim of the present work was to describe the *in vitro* culture of somatic cells derived from *P. onca* skin, using morphological analysis, trypan blue cell viability assay and metabolic activity by the 3-(4,5-Dimethylthiazol-2yl) -2,5-diphenyl tetrizoline bromide) or MTT. Thus, skin biopsy derived from peripheral ear region were recovered using surgical scissors from two males with age of 10 and 15 years, anesthetized and from zoos located in northeastern region of Brazil. Samples were transported for 3 to 5 h in minimal essential medium modified by Dulbecco (DMEM) supplemented with 2.2 g/L sodium bicarbonate, 10% fetal bovine serum and 2% penicillin and streptomycin solution, pH 7.4 at 4°C. In the laboratory, fragments (9.0 $mm^3$) were cultured under controlled atmosphere (38.5°C, 5% $CO_2$) and evaluated every 24 h. After the cells reached 70% confluency, the first cell subculture was performed to obtain the desired concentration (5.0 x $10^4$ cells/mL) for the MTT assay. Additionally, a pool of trypsinized cells was evaluated for viability with trypan blue. All data were expressed as mean ± standard deviation. Thus, the total culture time was 29 days for the samples derived from both individuals and from the eight fragments submitted to the culture (four fragments per animal) all presented adherence on the first day with cell growth around the explants from the ninth day (9.0 ± 2.8). Cells reached 70% confluence forming monolayer on day 12.5 ± 2.1. In general, from the morphological analysis, all cells had fusiform morphology with a centralized oval nucleus, showing to be similar to fibroblasts. As for the trypan blue test, a viability of 99.6% ± 0.6 was obtained. In the MTT assay, the percentage of 100% ± 14.9 was obtained in D5 and in the D7 100% ± 18.1 of metabolic activity. In conclusion, skin biopsy derived from jaguar peripheral ear region allowed the isolation of viable cells similar to fibroblasts and with high metabolic functionality, providing a valuable source for the somatic cell nuclear transfer, aiming at the genetic conservation of this species.

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# Medium and temperature optimization for the recovery of membrane integrity of swine spermatozoa submitted to electroporation

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Electroporation (EP) is an efficient method for transfection of sperm cells. The success of EP depends, among other things, on the use of the appropriate medium during PE and on cell recovery post EP. The membrane permeabilization in the PE should only be for a sufficient internalization of the desired substance and after EP, it is ideal that the cells remain in medium and temperature that allow their membranes to regenerate. Therefore, the aim of this study was to establish for the electroporation of swine spermatozoids under conditions of low and high stress the medium and incubation temperature post EP that provide greater recovery of plasma membrane integrity (RPMI). Dilution of inseminating doses of 10 males of commercial strains were in BTS to obtain samples with 200µl and $2x10^6$spzt / ml, which composed the experimental groups. After EP in two different conditions (500 volts, 250µs and 1 pulse (EP1) or 1000 volts, 500µs and 2 pulses (EP2), the samples were recovered in four different conditions: mTBM (modified Tyrodes Buffered Medium) at 17°C or 37°C, or BTS at 17°C. Propidium iodide (PI), one membrane impermeable fluorescent dye was the reporter molecule. Each sample was electroporated into Multiporator® (Eppendorf AG, Hamburg, Germany) using BTS at room temperature (22°C). Then, centrifuging of samples were at 9100 G for 3 minutes, the supernatant was removed and 200 µl of BTS or mTBM was added and incubated for 30 minutes at 17°C or 37°C according to their respective experimental group. Analysis of samples were by flow cytometry (BD Accuri™ C6) for measuring the rate of incorporation of PI, which occurred in three moments, before EP (PI0), just after EP (PI1) and 30 minutes after recovery (PI2). The calculation of RPMI rate was by the formula: RPMI = (PI2-PI0) – (PI1-PI0). The lowest value found indicates the best RPMI. For statistical analysis, PROC MIXED (SAS, version 9.2 for Windows) was used, with comparisons using the Turkey test, analysis of interaction between variables and significance level of 5% ($P < 0.05$). There was interaction between the medium and the temperature (p=0.0008). The BTS at 17°C had a RPMI of -9.94% ± 5.91, the only combination capable of recovering membranes above pre-recovery values, being more efficient than the mTBM at 17°C (32.5% ± 5.91 ($P < 0.0001$), but with no difference with BTS at 37°C (14,04% ± 6,61%) (P=0.0525) and mTBM at 37°C (10.38% ± 6.27) (p=0.1085). Interaction between the EP and the medium used (p=0.0471) was also observed. The mTBM medium when used together with EP2 presented worse results than the other groups, presenting RPMI of 35.82% ± 6.27, being the BTS medium with EP1 was 0.49% ± 6.27 (p=0.0022) and for EP2 3.60% ± 6.27 (p=0.0056) and the mTBM and EP1 medium was 7.06% ± 5.91 (p = 0.0018). This study demonstrated that, in general, the BTS medium, mainly at 17°C, was more efficient in recovering integrity of the plasma membrane of swine spermatozoa submitted to electroporation.

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# Reprogramming porcine fibroblast cells into induced pluripotent stem cells (piPSCs) using lentiviral vectors and exogenous pluripotency transcription factors *in vitro*

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Lentiviral vectors are commonly used for efficient transgenes and may be used for reprogramming different types of cells into both naïve and primed pluripotency states. Recent studies have reported the production of porcine induced pluripotent stem cells (piPSCs) however, no consensus in the literature has been reported between different protocols or interspecific exogenous transcription factors and pluripotent states the iPSCs in the porcine model. This study aims to evaluate the effect of murine leukemia inhibitory factor (mLIF), human basic fibroblast growth factor (bFGF) in piPSCs during in vitro reprogramming obtained through overexpression of murine pluripotency factors OCT4, SOX2, c-MYC and KLF4 (OSMK). Porcine fibroblasts were transduced with lentiviral polycistronic excisable vector containing the murine transcription factors (STEMCCA vector). Six days after transduction the cells were replated into mouse inactivated fibroblast feeder layers (MEFs) and cultured in iPS medium: KnockOut DMEM/F-12 medium with 20% KnockOut Serum Replacement (KSR, Cat#10828028, Invitrogen, EUA), 0.5 mmol L−1 L-glutamine, 1% NEAA, 0.1mmol L-1 β-mercaptoethanol, 1% Penicillin/Streptomycin. During the reprogramming period, the cells were divided into two group: First group, the culture medium 1 (M1) was supplemented with 4 ng mL-1 basic Fibroblast Growth Factor (bFGF, Cat # 13256029, Invitrogen, EUA) and 4ng ml-1 Leukemia inhibitory factor (mLIF, Cat # ESG1107, Millipore, EUA) and second group, the culture medium 2 (M2) supplemented only LIF. piPSCs cells generation were evaluated regarding its pluripotency state through immunofluorescence analysis, phosphatase alkaline and spontaneous differentiation. The results of piPS cell colony derivation showed that first colonies at day 12 post-transduction. The clonal lines generated were positive for alkaline phosphatase and these same colonies were OCT4 positive. The immunofluorescence analysis showed that all piPSCs were positive for OCT4, SOX2, NANOG, TRA1-60 and presented normal karyotypes. piPSCs generated with M1 presented expression of SSEA1 and TRA1-80. On the other hand, piPSCs generated with M2 presented SSEA4 expression. In vitro differentiation analysis showed that cells spontaneous differentiated when the bFGF and mLIF were removed from the medium. These cells were capable of deriving embryonic bodies after 15 days in culture. In M1 condition of culture, piPSCs differentiated into the three germ layers, proven by the expression of neuro-ilament 18, actin, and alpha-fetoprotein. Although, when M2 was used, the cells were not able to differentiate in any of the germ layers. We conclude that the lentiviral delivery of murine transcription factors promotes reprogramming of porcine fibroblast when mLIF and bFGF or only mLIF are used, however, the clonal pluripotent lines derived are different regarding the pluripotency-related characteristics. These preliminary results indicated that piPSCs generation in different culture conditions may contribute to a better understanding of the pluripotency acquisition and naïve/primed balance state in swine.

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# Systems biology analysis for the identification of biological processes and candidate genes for the prediction of oocyte donor cow quality for IVP

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The combination of OPU-IVP with modern genomic technology is foreseen to have a huge impact on cattle production. However, the complex biological mechanistic outcomes behind IVP are not fully understood. Systems biology provides a holistic view of the important biological mechanisms that control complex traits such as those related to IVP procedures. In this study, we applied a "Weighted Gene Co-expression Network Analysis" (WGCNA) to provide a better understanding of the biological mechanisms that control IVP performances and we used this information to identify candidate genes for the prediction of oocyte donor cow quality.

We sequenced total RNA from granulosa cells from aspirated oocytes collected from 23 individual slaughtered Holstein cows. A pool of oocytes from each animal was kept separately and used for IVP procedure to evaluate the performance of each donor cow, measured at blastocyst rate, kinetic score and morphology score. WGCNA of the RNA samples identified four groups of highly co-expressed genes called "modules" whose expression profiles were significantly correlated with the blastocyst rate (P-value <0.05). Functional analysis of the four modules highlighted a wide range of biological mechanisms (eg apoptosis, cell proliferation and development and oxidative stress) as well as important upstream regulators predicted to be activated (SMAD4, TP53, EGR1 and POR) or inhibited (TBX2, INSR and β-oestradiol),

The central genes in these modules as well as their upstream regulators are expected to be promising candidate genes to predict the oocyte quality of donor cows. These findings are expected to improve genomic-assisted IVP and to have a direct impact on cattle breeding.

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# Development of a laparoscopic ovarian biopsy pick-up method for goats

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Biopsy pick-up (BPU) has been considered a safe method to harvest ovarian fragments from live animals for research and/or clinical purposes. However, no studies have been reported on the use of BPU to collect in vivo ovarian tissue in goats. The goals of this study were: (I) to test different biopsy needle sizes to collect ovarian tissue in situ using the BPU method (Experiment 1), and (II) to study ovarian tissue features such as preantral follicle density, morphology, class distribution, and stromal cell density in ovarian fragments obtained by BPU (Experiment 2). In Experiment 1, goat ovaries (n=20) were collected in a slaughterhouse and subjected to in situ BPU. Three needles (16, 18, and 20G) were tested. In Experiment 2, the more efficient biopsy needle from Experiment 1 (16G) was used to perform laparoscopic BPU in goats (n=8). After analgesia, three incisions were performed for the insertion of a laparoscope, atraumatic forceps, and the biopsy needle. At least one ovarian fragment was obtained per ovary and subjected to classical histology procedure. All statistical analyses were conducted using R software version 3.0.2. Spearman correlation test, chi-square test, Kruskal-Wallis test and Wilcoxon Mann-Whitney test were applied when convenient. Data are presented as mean ± SEM and percentages, and the statistical significance was defined as $P<0.05$. In Experiment 1, the recovery rate was greater ($P<0.05$) using 16G and 18G needles compered to 20G needle. The mean weight of ovarian fragments by the 16G needle's (1.5 ± 0.1 mg) was greater ($P<0.05$) than those collected using the 18G (1.0 ± 0.1 mg) and the 20G (0.9 ± 0.2 mg) needle's. In Experiment 2, 62 biopsy attempts were performed and 52 ovarian fragments were collected (90% success rate). Overall, 2,054 preantral follicles were recovered from 5,882 histological sections. Mean preantral follicular density was 28.4 ± 1.3 follicles per $cm^2$. The follicular density differed among animals and ovarian fragments within the same animal ($P<0.05$). In addition, according to the variance component analysis, the histological sections contributed to the greatest variability (70%) of the total variance of follicular density compared to ovarian fragment (29%). The mean stromal cell density in the ovarian fragments was 37.1 ± 0.5 cells per 2500 $\mu m^2$, and differed ($P<0.05$) among animals. Moreover, a positive association ($r = 0.18$; $P<0.001$) between preantral follicle density and stromal cell density was observed. The percentage of morphologically normal follicles was 70.1 ± 1.2, and differed ($P<0.05$) among animals. The majority (79%) of the morphologically normal follicles were classified as primordial follicles, and differed ($P<0.05$) among animals and within each ovary. In summary, a laparoscopic BPU method has been developed to harvest ovarian tissue in vivo with a satisfactory success rate in goats. Furthermore, as previously reported for other species, this study described, for the first time in goats, a high heterogeneity in follicular density, morphology, class distribution, and stromal cell density in ovarian tissue collected by BPU.

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# The kinetics of first cleavages influences the energetic metabolism and the transcription pattern in bovine embryos produced *in vitro*

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The main goal of the present study was to evaluate how the kinetics of the first cleavages may influence the energetic metabolism and the transcription pattern in bovine embryos. For this purpose, embryos were produced in vitro following standard protocols. About 40 post insemination, embryos were evaluated for cleavage rate and classified as Fast (4 or more cells) and Slow (2 or 3 cells). Culture followed for 7 days in SOFaa medium at 38.5°C, 5% CO2 and saturated humidity. On D5, embryos were transferred to individual wells containing 20µL of the same medium. On D7, the medium was collected to quantify glucose, lactate and pyruvate consumption. The respective blastocysts were stained for the detection of reactive oxygen species (CellRox Green®, ThermoFisher Scientific – n=18) and evaluation of mitochondrial activity (MitoTracker Red CMXRos, ThermoFisher Scientific – n=18). Furthermore, blastocysts were also collected (24 embryos - 4 replicates) and submitted to mRNA extraction, cDNA synthesis and quantification of 96 transcripts of interest by RT-qPCR in the BioMark® HD system (Fluidigm, San Francisco). For statistical analysis, the variables were submitted to Kolgomorov-Smirnov normality test and later compared by Student t test. For gene transcription data, GAPDH was used as endogenous control for the calculation of ΔCt. Results show that cleavage rate is higher in Fast embryos (42.6% ± 5.3) than in Slow (26.7% ± 2.6). Similarly, conversion to blastocyst is also higher in the Fast group (40.9% ± 4.2 vs. 13.9% ± 3.6). Regarding the consumption of energetic substrates, we observed that Slow embryos consume more lactate (5.63 ± 0.50 vs. 7.82 ± 0.32, p = 0.01) and more pyruvate (6.18 ± 0), 16 vs. 6.79 ± 0.10, p = 0.012), but there was no difference in glucose consumption. In addition, Slow embryos also present higher mitochondrial activity (p = 0.0009) and higher amounts of ROS (p = 0.029). Finally, 24 of the 96 analyzed genes presented differences between groups (p <0.1), with 22 genes having the highest number of transcripts in Slow (ACSL1, ADCY6, ATF4, BID15, CASP9, CAT, G6PD, GPX1, GPX4, GSK3A, HMO1, HSF1, HSP90AA1, IGFBP4, NFKB2, PFKP, POU5F1, PRDX3, RGS2 and TFAM) and only 2 genes in Fast group (Dnmt3a and SDHA). These genes are related to oxidative metabolism, stress response and cell death. The results show that although slow embryos possess a more active metabolism, they also have lower rates of development. These data corroborate Leese's (2002) "silent embryo" theory, which says that less viable embryos have a compromised genome, and therefore, their more "active" metabolism is reflected in the higher consumption of oxygen and nutrients, which are used by repair mechanisms. Thus, these findings indicate that the kinetics of the first cleavages influence the pattern of metabolism functioning in pre-implantation embryos.

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# Inhibition of HSP90 associated to heat shock during *in vitro* maturation of bovine oocytes alters the relative amount of transcripts in 8-cell stage embryos

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HSP90 is a protein involved in cellular homeostasis and its inhibition during maturation reduces the oocyte developmental competence (Souza et al. 2015, Reprod. Fertil. Dev. 27:235). The present study investigated the relative amount of heat shock (*HSF1, HSP90, HSP40*) and totipotency (OCT4) transcripts in bovine embryos at 8-cell stage derived from oocytes exposed to an inhibitor of HSP90 (17AAG; 17-allylamino-17-demetoxigeldanamicina; Sigma, St Louis, EUA) associated to heat shock (HS) during *in vitro* maturation (IVM). Cumulus-oocyte complexes (COC) were allocated in four groups during IVM: Control - without both heat shock and 17AAG; HS - heat shock (41.5ºC) for the first 12h of IVM; 17AAG - 2µM of 17AAG for the first 12h of IVM, and 17AAG+HS - 2µM of 17AAG plus heat shock for the first 12h of IVM. *In vitro* maturation was performed in Nunc plates, containing 400µL of TCM199 medium (Invitrogen, Carlsbarg, USA) supplemented with porcine FSH (pFSH - Pluset, Lab. Callier, Espanha) and 10% estrus cow serum, and incubated under 5% $CO_2$, 95% humidity and 38.5ºC for 24h. The heat shock was performed under 7% $CO_2$, 95% humidity at 41.5°C. After maturation, oocytes were *in vitro* fertilized for 20h with $2x10^6$ spermatozoa/mL. The presumptive zygotes were cultured in four-wells plate with 500 µL of modified CR2aa medium supplemented with 2.5% FCS (Nutricell, Campinas, Brasil) in an incubator at 38.5ºC under 5% $CO_2$, 5% $O_2$, 90% $N_2$ and saturated humidity for 52h. Eight-cell stage embryos were washed three times in PBS plus 0.1% polyvinyl alcohol and then rapidly frozen in liquid nitrogen, and stored at -80°C. Three pools of 10 embryos per group where used for total RNA extraction with RNeasy Micro Kit (Quiagen,Valencia, CA, USA) and reverse transcribed using the SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA, USA). Relative quantification was performed by Comparative Ct quantification ($2^{-\Delta\Delta Ct}$) method relative to the sample with the highest delta Ct value in the control group (calibrator sample) and was based on primer efficiency. Analysis was performed by mixed model using the Proc Mixed command in the SAS 9.0 software. $P<0.052$ was considered significant and the relative amount values are presented as mean ± S.E.M. The relative amount of *HSF1* transcripts was higher ($p<0.052$) in 17AAG group than in Control and 17AAG+HS groups but similar to HS group. Higher ($p<0.03$) amount of *HSP90* transcripts was found in 17AAG+HS group than Control and HS groups, but similar to 17AAG group. No difference was found for *HSP40* and *OCT4* transcripts among groups. Those data show that despite inhibition of HSP90 during IVM can affect the expression of *HSF1* in 8-cells embryos, it does not have the same effect on expression of *HSP90*. In contrast, inhibition of HSP90 associated to heat shock influences the *HSP90* expression but has no effect on *HSF1* transcript. In conclusion, relative expression of genes in 8-cell stage embryos is influenced by the inhibition of HSP90 and heat shock during *in vitro* maturation of bovine oocytes. Financial support: CNPq, FAPEMIG and FAPES.

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# Characterization of AMH gene polymorphisms and its association with traits indicative of sexual precocity in Nelore heifers

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Anti-Müllerian Hormone (AMH) is a protein expressed in the gonads and is related to ovarian follicular development. AMH plays an important role to prevent premature depletion of the follicular reserve, by promoting balance between inhibition and promotion of follicular growth and development. Recently, Pierucci et al. (2016; Abstract Book of 18th ICAR, p. 85-86) characterized the presence of polymorphisms in the coding region of the AMH gene in Nellore heifers. The authors identified four SNP-type polymorphisms; one located in intron 2 (rs132972253) and three in exon 5 (rs527023314, rs722016629 and rs134387246), the latter three being qualified as synonymous mutations. The aim of this study was to analyze the association between polymorphisms in the exon region of the AMH gene and early pregnancy occurrence (EPO) and age at first calving (AFC) traits in Nelore females, and evaluate the genetic constitution of this population for this gene. Phenotypic data consisted of the verification of conception or not conception of 197 unrelated heifers exposed in the breeding season, aging 16 to 18 months. Allelic and genotypic frequencies were calculated by allele counting. Hardy-Weinberg Equilibrium and Linkage Disequilibrium (LD) were calculated by Chi-square test and regression, respectively. The effect of the genotypes identified for the AMH gene in EPO and AFC, as well as the mechanism of action of these polymorphisms (additive effect, dominance or over dominance), considering a significance level of 10%, were analyzed using the GLIMMIX procedure of SAS 9.3®. For analysis of EPO, we considered a binomial distribution model and for AFC, a linear model with normal distribution was considered. All three SNP in the present study were under Hardy-Weinberg equilibrium. Low LD values (<0.3) were detected in all of the exon 5 SNP pairs, indicating independent segregation, despite the short distance between loci. Only SNP rs134387246, located in the stop codon, exhibited significant value for both phenotypes (EPO = 0.059; AFC = 0.060). The heterozygous individuals showed a shorter AFC (in 75 days) compared to the average AFC of the homozygotes, representing the condition of over dominance. The results of the present study indicate that the marker rs134387246 may be one among many loci that affect reproductive traits in a non-additive manner. For the first time in literature, polymorphisms of the AMH gene were associated with sexual precocity traits, wherein one of the SNP demonstrated a positive influence on them and could be further used as a tool for selection of Nelore females, allowing acceleration of genetic gain and efficiency improvement of cattle herds.

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# Evaluation of L-carnitine supplementation on the production and vitrification of bovine embryos produced *in vitro*

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*In vitro* produced embryos (IVP) present low survival to conventional methods of cryopreservation. In order to improve production and survival rates after vitrification, L-carnitine (CA) was used in different doses and different cultivation moments of in vitro embryonic development. For this purpose, the oocytes IVM was performed in TCM199 medium supplemented with 25 mM of sodium bicarbonate, 1.0 µg/mL of FSH, 50 Ul/mL of hCG, 1.0 µg/mL of estradiol, 0.2 mM of sodium pyruvate, 83.4 µg/mL of amikacin and 10% of fetal bovine serum. After 24 hours of IVM, oocytes were co-incubated with semen in a Talp-IVF medium supplemented with 6 mg/mL of BSA for approximately 20 hours. The development culture (CIV) was performed in a SOFaa medium with 6 mg/mL of BSA and 2.5% of SFB. According to the design, CA was used in the concentrations of 0.0mM (control); 1.0 mM; 2.5 mM and 5.0 mM, from 96 or 144 hours after fertilization (hpf). All IVP cultures were performed in an incubator at 38.5 ºC and a CO2 atmosphere of 5% in air. At the seventh day, IVCs were evaluated for blastocysts rates, with the embryos being submitted to vitrification. The embryonic viability post-vitrification was evaluated by the re-expansion rate and embryos hatching after re-heating and cultivation for 48 hours within the same conditions of IVC. Embryos production had a completely randomized design with a factorial scheme 4 x 2 (four concentrations and two days) and four replicates, with data being transformed in arc sine and submitted to a variance analysis, with means compared by the Tukey's test at 1% probability, with the aid of the software SAS. Regarding categorical data analysis (expansion and hatching), the Chi-square test was used, considering the effects of concentration and day, as for P values equal or inferior to 0.01 ($p<0.01$), the differences between these effects were considered as significant. The moment of CA use did not interfere on blastocyst production ($P=0.3$), however the use of 2.5 mM resulted in a greater production of embryos (62.0±0.07%, $P=0.02$), followed by the groups 0.0 mM, 1.0 mM and 5.0 mM, respectively (53.6±0.02, 57.7±0.07 e 49.4±0.11, $P<0.05$). Both the embryonic re-expansion and hatching were influenced by the moment of application and the CA concentration. The use from 96 hpf (38.4 and 27.9) was better that 144 hpf (27.9 and 13.5), while the concentration of 2.5 mM resulted in a greater percentage of re-expanded and hatched embryos (37.7 and 27.0), when comparing to groups 0.0 mM, 1.0 mM and 5.0 mM, respectively (19.6 and 14.3; 22.8 and 15.7; 23.6 and 16.7 $P<0.05$). Considering the moment and concentration, the group 96 hpf/2.5 mM presented the greatest expansion rates of 60.7% ($P=0.008$) and hatching of 41.4% ($P=0.04$). Based on the obtained results, it was concluded that when using L-carnitine from 96 hpf with the concentration of 2.5 mM, the embryonic survival to vitrification is improved.

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# Protein profile evaluation of bull sex sorted sperm

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Sperm sorting to obtain samples with high percentage of gametes carrying X or Y chromosomes makes relevant the role of artificial insemination, maximizing genetic progress. The identification of distinct proteins in the sperm membrane with X and Y chromosomes could allow the development of a technique for sperm immunosexing. Therefore, the objective of this study was to assess the protein profile of sex sorted sperm by flow cytometry. Sex sorted sperm (n=6X and n=6Y samples) were used. Proteins were extracted and analyzed in mass spectrometry by data independent acquisition. The data were searched against Swissprot and trEMBL database in Bos taurus taxonomy. For the variables analysis the data were normalized, transformed (log2) and compared using tools available in Perseus. The Student t test was used to compare the variables results. Significance level of $p \leq 0.05$ and fold change $\geq 1.5$ were considered. There were 459 proteins common to both groups, 7 showed greater relative abundance between X and Y spermatozoa with fold change $>\pm 1.5$. The main proteins are FUN14 domain-containing protein 2 and NADH dehydrogenase [ubiquinone] iron-sulfur protein 7 mitochondrial are related to mitochondrial damage (MURRAY et al. J. Biol. Chem., v. 278, p. 13619-13622, 2003; CHEN et al. Autophagy, v. 12, p. 689-702, 2016) and their greater relative abundance in X may be related to lower sperm motility (BORO, Int. J. Appl. Res. v. 4, p. 460-462, 2016). In contrast the pyruvate dehydrogenase protein X component acts on the energy generation pathways, which was found in greater abundance in Y sperm and may be related to faster sperm motility (BORO, Int. J. Appl. Res. v. 4, p. 460-462, 2016). Similarly, predict dynein intermediate chain 2 axonemal is mostly responsible for sperm motility, and was too greater in Y sperm. Predict EF-hand domain-containing protein 1 is related to PLCζ activity, which produces calcium oscillation for embryonic development. Some outhors described that spermatozoa carrying the Y chromossome has greater embryonic growth rates (PERGAMENT et al. Hum Reprod, v. 9, p. 1730-1732, 1994). More studies are necessary to clarify the differential protein expression profile. Overall, the majority of proteins found was related to energy generation that leads an understanding of the greater motility in sperm carring Y chromossome. This study may be guidance for further experimental studies related to sex sorted sperm and a possible future development of the immunosexing.

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# Characteristics of H3K4me3 and H3K9ME at different stages of development of bovine embryos produced *in vitro*

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Post-translational modifications in histones are involved in the regulation of gene expression during the embryogenesis process (Boland, M.J. et al., Circulation Research, 115, 311-324, 2014). H3K4me3 is involved in the permission of gene transcription (Liu X. et al., Nature, 537, 558-562, 2016), while H3K9me is involved in the repression of gene transcription (Santos F. et al., Current Biology, 13, 1116-1121, 2003). The aim of the present study was to characterize the modifications of global H3K9me and H3K4me3 during embryonic development in vitro. The embryos were produced in vitro and conditioned at different stages of development for the investigation of these histone. For the Immunofluorescence analysis, were utilized confocal microscopy (5 Pascal, Zeiss, Germany). The quantification of the fluorescence signal intensity of H3K4me3 and H3K9me were carried in 15 blastomers for embryos (with the exception of the 4 cels, in which all the blastomers were analyzed by ImageJ). Thy fluorescence signal in the z direction was corrected (pattern) and the date were normalized in relation to the negative controls. The Shapiro-Wilk test indicated the normality of the date. Scott-knott at 5% of probability was utilizded when a significant difference was identified in ANOVA test (Program R version 3.3.1). In all stages of embryonic development, twice proteins, H3K4me3 and H3K9me, were present, showing their importance in transcriptional control during 4 cell (higher intensity of global H3K9me) and hatched blastocysts (higher intensity of H3K4me3). These epigenetic marks appear to be related to the regulation of pluripotency. In the early blastocyst period, there is no significant difference in the behavior of the marks, which suggests a state of preparation of the embryos for a period of great modifications, the cellular differentiation. H3K4me3 and H3K9me are present among the stages of 4 cells and hatched blastocyst, however their relation is inverse, so that in the stage of 4 cells, H3K9me showed higher intensity and from the blastocyst phase the H3K4me3 showed higher signal intensity. The behavior of the H3K4me3 was more dynamic during the pre-implantation development than H3K9me, with peaks in the early blastocyst phase and the hatched blastocysts, demonstrating higher influence of the trimethylation of the H3K4 during the developmental of the blastocysts. The early blastocyst phase appears to be the transition phase between these modifications, since there not significant difference between the signal intensity of the both histone epigenetic modifications.

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# Correlation between clarifide molecular markers and the final pregnancy rate of early heifers

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Molecular markers are a genomic tool, able to predict the reproductive potential of animals. The Nelore CLARIFIDE 2.0 uses the SNP Chip ZL5 with 25,458 markers for the Nelore breed. The objective of this study was to correlate the iMVPRep (bioeconomic reproductive index), with the final pregnancy rate of early heifers. The reproductive index simplifies the selection of multiple traits of maternal characteristics (cow productivity). Higher maternal index values indicate females or daughters with better reproductive performance and production characteristics. This index includes: age at first calving, probability of early calving, stayability, productivity gained, maternal ability at 120 days, weight at 210 days and weight at 365 days (Zoetis, Data on file, 2017). Were evaluated genomically (CLARIFIDE Nellore, Zoetis), 226 Nellore heifers, which after being weaned were submitted to feedlot supplementation for 150 days on a diet of 56% of bulky and 44% of concentrated for approximate consumption of 2.3% of body weight. Afterward they received supplementation with 3g/Kg of body weight until the end of the breeding season. All heifers, with an average age of 15 months, were exposed to the cycling induction protocol, previous to ovulation synchronization protocol and TAI. After insemination they were exposed to bulls on 1:30 ratio. The groups were divided by median according to the Rank iMVPRep. Group A (n = 116), 46% < in the Rank, animals with higher values of iMVPRep (158.83 ± 48.03); Group B (n = 107), 47% > in the Rank, animals with lower values of iMVPRep (32.11 ± 48.24). The heifers body weight at TAI day was adjusted to 450 days, being (297.7 ± 1.87) for Group A and (294.6 ± 1.87) for Group B, with no statistical difference (P = 0.23). The binomial variables (final pregnancy and TAI pregnancy rate) were analyzed by PROC GLIMMIX of SAS, with sire and technician as random variables, and the continuous variables (average weight adjusted to 450 days) were analyzed by PROC MIXED of SAS. A significant effect was considered when $P < 0.05$. There was an effect of reproductive index with the final pregnancy rate of heifers, 79.1% for Group A and 64.3% for Group B (P=0.04) and a trend in the TAI pregnancy rate, 49.2% for Group A and 35.4% for Group B (P=0.06). The animals identified by CLARIFIDE, with higher values of iMVPRep (greater reproductive potential), presented a higher TAI and final pregnancy rates comparing with animals with lower values of iMVPRep (lower reproductive potential), even with similar average body weight within groups. This data leads to a conclusion that heifers with greater iMVPRep had better reproductive performance and higher probability of early pregnancy. More studies are needed to elucidate the relationship between the parameters analyzed using genetic markers with reproductive performance (phenotypic) of animals.

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# Effect of sex on survival of bovine *in vitro* produced embryos vitrified by Cryotop

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Several studies have shown that male and female embryos are different not only in speed of development, but also for metabolism, gene expression, epigenetic patterns and stress response. This study aimed to evaluate if the cryopreservation effects caused on embryos may vary between gender. Oocytes obtained from slaughterhouse ovaries underwent IVM for 24 hours, were inseminated with 1 x $10^6$ spermatozoids/mL, co-incubated in IVF medium for 16-18 hours, and possible zygotes were cultured *in vitro* (IVC) for 8 days. Cleavage at D2 and blastocyst rates at D6, D7 and D8 were evaluated. At D7, grade I embryos at expanded blastocysts stage, according to IETS manual, were removed from IVC and divided in two treatments: control (C) and vitrified (V) by *Cryotop*. After warming process, embryos returned for additional 24 hours in ICV conditions, for survival (not degenerated embryos) and evolution rates evaluation. Afterwards, embryos from both treatments (C: n=129; V: n=165) were individually stored in DM-PBS with lysis buffer, at – 20 °C, for sex determination, that was assessed by polymerase chain reaction and confirmed in 1.5% agarose gel. Data were analyzed by Mann Whitney test ($P < 0.05$). Male (n=57 [44%] e n=89 [53.9%]) and female (n=72 [55.8%] e n=76 [46%]) embryos percentage were similar for both control group and vitrified one ($P > 0.05$), respectively. For the vitrified embryos difference between male and female data was not seen for survival rate (n=87 [55.1%] e n=71 [44.9%], respectively), evolution form expanded to hatched blastocyst rate (n=61 [52.4%] e n=50 [47.6%], respectively) and degeneration rate (n=2 [28.6%] e n=5 [71.4%], respectively). These results suggest that male and female embryos have the same vitrification tolerance for *Cryotop* method.

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# Efficacy between two protocols for isolation of primary culture of equine uterine tuba cells

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In the last decade, the primary culture of uterine tube (CTU) cells has become an effective, rapid, economical and reliable way to obtain information about the BEHAVIOR and modulation of different components of tubal fluid in THE fertilization PROCESS, Clarifying the biology of this process. In this sense, it´s proposed to compare the effectiveness of CTU isolation by two protocols; Isolation with Scraped with histological lamina and catheter cannulation. Twenty ovaries of mares from slaughterhouses were collected according to the modified Nelis technique (2013). These were transported to the laboratory in a solution of PBS, penicillin and streptomycin (Penstrep®) and amphotericin (Fungizone®) at 4°C. The uterine tube (TU) was isolated and the portion between the tubal uterine union and up to two centimeters of the isthmus ampullary union was used. In group A (n = 10) cells were collected by scraping with light pressure from a histological slide over the entire length of the duct. The uterine tube (TU) was isolated and the portion between the tubo-uterine junction and up to two centimeters of the isthmic-ampulla junction was used. In group A (n = 10) cells were collected by scraping with light pressure from a histological slide over the entire length of the duct. In group B (n = 10) each tube was cannulated with a 24G catheter in the ampullary portion and approximately 500 μL of the previously mentioned solution was introduced by moderately massaging and then withdrawing all of its contentes. The fluid obtained from both techniques was centrifuged at 200xg for 10 minutes, the pellet formed was cultivated in 60mm Petri dishes containing TMC 199 medium (90%), fetal bovine sérum (10%), pen-strep (1.5%) and fungizone (1.2%). The medium was changed every 48 hours for a period of 4 days. In the preliminary analyzes, confluence, colony formation (explant), ciliary movement and amount of cell debris were evaluated. In group B, the amount of cell debris was lower, whereas in group A, confluence was better, suggesting that the extraction of epithelial cells with catheter aid significantly reduced the amount of cellular debris and the possibility of contamination, favoring the visualization and the management of explants. The ciliary movement, one of the most important characteristics, was present in both groups, and increased throughout the days. In the scraping technique, group A obtains a high amount of cell debris with a confluence of 50% in 48 hours. It is believed that the difference between the groups is due to the applied mechanical pressure, in order to give off more quantity when the force increases. Likewise, more aggressive techniques such as the use of enzymes to digest the tissue or scraping of the tubal epithelium can obtain more cells. According to the objective of the present work, it is believed that to obtain 100% viable explants with ciliary movement, it is necessary to cultivate for a time superior to 72 hours, contrasting with cattle, where the research group has observed with the same protocols viability and Ciliary movement with 24 hours of culture. It is concluded that the two methods are effective and can be used as validated protocols for obtaining equine explants. It is concluded that the two methods are effective and can be used as validated protocols for obtaining equine explants.

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# PTGS2 Expression in cumulus cells is a potential biomarker of oocyte quality independently of patient's clinical variables

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Gene expression in Cumulus cells (CCs) have previously been suggested as a predictive tool for oocytes quality in several studies. Still, there is little consensus about which biomarkers would actually be clinically efficient and applicable (Fragouli, Human Reproduction Update, 20:1-11, 2014). We analyzed CCs gene expression data considering the patient's clinical characteristics and the oocyte's potential, with the aim to identify possible biomarkers of oocyte competence independently of patient's clinical characteristics. Pooled CCs samples were obtained from 29 patients submitted to ICSI procedure. The oocytes corresponding to the samples were accompanied until day 5 after ICSI, and samples were divided in Good Quality group (GQ) (n=11) and Poor Quality group (PQ) (n=18) accordingly to percentage of blastocyst formation (GQ= 50% or more of the sample's embryos generated blastocysts; PQ= less than 50% of the sample's embryos generated blastocysts). All embryos from the same patient were cultivated together. Each sample was submitted to reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) on StepOnePlus™ (Applied Biosystems, USA). Oligonucleotides were selected to be complementary to the human sequence of Anexin 1 (ANXA1), Prostaglandin-endoperoxide synthase 2 (PTGS2), Glutathione Peroxidase 4 (GPX4) and Glutathione-S-Transferase 1 (GST1). The relative expression was calculated by the 2-ΔΔCT method (Livak and Schmittgen, Methods, 25:402–408, 2001). Patient's clinical data (infertility diagnosis, BMI, age and stimulation protocols) and experimental data were combined using mice package (Buuren and Groothuis-Oudshoorn, Journal of Statistical Software, 45, 2011) in a multiple regression model built using the percent of blastocysts as dependent variable and clinical data as independent variables. Test models composed of clinical variables and each assay data were compared against the baseline model. All procedures and computations were performed in R statistical environment. Differences within the experimental groups were determined by Mann-Whitney test (GraphPad® Software 5.0). When submitted trough Mann-Whitney test, only ANXA1 expression, that has antiinflamatory properties, shown to be significantly different between GQ and PQ groups ($P<0.05$), being elevated in GQ group. Gene expression results were then submitted to linear regression analysis, which indicated that ANXA1 expression levels was not a potential predictor of oocyte quality when we considered clinical information. Contrarily, PTGS2 expression levels are overexpressed in GQ group ($P<0.05$), and this significance is independent of the clinical variables of each patient. As far as we are aware, this is the first study suggesting biomarkers found in CCs that predicts blastocyst formation potential regardless of patients age, diagnosis, BMI and stimulation protocol.

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# Gene expression of VEGF in the corpus luteum of rats that ingested Black Tea (Camellia Sinensis (L.) Kuntze)

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Black tea is one of teas derived from the Camellia sinensis plant. Some studies determined that catechins of decreased the gene expression of Vegf (gene of vascular endothelial growth factor) in tumor, however the influence of black tea in the VEGF of the ovary have not been studied until now. The VEGF is an important angiogenic factor in the reproductive organs.This study aimed to verify the effects of intake of black tea on the relative abundance of Vegf mRNA in rats. For this purpose, the rats were divided into two groups, with 30 animals in each group: control group (CT), which received water, and black tea intake group (BT) ad libitum at the water bottle. Black tea was prepared daily at a concentration of 2.5% by the addition of boiling water in the pure extract of black tea and subsequently filtered, the animals consumed on average 27.56 mL/day of black tea.The ovaries were collected from 10 animals in each group at the end of every month, for three consecutive months, stored in TRIzol® (Thermo Fisher Scientific, California, USA) in a freezer at -80°C and the relative abundance of VEGF mRNA were subsequently evaluated for qPCR. Three endogenous genes were tested: beta-actin, Gapdh (glyceraldehyde-3-phosphate dehydrogenase), Hprt-1 (hypoxanthine-guanine phosphoribosyltransferase) and Rps-18 (18S ribosomal protein). Hprt-1 was the most stable gene and therefore was used as the normalizer of the reaction.The data were evaluated using the Mann-Whitneytest between groups at different collection moments ($p < 0.05$). The means and standard errors of relative expression of Vegf were: CT = 1.06±0.13 and CP = 2.09±0.18 (first month); CT = 1.08±0.14 and CP = 0.94±0.18 (second month); and CT = 1.05±0.12 and CP = 1.36±0.35 (third month). The relative abundance of mRNA of Vegf was higher in the group that consumed black tea in the first month of experiment ($p=0,0048$), in the other months no statistical difference was observed between groups. It wasconcluded that the black tea intake increases the Vegf expression in the corpus luteum of Wistar rats only in the beginning of the consumption, after that, the expression normalizes. It was concluded that the ingestion of black tea increases the expression of Vegf in the corpus luteum of Wistar rats only in the first month of consumption in relation to the control, in the following months the expression does not differ between the two groups. Apparently, the increase in the relative abundance of Vegf mRNA in the first month demonstrates a molecular regulation of the body to maintain the physiological ovarian angiogenesis, but further studies should be performed to prove the safety of black tea for female reproduction. FAPESP (2010/20274-0).

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# Conceptus elongation in beef heifers with superior uterine capacity for pregnancy

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Infertility and subfertility represent major problems in domestic animals and humans. To select animals with intrinsic differences in pregnancy loss, beef heifers were subjected to serial embryo transfer and classified based on day 28 pregnancy rates as high fertile (HF=100%), subfertile (SF=25-33%), or infertile (IF=0%). Studies using in vivo-produced embryos established that preimplantation conceptus survival and growth to day 14 was not compromised in the SF and IF heifers (Geary, Biol Reprod, 95:47 2016). Thus, the observed difference in fertility was hypothesized to manifest during conceptus elongation and pregnancy recognition. Two in vivo-produced embryos were transferred into HF (n=21), SF (n=10), and IF (n=5) heifers on day 7. On day 17, the uterus was flushed and endometrium collected. Binary data were analyzed by logistic regression using the LOGISTIC procedure of SAS. Continuous data were analyzed by ANOVA using the GLM procedure or in a Poisson regression repeated measurements model using GENMOD procedure of SAS. Conceptus recovery rate was higher ($P<0.05$) in HF (71%) and SF (90%) than IF (20%) heifers. Interferon tau (IFNT) in the uterine flush was quantified by ELISA. Conceptus length was positively ($P<0.01$) correlated ($R=0.79$) with IFNT in the flush. IFNT was greater ($P<0.05$) in uterine flush from pregnant HF (148+54 ng/mL) compared to SF (74+67 ng/mL) and IF (0 ng/mL) heifers. Conceptuses from HF (x=10.6 cm, range=1.2-32.2 cm) were longer ($P<0.01$) than SF (x=4.7 cm, range=1.5-13.5 cm) or IF (<0.1 cm) heifers. Total RNA was sequenced (n=5 per group) from the day 17 endometria of open or nonpregnant (NP) HF, SF and IF heifers and pregnant (P) HF and SF heifers as well as 17 HF conceptuses and 10 SF conceptuses. RNA-Seq data was analyzed by EdgeR-robust analysis. There were 96 differentially expressed genes (DEGs; FDR $P<0.05$) in NP endometrium; several DEGs encoded proteins involved in immune responses or present in immune cells. Comparison of P and NP endometrium in HF or SF heifers found 3,422 and 1,095 DEGs, respectively, but no difference in IFNT-stimulated genes. There were only 168 DEGs in the endometrium of P HF and SF heifers, but the response to pregnancy was significantly diminished in SF heifers. There were 1,287 DEGs between HF and SF conceptuses. In contrast, only 3 DEGs were detected in long (9.8-32.2 cm) versus short (1.2-6.9 cm) HF conceptuses. Several transcripts encoding secreted proteins and involved in lipid metabolism were dysregulated in the SF as compared to HF conceptuses. Many of the down-regulated genes are associated with embryonic lethality in other species. These studies support the idea that the uterine environment directly affects conceptus survival and elongation during the establishment of pregnancy and asynchronous conceptus-endometrial interactions result in pregnancy loss after conceptus elongation during the implantation and embryogenesis phase of early pregnancy. Supported by NIH R01 HD072898.

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# Identification of isoform PR-B in canine corpus luteum through the quantitative analysis of RNA sequencing (RNA-seq)

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Progesterone and its receptor are involved in the regulation of gene expression and can affect cell proliferation and differentiation in the canine corpus luteum (CL). The identification of progesterone receptor (PR) isoforms is fundamental to characterize the role that each isoform exerts in CL function. The objective of this study was to identify possible PR isoforms during non-gestational diestrus in the canine species, using the RNA-seq technique. For this purpose, the corpus luteum of ovaries from bitches that had undergone salpingo-hysterectomy were used on days 10, 20, 30, 40, 50, and 60 (n = 3/group) after ovulation. Preparation of the libraries and sequencing of the new generation followed the TruSeq RNA Sample Preparation Guide protocol described by Illumina. The reads were mapped against the reference genome (Canis_familiaris.CanFam3.1.75.dna.toplevel.fa) using the Hisat program ('Our Galaxy' - ETH-Zurich) which generates files in BAM format (Binary Alignment/Map). The sequencing data were statistically analyzed using the Cuffdiff program, a Cufflinks pipeline package, with the relative abundance of the transcripts measured in FPKM (Fragments per kilobase of exon per million fragments mapped). The BAM format files were indexed in IGV visualization software (Integrative Genomics Viewer) (Robinson et al.; Nat Biotechnol.; 29:24-26,2011), the reference genome being selected and the PR gene identified. From the alignment data generated in PR, it was possible to verify the presence and relative expression of the isoform by Sashimi plots, in which each exon junction is illustrated and the reads are enumerated. The Sashimi plot identified only the progesterone B receptor isoform (PR-B) in all periods, indicating a greater expression on days 10 and 20 after ovulation. This result was not expected, we also considered find the isoform of the progesterone receptor A (PR-A), since the secretion of the hormone progesterone reaches maximum values at this stage and the possibility of different isoforms was assumed. However, the result offers prospects for further studies on the role of the PR-B isoform in relation to proliferation and cell survival in the canine corpus luteum during diestrus.

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# New insights into the proteomic abundance and the action of L-arginine during *in vitro* sperm capacitation of cattle

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The aim of this work was to evaluate the proteomic changes of bovine spermatozoa after heparin-induced *in vitro* sperm capacitation with the addition of L-arginine (L-arg) using the shotgun approach. It was also evaluated the sperm capacitation pattern, membrane integrity, mitochondrial activity, sperm motility and vigor to confirm the effect of L-arg during *in vitro* capacitation. The evaluations were respectively assessed by chlortetracycline staining, H342/PI, JC1, optical microscopy, and the proteomic abundance by nUPLC-MS/MS analysis. Sperm cells from three Nellore bulls (*Bos taurus indicus*) went through *in vitro* capacitation for 3 hours in sp-TALP medium supplemented with 20 µg/mL heparin (Control), or with 20 µg/mL heparin plus 1 mM L-arg (treatment). Data were subjected to analysis of variance (Proc GLM) and the averages compared by SNK test at 5% probability. When comparing to Control, the percentage of sperm motility was higher in the capacitation group treated with L-arg (67.50 ± 8.66% vs 55.00 ± 7.98%, $P < 0.05$) and there was an increase in the percentage of capacitated pattern (70.30 ± 1.85% vs 57.84 ± 2.15%, $P < 0.05$). After 3 hours of incubation, sperm capacitated with L-arg showed higher mitochondrial potential and showed increased membrane integrity comparing to Control (77.15 ± 5.02% vs 57.72 ± 3.13% and 57.15 ± 4.71% vs 42.85 ± 4.71%, $P < 0.05$). The proteomic approach identified 367 proteins in the bovine sperm after the *in vitro* capacitation. Forty were found to be differentially abundant between Control and treatment ($P < 0.05$), one was abundant only in the treatment with L-arg ($P < 0.05$) and 326 were unchanged ($P > 0.05$). Eleven proteins were upregulated and 29 were downregulated in treatment comparing to Control. In conclusion, the addition of L-arg to the culture medium in presence of heparin *in vitro* showed different protein abundance pattern, and increased the bovine sperm quality and the percentage of capacitated sperm. This proteomic change may be closely linked to the molecular mechanisms involved in the action of L-arg on the *in vitro* sperm capacitation of cattle. Further investigation should be performed to determine whether if these findings have any clinical value for fertility/infertility assessment.

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# Transcripts levels of enzymes involved in histone acetylation in bovine oocytes of different competences

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Oocyte competence refers to the ability of an oocyte to undergo maturation, to be fertilized and to have normal embryonic development. Studies have reported the importance of enzymes involved in the acetylation of histones in oocyte during maturation and their possible association with oocyte competence. The aim of the present study was to analyze the expression profile of genes involved in histone acetylation and deacetylation in bovine oocytes of different competence levels in maturation. COCs were recovered from 1.0-3.0mm (less competent) and 6.0-8.0mm diameter follicles (more competent) dissected from the ovarian cortex. Oocytes from each group were matured in vitro for 0, 8 and 24 hours and stored for gene expression analysis. Total RNA was extracted from 4 pools of 15 oocytes, from each treatment at each maturation time. The levels of the gene transcripts involved in acetylation (HAT1, KAT2A) and histone deacetylation (HDAC1, HDAC3) were determined by qPCR, being the expression values normalized by the constitutive gene PPIA. Data were analyzed by ANOVA, and the means of each treatment were compared by Tukey test at the significance level $p < 0.1$. The results showed that the expression pattern of the genes studied was similar ($p > 0.1$) for more and less competent oocytes, not change during maturation. With the exception of the HAT1 gene, in which its transcripts increased ($p=0.05$) between 0 and 8 hours of maturation in the most competent group. When the different groups were compared at the same maturation time, the most competent group presented higher expression ($p=0.06$) of HAT1 and HDAC1 ($p=0.03$) at 8 hours of maturation than the less competent group. The other genes had similar expression in the different treatments ($p > 0.1$). It can be concluded that transcription of HAT1 gene had occurred during maturation in the most competent group, and that this group shows a improve expression of the HAT1 and HDAC1 genes at 8 hours of maturation than the less competent, suggesting that these can be used as markers for oocyte competence.
Support: Embrapa and FAPEMIG.

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# The use of linoleic acid in *in vitro* culture of bovine embryos and its effects on production and survival to vitrification

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In the attempt to produce in vitro bovine embryos more resistant to vitrification and to maintain the embryo production levels, the trans-10; cis-12 conjugated linoleic acid (CLA t10, c12) was use in different dosages and different moments of culture. For this purpose, the oocytes IVM (in vitro maturation) was performed in TCM 199 medium supplemented with 25 mM of sodium bicarbonate, 1.0 µg/mL of FSH, 50 UI/mL of hCG, 1.0 µg/mL of estradiol, 0.2 mM of sodium pyruvate, 83.4 µg/mL of amikacin and 10% of bovine fetal serum. After 24 of IVM, oocytes were co-incubated with semen in a Talp-IVF medium supplemented with 6 mg/mL of BSA for approximately 20 hours. The in vitro culture (IVC) was performed in a SOFaa medium with 6 mg/mL of BSA and 2.5% of SFB. According to the experimental design, CLA t10, c12 concentrations were 0.0 µM (control); 50.0 µM; 100.0 µM and 150.0 µM from 96 or 144 hours after fertilization (hpf). All IVP (in vitro production) cultures were made in incubators at 38.5 ºC and CO2 atmosphere of 5% in air. At the seventh day of IVC the produced embryos indexes were evaluated, being the blastocysts of good quality submitted to vitrification. The embryo viability post-vitrification was evaluated by the re-expansion and hatch rate of embryos after re-heating and cultivation for 48 hours at the same IVC conditions. The embryo production had a completely randomized design with a factorial scheme 4 x 2 (four concentrations and two days) with six replicates and data were transformed in arc sine and submitted to a variance analysis, with means compared by the Tukey's test at 1% probability, with the aid of the software SAS. The Chi-square test was used for the analysis of categorical variables (expansion and hatching), considering the effects of concentration and day, as for P values equal or inferior to 0.01 ($p<0.01$), the differences between these effects were considered as significant. Were treated from 96 hpf 1564 zygotes, 0.0 µM (278); 50.0 µM (401); 100.0 µM (448), 150.0 µM (437); and from 144 hpf 1566 zygotes, 0.0 µM (278); 50.0 µM (469); 100.0 µM (441), 150.0 µM (378). Using CLA t10, c12 did not alter the in vitro production of embryos, regardless of dosages 0.0 µM (56.1±0.08), 50.0 µM (50.1±0.06); 100.0 µM (50.8±0.05) and 150.0 µM (50.1±0.08) or the moment of application 96 hpf (51.2±0.07) or 144 hpf (50.1±0.08) (P=0.4). The embryos' re-expansion and hatching after vitrification was positively influenced with the use of CLA t10, c12 with any dosage 50.0 µM (63.6 e 43.8%), 100.0 µM (64.6 e 39.6%) and 150.0 µM (70.3 e 53.3%) when comparing to the control group (34.7%; 16%) and to the start day of supplementation, with 96 hpf being more efficient than 144 hpf (70.4% and 50% vs. 48.2% and 28.4%, respectively), regardless of the used concentration (P=0.01). Considering both the moment and concentration, the group 96 hpf/150 µM presented the greatest expansion (85.5%) and hatching (70.9%) rates post-vitrification ($P<0.05$). Based on the results, it was concluded that when CLA t10, c12 is used from 96 hpf at the concentration of 150 µM, the embryo survival to vitrification is improved, without impairing production.

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# Protein profile of ovarian follicular fluid in brown brocket deer (Mazama Gouazoubira): Preliminary results

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The brown brocket deer (Mazama gouazoubira) is a species of cervidae found in the Northeast Brazil. This species already presents difficulties with the changes of its habitat and antropic actions. Therefore, programs that seek the conservation of this species may benefit from reproductive techniques, such as the in vitro embryo production. Thus, it is interesting to carry out studies on the proteomics of follicular fluid in order to understand both development and maturation of oocytes. The aim of this study was to quantify the total proteins and to describe the fluid protein profile two follicular categories: I (< 3.5 mm) and II (> 3.6 mm). For this, four adult females were submitted to a hormonal treatment with progesterone, estradiol benzoate and equine chorionic gonadotrophin. Females were starved for 36 h and submitted to an inhalation anesthesia. Follicular aspiration was performed by videolaparoscopy and samples were centrifuged (3000xg for 20 min) individually according follicular category size. The supernatant was stocked -80°C for posterior use. Evaluation of protein concentration was performed by the Bradford method. Also, samples were submitted to SDS-PAGE electrophoresis, using a total of 15 µg proteins/well. Gels were stained with Comassie Blue G-250 and images were analyzed using Software Quantity One 4.5 (Bio Rad, USA). The main bands were submitted to tryptic digest and tandem mass spectrometry (ESI-Q-Tof) and identified using the MASCOT software. Statistical analysis was performed using Student t test ($p < 0.05$). No significant differences were observed for total amount of protein (mean ± sd) between category size: 49.20 ± 22.76 µg/µL (category I) vs 56.70 ± 27.38 µg/µL (category II). Analysis of gels showed the following distribution: 180-115 kDa (29%), 82-64 kDa (36%), 49-19 kDa (28%) and 15-6 kDa (7%). According to molecular weight, the major proteins were: haptoglobin, immunoglobulin gamma and complement C3 (180-115 kDa); ceruloplasmin X1, immunoglobulin G1, plasminogen, apolipoprotein A-1, alpha 1-β glycoprotein and serpine (82-64 kDa); serotransferrin, apolipoprotein A-1 and complement C3 (49-19 kDa) and hemoglobin subunits beta-3 and alpha-like (15-6 kDa). This study provides the first description of the proteome in brown brocket deer ovarian follicular fluid. Studies in this area should continue in an attempt to identify potential markers of oocyte quality in this species.

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# Quantitative proteomic profiling of bovine follicular fluid during follicle development

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Follicular fluid (FF) constitutes the microenvironment of follicles and includes various biologically active proteins that can affect follicle growth and oocyte fertilization. The aims of this study were to investigate the proteome profile and functional overview of bovine FF during different stages of follicle development and to evaluate the association of the identified proteins with local steroids concentration. Eighteen healthy non-lactating Holstein cows were used in this experiment. Cows had their estrous cycles synchronized with Ovsynch added with an intravaginal progesterone device. Transrectal ultrasonography (MyLab30, Esaote, Genova, Italy) was performed every 12h to monitor the follicular dynamics. Follicles were individually aspirated at pre-deviation (F1~7.0mm); deviation (F1~8.5mm); post-deviation (F1~12.0mm); and pre-ovulatory stages of follicle development, which were confirmed by measurement of follicular estradiol and progesterone concentrations by ELISA. The FF from nine cows were selected for proteomic analysis. After albumin depletion, triplicates of pooled FF were reduced, alkylated, and digested with trypsin. The resulting peptides were labelled with TMTsixplex (Pierce, Rockford, USA) and quantified using LC-MS/MS (Orbitrap Elite, Thermo, San Jose, USA). Proteomic data were compared by PROC GLM of SAS (SAS Institute, Cary, USA) and associations between steroids concentrations and relative abundance of the differentially expressed proteins were tested by Pearson's correlation test. A total of 143 proteins was identified and assigned to a variety of biological processes, including biological regulation, response to stimulus, metabolic processes, defense response, and transport. Twenty-two differentially ($P<0.05$) expressed proteins were found between stages indicating intrafollicular changes over follicle development, with presumed deviation time critical to modulate the protein expression. Follistatin, inhibin, serglycin, spondin-1, fibrinogen, and anti-testosterone antibody were found to be relatively more abundant during early stages of follicular development. In contrast, apolipoprotein H, alpha-2-macroglobulin, plasminogen, antithrombin-III, and immunoglobulins were up-regulated after deviation. Amongst the differentially abundant proteins, 19 were found to be associated with steroidogenesis. Canonical pathways analysis using IPA software (Qiagen, Redwood City, USA) highlighted the occurrence in FF of functional networks in a differential temporal balance and control, including activation/inhibition of the acute phase response, coagulation system, complement system, liver/retinoid X receptor activation, and biosynthesis of nitric oxide and reactive oxygen species. The results provide new insights into the size-dependent protein changes in ovarian follicle microenvironment, associated with changes in local steroids concentrations, which are critical for follicle maturation and may influence follicular function.

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# *In vitro* embryos production in bovine after metaphase plate nuclear transfer

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Studies have shown that damages of the cytoplasmic organization is one of the main alterations caused by vitrification in bovine oocytes, being one of factors responsible for the inefficiency of this technique. Genomic nuclear transfer (GNT), in which the DNA of a damaged oocyte is transferred to a viable oocyte cytoplasm with the aid of a micromanipulator, is an alternative to rescue genetic material from oocytes with compromised cytoplasm, such those that have been submitted to vitrification. The objective of this study was to evaluate the viability of the GNT technique in bovine oocytes. Two experiments were performed. In the first experiment, the ability of reconstructed structures to develop to blastocyst stage after Parthenogenetic activation (PA) was evaluated. The second experiment aimed to evaluate the capacity of the reconstructed structures to be fertilized and to determine if the sperm concentration could affect fertilization rate. For the first experiment, cumulus-oocyte complexes (COCs) obtained from slaughterhouse ovaries were matured for 21 hours and distributed into three groups: 1) previously enucleated cytoplasm (n=275) reconstructed with a metaphase plate from another oocyte (GNT-MP) and submitted PA; 2) PA control (n=141)and 3) IVP control (n=204). In the second experiment, COCs were matured, micromanipulated and divided into 3 groups: 1) GNT-MP fertilized with 1x106sptz / ml (n=64); 2) GNT-MP fertilized with 0.5x106sptz / ml (n=63); 3) Control IVP (n=92; fertilized with 1x106sptz / ml). After 18 hours of fertilization, the structures were denuded, fixed in acetic acid: alcohol (1: 3) for 48 hours and stained with lacmoid. Oocytes were then classified as fertilized, unfertilized, polyspermic and abnormal. The chi-square test was used for the rates of fertilization and blastocyst production considering the value of $P \leq 0.05$. In the first experiment, no difference was found between the control PA and control IVP groups for both cleavage (83% and 80.4%) and blastocyst rates (46.1% and 38.7%). However, the GNT-MP group had lower cleavage (63.4%) and blastocyst rates (18.8%) compared to the two control groups. In the second experiment, the fertilization rate of the control group (76.1%) was higher than the fertilized GNT-MP with 1x106sptz/ml (46.9%) and GNT-MP fertilized with 0.5x106sptz / ml (46%), which did not differ from each other. The polyspermic rate was similar between the control group IVP (18.5%) and the GNT-MP groups either fertilized with 1x106sptz / ml (17.2%) or with 0.5x106sptz / ml (17.5%). It can be concluded that the structures reconstructed by the GNT-MP technique are capable of developing into embryo, and they can be fertilized without increasing the polyspermic rate. Therefore, it is a possible tool for the use of cryopreserved bovine oocytes.

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# Regulation of phospholipase C activity reduces premature capacitation of cryopreserved ovine semen

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The objective of the present study was to control the early capacity of cryopreserved ovine spermatozoa by the regulation of phospholipase C (PLC) activity. In Test I, spermatozoa in natura were maintained at 37°C for 4 h in Botubov ® diluent (fraction I) supplemented with 0 (control), 10, 20 or 30 μM of U73122 (Sigma), a PLC inhibitor, in the presence or absence of Glycerol. Parameters of sperm kinetics were evaluated during the incubation period in order to select the doses of the inhibitor to be tested in the cryoprotectant solution (Test II). The cryopreserved ejaculates in Test II were diluted in Botubov (single fraction), plus 0 (control), 10 and 20 μM of U73122. The thawed samples were evaluated for sperm kinetics by the Computer Assisted Sperm Analysis (CASA) system and for membrane integrity by the association of fluorescent propidium iodide, PSA-FITC and JC-1 probes. In addition, evaluations of sperm capacitation and acrosomal reaction were carried out using the fluorescent probe chlortetracycline hydrochloride (CTC) before and after cryopreserved semen samples were submitted to induction of in vitro capacitation in TALP sp medium supplemented with heparin for 4 h Incubation at 37 ° C. All the results were submitted to analysis of variance and Tukey's test, at a 5% probability level. Test I revealed a dose-dependent and time-dependent effect of the PLC inhibitor on the in natura sperm of sheep. In addition, the presence of glycerol enhanced the toxic effect of U73122. In test II, U73122 did not affect any of the kinetic parameters evaluated by the CASA system, nor did the number of spermatozoa with intact or mitochondrial plasma membranes, intact or damaged. However, the evaluations performed immediately after thawing showed that the use of 10 or 20 μM of the inhibitor reduced the percentage of spermatozoa capacitated and with acrosome reacted, in relation to the control group ($p < 0.05$). After induction of the in vitro capacitation, there was a reduction ($p < 0.05$) in the number of unprocessed spermatozoa in all treatments suggesting a reversible effect of U73122 on the process of sperm capacitation and acrosomal reaction. It is concluded that the regulation of PLC activity by the use of U73122, was shown to be efficient in reducing premature capacitation of ovine spermatozoa. In vivo tests should be performed to certify the effect of treatment on the pregnancy rate.

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# L-carnitine supplementation during vitrification did not improve survival and quality rates, but altered CrAT and PRDX1 expression in *in vivo*-produced ovine embryos

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Embryo cryodamage is observed mainly at metabolic and molecular aspects and it impairs post warming quality and survival rates. This study aimed to evaluate the effect of L-carnitine (LC) supplementation during either vitrification or post warming solutions on the 6-7th day of in vivo-produced ovine embryos. LC (3.72 mM) was added to vitrification (Experiment 1; C1: control; LC1: supplemented embryos) or warming solutions (Experiment 2; C2; LC2). In vitro culture (IVC) of warmed embryos was performed for 72 h at 38,5 °C, 5% CO2 and 5% O2 to evaluate survival rates in both Experiments. In Experiment 1, reactive oxygen species (ROS) levels were measured by CellROX Green staining, total cell number (TCN) by Hoechst 33342, number of apoptotic cells by caspase-3 immunofluorescence staining protocol, apoptotic index evaluation in both groups. Gene expression analysis of carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2), carnitine O-acyltransferase (CrAT) and peroxiredoxin 1 (PRDX1), were performed by RT-qPCR (ACTB as endogenous control) in Experiments 1 and 2 and results were compared to fresh embryos (FE). Averages of survival rates were compared by the Chi-Square test. Means of TCN, apoptotic cells, apoptotic index and fluorescence intensity were compared by Student's t-test, at 5% significance level. Survival rates were similar between groups ($p > 0.05$) in Experiments 1 (68.7%, C1 vs 81.8%, LC1) and 2 (48.5%, C2 vs 64.7%, LC2). In Experiment 1, ROS levels at 24 h of IVC (85.83 ± 68.37 x 1010, C1 vs 89.04 ± 84.48 x 1010, LC1), total cell number at 24 h (89 ± 22, C1 vs 82.2 ± 28, LC1) and 72 h (86 ± 19.9, C1 vs 68.5 ± 25.26, LC1), apoptotic cells (3.75 ± 1.48, C1 vs 4.50 ± 4.72, LC1) and apoptotic index (4.37 ± 1.45, C1 vs 5.23 ± 4.72, LC1) at 72 h of IVC did not differ ($p > 0.05$) between C1 and LC1. Gene expression analysis showed no differences in CPT1 and CPT2 mRNA relative abundance in embryos of both experiments compared to FE, however, CrAT was downregulated ($p < 0.05$) in C1 and PRDX1 was downregulated ($p < 0.05$) in both C1 and LC1, compared to FE. Moreover, CrAT and PRDX1 were upregulated ($p < 0.05$) in C2 and CrAT was downregulated ($p < 0.05$) in LC2, in relation to FE. In conclusion, although the short-term LC supplementation at 3.72 mM during cryopreservation did not improve post-warming survival and morphological parameters of the evaluated embryos, it was able to modulate expression of genes related to energy homeostasis (CrAT) and oxidative stress (PRDX1), proving to be beneficial, in both forms of supplementation, to in vivo-produced ovine embryos.

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# Survival rates of cryopreserved murine blastocysts exposed to heat stress at 8-cells stage

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Sublethal stress has been reported as inducer of gametes and embryos response, providing cell protection to a subsequent stress. Experiments showed that different treatments using pH modifications, heat and cold shock, osmotic challenge, high environmental pressure, and nutrients starvation lead embryos and gametes to produce different proteins than normally sintethyzed in homeostatic conditions in order to keep favorable conditions facing a next stresfull situation. Within this observation, many researchers started to experiment sublethal stress as protector treatment for cryopreservation. The aim of this experiment was to investigate the use of heat stress of environmental temperature as 8-cells embryos stressor to improve cryopreservation rates at blastocyst stage. Embryos at 8-cells stage were recovered from six weeks old superovulated Mus musculus domesticus females at day 3 pregnancy. Two hundred and twelve embryos were randomly segregated in control (C) and experimental (B) groups. Eight-cells embryos from B group were maintained during 4 hours at 21ºC while control embryos were immediately after recovery in vitro culured in mKSOM media + 0.4% BSA at 37ºC under atmosphere of 5% $CO_2$, 5% $O_2$, 90% $N_2$ and satured humidity. After been exposed to environmental temperature, group B embryos were transferred to the same in vitro conditions as the control group embryos for 48h. Then, embryos from both experimental groups that reached blastocyst stage were cryopreserved in 0.25 mL straws using a classical frozen curve: first, blastocysts were exposed to 1.6 mol of ethylene glicol + mPBS + 0.4% BSA, then cooled at 2 ºC/min to reach seeding (-6 ºC) temperature and then they were cooled at -3 ºC/min until reach - 35 ºC, when they were transferred to liquid azote. Embryo development and expansion rates were compared using Chi-square test ($P < 0.05$) 24h after thawing. Eight-cell embryo developmental rates to blastocyst stage showed no difference among control (95.0% - 92/97) and experimental (95.4% - 110/115) group. Cryopreserved blastocyst expansion rate of stressed embryos was significantly higher (84.5% - 93/110) than cryopreserved control embryos (72.8% - 67/92; $P < 0.05$). We concluded that a simple stress condition like maintaining embryos at environmental temperature (21ºC) can induce a heat stress response that could be usefull to enhance embryo survival after cryopreservation.

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# Use of Doppler ultrasonography in embryo transfer programs in the equine species

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In the equine industry, the increase of reproductive efficiency is necessary to reach better results, in addition to increased rate of genetic improvement of livestock. The demand for new biotechnology of assisted reproductive techniques has increased substantially. Ovarian and uterine evaluation of mares by Doppler mode ultrasound helps in the selection of animals that will participate in embryo transfer programs, correlating luteal vascularity with serum levels of progesterone. The aim with this study was to observe results of the assessment of recipients using Doppler mode ultrasound, rectal palpation, cervical evaluation and ultrasound B Mode, in order to compare pregnancy rates between recipients selected by using B Mode ultrasound and using Doppler Mode ultrasound. In this study, follicular dynamics was controlled by rectal palpation and ultrasound examination every day. Two groups of recipients was divided where the first group of recipients (n=14) mares were evaluated by rectal palpation, cervical assessment and B mode ultrasound, while in the second group of recipients (n=15), same parameters were used (rectal palpation, cervical assessment and B mode Ultrasound) and a Doppler Mode Ultrasound examination was added. The evaluations were performed during the selections of recipients at the time of embryo implantation, collected from donor mares at D8 post-ovulation. For statistical analysis calculation Fisher exact test was used. There were 42 embryo collections with a recovery rate of 29 embryos (69%), resulting in 20 pregnancies (68.9%). From these 29 embryos implanted, 14 recipients were evaluated by B Mode Ultrasound where 7 (50%) resulted in positive pregnancy and 15 recipients were evaluated by Doppler Mode Ultrasound and of these, 13 (86.6%) resulted in pregnancy. Despite the low number (n) of recipients evaluated, it was possible to observe a pregnancy rate statistically superior in those animals assessed with Doppler Mode ultrasound compared to the recipients assessed only with B mode ultrasound. In conclusion, the results of this study demonstrated that Doppler Mode Ultrasound presents a large potential for its use and can serve as an important tool in order to optimize selection of recipients that will receive embryos in commercial embryo transfer programs.

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# Vitrification of bovine ovarian fragments associate with resveratrol

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In the attempt to minimize morphological damage and the production of metabolites caused by reactive oxygen species from the cryopreservation process, studies have suggested the addition of antioxidants such as catalase, α-tocopherol, trehalose, and resveratrol in vitrification/heating solutions. Resveratrol regulates the expression of SIRT-1 and mitochondrial activity. The aim of this study was to analyze the effect of resveratrol on preantral follicles morphology and, the tissue viability of ovarian fragments from bovine fetuses after vitrification/warning procedures. The ovaries were fragmented and distributed to the control, vitrified and vitrified with resveratrol groups. Preantral follicles were quantified and classified according to the developmental stage. The proportion of normal follicles differed between treatments ($P < 0.05$) and the group of follicles vitrified with resveratrol was superior to the vitrified without the addition of the antioxidant. In resveratrol group, the class of primordial follicles had a higher proportion ($P < 0.05$) of viable follicles. In contrast, the secondary follicle class presented the lowest proportion of normal follicles in both treatments. In addition, a negative association ($P < 0.05$) was observed between the proportion of viable follicles and the stage of follicular development. The probability of finding viable follicles was higher in the group of vitrified follicles in the presence of resveratrol. Moreover, primordial follicles of the resveratrol group had 2.5 times more likely to be viable after vitrification. The chance of observing normal follicles was greater ($P < 0.05$) in the early stages of follicular development. The diameter of the transitional follicles and their respective oocytes were lower when submitted to the vitrification process with resveratrol ($P < 0.05$). In the primary follicle class, follicular and oocyte diameters were similar among the studied groups. The tissue viability performed with confocal microscopy technique evaluated the levels of fluorescence related to reactive oxygen species levels and degeneration levels emitted by diihidrochlorfluorescein and propidium iodide. The fluorescence levels indicating cell degeneration in the group of vitrified fragments with the addition of resveratrol were similar ($P > 0.05$) to the control group. The reactive oxygen species were similar between the vitrified and vitrified groups with the addition of resveratrol. Also, the control group presented a higher level of reactive oxygen species ($P < 0.05$) compared to the other groups. The fluorescence intensity emitted with the use of both probes decreased ($P < 0.05$) with the increased tissue depth. In linear regression analysis, there was a negative correlation between fluorescence intensity and tissue depth. Furthermore, there was a positive association ($P < 0.01$) between degenerate cell levels and the rates of reactive oxygen species produced in treatments with different depths of ovarian tissue, regardless of treatment and tissue depth. Treatment groups showed similar levels of reactive oxygen species. Moreover, the proportion of fragments with high levels of reactive oxygen species was higher in the control group compared to the vitrification treatments. In conclusion, the ovarian tissue fragments of vitrified with antioxidant resveratrol presented a better follicular morphology and tissue viability.

A085E TAI/FTET/AI

# What could be the impact of cervical mucus removal after oestrus synchronization on artificial insemination outcome in INRA180 prolific sheep?

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**Keywords**: Oestrus synchronization, mucus, artificial insemination.

Artificial insemination (AI) is an important tool that improves the chance of using superior rams (Arranz et al., Renc. Rech. Rum, 15, 359-362, 2008) and helps to control genitally diseases. During the AI process, the administration of exogenous progestagen to synchronize oestrus in ewes is one of the most important steps. However, the administration of such hormones has resulted in contradictory reports of both increased and decreased mucus production, which could affect the outcome of AI. The present work aimed to study the effect of mucus removal after oestrus sychronisation of INRA180 prolific adult ewes. A total of 84 ewes (2.5 to 3 years old) that have been managed under natural reproduction system were used to make 3 groups: group 1 (control) reproduce naturally, group 2 inseminated without removing the mucus and group 3 inseminated after mucus removal. For each group, two different doses of eCG (250 IU vs 300IU) have been used. The ewes were treated with intravaginal progesterone sponges (20 mg Flurogestone acetate, Pharmavet) for 14 days, and then injected with equine chorionic gonadotropin (eCG) at 250 IU or 300IU (Folligon®, Pharmavet) during Jun 2016. They were naturally mated (n=20 with 250 IU, and n=20 with 300 IU) or inseminated after removing the mucus (n=12 with 250 IU, and n=12 with 300 IU) or not (n=12 with 250 IU, and n=12 with 300 IU). The mucus was removed from animals in standing position using vaginal speculum. A split-plot design was adopted and all analyses were performed using JMP SAS v11. Fertility and prolificacy data were assessed by $\chi 2$ analysis of contingency tables. The results showed that the natural mating revealed the highest fertility rates (80 to 90%). In both 250 IU and 300 IU groups, the treatment (removing the mucus or not) was highly significant ($P<0.05$). The conception rates were 32% and 40% respectively for the ewes receiving 250 IU and 300 IU of eCG. The mucus removal has significantly improves the fertility as this parameter increased to 59% and 67% respectively in 250IU and 300 IU. Recently, it has been reported that the cervicovaginal mucus proteome of the ewe undergoes natural variation across the oestrous cycle, and is significantly altered by progesterone synchronisation (Maddison et al., Journal of Proteomics, 155, 1-10, 2016,). This could explain a part of the result obtained in this work. The prolificacy was not improved ($P>0.05$) and it varies from 1.44 to 1.89. In conclusion, the present study revealed that it is possible to improve the fertility rate after artificial insemination by means of mucus removal after oestrus induction. Further protocols are planned on a large group of animals and will focus mainly on the eCG doses (300 IU) showing the greatest fertility rate. A part of this work was supported by CNRST of Morocco (PPR15/47).

A086E TAI/FTET/AI

# Comparative study of ewe's cervixanatomy of two Moroccan breed: A necessary step before the artificial insemination

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**Keywords**: Boujaâd ewes, D'man ewes, cervix anatomy.

The anatomy of the cervix represents a major constraint for developing the trans-cervical artificial insemination (TCAI) in sheep. Thus, the aim of this study was to compare this parameter in two Moroccan indigenous breeds. A total of 250 uteri Boujaâd (non-prolific,n = 187) and D'man (prolific, n = 64), with different ages (2,4,6 and 8 teeth) (Hemming, J.Wildl. Manag. 33, 552–558,1969) were collected from slaughterhouses at Bejaad and Errachidia respectively. The uteruses were transported at 5°C to the laboratory, and were cleaned in order to perform various measurements. For each sample, the weight of the uterus, length of the cervix, depth of penetration (using the artificial insemination gun), cervical grade (Kershaw et*al*.,Theriogenology 64, 1225-1235, 2005), and the number of cervical rings were recorded.All data were analyzed using the Statistical Analysis System software JMP (SAS version 11), by means of a factorial design ANOVA. The statistical model included the breed and age as fixed effects. When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SD.The cervical grade data were assessed by $X2$ analysis of contingence tables. Independently on the ewes age, this study showed significant differences in the cervical grades between the two breeds. According Kershaw et *al*., (Theriogenology 64, 1225-1235, 2005) the grades were as follow: grade 1 (11.90%) vs (12.82%), grade 2 (52.38%) vs. (69.23%) and grade 3 (35.71%) vs. (17.95%) respectively for Boujaâd and D'man. Furthermore, the average length of the cervix was 54.47 ± 13.60 mm for Boujaâd ewes instead of 41.53 ± 9.54 mm for D'man ($P <0.05$). In addition, there were significant differences between breeds in the number of cervical rings (4.56 ± 1.32 for Boujaâd *vs*.3.91± 1.09 for D'man), weight of the uterus (46.04± 18.21 g for Boujaâd *vs*.37.39±13.15 g for D'man) and the depth of penetration percentage (34.18% for Boujaâd *vs*.41.70% for D'man). While the penetration depth of the insemination gun was not significantly different between the two breeds(18.06± 7.95 mm). Age had a significant effect on all studied parameters within each breed. Generally, in ageing ewes, the cervix tended to become longer with loose folds. As a conclusion, there was an apparent difference in the complexity of the cervix between Boujaâd and D'man breeds and between age within each breed, with a marked complexity in the Boujaâd ewe,which may make trans-cervical artificial insemination more difficult in this breed.

A087E TAI/FTET/AI

# Analysis of sperm-induced neutrophil extracellular traps (NETs) formation in the bovine system

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**Keywords**: sperm, PMN, neutrophil extracellular traps (NETs).

The natural site of semen deposition is the vagina in cattle and sperm migrate into the uterus leaving the bulk of the seminalplasma (SP) behind. However, current artificial insemination introduces variable amounts of SP into the uterus, which naturally remains in the vagina. While neutrophils combat microbial contamination and eliminate excess/dead sperm, their presence at the time of semen deposition reduces fertility. Beside phagozytosis and secretion of immune modulators, polymorphonuclear neutrophils (PMNs) are able to form "Neutrophil Extracellular Traps" (NETs) extruding their DNA into the extracellular environment. These are web-like structures, mainly composed of chromatin. Bovine PMN were isolated via Ficoll gradient centrifugation from peripheral blood. Frozen/thawed sperm cell suspensions (SCS) of bulls with proven fertility were used. The visualization and identification of NETs was achieved by scanning electron microscopy (SEM) or via fluorescence microscopy analysis, respectively. For NET induction, PMN and SCS were co-cultured for different time points (0, 15, 30, 45, 60, 120, 180 min). NET induction of sperm and supernatant alone was also measured. Zymosan was used to induce the formation of NETs in bovine PMN as positive control. Quantification of NETs formation was performed by spectrofluorometric analyses using an automated plate monochrome reader (Varioscan Flash; Thermo Scientific). Scanning electron microscopy as well as fluorescence microscopy analyses revealed that the exposure of bovine PMN to frozen/thawed bovine SCS trigger the formation of NETs. After quantification, fluorescence intensities (FI in arbitrary units, AU) indicate that sperm alone led to significantly reduced fluorescence intensities suggesting that the extender and the remaining seminal plasma are affecting NET formation to a higher extent. A significant increase in FI was seen until 60 min of incubation indicating that NET formation might be finished at that time point. Furthermore, no differences in FI were assessed with motile or immotile sperm indicating that the release of NETs is independent on sperm motility. These data show that bovine sperm are able to induce NETs formation. The financial support of the Förderverein Bioökonomieforschung e.V. (FBF) is gratefully acknowledged.

A088E TAI/FTET/AI

# Automated activity monitoring of estrus in recipient heifers: a retrospective study in an embryo transfer center

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**Keywords**: automated activity monitor, recipient, estrus.

For several years, EVOLUTION has set-up a new strategy to optimize its selective breeding programs by genotyping embryos after biopsy, keeping them frozen until their transfer once selected based on their estimated breeding value. Since only the best embryos are transferred, the management of recipients become essential and to succeed in this task, a recipients center has been opened to transfer embryos from our dairy breeding programs. The aim of this retrospective study is to highlight some zootechnical results obtained with an automated activity monitoring of estrus in recipient heifers. 209 Holstein heifers weighting more than 400 kg were used to receive frozen biopsied embryos. Most of them wear activity monitors (HEATIME®) and their heats were synchronized by groups of 5 to 15 using the following protocol: D0, Norgestomet implant insertion, Norgetsomet and Buserelin injection (CRESTAR Pack®) – D10 Cloprostenol (ESTRUMATE®) injection – D11 implant removal – D12 to D14 heat observation. The objective of this protocol is to transfer embryo between 6 and 7 days after heat detection, at D19. 383 heats were detected and followed by a transfer for 295 of them while the full activity profile was recorded for 219 of them. Each heifer was allowed up to 3 transfers before leaving the breeding program. The HEATIME® profile allows the record of the beginning of the peak activity, the increase of activity at the peak and the duration of the peak activity. The pregnancies were checked by ultrasonography at day 30 and confirmed between day 55 and day 65. 419 synchronization protocols were performed : 87 heifers were synchronized once, 62 twice, 40 three times and 20 more than three. Regarding the beginning of activity peak, 2% occurred in less than 12 hours after implant removal, 12% between 12 and 24 h, 41 % between 24 and 36 h, 17% between 36 and 48 h, 17% between 48 and 72 h, 9% between 72 and 96 h and 2% after more than 96 h. The mean activity increase at the peak during heat is 85 % ±16 and the mean of peak activity duration is 14.5 h ±6.1. For the 295 transfers, the pregnancy rate is 56%. This rate differs according to intensity of peak activity ($P<0.05$) with 42.5% when the increase was strictly less than 90 % and 57.5% for increase of 90% or more. No effect of protocol or transfer rank can be observed. No significant effect either of the recipient's stage could be shown as the pregnancy rates are 54 % (7/13), 61 % (27/44), 56% (128/227) and 45 % (5/11) for transfers done between [4-5[, [5-6[, [6-7[ and [7-8] days after the heat, respectively. This kind of automated activity monitoring allows us to have detailed description of recipient's heats which is not the case when recipients are in partners' farms. Recorded information will allow us to discriminate more closely the recipients based on estrus characteristics or stage.

A089E TAI/FTET/AI

# Progesterone concentrations on the day of re-insemination on farms using artificial insemination services and on herd-owner insemination farms

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**Keywords**: cow, oestrus, pregnancy.

The objective was to examine whether training background of artificial inseminator (herd-owner inseminators=OWNER, AI technicians=AI-T and fertility consultants=FC) has an effect on pregnancy rate and if additional training helps AI professionals to detect cows not in oestrus. A total of 1584 re-AI occasions on 754 farms were included. Whole milk samples were collected for progesterone (P4) analysis (RIA) from all cows submitted to re-AI and data including farm type, previous breeding attempts, oestrus signs and evaluation of uterine tone, slipperiness of the cervix and co-operation of the cow was collected. Further breeding attempts and next calving or culling date were sought from registers. AI occasions were divided into three categories based on P4 concentrations; <6 nmol/l: no luteal activity and cow could be in oestrus, 6 to 10 nmol/l: some luteal activity and >10 nmol/l: high luteal activity and cow was not in oestrus. A proportion of 7.7% of cows offered for re-AI had P4 concentration >10 nmol/l. There was no statistical difference between OWNERs and farms using AI service. OWNER farms chose for AI more cows with intermediate P4 values than farms using AI service (OWNERs: 82.7% <6 nmol/l, 9.8% from 6 to 10 nmol/l and 7.5% >10 nmol/l and farms using AI-service: 86.2%, 5.9% and 7.8%, respectively). AI-Ts recommended no AI significantly less than FCs: 1.6% versus 4.9%. Both groups were equally right: 71% and 68% of cows recommended no AI had high P4 concentration. Finally, in the three P4 categories, AI-Ts and FCs inseminated 86.3%, 6.5%, 7.2% and 89.2%, 5.5%, 5.3% of cows, respectively. As of more courageous rejection of cows with high P4, FCs inseminated statistically significantly more cows at <6 nmol/l and less cows at 6 to 10 nmo/l than OWNERs. 36.7% of cows finally inseminated got pregnant and there was no significant difference between OWNERs and farms using AI service (37.1% versus 36.4%). FCs had significantly higher pregnancy rates than AI-Ts (39.6% versus 32.6%). The proportion of cows inseminated during the luteal phase has increased in Finland from 4.4% (Laitinen 1983, Oestrus confirmation, pregnancy diagnosis and postpartum ovarian follow-up of the Finnish dairy cows by milk progesterone assay: Effects of breed, season, feed and sampling on milk progesterone levels, PhD thesis) to 7.7% in the past 30 years. This should be taken into account in the education of AI professionals and OWNERs. The ability of the inseminators to detect the cows not in oestrus and to reject them can be strengthened through training. More accurate rejections yield a higher pregnancy rate. The best indicative and predictive oestrous signs detected by the inseminator at the time of AI are uterine tone and consistency of the vaginal mucus. Behaviour of the cow at AI did not predict the P4 concentration. Acknowledgements The study was supported by the MAILI project of Savonia University of Applied Sciences financed by the European Union.

A142E OPU - IVF and ET

# Addition of seminal plasma reduces binding of stallion spermatozoa to bovine oocytes

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**Keywords**: seminal plasma, stallion, binding assay.

Equine *in vitro* fertilization (IVF) is currently not a repeatable process; a heterologous zona binding assay (HZBA) using *in vitro* matured (IVM) bovine oocytes could be an alternative to test the fertilizing capacity of stallion spermatozoa. Survival of stallion spermatozoa during cryopreservation varies considerably between individuals. Seminal plasma (SP) has been shown to repair cryodamage to sperm membranes (Bernardini et al., Theriogenology 76:436-447; 2011) and therefore may influence binding to the zona pellucida. Objective: To investigate the effect of adding SP from "good" (GF) or "bad" (BF) freezer stallions on sperm binding capacity. Ejaculates (one from each of six stallions) were processed by Single Layer Centrifugation (SLC) to remove SP and were frozen using the standard protocol at a commercial stud (Schober et al., Theriogenology 68:745-754; 2007). Straws were thawed at 37°C for 30s; the contents were gently layered on a low density colloid and were centrifuged to separate spermatozoa from cryoprotectant. The pellet was harvested and resuspended in modified Whitten's capacitation medium (MW) containing sodium bicarbonate and BSA. Salt-stored IVM bovine oocytes with intact zona pellucida, were obtained from several batches of ovaries, pooled and stored until needed. They were washed several times in prewarmed PBS/PVA, equilibrated for one hour in 37°C and transferred in groups of 25-27 to four-well plates containing: i) control (C) 500 µL MW (n=152); ii) 500 µL MW supplemented with 5% pooled BF-SP (BF) (n=161); or iii) 500 µL MW containing 5% pooled GF-SP (GF); (n=164). Sperm samples (final concentration $5\times10^6$ spermatozoa/mL) were added to the drops; the plates were incubated for 14-18 h in 38°C in 5% $CO_2$ incubator, 95% humidity atmosphere. The sperm-oocyte complexes were pipetted several times then rinsed gently three times to remove loosely attached spermatozoa. They were fixed in 2% (V/V) paraformaldehyde in PBS/PVA overnight at 4°C, washed, stained with Hoechst 33342 (5 µg/mL) and mounted under anti-fade medium (Vectashield) on glass slides. The coverslip was sealed with nail polish and allowed to dry. The number of spermatozoa bound to the zona pellucida (ZP) was assessed using confocal microscopy at 200x. Data were analyzed by General Linear Model using the SAS® software (version 9.3); significance was set to $P \leq 0.05$. All values are LSMEAN ± S.E. The number of spermatozoa bound to ZP was higher in C than in BF or GF (C 21.89±0.67; BF 2.86±0.65; GF 2.50±0.64; C vs. BF $P\leq0.0001$; C vs GF P $P\leq0.0001$). No differences were found between BF and GF. In conclusion, addition of SP impaired stallion sperm binding to the zona pellucida of bovine oocytes, independently of whether the SP came from a good freezer or a bad freezer. This effect may be due to the presence of sperm decapacitation factors in the SP.

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A143E OPU - IVF and ET

# Biobanking the first collection of oviductal and uterine fluid from hysterectomised patients

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**Keywords**: biobank, human reproductive fluids, surgery patients.

The safety of procedures in assisted reproductive technologies (ART) and the effect of culture conditions on embryo and fetal development, it is raising a great deal of concerns, mainly due to the lack of information about the formulations of commercially available culture media used in human IVF/ICSI treatments. As it was already described, the different IVF culture media influence the rates of successful implantation, pregnancy and birth weights (Kleijkers, Human Reproduction, Vol.31, No.10 pp. 2219–2230, 2016). It has been recently shown that culture media supplemented with natural female reproductive fluids have improved IVF efficiency, morphological embryo quality and epigenetic reprogramming profiles in pig blastocysts, compared with culture media without these supplements (Canovas, Elife, 6: e23670, 2017). This has encouraged the development of strategies that allow a noninvasive collection of reproductive fluids in humans, in order to validate them as supplements in the future. The first objective of this study was the development of a method to collect human oviductal and uterine fluids. A second objective was the initial characterization of reproductive fluids by measuring volume, protein concentration (Bradford Reagent, Sigma, Madrid, Spain), osmolality (Wescor Vapro 5520 Vapor Pressure Osmometer) and pH (pH OxyMini FOR PRESENS, Germany). The fluids were collected from 33 premenopausal women undergoing a total abdominal hysterectomy in the scheduled gynecological surgery of 'Virgen de la Arrixaca' University Clinical Hospital, whose indication was a benign uterine pathology. The oviductal fluid was collected according to the method previously described in Carrasco et al. (Reproduction, 136: 833–842, 2008). The collection of uterine fluid was carried out with a device normally used for mucus sampling. Once collected, the fluids were centrifuged at 7000 g for 10 min at 4°C to remove cellular debris and stored at −80°C in BIOBANC-MUR IMIB. It was possible to collect a mean volume of 23.9 ± 14.6 µl (n=22) of oviductal fluid and 62.8 ± 33.0 µl (n=26) of uterine fluid. The mean total protein concentration was 30.9 ± 14 µg/µl (n=22) for oviductal fluid and 48.9 ± 17.9 µg/µl (n=26) for uterine fluid. Mean value of osmolality was 316.6 ± 35.9 mmol/kg (n=22) for the oviductal fluid and 283.8 ± 69.5 mmol/kg (n=22) for the uterine fluid. Finally, mean pH values for oviductal and uterine fluids were 7.4 ±0.7 (n=22) and 7.8 ±0.3 (n=22), respectively. Although the selected methods allowed the reproductive fluids collection, they should be improved in order to obtain higher volumes without endometrial damage, to perform clinical trials that could validate their use as a supplement in culture media for ART. Besides the volume limitations, we can conclude that it is possible to establish a biobank of reproductive fluids, which meets sanitary conditions and legal requirements for research and future medical applications.

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A144E OPU - IVF and ET

# Seminal plasma proteins increase *in vitro* fertility rate of frozen-thawed ram semen

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**Keywords**: apoptosis, capacitation, fertility.

Ram seminal plasma proteins (SPP) have an antiapoptotic effect (Mendoza et al., FEBS J 279: 62-63, 2012) and can protect spermatozoa from the cryopreservation damage (Barrios et al., Biol. Reprod. 63: 1531-1537, 2000). Therefore, their use in frozen-thawed seminal doses might increase fertility results. In order to test this hypothesis, ram semen was frozen after adding SPP. SPP were obtained by semen centrifugation at 12000 x g for 5 min at 4 °C, the supernatant was loaded in >3 kDa filters (Filtron Tech, Northborough, MA, USA) and centrifuged for 6 h at 3000 x g at 4 °C. Protease and phosphate inhibitors (Sigma Chemical Co, St. Louis, MO, USA) were added and SPP were stored at -20 ºC until use. Ram semen collected from nine Rasa Aragonesa rams using an artificial vagina was frozen in plastic straws with (P) or without (NP) 40 mg/ml SPP, in a Tris-glucose-citric acid-egg yolk based medium (Evans, Aust. J. Biol. Sci. 41: 103-116, 1988), following the Fiser's et al. method (Theriogenology 28: 599-607, 1987). After thawing at 37ºC for 30 sec in a water bath, seminal parameters of frozen-thawed samples (P and NP) and a fresh semen sample (control, C) were analyzed (n=4). Viability (by the double staining with carboxyfluorescein diacetate/propidium iodide (Sigma Aldrich; Harrison and Vickers, J Reprod Fertil 88: 343-352, 1990)) and apoptotic markers (phosphatidylserine translocation by FITC-Annexin V (Thermo Fisher Scientific, Waltham, MA, USA) combined propidium iodide, and DNA damage by TUNEL assay (Sigma Aldrich, San Luis, MO, USA)) were measured by flow cytometry. The capacitation state was assessed by the chlortetracycline staining (Grasa et al., Reproduction 132: 721-732, 2006), and fertility by IVF of ewes' oocytes (n=103, 99 and 98 for P, NP and C, respectively in 4 replicates) and subsequent embryo development (Forcada et al., Span J Agric Res 11: 366-370, 2013). Obtained results were analyzed by chi-square test (SPSS Statistics, IBM analytics, Armonk, NY, USA). The frozen-thawed processes lowered ($P<0.05$ when P and NP are compared to C) both sperm viability (21.6±7.6% in P, 22.8±7.4% in NP and 61.6±4.3% in C) and the rate of viable spermatozoa without phosphatidylserine translocation (15.1±8.4%, 15.3±9.5% and 27.0±8.1% for P, NP and C, respectively). No differences were found in DNA damage (9.2±2.6%, 12.2±5.8% and 7.2±0.9% for groups P, NP and C, respectively). The addition of SPP resulted in significant differences in the rate of non-capacitated spermatozoa (11.5±0.5% in P, 5.0±1.1% in NP, and 58.0±7.3% in C; $P<0.05$ for all groups), which was reflected in a higher *in vitro* fertility rate (88.5%, 72.2% and 98.7% for P, NP and C, respectively; $P<0.05$) and embryo cleavage (67.0%, 51.5% and 76.5% for P, NP and C, respectively; $P<0.05$ when NP is compared with P and C). Furthermore, blastocyst rate was also higher in groups P and C when compared with group NP (53.6%, 33.3% and 51.3% for P, NP and C, respectively; $P<0.05$). In conclusion, ram SPP can increase fertility results after frozen-thawed procedures. Grants: DGA A26.

A145E OPU - IVF and ET

# Effect of reproductive tract O2 levels during *in vitro* fertilization and porcine embryo culture

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**Keywords**: oxygen, *in vitro* fertilization, embryo culture.

Oxygen concentration is a key factor for many physiological reactions in cells. However, most of the *in vitro* processes during pig embryo production are performed under environmental $O_2$ conditions (20%). These levels are far away from those recently measured in oviduct and uterus of sows and gilts (7 and 10%, respectively) (López Albors *et al*., Society for Reproduction and Fertility, vol. 2, P045, 2015). Indeed, 20% $O_2$ have a negative impact on embryo development in several species (Mantikou *et al*., Hum Reprod Update, vol. 19 (3), p. 209, 2013). Therefore, the effect of atmospheric vs. reproductive tract $O_2$ concentration during IVF and embryo culture (EC) in pig was compared. Porcine oocytes collected from gilts at slaughterhouse were *in vitro* matured and, 44 hours later, *in vitro* fertilized with sperm selected by swim up (Cánovas *et al*., eLife, vol.6, p. e23670, 2017). Gametes were co-cultured (2000 spz/oocyte) in TALP medium with 1% oviductal fluid from the late follicular phase (NaturARTs® PIG OF-LF, Embryocloud, Murcia, Spain), from 0 to 8 hours post insemination (hpi). Putative zygotes were cultured until blastocyst stage in NCSU23 medium with 1% oviductal fluid from the early luteal phase (NaturARTs® PIG OF-EL) from 8 to 48 hpi and 1% uterine fluid (NaturARTs® PIG UF-EL) from 48 to 180 hpi. Two groups were distinguished depending on whether 20% $O_2$ or 7% $O_2$ was used during IVF and subsequent EC. After 18-20 hpi, putative zygotes (182/653 from the 20% $O_2$ and 174/641 from the 7% groups) were fixed and Hoechst stained to evaluate IVF by fluorescence microscopy. After 48 hpi, cleavage rate was assessed. After 180 hpi, kinetic of development was evaluated classifying blastocysts as early, late, hatching or hatched. Later, they were fixed and Hoechst stained to quantify the number of nuclei in each blastocyst by fluorescence microscopy. Data were analysed by one-way ANOVA. A P-value $<0.05$ was considered to denote statistical significance. Oocytes fertilized under 7% $O_2$ showed the same penetration and monospermy rates, mean number of spermatozoa inside oocytes, and attached to the zona pellucida than oocytes fertilized under atmospheric $O_2$ levels. However, embryos cultured under 7% $O_2$ showed a significant increase in cleavage rate (60.0 ± 2.3%) compared with those cultured under 20% $O_2$ (32.0 ± 2.2%). Embryos cultured under 7% $O_2$ showed also a higher mean number of cells per blastocyst (88.9 ± 5.9) compared with those cultured under 20% $O_2$ (59.0 ± 5.0). Although no significant differences were observed for different embryo developmental stages between the groups due to the limited number of blastocysts (25 from the 20% $O_2$ and 50 from the 7% groups), absolute values for hatching and hatched blastocysts were larger in embryos cultured under 7% $O_2$ than under 20%. Overall, $O_2$ is an important factor to take in consideration during ART. The use of $O_2$ levels closer to those found in the reproductive tract not only enhances embryonic development but also improves the quality of the blastocysts produced.

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A146E OPU - IVF and ET

# The effect of the presence or absence of a cavity in the corpus luteum on progesterone concentrations and pregnancy rate in heifers following embryo transfer

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**Keywords**: corpus luteum, recipients, pregnancy rate.

The aim of the study was to compare pregnancy rate and concentration of progesterone (P4) in embryo recipients in which the presence of compact corpus luteum ($CL_{com}$) or corpus luteum with a cavity ($CL_{cav}$) was observed at the day of embryo transfer. 79 heifers recipients were used in the study. Oestrus was synchronized with two i.m. injections of 25 mg of dinoprost tromethamine (5 ml of Dinolytic, Zoetis, Warsaw, Poland) administered at 14 day intervals. On Day 7 after oestrus, the ovaries were examined with the use of ultrasound (linear probe, 7,5 Mhz, iScan, Draminski). Corpora lutea were divided based on the presence or absence of a cavity into $CL_{com}$ (n=187) and $CL_{cav}$ (n=92). With the use of ultrasonography, the diameter, area and volume of CL and cavities (where present) were measured. Simultaneously, blood samples were taken from 41 heifers (25 recipients with $CL_{com}$ and 16 recipients with $CL_{cav}$). Serum concentrations of P4 in the samples were evaluated by RIA. Fresh embryos (one embryo per recipient) were placed into the ipsilateral horn of the uterus. Pregnancy was diagnosed by ultrasonography 2 months after embryo transfer. Data were analysed by ANOVA and logistic regression using the STATISTICA 9,0 software PL. The mean diameter, area and volume of $CL_{com}$ and $CL_{cav}$ were 21.7 ± 2.57 mm vs. 23,0±2,56 ($p<0,001$), 384.6 ± 94.5 $mm^2$ vs. 458.8 ± 98.4 $mm^2$ ($p<0,0001$) and 7301.7 ± 2416 $mm^3$ vs. 8849.5 ± 2579 $mm^3$ ($p<0,0001$), respectively. The mean cavity diameter, area and volume were 9.4 ± 2.91 mm, 140.6 ± 50.4 $mm^2$ and 1177.5 ± 296 $mm^3$, respectively. The area and volume of luteal tissue were greater in $CL_{cav}$ compared to $CL_{com}$. Mean concentrations of P4 12.1 ± 3.58 and 8.1 ± 3.96 ng/ml in $CL_{cav}$ and $CL_{com}$, respectively ($p<0.0001$). Pregnancy rate two months following embryo transfer were 51.1% and 34.7% for $CL_{cav}$ and $CL_{com,}$ respectively ($p<0.02$). In recipients with $CL_{cav}$, transfer to the right uterine horn resulted in a pregnancy rate of 41.8% compared to 59,5% for the left uterine horn ($p>0,05$). For recipients with $CL_{com}$ pregnancy rate following transfer to the right horn was 30.4% compared to 37.3% for transfers to the left horn ($p>0,05$). Moreover, when P4 concentration was higher than 10.88 ng/ml in 87.5% of CL there was a cavity, whereas when P4 concentration was lower or equal to 10.88 ng/ml in 88% CL was compact ($p<0.01$). The presence of cavities in the CL 7 days after ovulation appears to have a beneficial effect on the results of fresh embryo tranfer in recipients. Regardless of the type of CL, the placement of the embryo in the left horn of the uterus provided a higher percentage of pregnancies. However, in the case of $CL_{cav}$ placing the embryo in the left horn resulted in higher pregnancy rate than $CL_{com}$. It seems possible to predict the occurrence of cavity inside the CL basing on the P4 concentration in the blood.

A147E OPU - IVF and ET

# Bovine *in vitro* maturation medium with different protein supplementation influences the maturation and fertilization rates

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**Keywords**: bovine follicular fluid, IVM, IVF.

While some studies show beneficial outcomes on the use of bovine follicular fluid (bFF) in in vitro maturation (IVM), others display neutral or even detrimental effects. The main problem is related to the inhibitory effect on the meiosis resumption when high concentrations are used (Kim, Theriogenology, 45, 798, 1996). We hypothesized that inactivation of bFF might avoid this feature, thus we conducted 2 experiments (Exp) to evaluate the effect of bFF (either heat-inactivated or not) on the oocyte competence assessed by different parameters related to the nuclear and cytoplasmic maturation (Exp1) and the IVF efficiency (Exp2). Cumulus-oocyte complex's (COC) were obtained from slaughterhouse ovaries and IVM was performed using TCM-199 with 10% of either Fetal Bovine Serum (Control), bFF or bFF heat-inactivated (bFFin – 30' 56ºC). COC's were incubated for 22-24h and either denuded for Exp1, or submitted to IVF for Exp2. Frozen semen was used for IVF in TALP medium and incubated for 20-22h with oocytes. After fixation and Hoechst staining, oocytes and zygotes were evaluated under a fluorescence microscopy to assess nuclear status or fertilization parameters. In addition, cumulus cell expansion was measured in fresh oocytes before and after IVM. Total number of oocytes and replicates were as follows: 387 within 4 replicates for nuclear status; 432 within 3 replicates for cumulus expansion; 691 within 5 replicates for IVF. Data were analysed by one-way analysis of variance (ANOVA) and Tukey test with a level of significance $p<0.05$. The software used was IBM SPSS Statistics (v22.0). Values are percentages ± S.E.M. In Exp1, the nº of oocytes reaching metaphase II and showing a clear polar body were not significantly different among groups (69.29±3.91 for control, 68.29±4.21 for bFF and 68.55±4.19 for bFFin). Cumulus cell expansion showed no statistical difference between groups. In Exp2, the sperm penetration rate wasn't significantly different between control and bFF (91.29±1.9 and 83.37±2.5 respectively) but it was between control and bFFin (78.41±2.7). Monospermy, mean nº of penetrated sperm per oocyte (S/O) and male pronucleus formation (MPN) showed no significant differences among groups. Mean nº of sperm bound to the zona pellucida (S/ZP) was different between groups, with bFF showing the lowest value (1.98 S/ZP) and control the highest (4.5 S/ZP). The efficiency of the IVF was also different with the highest value for control 73.45±3.0 and the lowest for bFFin 59.49±3.2. However, there were no significant differences between bFF (63.48±3.2) and the other groups. In conclusion, adding bFF/bFFin to the IVM medium did not improve nor decreased maturation rates. However, IVF efficiency was lower when using bFFin but not when untreated bFF was used. Most likely, as others studies have shown (Collins, Theriogenology, 43, 1, 189, 1995), heating might inactivate some crucial heat-labile proteins that will further influence the ability to form a viable embryo.

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A148E OPU - IVF and ET

# *In vitro* viability and developmental competence of porcine morulae stored in liquid state for up to three days

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**Keywords**: embryo storage, porcine, morulae.

The use of vitrified porcine embryos for non-surgical embryo transfer (Ns-ET) programs has disadvantages, as complying with strict air regulations for handling and transport of dewars, the risk of devitrification during transport, the need of $LN_2$ in the recipient farms or the number of embryos needed per Ns-ET. These drawbacks call for alternative procedures for short-term embryo storage in liquid state. This study aimed to evaluate storage of in vivo derived pig morulae in liquid state for up to 72 h on their further in vitro development. In Experiment 1, morulae (N=228) were stored at 25ºC or 37ºC in TL-HEPES-PVA defined medium (DM) or NCSU23-HEPES-BSA semi-defined medium (S-DM) for 48 h. After storage, embryos were assessed for viability (embryos with appropiate morphology according to the International Embryo Transfer Society criteria) and development, and then conventionally cultured (NCSU23-BSA-fetal calf serum, 38.5°C, 5% of $CO_2$ and 95% humidity) for 48 h to assess their hatching competence. Non-stored morulae (N=44) cultured under conventional conditions were used as controls. Differences among groups were analyzed using Fisher's exact test. At 48 h of storage, DM at 25ºC was detrimental ($P<0.05$) for embryo viability (73.9%) compared to the control (93.2%) and the rest of the experimental groups (90.9% to 98.3%). Following conventional culture, S-DM at 37ºC was the only group able to maintain embryo viability in a percentage similar to the control group (96.7%). Embryo development at 48 h of storage was delayed ($P <0.001$) in all experimental groups compared with the controls, being the delay more severe at 25ºC. Most embryos stored at 37ºC reached blastocyst stage but, unlike controls, none of them hatched at the end of storage. After conventional culture, the hatching rate of embryos stored in S-DM at 37ºC was similar to that of controls (85.0%) but higher ($P<0.01$) than for the other groups (9.1% to 23.8%). In Experiment 2, morulae (N=59) were stored at 37ºC in S-DM for 72 h, assessed for viability and development, and conventionally cultured for 24 h. Non-stored morulae (N=50) cultured under conventional conditions were used as controls. There were no differences in embryo viability between S-DM and controls at the end of storage (98.3% vs 90.0%, respectively). Moreover, all viable embryos from S-DM group remained viable after 24 h of conventional culture. Although there was a development delay in the stored embryos compared with the controls, some stored embryos (6.9%) hatched at the end of storage. The hatching ability after conventional culture was similar for stored and control embryos (65.5% and 70.4%, respectively). In conclusion, morulae stored in S-DM at 37°C for up to 72 h maintain in vitro viability and developmental competence. In addition, most blastocysts derived from stored morulae conserved intact the zona pellucida at the end of storage. These findings open new possibilities for porcine embryo transport in liquid state. Supported by MINECO-FEDER (RTC-2016-5448-2 and AGL2015-69735-R) and Seneca Foundation (19892/GERM/15).

A149E OPU - IVF and ET

# Comparison of two culture conditions during maturation on *in vitro* development of sheep embryos

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**Keywords**: *Ovis aries*, static culture, dynamic culture.

Over the last two decades, the most studied variables to improve embryo development in vitro include the chemical composition of culture media. In fact, these approaches have proven to be beneficial and have contributed to improve success rates after assisted reproduction. However, not only the chemical requirements should be considered, but potential physical requirements may also be important factors in the continuous search for improving in vitro conditions. The objective of this study was to evaluate the effect of two culture systems (static and dynamic) during oocyte maturation in early ovine embryonic development. A total of 338 oocytes were obtained by aspiration of ovaries collected from a slaughterhouse. The oocyte control group (T1, n = 165) was placed for 24 h in a static culture, while another group (T2, n = 173) underwent dynamic culture receiving orbital movement with the aid of an electric stirrer agitator (AGO-1016, PRENDO, Mexico), for 5 seconds every 60 minutes for 24 hours. In both treatments the same maturation medium was used (TCM-199; In vitro S.A., Mexico), which was supplemented with 10% fetal bovine serum (Microlab, Mexico), 5 µg mL-1FSH (Folltropin, Vetoquinol, USA), 5 IU mL-1 hCG (Chorulon, Intervet, Colombia), 1 µg mL-1 17-β estradiol (Estrol, Pharmavet Argentina) and 50 IU heparin / mL (PISA, Mexico). The oocytes were fertilized with fresh semen using 55x106 mL-1 spermatozoa in medium TALP-Hepes (In vitro S.A., Mexico) and 18 hours later both groups were placed in Cleavage medium (COOK Medical, Australia), 60 hours later they were placed in Blastocyst medium (COOK Medical, Australia), performing the same management in both treatments. Embryo development was carried out in a CO2 incubator at 38.5 °C, 5% CO2 and 95% humidity. The size and development of the embryos was measured with an inverted microscope and a camera (AmScope) 144 hours after fertilization. The criterion for evaluating maturation in the cumulus-oocyte complexes (COCs) was by identifying the polar corpuscle and the level of expansion of the granulosa cells Fertilization was evaluated by the first cell division at 30 hours after performing Change to Cleavage medium (i.e.: 48 hours post insemination). The percentage of maturation, fertilization and blastocysts yield was calculated based on the initial number of COCs of each treatment. The means were compared by Student's t-test and chi-square according to the type of variable, using SAS. The percentage of maturation rate was higher ($P < 0.05$) in oocytes that underwent dynamic culture compared to static culture (78.3 ± 2.6 vs. 71.3 ± 2.7%). However, fertilization rate (72.8 ± 8.3 vs. 67.3 ± 13.0%), blastocyst yield (39.3 ± 6.8 vs. 36.36 ± 11.5%), and blastocyst diameter (166.5 ± 3.4 vs. 163.8 ± 2 µm) were similar ($P > 0.05$) in T1 and T2 groups. In conclusion, under the conditions of this study the use of dynamic culture for maturing sheep oocytes only improved maturation rate without any effect on embryo development.

A150E OPU - IVF and ET

# The addition of ascorbic acid to the vitrification-warming media enhances the cryotolerance of in vitro produced porcine blastocysts

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**Keywords**: ascorbic acid, blastocysts, vitrification.

It is known that vitrification and warming procedures disturb the oxidation-reduction status increasing intracellular reactive oxygen species levels in porcine blastocyst. This study aimed to assess the effects of adding ascorbic acid (AsA) as antioxidant to vitrification-warming media on the post-warming survival and quality of IVP porcine blastocysts. Immature oocytes (N=3600) collected from prepubertal gilts were cultured in maturation medium supplemented with 10 IU/mL eCG and 10 IU/mL hCG for 22 h and then for an additional 22 h in maturation medium without hormonal supplements. Mature oocytes were inseminated with thawed sperm (1000 spermatozoa per oocyte) in fertilization medium for 5 h. Presumed zygotes were cultured in glucose-free embryo culture medium (supplemented with pyruvate and lactate) for 2 days and in embryo culture medium containing glucose for an additional 4 days. Blastocysts were vitrified and warmed with the superfine open pulled straw method using TL-HEPES as basic medium and ethylene-glycol and dimethyl sulfoxide as cryoprotectants (Sanchez-Osorio et al. Theriogenology, 2010, 73:300-308). We added 50 µg/mL of AsA both vitrification and warming media (VW+ group). Control group media were not supplemented with AsA. After warming, VW+ (N=281) and control (N=307) blastocysts were cultured for 24 h to assess embryo survival (ratio of blastocysts that reformed their blastocoelic cavities at the end of culture to the total number of embryos cultured) and hatching rates. To evaluate the quality of vitrified-warmed blastocysts, the number of inner cell mass (ICM) and trophectoderm (TE) cells was determined in each embryo using a differential staining based on an indirect immunofluorescence reaction. For that, a primary antibody (anti-CDX2), which specifically binds TE cells, and a secondary antibody (anti-Mouse IgG) conjugated with alexa Fluor® 568 that emits red fluorescence were used. Afterwards, all blastocysts cells were counterstained with the DNA-binding fluorochrome Hoechst-33342 to identify the ICM cells that displayed only blue fluorescence. Stained blastocysts were examined under fluorescence microscopy. Results are expressed as means ± SD of six replicates, and differences between groups were analyzed by an unpaired Student's t-test corrected for inequality of variances (Levene's test). The VW+ group showed a higher ($P < 0.02$) survival rate (51.1 ± 20.9%) than the control group (34.8 ± 21.4%). However, there were no differences between groups in hatching rates (10.7±12.0% vs. 6.0±8.1%). There were also no differences between VW+ and control blastocysts in terms of ICM (14.5 ± 6.5% vs. 16.4 ± 7.5%) or TE (44.2 ± 18.2 vs. 46.3 ±12.1%) cells. In conclusion, the addition of 50 µg/mL of AsA to vitrification-warming media considerably enhances the cryotolerance of IVP porcine blastocysts but does not affect the quality of embryos in terms of number of cells in the ICM or TE. Supported by Séneca Foundation (19892/GERM/15).

A151E OPU - IVF and ET

# Haematological and blood biochemical parameters in piglets derived from embryo transfer

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**Keywords**: porcine, *in vivo*, embryos.

Porcine embryo transfer (ET) has an important role in pig industry because it allows the transport of genetic material, avoiding the risk of diseases dissemination. However, there are not many studies that assess the characteristics of piglets born by ET and information on the haematological and biochemical parameters in the newborns is limited. Since those parameters can be indicators of metabolic disorders and other pathologies, we aimed to compare haematological and blood biochemical parameters from piglets obtained by ET (ETp, n=22, 2 litters) vs. piglets obtained by artificial insemination (AI) (AIp, n=27, 2 litters). For this study, sows were used with the same genetics, feeding and housing conditions and they were inseminated with the same Large White boar semen doses. *In vivo* produced embryos (7 days after AI) were transferred to recipient sows by non-surgical methodology (DeepBlue® Porcine ET catheter, Minitübe, Tiefenbanch, Germany). Piglets were weighed and blood samples were collected on days 3 and 15 after birth. Blood samples were analyzed by haematology analyzer (Siemens ADVIA® 120, Tarrytown NY, USA) and clinical chemistry analyzer (Olympus AU400, Tokyo, Japan). Statistical analysis was performed using Systat Software (v. 13, San Jose CA, USA) by ANOVA considering day of birth and group (ETp and IAp) as factors, and litter as covariable. Differences were considered to be statistically significant when $P \leq 0.05$. Sex of the piglets and weight were not different between ETp and AIp after birth. An increase in the number of white blood (WBCB), red blood cells (RBC), RBC distribution width (RDW), mean platelet volume (MPV), platelet component distribution width (PCDW) and platelet mass distribution width (PMDW) was detected in ETp in comparison to AIp. On the other hand, a reduction in platelets counts (PLT), plateletcrit (PCT), mean PLT component (MPC) and corpuscular haemoglobin concentration mean (CHCM) were observed in ETp. Furthermore, higher alkaline phosphatase (ALP) values were observed on day 3 in ETp, while gamma-glutamyl transferase (GGT) values increased on day 3 and 15. No significant differences were observed on the other parameters measured. In conclusion, these preliminary results (derived from 4 litters) suggest that changes in the haematological and biochemical parameters are associated to the ET, although there are not differences from the reference values in piglets (Ventrella, BMC Veterinary Research, 13: 23; 2017). The alterations in platelets related parameters could be explained by an immunological platelet injury, probably associated to presence of maternal antibodies incompatible with platelet antigens from the piglets (Forster, Can Vet J. 48:855-7; 2007). This hypothesis must be confirmed with further studies. Currently, we are evaluating the clinical significance of the data, as well as the gene expression and DNA methylation changes in blood cells and placental tissue from these animals. Supported by MINECO-FEDER (AGL 2015-66341-R) and Fundación Séneca, 20040/GERM/16.

A152E OPU - IVF and ET

# Supplementation of α-tocopherol in two sheep breeds: effect on *in vivo* embryo production

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**Keywords:** ewes, α-tocopherol, superovulation.

The efficiency of a multiple ovulation and embryo transfer (MOET) program depends on the number of good quality embryos obtained, some studies suggest that supplementation with antioxidants can help to improve embryo quality. The objective of the study was to evaluate the effect of α-tocopherol supplementation on embryo quality of a MOET program in two sheep breeds. In total 43 females were superovulated, from which 12 Charollais and 12 Dorper were treated with 500 IU of α-tocopherol given 60 h before sponge removal, while 11 Charollais and 8 Dorper were not treated (0 IU). The ewes were synchronized with intravaginal sponges containing 20 mg FGA for 12 days and on day 10th were superovulated with a purified source of follicle stimulating hormone. Estrus was detected with teaser rams and ewes in estrus were inseminated by laparoscopy 18 h after estrus onset with 4 doses of fresh semen containing $100x10^6$ spermatozoa each. Embryo recovery was attempted 7 d after estrus by laparotomy. Ovulation rate, recovery rate, fertilization rate, and embryo quality were measured. The results were analyzed using ANOVA and t-test for means comparison or Chi-square tests as it was required. There was no effect ($p>0.05$) of α-tocopherol application, breed or their interaction on ovulation and recovery rates. Fertilization rate was similar ($p>0.05$) among ewes treated or not with α-tocopherol, but was higher ($p<0.05$) in Dorper than Charollais ewes (45.10 vs. 36.42%). Embryo quality was similar ($p>0.05$) among breeds, but lower ($p<0.05$) in treated (53.91%) than non-treated (70.33%) α-tocopherol ewes. The same trend occurred in the two breeds. In conclusión, the application of α-tocopherol did not improve fertilization rate and embryo quality of superovulated ewes under the conditions of the study.

A153E OPU - IVF and ET

# The STEINER OPU System: A new autoclavable device for flushing follicles in equine oocyte collection

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**Keywords:** eqine OPU, flushing follicles, new autoclavable device.

In equine IVF, oocyte collection is particularly challenging. Because equine IVF veterinarians must perform in vitro maturation (IVM), collecting oocytes from unstimulated follicles, it is essential to optimize the follicular flushing technique. The STEINER-TAN Needle (available in 17-, 19- and 21-gauge sizes) System was developed as a result of many years of research (Rose and Laky 2013. J Assist Reprod Genet. 30: 855-860) and practical work conducted in the field of human in vitro fertilization (IVF) (Schenk et al. 2016. J Assist Reprod Genet. 34:283-290.). It is manufactured by IVFETFLEX.com Handelsgmbh & Co KG (Graz, Austria). This needle combines the advantages of both single lumen and double lumen needles in that the option of flushing follicles is retained. Double lumen needles (12-gauge) are currently used for equine IVF in combination with epidural anesthesia and sedation. The STEINER (ovum pick-up) OPU System could also potentially be useful to veterinarians due to the fact that it is autoclavable and has the same functional properties as the low-cost STEINER-TAN Needle System currently used in human IVF. In this study, an autoclavable device (Steiner OPU System) was adapted to meet the needs of the veterinarian and demands of the IVF market. A 15-, 16-, or 17-gauge disposable needle or EchoTip® autoclavable needle, approximately 10-15 cm in length, is attached via a male luer lock to distal end of a piece of metal tubing. The aspiration tubing is inserted into this tubing with open end a few millimeters (proximal) from the male luer lock, enabling the free flow of fluid between outer (flushing) and inner (aspirating) tubing and facilitating follicle flushing. Female luer locks are attached to the aspiration and flushing tubing at the proximal end. The complete length of the tubing is 45 cm. This system can be used in combination with an autoclavable needle guide and elongated with a vaginal probe (available from IVFETFLEX.COM and manufactured to fit any US probe on the market). This tubing may be flushed manually. For optimal temperature control, a STEINER flush/valve, which is a flushing pump with a syringe warmer fitted for 50-cc syringes, may be used (also available from IVFETFLEX.COM), two models (mechanical or electrical) of which are available. Based on our research findings in humans, we hypothesize that the newly-designed OPU system could potentially be used by veterinarians to facilitate IVF in horses and openly welcome opportunities for scientific collaboration. Our results using this needle for oocyte retrieval in humans (Rose BI and Laky DJ. 2013. J Assist Reprod Genet. 30: 855-860) have led us to the conclusion that this new system could have significant advantages for IVF in horses over existing systems because it reduces pain (use of a much smaller OPU needle), obliterates the need for epidural anesthesia, allows for better temperature control (use of the syringe warmer), and helps keep the costs of the OPU procedure low.

A154E OPU - IVF and ET

# Effect of resveratrol-cyclodextrin complex supplementation during oocyte maturation or embryo culture *in vitro* in bovine

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**Keywords**: resveratrol, embryo, bovine.

The damaging effects of reactive oxygen species on *in vitro* embryo production have been widely studied on the past decade. Thus, many antioxidants such as resveratrol have been added to the *in vitro* production media mimicking endogenous antioxidants in an attempt to decrease their negative impact. Resveratrol has been reported to have a positive effect when added to *in vitro* maturation (0.1- 10 µM) or to culture media (0.25- 1.0 µM) in bovine and porcine *in vitro* embryo production. Higher concentrations of resveratrol in the embryo culture medium had been proved to have toxic effects on the developing embryos. Methyl ß-cyclodextrin, a group of cyclic oligosaccharides, has been used to improve the solubility of drugs. The present study evaluates the effect of the complex resveratrol-cyclodextrin during *in vitro* oocyte maturation (IVM) or *in vitro* embryo culture (IVC) on developmental competence and quantitative changes in gene expression of developmental important genes. In experiment 1, a concentration of 1 or 10 µM resveratrol (R1 or R10 respectively) diluted in 0.001% cyclodextrin was added to IVM media (TCM-199+10% FCS) and after 24 h a representative number of oocytes (n=330) were fixed to examine maturation level or snap frozen for gene expression analysis by RT-qPCR (n=120). The remaining were *in vitro* fertilized and cultured in SOF+3 mg/ml BSA to the blastocyst stage (n=1293). In experiment 2, 744 *in vitro* produced zygotes were cultured in SOF+3 mg/ml BSA supplemented with 0.5 or 1 µM resveratrol (R0.5 and R1 respectively) diluted in 0.0001% cyclodextrin. In both experiments, cleavage rate and blastocyst yield were recorded and blastocysts on Day 7 and 8 of the experiment 2 were snap frozen for gene expression analysis. A group without complex resveratrol-cyclodextrin (control$^-$) and a group with cyclodextrin (control$^+$) were included during IVM and IVC. A higher percentage of oocytes remained arrested in germinal vesicle when 10 µM resveratrol was added to the IVM medium (16.6 ± 2,6 %) compared to R1 (9.03 ± 0.6) and control groups (4.22 ± 0.6 and 10.3 ± 0.7 for control$^-$ and control$^+$ respectively ANOVA, $P<0.05$). No differences were found in cleavage rate or blastocysts yield between groups in both experiments. Regarding gene expression in oocytes, 10 µM of resveratrol during IVM decreased the expression of genes involved in competence of oocytes and subsequent embryo development (*NLRP2* and *BMP15* and *POU5F1,* ANOVA, $P<0.05$). Moreover, the expression of *BAX* was lower in oocytes treated with resveratrol compared to control$^-$group (ANOVA, $P<0.05$). Blastocysts produced with 0.5 µM f resveratrol showed a positive effect on the expression of genes related to lipid metabolism (*LIPE, CYP51, PNPLA2 and MTORC1)* compared to control groups (ANOVA, $P<0.05$) indicating that resveratrol could decrease lipid accumulation leading to a higher survival rate after vitrification. Further studies are needed to study the long-term effects of resveratrol supplementation on *in vitro* embryo production.

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A180E Folliculogenesis, oogenesis, and superovulation

# Effect of slow-release FSH on embryo recovery in dairy cows

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**Keywords**: bovine, superovulation, embryo flushing.

The objective was to study if a slow-release FSH superovulatory treatment (SLOW/2FSH) differs from the traditional 4-day FSH superovulatory treatment (TRAD/8FSH) in the numbers and grades of viable embryos and the numbers of degenerated embryos and unfertilized ova (UFO). Reduction of FSH treatments from eight to two diminishes restraining, discomfort and pain to the cows and labor and human errors in administration of treatments.Eight dairy cows (parity 1 to 4) were randomly designated into the TRAD/8FSH and SLOW/2FSH protocols in a cross-over study. First, the oestrous cycles of the cows were synchronized using 25 mg of prostaglandin (PG) (Dinolytic vet. 5 mg/ml, Zoetis Finland Oy, Finland) i.m., and 9 to 12 days after the synchronized oestrus either TRAD/8FSH or SLOW/2FSH treatment was initiated. TRAD/8FSH treatment consisted of eight declining i.m. doses (total 1000 IU) of FSH (Pluset vet, Laboratorios Calier, S.A., Spain) for four days at 6:00 h and 18:00 h. SLOW/2FSH treatment consisted of Pluset combined with hyaluronic acid (Hyonate®vet 10 mg/ml, Bayer Animal Health GmBH, Germany), 666 IU i.m. as 1st treatment at 6:00 h on the first treatment day and 334 IU i.m. as 2nd treatment 48 hours later. In the evenings of the 3rd and 4th day of each treatment, the cows were treated with 25 mg of PG and were inseminated after induced oestrus three times 12 hours apart, beginning 12 h after standing oestrus (= Day 0). Embryos were flushed non-surgically on Day 7. After flushing, CIDR devices (CIDR depot 1.38g, Zoetis Finland Oy, Finland) were inserted in the vaginae, removed after 12 days and a day before removal, 25 mg of PG was administered i.m. After this induced oestrus, the second cross-over run of the experiment was initiated 9 to 12 days later. The numbers of viable embryos, degenerated embryos and UFO were counted. All viable embryos were graded following IETS recommendations and cool-transported in straws to be analyzed in another study for their survival after 1, 3, 5 or 7 days in +4°C storage. Results are depicted as percentages of totals and averages ± SDs. Paired t-test was used to define difference between treatments. The number of viable embryos did not significantly vary between treatments (p=0.47). The TRAD/8FSH treatment yielded an average of 12.50±7.11 and SLOW/2FSH treatment an average of 10.13±4.67 viable embryos. In the TRAD/8FSH treatment, 82.0% (100/122) of the recovered structures were viable embryos, 6.5% (8/122) were degenerated embryos and 11.5% (14/122) were UFO. In the SLOW/2FSH treatment, 86.2% (81/94) were viable embryos, 8.5% (8/94) were degenerated embryos and 5.3% (5/94) were UFO. In the TRAD/8FSH and SLOW/2FSH treatments, 72.0% and 79.0% were grade I, 16.0% and 12.3% were grade II and 12.0% and 8.6% were grade III viable embryos, respectively. The results indicated no difference in the average number of viable embryos between treatments. However, slow-release FSH treatment yielded a higher percentage of viable embryos and less UFO than the traditional FSH treatment and therefore warrants further investigation. Olvi Foundation is acknowledged for funding the research.

A181E Folliculogenesis, oogenesis, and superovulation

# Effects of docosahexaenoic acid on bovine granulosa cells in vitro: involvement of FFAR4 receptor

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**Keywords**: n-3 PUFA, lipid, signalling pathways.

Previous studies suggest a beneficial effect of dietary fish oil supplementation (enriched in n-3 polyunsaturated fatty acid (PUFA) on reproductive variables in dairy cows. These PUFA influence female reproduction by acting at the uterine and ovarian levels (Leroy JL, et al. Reprod Domest Anim. 49:353-61; 2014). Recently, we showed that docosahexaenoic acid (DHA, the most active n-3 PUFA) was able to affect oocyte quality by increasing blastocyste rate after *in vitro* maturation and fecundation (Oseikria M, et al. Theriogenology. 85:1625-1634.e2; 2016), but no data is available on its potential effects on ovarian somatic follicular cells. Our objectives were to assess the effect of DHA on proliferation, steroidogenesis and signalling pathways in bovine granulosa cells (GC). The potential involvement of the receptor FFAR4 in the effects of DHA was investigated in bovine GC through FFAR4 expression and FFAR4 agonist (TUG-891) assessment in functional studies. Primary GC cultures were performed after dissection of ovarian small follicles (3-6 mm) collected from slaughterhouses. Recovered GC were cultured in serum-free McCoy's 5A medium with insulin (10 µg/L) in absence or presence of DHA (1, 10, 20 or 50 µM) or TUG-891 (1, 10 or 50 µM) for the appropriate times. Fatty acid composition of total lipids in GC after 24h DHA treatment was assessed by gaz chromatography. Cell proliferation after 24h and steroidogenesis after 48h were measured by tritiated thymidine incorporation in cells and by ELISA of secreted progesterone and estradiol in culture medium, respectively. Phosphorylation of MAPK14, AMPK, MAP1/3 and AKT signalling pathways were assessed by western Blotting in GC treated with DHA for 5 to 60 min. These parameters were statistically analysed using either Kruskal-Wallis test or non parametric permutational ANOVA. We showed that FFAR4 mRNA and protein were expressed in bovine GC. GC proliferation was stimulated after 10 and 50 µM DHA treatment and a similar increase was observed with TUG-891 at 1 and 50 µM. Progesterone secretion was enhanced after 20 and 50 µM DHA supplementation, whereas a slight decrease was observed with TUG-891 at 1µM. Estradiol secretion was increased after DHA 1, 10 and 20 µM treatment, whereas no effect of TUG-891 was reported. The DHA content in total lipids was increased in GC supplemented with 10 and 50 µM DHA for 24h compared to control GC. DHA had no effect on MAPK1/3, AKT and AMPK phosphorylation, whereas it stimulated transiently MAPK14 phosphorylation after 30 min DHA treatment at 10 µM and 50 µM similarly to TUG-891. In conclusion, this work showed that DHA is able to highly incorporate the GC total lipids after 24h supplementation. Moreover, both DHA and TUG-891 stimulated similarly GC proliferation and MAPK14 phosphorylation, whereas only DHA increased steroid secretion from GC, suggesting that DHA could influence female fertility by acting on GC partly through FFAR4 and MAPK14 pathway for GC proliferation and through other mechanisms on steroidogenesis.
Funding: INRA, Region Val-de-Loire (BOVOMEGA3).

A182E Folliculogenesis, oogenesis, and superovulation

# Polymers used to reduce a number of fsh-injections during superstimulation treatement for superovulation induction in cows

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**Keywords**: bovine, FSH, prolongators.

Over 50% of more than 1 million bovine embryos presently produced in the world are in vivo derived. Induction of superovulation has been one of the major methods for embryo production for the past 40 years. However, there were and still are many attempts to develop protocols allowing fewer injections and to find substances prolonging FSH effects during superovulation induction in cows. In our work we examined the ability of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) to affect the release of FSH after injection and to act as prolongators during hormonal treatment. To achieve this goal, two experimental groups of cows were treated with FSH-Super (LLC Agrobiomed, Russia) plus either PVA or PEG. Animals were assigned semi-randomly to two experimental groups. Each of the two working compositions was prepared directly before injection. To inject a single animal the necessary amount of polymer (either 0.9 g of PVA or 2.5 g PEG) was mixed with 1000 IU of FSH and dissolved in 7.5 ml of 0.9% NaCl solution. Animals of the first group (group I; n=98) were injected once with FSH plus PVA. Animals of the second group (group II; n=96) were injected once with FSH plus PEG. Subcutaneous injection of FSH plus either PEG or PVA was conducted once at the 10th day of the cycle, if there was a well-defined corpus luteum on one of the ovaries. The injection was located in the shoulder blade area. Injection of the PGF2a (0.5 mg) was conducted intramuscularly 48 hours post FSH injection. Artificial insemination was conducted 48 hours after PGF2a injection and was repeated two more times at 12 h intervals. On day 7 after the first insemination, corpora lutea were determined by trans-rectal palpation and embryos were collected by a standard non-surgical flushing procedure. Embryo quality was assessed according to IETS Manual. Portion of cows with a reaction to treatment was significantly higher ($P<0.05$) in the second group (86.4%) than in the first group (74.5%). Total number of ovulations and collected embryos were 1080 and 811 (14.2±8.2 and 10.7±8.1 per donor, respectively) in group I and 1181 and 922 (14.2±7.4 and 11.1±6.6 per donor, respectively) in group II. Relative portion of grade 1-2 embryos in each group differed significantly (64.2% in group I and 79.0% in group II; $P<0.001$). Total number of the collected embryos of grade 3 and lower was 114 in group I and 89 in group II. Their relative number was also significantly larger in group I compared to group II ($P<0.05$). There was also a significantly larger ($P<0.001$) portion of oocytes collected in group I (21.7%) than in group II (11.3%). The results of our study indicate that treatment of cows with a single injection of FSH plus PEG results in higher flushing outcomes after superstimulation compared to single treatment with FSH plus PVA. Thus PEG may be a more effective agent for optimization of the superovulation induction procedure using FSH in cows.

A202E Physiology of reproduction in male and semen technology

# Glycosaminoglycans isolated from follicular fluid reduce PKA activity during capacitation in porcine spermatozoa

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**Keywords**: Glycosaminoglycans, hyaluronic acid, porcine spermatozoa.

Glycosaminoglycans are linear polysaccharides comprised of repeating hexosamine-containing disaccharides that are found in the female genital tract and follicular fluid. Different studies showed that they may be involved in capacitation and acrosome reaction *in vivo* and their presence in sperm reservoir could maintain membrane stability and viability due to their interaction with sperm membrane (Tienthai, Journal of Reproduction and Development, 61, 245-250, 2015). The aim of this study was to investigate the effect of hyaluronic acid (HA) and glycosaminoglycans (GAGs) isolated from folicullar fluid (G-FF) and cumulus oophorus secretions obtained after oocyte porcine *in vitro* maturation (G-COS) on PKA activity and acrosome status after *in vitro* capacitation. Glycosaminoglycans were isolated from COS and FF (from ovaries in the periovulatory phase) by protease digestion, lipid extraction and by different precipitation conditions according to Bellin and Ax (J Dairy Sci 70:1913–1919. 1987). Spermatozoa from five fertile boars (N=5) were incubated for 3 h in TALP (at 38.5ºC and 5% $CO_2$) or PBS (38.5ºC in air) supplemented or not with G-FF, G-COS or HA (100 ug/mL or 500 ug/mL). PKA activity was assessed by Western Blot using anti-PKA antibody (9624, Cell Signaling Technology, Massachusetts, USA). The relative optical density (R.O.D.) was quantified with ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK). Viability was evaluated by propidium iodide 500 mg/mL (Sigma P 4170, Madrid, Spain) and carboxifluorescein 0.46 mg/mL (Sigma D6883, Madrid, Spain), while the acrosome status was analyzed by fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FICT). In each case, 200 spermatozoa were evaluated. Data were analyzed by one-way ANOVA followed by Dunnett´s multiple comparisons test ($P < 0.05$). PKA activity was significantly reduced after the incubation in TALP supplemented with G-FF compared to control ($1.74 \pm 0.39$ and $3.77 \pm 0.33$, respectively). No effect was observed after the incubation with HA (100 ug/mL or 500 ug/mL) and G-COS. Spermatozoa incubated in TALP showed the lowest percentage (50.83%±3.02) of viability and the highest percentage of acrosomal damage (3.83%±0.54) than spermatozoa incubated in PBS groups (75.66%±1.71 and 1.66±0.40, respectively) ($P<0.05$). However, the addition of HA or GAGs had not effect on this parameter. These results provide evidence that the supplementation of GAGs from FF might prevent sperm capacitation by reducing PKA activity. Nevertheless, GAGs were not able to maintain viability and to protect acrosomal damage of sperm.
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A203E Physiology of reproduction in male and semen technology

# Effect of Saffron extract on Boujaâd ram semen liquid storage

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**Keywords**: Boujaâd ram, semen, aqueous saffron extract.

The objective of this study was to evaluate the effect of aqueous saffron extract addition (ASE) (6%) to skim milk and tris on the Boujaâd rams (3-4 years) sperm liquid storage. To achieve this goal, this work was divided into two steps; step 1: Boujaâd ram semen was extended in skim milk based extender supplemented or not with 6% of ASE and stored at 15°C during 24h. While for the step 2: Boujaâd ram semen was extended in Tris egg yolk based extender supplemented or not with 6% of ASE and stored at 5°C during 24h. Ejaculates were collected once a week during 4 weeks for each step using an artificial vagina. Samples were extended to reach a final concentration of $0.8x10^9$ spermatozoa/ml. Then evaluated at different storage times (0 and 24h). A computer-assisted sperm motility analysis (ISAS, version 1.0.17) was used to determine total (TM), progressive motility (PM) and linearity (LIN). Nigrosine-eosin staining, hypo-osmotic swelling test (HOST), and Malondialdehyde (MDA) concentrations were used to determine viability, membrane integrity and lipid peroxidation. The statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. A factorial design ANOVA analyzed the data of extended semen quality parameters. The statistical model included the addition of saffron extract, and storage periods (0 and 24h). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SD. In the first step, it was observed that at 0h no significant difference was recorded between the two treatments (Control vs ASE addition) regarding all studied quality parameters: (PM, TM, LIN, viability, HOST and MDA: 79.2±1.66%, 94.25±1.51%, 63.1±3.45%, 95.97±2.89%, 89.41±1.84%, 1.6±0.08 TBARS, nmol/$0.810^9$ sperm) respectively. While, at 24h it was found that ASE significantly improved the PM (71.23±1.27 vs 74.64±1.25%), TM (91.93±1.2 vs 94.14±1.38%), LIN (61.65±1.83 vs 65±0.83%), viability (92.4±1.47 vs 94.6±0.97%) and HOST (77.9±1.88 vs 81.2±1.08%). As it decreased the MDA production (3.1±0.06 vs 2.6±0.05 TBARS, nmol/0.8 x $10^9$ sperm), compared to the control. In the second step; at 0h, the addition of 6% of ASE to the tris eggs yolk extender increased significantly the PM (68.6±3.45 vs 78.6±2.14%). While TM, LIN, viability and HOST, were not affected by this supplementation. At 24h, ASE significantly improved the PM (40.77±3.79 vs 64.67±3.97 %), LIN (28.5±2.93 vs 41.2±1.47 %), viability (89.7±4.55 vs 91.8±2.31%), HOST (65.9±2.93 vs 74.5±1.46%), and decreased MDA production (3.27±0.8 vs 1.81±0.6 TBARS nmol/0.8 x $10^9$ sperm) compared to control. While TM was not influenced by the treatments. In conclusion, the addition of the ASE (6%) improved the quality of Boujaâd ram sperm conserved, either at 5°C in Tris egg yolk or at 15°C in skimmed milk based extenders. A biochemical characterization of the aqueous extract and a confirmatory study using artificial insemination are necessary to complete this work.

A204E Physiology of reproduction in male and semen technology

# Measurements of ram sperm quality under anaerobic and aerobic liquid storage conditions

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**Keywords**: ram semen, liquid storage, aerobic.

The aim of the present study was to assess sperm quality of INRA180 ram, stored in skim milk extender (SME) at 5°C under aerobic and anaerobic conditions. Ejaculates were collected once a week during 9 weeks from four INRA180 rams, using an artificial vagina. The ejaculates containing spermatozoa with more than70 % total motility and concentrations higher than 3 x $10^9$ spermatozoa/ml were pooled. The pool was divided into two parts and each part was extended in SM under aerobic or anaerobic (in sterile syringes) conditions, to reach a final concentration of 0.8 x $10^9$ spermatozoa/ml. Samples were evaluated at different storage times; 0, 24, and 48 h. A CASA system was used to determine total motility (TM%) and progressive motility (PM%). Other tests such as nigrosine–eosin staining, Diff-Quick staining, hypo-osmotic swelling test (HOST), and Malondialdehyde (MDA) concentrations were used to determine viability(%), morphology (%), membrane integrity (%) and sperm lipid peroxidation. The statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. A factorial design ANOVA analyzed the data of extended semen quality parameters. The statistical model included the fixed effect of storage conduction (aerobic vs anaerobic), and storage periods (0, 24 and 48 h). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SE.The results of PM (71.72±1.34%), TM (90.78 ± 1.03%), viability (97.5±0.52%), abnormality (2.44±0.21%) and membrane integrity (94.5±0.84%), showed that, at 0h, there was no difference between the two storage conditions (anaerobic vs aerobic). However, lipid peroxidation was significantly higher in aerobic condition(0.59±0.03 TBARS, nmol/$10^8$ sperm) compared to the anaerobic one (0.47±0.03 TBARS, nmol/$10^8$ sperm). At 24 h, semen stored in the anaerobic condition shows the highest PM (64.22±1.17 vs 53.88±2.31%), membrane integrity (88.44± 0.68 vs 84.22±0.95%) and the lowest lipid peroxidation (1.32±0.01 vs 2.05±0.08 TBARS, nmol/$10^8$ sperm) compared to the aerobic storage ($p<0.05$). While the TM (80.72±1.22%), viability (91.14±0.91%) and abnormality (6.25±0.31%) were not affected by the storage condition ($P>0.05$). At 48 h, the best semen quality results were obtained in anaerobic condition. And that concerned; PM (50.66±1.75 vs 33.66±1.12%), viability (81±0.46 vs 76.22±1.31%), abnormality (10.5±0.32 vs 13.05±0.52%), membrane integrity (63±1.49 vs50.44±1.12%) and lipid peroxidation(1.35±0.03 vs 3.56±0.09 TBARS, nmol/$10^8$ sperm) compared to aerobic one. Whereas, for the TM (74.28 ±2.21%) is was not affected by the two treatments. In conclusion, INRA180 ram semen stored at 5 °C in a skim milk-based extender exhibited highest quality parameters under anaerobic exposure compared to aerobic exposure.

A205E Physiology of reproduction in male and semen technology

# Cyclin/Cdk complexes are involved in control of actin dynamics during boar sperm capacitation

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**Keywords**: sperm capacitation, Cyclin/Cdk complexes, Aminopurvalanol A.

Mammalian spermatozoa are virtually infertile immediately after ejaculation and will only reach their full fertilizing ability after they reside within the female genital tract for hours to days, depending on the species. This process of capacitation implies marked changes in the whole biochemical machinery expressed by spermatozoa. Thanks to the adoption of high-throughput technologies (Chronowska, *Biomed Res. Int.* vol 2014 (2014)) it was demonstrated that male gametes express proteins involved in cell cycle control, that are thought to be not present or active in sperm cells (Hydbring et al., *Nat. Rev. Mol. Cell Biol.* 17, 280–292, (2016)). To identify the cell cycle proteins potentially involved in sperm capacitation by a computational modelling approach and to assess their actual role in vitro capacitation and IVF by inhibiting these with a potent and specific inhibitor of the identified proteins, Aminopurvalanol A (AA). Cell cycle network was created and analysed using Cytoscape 3.3.0, by previously using the pathway database Reactome as data source. All the chemicals were purchased by Sigma Aldrich and were of the purest analytical grade. Semen samples were processed using a validated protocol (Barboni et al., *PLoS One* **6**, e23038 (2011)) and spermatozoa were incubated under capacitating conditions with or without AA at different concentrations (20, 10 and 2 μM) during 4 hours. Then, acrosome integrity (PSA staining), actin polymerization (Phalloidin staining), tubulin relocation (immunocytochemistry assays), membrane lipid remodelling (FRAP), and fertilizing ability (IVF) were evaluated in vitro. The network representing the molecules involved in cell cycle control was created by using an in silico approach (Bernabò et al., *OMICS* 19, 712–21, (2015); Bernabò et al., *BMC Syst. Biol.* **5**, 47, (2011a)). Among the whole proteins involved in cell cycle, it was possible to highlight the central role of Cyclins/Cdk in signal transduction during capacitation. With this information, in vitro experiments were performed to confirm the finding. By adding the Cyclins/Cdk inhibitor AA at different concentrations (20,10 and 2 μM) during capacitation it was possible to evince a dose-dependent inhibition of actin polymerization (phalloiding staining), with the consequent loss of acrosomes (PSA staining) and a decrease of in vitro fertilizing ability of spermatozoa (IVF), far-reached with the highest concentrations of AA ($p<0.05$). Otherwise, AA showed not to interfer with membrane lipid remodelling (FRAP analysis, DILC12 staining) or cytoskeleton tubulin dynamics (immnunocytochemistry assays). Cyclin/cdk complexes could be a new element in control system of actin polymerization during boar sperm capacitation. This data could revamp the knowledge on biochemistry of capacitation and could suggest new perspectives in studying male infertility. Marina Ramal Sanchez is granted by Marie Sklodowska-Curie ITN REP-BIOTECH 675526, European Joint Doctorate in Biology and Technology of the Reproductive Health.

A206E Physiology of reproduction in male and semen technology

# Variation of melatonin, testosterone and antioxidant enzymes in seminal plasma of three ram breeds under tropical conditions

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**Keywords**: Ram seasonality, rainy season, drought season.

In temperate regions, pineal melatonin and photoperiod regulates sheep seasonality (Chemineau *et al. Reprod Dom Anim* 43 (Suppl. 2), 40–47. 2008). In tropical conditions, with equal duration of day light, small ruminant reproduction is regulated by the annual cycle of rainfall and food availability more than photoperiodic changes (Morales *et al*. *Small Rum Res* 137, 9-19. 2016). Therefore, melatonin must be playing other functions, like the regulation of the antioxidant defense system (Mayo *et al. Cell Mol Life Sci.* 59, 1706–1713. 2002). Although melatonin, testosterone and antioxidant enzymes (AE) are present in the ram seminal plasma of seasonal breeds (Casao, *et al. Reprod Biol Endocrinol* 8, 59. 2010), there is no information on hormonal concentration and AE activity in the ram seminal plasma from tropical regions such as Colombia, located at equatorial level, with a bimodal regime of rain in the Andean region. Thus, the aim of this study was to evaluate the variation of melatonin, testosterone and AE in the seminal plasma form three sheep breeds (Colombian Creole, Romney Marsh and Hampshire) under tropical conditions. Semen from twelve rams (four rams from each breed) was collected weekly for one year by artificial vagina. Sires were housed at the National University of Colombia, located in Mosquera (4°40'57'' N. 74°12'50'' W) at 2510 m above the sea level. Seminal plasma was extracted by double centrifugation at 9000xg for 10 min at 4ºC, filtered through a 0,22 µm Millipore membrane (Merck, Darmstadt, Germany) and kept at -20 ºC until use. Melatonin and testosterone concentration were measured by a commercial competitive immunoassay (Direct saliva melatonin ELISA kit, Bühlmann Laboratories AG, Switzerland and Testosterone-ELISA, DiaSource InmunoAssays S.A., Belgium), following the manufacturer's instructions. The activity of catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRD) were analyzed as previously described (Casao *et al. Anim Reprod Sci* 138, 168–174. 2013). Results were grouped into four environmental seasons; two rainy (March-May, September-November) and two dry (December-February, June-August) and analyzed by two-way ANOVA followed by Bonferroni post-test (GraphPad Software, La Jolla, CA, USA). Melatonin concentration was lower ($P<0.05$) in the March-May rainy season (25.8±0.7 pg/mL) than in the other seasons (32.5±1.5 pg/mL for Sep-Nov, 35.2±1.7 pg/mL for Dec-Feb and 36.3±1.8 pg/mL for Jun-Aug). Testosterone showed higher concentrations ($P<0.05$) in Romney Marsh and Hampshire breeds than in Creole (3.4±0.1 and 3.5±0.2 *vs*. 2.3±0.3 ng/mL) during the June-August dry season. GPx activity were higher ($P<0.05$) between Sep-Nov (10.4±0.9 nmol/min.mL) and Jun-Aug (10.6.±1.3 nmol/min.mL) compared with Dec-Feb (5.2±0.9 nmol/min.mL) and Mar-May (5.4±1.1 nmol/min.mL). In conclusion, melatonin, testosterone and antioxidant enzymes are present in seminal plasma of rams under tropical conditions, and show seasonal or breed differences. Grants: Colombia 110157635854 and Col576-2012, Spain AGL2014-57863-R and AGL2013-43328-P.

A207E Physiology of reproduction in male and semen technology

# Effects of short exposure of bull semen to Roundup? on sperm kinetics and on *in vitro* embryo production

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**Keywords**: Roundup?, semen, embryo.

Common toxicants, such as herbicides, are considered as potential threats for fertility. The aim of the present study was to investigate the impact of a low concentration of the herbicide Roundup® (1ppm of Roundup®, -Monsanto-corresponding to 360ng/ml of the active ingredient, glyphosate), on sperm motility of frozen-thawed bull semen and on the subsequent *in vitro* bovine embryo production. In experiment 1, frozen semen samples, from the same bull and ejaculation, were allotted in two groups [treated (R) and controls (C), each n=3, in three replicates)] and separated by swim–up. In group R, the swim-up medium was modified with the addition of 1ppm of Roundup®. After 1 hour, all semen samples were centrifuged for 5 min at 10000r/min, the supernatant was discharged, 800 μl of new swim-up medium was used to reconstitute the semen pellet, and samples were evaluated for kinetics by CASA [(progressive, immotile, rapid, medium, slow moving spermatozoa, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), beat cross-frequency (BCF), amplitude of lateral head displacement (ALH) and wobble (WOB)]. In experiment 2, immature cumulus oocyte complexes (COCs n=494, 4 replicates) were obtained by aspirating small-medium size follicles (2-6mm) from ovaries of slaughtered cows. COCs were matured in TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml EGF at 39°C under an atmosphere of 5% $CO_2$ in air with maximum humidity. Matured oocytes were divided in two groups, (control (C) n=162 and treated (R) n=332) and were inseminated using frozen-thawed swim-up separated bull sperm (as in exp 1) at a concentration of 1 x $10^6$ spermatozoa/ml. Treated sperm was exposed to the herbicide only for 1 hour; this was during the swim-up process. Gametes were co-incubated at 39°C under an atmosphere of 5% $CO_2$ in air with maximum humidity. At approximately 20 h post insemination (hpi), presumptive zygotes were denuded, transferred to 25 ml culture droplets under mineral oil, and cultured in SOF supplemented with 5% (v/v) FCS at 39°C in 5% $CO_2$, 5% $O_2$ and max. humidity. Cleavage and blastocyst formation rate were evaluated at 48 hpi and on days 7, 8 and 9 respectively. Comparisons on semen kinetics were analyzed by t-test, while cleavage and blastocysts formation rates were carried out by $\chi^2$; in all cases significance was set at 0.05 level. Sperm evaluation by CASA revealed no difference in any of the parameters studied. Cleavage rate was similar between groups (C: 88.3%, R: 90,9%, p=0.5) while in group R blastocyst formation rates were steadily lower than that of group C (day 7, C: 24.7 ± 3.6% , R:14.5±4.8%; day 8 C:29.7±3.5%, R:18.1±5.8%; day 9 C: 32.8±3.5% , R:18.1±5.8%; in all cases $p<0.02$). These results imply that short exposure to roundup brings about alterations to sperm that are expressed during early embryo development. Further research is underway to evaluate sperm of DNA integrity and to assess embryo quality.

A208E Physiology of reproduction in male and semen technology

# Evaluation of exogenous DNA integration and fertilization ability of transfected boar spermatozoa

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**Keywords**: sperm, transfection, embryo.

The use of boar spermatozoa as vectors for introduction of exogenous DNA into the oocyte during fertilization could be an alternative and simple method for generation of transgenic pigs. So far, the results of using sperm as DNA vectors are very controversial. The aim of this study was to evaluate the integration of gene construct and the ability of fertilization by transfected boar spermatozoa. The boar sperm was transfected by direct incubation with the p12hGH-GFPBsd gene construct labeled with rhodamine. We investigated sperm of 92 ejaculates collected from 30 boars of which 16 ejaculates from 4 boars were selected for transfection. Sperm motility was evaluated after centrifugation and transfection. The presence of the gene construct in transfected boar spermatozoa was detected by fluorescence *in situ* hybridization (FISH) by assessment of a positive fluorescence signal for rhodamine. The control group consisted of non-transfected boar spermatozoa hybridized with labeled construct. Boar sperm after transfection was used for *in vitro* fertilization of pig oocytes. For this purpose, sperm motility was evaluated before and after capacitation. The control group consisted of capacitated, non- transfected sperm. Selected semen from 3 boars after transfection and capacitation, exhibiting the best motility parameters was used for *in vitro* fertilization (IVF) of oocytes. Presumptive zygotes were cultured in the NCSU-23 medium up to the blastocyst stage. The percentage of potential cleaved zygotes, morulae and blastocysts was evaluated and the presence of the gene construct in embryos was assessed by observing a positive fluorescence signal for rhodamine. After transfection a decrease of sperm motility was observed. The gene construct was detected in 7138 (47.1%) out of 15981 analyzed transfected spermatozoa. Two types of transgene fluorescence were observed: a single fluorescence signal specific to a given transgene, and a fluorescence signal indicating transgene-coated sperm. Among the control group, the signal was present in 2082 (15.3%) analysed cells, which should be regarded as a false-positive result obtained from the fluorochrome attached to the probe. After capacitation of transfected boar sperm, slight differences in seminal motility were observed in comparison to the control group. As a result of IVF with transfected sperm, out of 77 (59.68%) presumptive zygotes 19 (24.64%) and 9 (11.68%) developed to the morula and blastocyst stage, respectively. The development of the other presumptive zygotes have been stopped at the stage of 3-4 blastomers. The presence of the gene construct in evaluated embryos was not detected. Our results confirm the ability of boar transfected spermatozoa to bind exogenous DNA. However, in embryos obtained after in vitro fertilization with DNA transfected spermatozoa no gene expression was detected. Further studies are required to determine the transfection efficiency using ICSI technique.
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A209E Physiology of reproduction in male and semen technology

# Effect of foodborne contaminants on sperm fertilization competence and embryonic development

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**Keywords**: AFB1, ATZ, sperm.

There is growing concern about the effects of environmental toxins on human and domestic animal reproduction. Among the toxin sources, food products can potentially be contaminated with mycotoxins and/or pesticides. Aflatoxins are poisonous by-products of the soilborne fungus *Aspergillus*, found in food products such as maize, rice and wheat. Of these, aflatoxin B1 (AFB1) is the most toxic. Atrazine (ATZ) is an herbicide, that is extensively used to control weeds in broadleaf and grassy crops. ATZ is considered a ubiquitous environmental contaminant and is frequently detected in the ground, surface water and various types of crops. In the body, ATZ is metabolized to diaminochlorotriazine (DACT), which is further detected in the urine, serum and tissues. Exposure to ATZ and DACT impaired sperm viability, acrosome reaction and mitochondrial function (Komsky-Elbaz, Reprod Toxicol 67:15, 2017). Furthermore, exposure to AFB1, ATZ or DACT resulted in DNA fragmentation in sperm. However, these toxins' effects on fertilization have never been examined. Examine the effects of AFB1, ATZ and DACT on sperm fertilization competence and early embryonic development. Cumulus oocyte complexes (n=30–60/group; 6 replicates) were aspirated from bovine ovaries, in-vitro matured (22 h) and fertilized (18 h) with fresh semen. Before fertilization, sperm was capacitated in the absence (control) or presence of AFB1 (10 µM; Cayman Chemical, MI, USA), ATZ (0.1 or 1 µM) or DACT (1 or 10 µM; Chem Service Inc., PA, USA). Cleavage into 2- to 4-cell-stage embryos and blastocyst-formation rates were evaluated 42 h and 7 days post-fertilization, respectively, using JMP-7 software (SAS Institute Inc., 2004, Cary, NC, USA). Cleavage and blastocyst-formation rates, and distribution of blastocysts to various developmental stages were compared by chi-square test followed by Fisher's exact test. Data are presented as means. Exposing sperm to 10 µM AFB1 pre-fertilization reduced the proportion of embryos that cleaved to the 2- to 4-cell stage relative to controls (70.9 vs. 85.1%). Similar findings were noted when sperm was exposed to 1 µM ATZ (60.7 vs. 91.3%), 1 µM DACT (72.6 vs. 85.1%), or 10 µM DACT (64.6 vs. 85.1%) relative to controls, respectively ($P < 0.005$). Blastocyst-formation rate was significantly lower when oocytes were fertilized with sperm exposed to ATZ (0.1 or 1 µM; 7.7 and 8.7%, respectively) or DACT (1 or 10 µM; 20.5 and 16.6%, respectively) relative to controls (30.4%; $P < 0.04$). Blastocyst-formation rate did not differ from controls after fertilization with sperm exposed to AFB1. Distribution into different embryonic stages differed among groups, with significantly lower rates of development to the blastocyst stage for ATZ-treated sperm (0.1 or 1 µM; 6.6 and 8.7%, respectively) relative to controls (16.1%; $P < 0.05$). The findings reveal risk associated with exposure of sperm to foodborne contaminants. Even relatively low doses of AFB1, ATZ or DACT and short time exposure impair fertilization and blastocyst formation.

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# Heat stress effects on reactive oxygen species production and lipid peroxidation in bovine spermatozoa

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**Keywords**: oxidative stress, semen analysis, heat stress.

The aim of this study is to elucidate the effects of heat stress on oxidative status in bovine spermatozoa by quantifying reactive oxygen species (ROS) and lipid peroxidation (LPO). Heat stressed (HS) and non-heat stressed (NHS) frozen bovine semen samples were examined. HS semen, collected 14 to 42 days after artificial scrotal insulation, showed lower protamination and motility, and changes in the methylation of paternal pronuclei (Rahman et al., Theriogenology, 76, 1246–1257, 2011). Spermatozoa were passed through a discontinuous Percoll gradient (45/90% (v/v); VWR International) and adjusted to a final concentration of $2.5x10^6$ cells/ml in PBS. 100mM (final concentration) of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, USA) was added to the sperm samples to stain ROS and incubated at 37ºC for 15 minutes. Dead cells were stained with 1.5µM (final concentration) of propidium iodide (PI) and analyzed using a Cytoflex flow cytometer (Beckman Coulter, Brea, USA) (n=4). 10µM (final concentration) of BODIPY 581/591 C11 (Thermo Fisher, Waltham, USA) was added and incubated at 37ºC for 15 minutes before Cytoflex analysis (n=4). It emits red fluorescence in the non-oxidized state, shifting to orange and green after LPO. 5mM (final concentration) of Luminol sodium salt (Sigma-Aldrich, St. Louis, USA) was added to measure chemiluminescence for 15 and 30 minutes at 37ºC using a luminometer (n=3). PI, DCFH-DA and BODIPY signals were acquired screening a minimum of 5000 spermatozoa per sample. Data were analyzed using the Student's t-test ($p \leq 0.05$) and Spearman correlation. A significantly higher percentage of $PI^+$ dead cells was present in HS (30.7%) than in NHS semen (21.9%). No differences were observed in the percentage of DCFH-$DA^+$ cells between HS and NHS semen. However, a higher mean fluorescence intensity (MFI) was observed in HS compared to NHS semen. Significant differences were observed in BODIPY between red stained HS (83.6%) and NHS (91.3%) sperm cells, although no significant differences were observed in the percentage of green stained cells. This shift towards green fluorescence was higher in HS compared to NHS semen. Although no significantly higher ROS production in HS compared to NHS semen was observed after luminol quantification, there was a positive correlation between ROS production (luminol) and LPO (BODIPY green) ($r=0.82$, $p=0.01$).
The survival rate of sperm cells was higher in NHS than in HS semen, while a higher LPO and ROS production were observed in HS compared to NHS semen. Further evaluations are needed to better understand these effects of heat stress on the oxidative status of bovine spermatozoa.

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# Effect of antioxidants and thawing rates on the quality of cryopreserved camel sperm

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**Keywords**: camel sperm, cryopreservation, antioxidants.

The viscous seminal plasma of camel semen may prevent penetration of cryoprotectants into spermatozoa, making cryopreservation difficult. This study examined whether addition of antioxidants catalase (CAT), carnitine (CARN) and glutathione (GSH) pre-freezing could improve post-thaw semen quality, and/or increasing thawing temperature from 30°C (30 sec) to 60°C (10 sec). Two ejaculates were collected from each of four fertile dromedary males. Spermatozoa were separated from seminal plasma by single layer centrifugation (SLC) and sperm pellets were resuspended in freezing extender supplemented with individual antioxidants or control without antioxidants for freezing in liquid nitrogen. One straw from each treatment was thawed at 60°C for 10 sec and the other at 30°C for 30 sec; sperm quality was evaluated at 0, 1 and 2h post thawing. Parameters evaluated were total motility (TM), progressive motility (PM) and kinematics: ALH (lateral head displacement), BCF (beat cross frequency), LIN (linearity), STR (straightness), VAP (average path velocity), VCL (curvilinear velocity), VSL (straight line velocity) using computer-assisted semen analysis, membrane integrity and acrosome integrity (eosin-nigrosin), and membrane functionality (HOST-test). Normality of the samples was analysed by Saphiro-Wilk test. Normally distributed samples were analysed using ANOVA (Antioxidants) or t-Student (Thawing rates). Non-normally distributed data were analysed using Kruskal-Wallis (Antioxidants) or Mann-Whitney (Thawing rates). Values are means ± SEM. There were no differences among treatments at 0h for any parameters. A significantly higher TM was observed at 1h post thawing (P=0.009) for CAT (37%) and CARN (32%) compared to control (26%). After 2 h, TM was significantly higher (P=0.001) for CAT, CARN and GSH (27%, 25%, 23%, respectively) compared to control (15%). VAP 1h post thawing was increased for CAT (72 µm/s) and CARN (67 µm/s) compared to control (62 µm/s) (P=0.014). At 2h post thawing the following parameters were significantly higher for CAT, GSH and CARN: PM (9%, 11%, 9%, respectively; P=0.004), ALH (7 µm for each; P=0.015), VAP (65 µm/s, 66 µm/s, 62 µm/s; P=0.014), VCL (136 µm/s, 139 µm/s, 134 µm/s; P=0.019), VSL (43 µm/s, 46 µm/s, 42 µm/s; P=0.013) compared to control (4%, 6 µm, 54 µm/s, 117 µm/s, 36 µm/s, respectively).
Thawing at 60°C for 10 sec gave significantly higher TM at all time points (P<0.01), 40%, 35%, 26% at 0, 1 and 2 h compared to 37ºC for 30 sec (34%, 29%, 19%, respectively). Greater values were also observed at 60ºC for PM at 0 h (18%; P=0.002), 1h (P=0.007; 16%) and 2h (10%; P=0.013) compared to 37ºC (13%, 12%, 7% respectively). Thawing at 60º C increased the values of ALH (7.9 µm; p=0.033), STR (68%; P=0.034) and VCL (149 µm/s; P=0.004) at 1h compared to 37ºC (7.6 µm, 67% and 137 µm/s, respectively). No differences were observed in membrane or acrosome status for the different thawing rates.These results suggest that antioxidants exert a protective effect during cryopreservation of camel spermatozoa; it is better to thaw camel semen at 60°C for 10 sec compared to 37°C for 30 sec.

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# Mouflon *(Ovis musimon)* sperm cryosurvival is better at the end of the rutting season coinciding with low plasma testosterone concentrations

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**Keywords**: semen, cryopreservation, seasonality.

Cryosurvival of spermatozoa in Iberian ibex (*Capra pyrenaica*) is poorer at the onset and in the middle of the rutting season, when plasma testosterone levels are the highest, than at the end of the rutting season coinciding with fall of testosterones levels. We hypothesized that high plasma testosterone concentration might have a negative effect on sperm cryosurvival, and thus a similar situation may be found in other wild ruminants, such as the mouflon (*Ovis musimon*). Sperm samples were obtained from 22 mouflons, using the transrectal ultrasound-guided massage of the accessory sex glands technique. Samples were collected during autumn (October) when plasma testosterone concentrations are high, and at the end of the rutting season (January), when levels of testosterone tend to decrease to basal levels. Sperm motility was assessed with a computer-aided sperm analysis system. Membrane integrity and acrosomal status were evaluated by fluorescence and by the eosin-nigrosin technique. Morphological abnormalities and acrosome integrity were evaluated in samples fixed in buffered 2% glutaraldehyde. Each sample was cryopreserved following two different protocols. Ejaculates were diluted using a Tris-TES-glucose-based medium with 6% egg yolk and two different cryoprotectants: glycerol 5% for the traditional freezing protocol in straws in liquid nitrogen vapors (frozen sperm), and sucrose 100 mM for the ultrarapid freezing protocol in pellets (vitrified sperm). Plasma testosterone concentrations were measured by radioimmunoassay. Statistical analysis was performed by one-way ANOVA. Sperm quality of frozen-thawed and vitrified-warmed samples was higher in January, when levels of testosterone are decreasing, than in October. Plasma testosterone concentration was higher ($P<0.01$) in October (5.49 ± 1.33 ng/ml) than in January (1.02 ± 0.55 ng/ml). There were no differences in fresh sperm variables between samples collected in October and in January. Frozen-thawed sperm cryopreserved in January had a total sperm motility, curvilinear velocity (VCL), average path velocity (VAP), amplitude of lateral head displacement and beat-cross frequency greater than samples collected in October ($P<0.05$). Sperm viability was also higher in frozen-thawed samples collected in January than in October ($P<0.05$). Vitrified-warmed sperm had a VCL ($P<0.01$), VAP ($P<0.01$), straight-line velocity ($P<0.05$) and viability ($P<0.05$) higher in samples collected in January than in October. These results confirmed the hypothesis that the pick of plasma testosterone concentration that occurs in October, could affect negatively to mouflon sperm cryosurvival.

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# Melatonin has a protective role against cryocapacitation of ram spermatozoa

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**Keywords**: melatonin, sperm cryopreservation, apoptosis.

Melatonin is present in the ram seminal plasma and in the male reproductive tract. In vitro treatments with melatonin have shown a direct effect on ram spermatozoa by decreasing apoptosis-like changes, affecting capacitation, and increasing the IVF results. Therefore, this study raises the hypothesis that melatonin could have a protective effect against cold-shock in ram spermatozoa, avoiding changes related to cryocapacitation such as the increase in apoptosis indicators that sperm suffer at low temperatures. Briefly, two successive ejaculates were collected three times per week (from February until June) from nine healthy *Rasa Aragonesa* rams (2-6 years old) of proven fertility with the aid of an artificial vagina. Second ejaculates were pooled and processed together in order to eliminate individual differences. After sperm selection by a swim-up method, aliquots of 300 µl were incubated with 100 pM, 10 nM and 1 µM melatonin (Sigma-Aldrich Co., dissolved in PBS with 0.1 ‰ DMSO) for 30 min at room temperature. Then, samples with melatonin plus a control sample with 0.1 ‰ DMSO were directly cooled at 5 °C on ice-bath for 10 min followed by 5 min at 37 °C (cold-shock treatment). The following sperm functionality parameters were analyzed in the swim-up sample and in cooled samples (with and without melatonin): motility, using a CASA system (ISAS 1.04; Proiser SL, Valencia, Spain); membrane integrity (CFDA/PI stain); capacitation state related to intracellular calcium distribution by chlorotetracycline (CTC) staining, and tyrosine phosphorylation of membrane proteins by SDS-PAGE immunoblotting. The apoptotic markers assessed were phosphatidylserine (PS) translocation (Annexin V/PI stain); DNA damage by TUNEL (In situ cell death detection kit, ROCHE), and caspase activity (Vibrant® FAM™ Caspase-3 and -7 Assay, Invitrogen). Eight replicates were performed and data were compared by Chi-squared test. The obtained results indicate that cold-shock produced a significant ($P<0.001$) decrease in all the seminal quality parameters, except in DNA damage. The addition of 100 pM and 10 nM melatonin before cooling decreased significantly ($P<0.05$) the percentage of capacitated spermatozoa (51.71% ±2.55 and 49.86% ±2.83 respectively) comparing to the control sample (57.43% ±2.88). The lowest dose of melatonin (100 pM) reduced the PS translocation and caspase activation compared to the control sample without hormone. The addition of melatonin did not result in significant differences in motility, membrane integrity or tyrosine phosphorylation. It can be concluded that melatonin at low doses (100 pM) is able to prevent, at least partially, the cold-associated apoptosis and premature capacitation-related changes. Grants: CICYT AGL 2014-57863-R and DGA 2016-A26 FSE.

A214E Physiology of reproduction in male and semen technology

# Effect of seminal plasma on cytokine production from bovine endometrial epithelial cells in culture

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**Keywords**: bovine seminal plasma, uterine cell, cytokine response.

Seminal plasma (SP) is involved with immune-regulation in the female reproductive tract through specific cytokines. Variations in fertility among bulls could be due to SP. The objective was to investigate the type and level of cytokine response bovine endometrial epithelial cells (bEEC) in culture (passage 5) after challenge with SP. Donor bulls were categorized as below average (L) or above average fertility (H) according to an index based on the 56-day non-return rate from at least 1,000 artificial inseminations. Bulls of average fertility scored 100. The L-bulls had a score of≤ 92 (n=2) and H -bulls > 104 (n=3). Approximately 5 – 13 x $10^5$ bEECs per flask were challenged with 1% or 4% SP from L- or H-fertility bulls (L1, L4, H1, H4, respectively) or 1% or 4% PBS as control (C1, C4) in 13 replications with cells from 8 uteri. After 72h, the total number of cells, stained with trypan blue, was counted in a Burker hemocytometer. The supernatant was analysed for transforming growth factor beta (TGF-β1, TGF-β2 and TGF-β3) by Luminex (MILLIPLEX™ MAP, Merck Millipore, USA) and Interleukin 8 (IL-8) by ELISA (Bovine IL-8, MABTECH, Sweden). The concentration of each cytokine was calculated (pg/million cells). Data were analysed using the mixed model in SAS® (Proc Mixed, SAS® 9.3, USA). Fertility of bull, concentration of SP, and their interaction were fixed parts of the model, with cytokine response as variable parameter. Cow and cow interaction with replication were used as random factors. Post-hoc comparisons were adjusted for multiplicity using Tukey's, and the Contrast option was used to analyze individual differences. All values are presented as LSMEAN ± SEM. Challenge had significant effects on cytokine production (TGF- β1, TGF- β2 and IL-8) due to fertility of bull ($p<0.0001$), concentration of SP ($p<0.0001$) and the interaction between both factors ($p<0.0001$). There were no differences in TGF- β1, TGF- β2 and IL-8 production after challenge with L1 (4.8±3.2, 52.2±19.7, 18.0±5.1; x$10^3$ respectively) and H1 (7.1±3.0, 38.9±19.0, 18.4±4.7 x$10^3$ respectively) compared to C1 (3.5±3.0, 27.1±18.9, 17.5±4.7 x$10^3$ respectively) and C4 (3.2±3.1, 25.3±19.5, 17.1±4.9 x$10^3$ respectively). A higher production of TGF-β1, TGF-β2 and IL-8 ($p<0.0001$) resulted from challenge with L4 (20.6±3.1, 136.3±20.5, 54.8±4.9 x$10^3$ respectively) or H4 (18.6±3.2, 106.6±19.9, 44.6±5.1 x$10^3$, respectively); challenge with L4 SP was differed from H4 ($p<0.05$). For TGF-β3, fertility of bull ($p<0.05$), concentration of SP ($p>0.05$) and the interaction between factors were significant ($p<0.01$). The highest production of TGF-β3 was found in L4 (2.4±0.4 x$10^3$) than H4 (1.2±0.4 x$10^3$) ($p<0.05$), L1 (0.6±0.4 x$10^3$) ($p<0.01$), H1 (0.4±0.4 x$10^3$) ($p<0.01$), also C1 (0.5±0.4 x$10^3$) and C4 (0.2±0.4 x$10^3$) ($p<0.01$). In conclusion, higher concentrations of SP stimulated more cytokine production; 4% SP from L-bulls stimulated more TGF-β1, TGF-β2, TGF-β3 and IL-8 production than SP from H-bulls, which could be associated with impaired cell adhesion or cell damage.

A215E Physiology of reproduction in male and semen technology

# Comparison of the apoptotic like-changes in boar semen before and after in vitro capacitation

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**Keywords**: boar, apoptosis, spermatozoa.

Boar semen is very sensitive to all biotechnical modifications, including *in vitro* capacitation and *in vitro* fertilization. Moreover, the quality of boar semen may affect the efficiency of *in vitro* fertilization. However, the process of capacitation required by spermatozoa to acquire the fertilization ability is impaired in the IVF system. Presence of the apoptotic-like changes (ALC) in capacitated spermatozoa can lead to decreased fertility. The assessment of ALC can be useful for estimating sperm ability for fertilization. The purpose of this study was to determine the ALC in boar spermatozoa before and after *in vitro* capacitation. Semen was obtained from 11 boars of different breeds with normal fertility from the AI Center in Klecza Dolna. The sperm capacitation took place during incubation in a medium based on TCM-199 (Sigma, Germany) for 1 hour, at 39°C and 5% $CO_2$ in the air. For assessment of ALC, semen was incubated with the fluorophore YO-PRO-1 (Vybrant Apoptosis Assay Kit, Molecular Probes, USA) (Trzcinska et al, Anim Reprod Sci, 124, p 90-97, 2011) in the dark for 20 – 30 minutes, in room temperature before and after *in vitro* capacitation. After incubation, the semen was analysed under a fluorescent microscope. Statistical analysis was performed using the t-test. The mean percentage of viable spermatozoa before capacitation ranged from 65 to 91% (mean 78.4%; P>0.01), while after capacitation from 44 to 88% (mean 61.6%; P>0.01). The viable spermatozoa with ALC before capacitation oscillated between 0.5 and 9% (mean 4.4%) and after capacitation 0 and 21% (mean 5.9%). The percentage of nonviable spermatozoa before capacitation ranged from 4 to 32% (mean 16.5%; P>0.01), while after capacitation from 8 to 48% (mean 32.6%; P>0.01). When boar semen before *in vitro* capacitation was compared with semen after capacitation, an increase in the percentage of cells with ALC was detected accompanied by a significant decrease in nonviable spermatozoa. In conclusion in vitro capacitation of boar semen resulted in an increase in the percentage of apoptotic-like changes which may result in a decrease in fertility.
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A216E Physiology of reproduction in male and semen technology

# Progesterone-induced changes in the ram sperm acrosome reaction are inhibited by the antagonist mifepristone

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**Keywords**: mifepristone, acrosome reaction, ram spermatozoa.

Progesterone (P4) is an important hormone regulating the reproductive functions and may exert rapid non-genomic effects on sperm functionality. P4 may stimulate sperm hyperactivation, chemotaxis, in vitro capacitation and the acrosome reaction in several species. Most of these rapid actions could be mediated by P4 binding to membrane receptors on the sperm surfaces. The aim of this study was to investigate the effect of progesterone on ram sperm functionality and to elucidate whether these actions are mediated by its binding to sperm membrane receptors. For this purpose, sperm samples were incubated in capacitating conditions in the presence of P4 with or without mifepristone, a progesterone receptor antagonist. Briefly, two successive ejaculates were collected three times per week from nine males (2-6 years old) with the aid of an artificial vagina, and second ejaculates were pooled and processed together in order to eliminate individual differences. After sperm selection by a swim-up-dextran method, samples were incubated for 3 h at 39 °C, 5% $CO_2$ and 100% humidity in a high-cAMP medium (cocktail), already successfully demonstrated for capacitating ram spermatozoa. After pre-incubation with 4 or 40 µM mifepristone (dissolved in PBS with 0,1‰ DMSO), 1 µM P4 was added to samples. Two samples without P4 were included as controls, the cocktail-sample and another one incubated in TALP medium. Analyzed parameters were sperm motility (CASA), plasma membrane integrity (CFDA/PI stain), capacitation status by chlortetracycline (CTC) staining and tyrosine phosphorylation of membrane proteins (by western blotting), and phosphatidylserine (PS) translocation (Annexin V/PI stain) as an apoptotic marker. Data were compared by Chi-squared test. The results obtained showed that the presence of P4 in capacitating conditions led to a significantly higher percentage of acrosome-reacted spermatozoa compared with the sample without hormone (43.3±3.5% vs. 24.0±1.1). Pre-incubation with mifepristone significantly inhibited the increment in rate acrosome-reacted sperm rate (17.0±1.5 and 16.0±1.5 for 40 µM and 4 µM mifepristone, respectively) and also decreased the percentage of sperm with phosphatidylserine translocation (20.7±3.4, 24.7±2.9 vs. 35.7± 2.4 for 40 µM, 4 µM and without mifepristone, respectively). However, mifepristone did not affect the percentage of motile and with integral plasma membrane spermatozoa. In conclusion, this study showed that P4 induces the acrosome reaction and apoptotic changes in ram spermatozoa and that these actions are mediated by sperm progesterone receptors, given that mifepristone is able to decrease both the acrosome reaction and apoptotic markers.
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# Effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin j2 (PGJ2) on sperm motility and binding to *in vitro* cultured oviductal epithelial cells (OEC). A preliminary study in porcine

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**Keywords**: spermatozoa, boar, prostaglandin J2.

The effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) on its receptor, the sperm peroxisome proliferation-activated receptor gamma (PPARγ), has been linked to numerous biological activities, including boar sperm capacitation (Soriano-Úbeda, 2013, The 1st EPICONCEPT Annual Meeting, Antalya, Turkey). The aim of this work was to evaluate the effect of PGJ2 at 10 µM [Santoro, J Exp Biol, 216(6), 1085-1092, 2012] on sperm viability, motility and binding to *in vitro* cultured oviductal epithelial cells (OEC). Spermatozoa samples were obtained from 4 ejaculates of 2 to 3 years-old boars of proven fertile. OEC were obtained from oviducts of 6 to 7 month-old sows slaughtered at an abattoir and cultured in monolayer (López-Úbeda, Asian J Androl, 18, 1-18, 2016). For viability and motility analysis, spermatozoa were incubated in a capacitation medium (TALP) in presence (PGJ2) or absence (ø) of 10 µM of PGJ2 (D8440, Sigma-Aldrich®, Madrid, Spain). The viability was analyzed at 1 and 30 min of incubation using the eosin-nigrosin staining. The motility parameters were determined by computer-assisted sperm analysis (CASA) at 1, 5 and 30 min of incubation. The binding to OEC was evaluated after insemination with $1x10^5$ spermatozoa/ml stained with bisbenzimide and 30 min of co-culture in TALP in PGJ2 and ø groups. After co-culture, inseminated monolayers were fixed with paraformaldehyde and analysed under fluorescence microscopy at 10x determining the number of attached spermatozoa to OEC per square millimeter (spz/mm$^2$). Independent t-test ($p<0.05$) were performed and the results were expressed as mean ± SD for viability and motility parameters or SEM for binding to OEC. The viability of spermatozoa was not affected by the presence of PGJ2 both at 1 and 30 min of incubation. Several CASA parameters were influenced by PGJ2 presence throughout the incubation. At 1 min, PGJ2 produced a higher percentage of motile spermatozoa (%Mot: PGJ2: 93.9%; ø: 90.7%), progressive spermatozoa (%Prog: PGJ2: 46.6%; ø: 43.6%), curvilinear velocity (PGJ2: 146.3±89.6 µm/s; ø: 131.3±81.2 µm/s) and amplitude of lateral head displacement (ALH: PGJ2: 3.1±1.9 µm; ø: 2.8±1.6 µm). At 5 min, PGJ2 showed a higher linearity (LIN: PGJ2: 38.5±25.9%; ø: 32.7±24.0%) and wobble (WOB: PGJ2: 56.3±22.7%; ø: 53.8±21.8%), whereas at 30 min, none of the CASA parameters showed statistical differences between groups. When PGJ2 was added to OEC-spermatozoa co-culture, the number of bound spermatozoa was 27.5% lower than in ø (PGJ2: 85.2±3.4 spz/mm$^2$; ø: 117.5±3.6 spz/mm$^2$). Concluding, PGJ2 did not affect sperm viability, however it produced an immediate effect on motility, which probably increased the energy expenditure of the spermatozoa via activation of PPARγ, allowing a lower binding of spermatozoa to OEC. Supported by MINECO-FEDER AGL2012-40180-C03-01 and AGL2015-66341-R.

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# NOS/NO modulate the protein phosphorylation on serine and threonine residues during boar sperm capacitation

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**Keywords**: nitric oxide, sperm, capacitation.

Spermatozoa need to undergo a series of functional changes before they can fertilize, which constitute the process known as capacitation. This involves the early activation of protein kinases and the inactivation of protein phosphatases [Battistone MA, Mol Hum Reprod. 19(9):570-80; 2013]. It has been reported that NO can be generated by spermatozoa during capacitation and it can modulate this process through protein S-nitrosylation [Lefièvre L, Proteomics. 7(17):3066-84; 2007], activation of the cAMP/PKA pathway [Belén Herrero M, Free Radic Biol Med. 29(6):522-36; 2000], but also by increasing the cGMP concentration [Murad F, Recent Prog Horm Res. 49:239-48; 1994; Wiesner B, J Cell Biol. 142(2):473-84; 1998]. High levels of cGMP may inhibit cAMP degradation, which leads in turn to PKA activation [Kurtz A, Proc Natl Acad Sci U S A. 95(8):4743-7; 1998]. The aim of this study was to further investigate the NO's involvement in PKA activation during the *in vitro* capacitation of boar spermatozoa. For this purpose, ejaculated sperm were incubated for 1 hour in capacitating and non-capacitating conditions (TALP medium [Rath D, J Anim Sci. 77(12):3346–52; 1999] and PBS (D1408, Sigma-Aldrich, St. Louis, USA), respectively). The media were supplemented with 100 μM S-Nitrosoglutathione (N4148, Sigma-Aldrich, St. Louis, USA), a NO donor, and two NOS inhibitors: 10 mM $N^G$-Nitro-L-arginine Methyl Ester Hydrochloride (483125, Merck, USA) and 10 mM Aminoguanidine hemisulfate salt (A7009, Sigma-Aldrich, St. Louis, USA). The pattern of protein phosphorylation on Ser and Thr residues was evaluated by Western blotting. The antibodies used in this study were: rabbit monoclonal antibody anti-protein kinase A (9624, Cell Signaling Technology, Beverly, USA, 1:2000) and goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology, USA, 1:10000). The relative amount of signal in each membrane was quantified using the ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK). Our results indicated that when capacitated in the presence of NOS inhibitors, spermatozoa showed a lower Ser and Thr phosphorylation pattern than those capacitated with or without the NO donor. This effect was not observed under non-capacitating conditions. In conclusion, this study provides additional evidence that NOS/NO plays a role in regulating the phosphorylation of Ser and Thr residues during sperm capacitation in porcine. Supported by European Union, Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526 and MINECO-FEDER (AGL 2015-66341-R).

A253E Embryology, developmental biology, and physiology of reproduction

# Possibilities of preserving the local cow breeds in Latvia

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**Keywords**: cow endometrium, reproduction, embryo transfer.

Latvian Brown (LB) and Latvian Blue (LZ) are local Latvian breeds of the bovine gene-fund (GF) and therefore unique and characteristic only to Latvia. In 2016, only 152 LB and 313 LZ cows were registered as GF animals. So far, natural breeding and artificial insemination (AI) have been the methods of conserving GF in Latvia. Majority of the GF animals are at advanced age, they live on small farms without calculated feeding ration, and therefore may suffer from reproductive disorders. In 2017, through ERAF project Nr. 1.1.1.1/16/A/025 „Latvian Brown and Latvian Blue cow gene pool conservation using embryo transfer (ET) and related biotechnologies", ET will be restarted in Latvia after 35 years of interruption. The aim of this investigation was to consider possibilities of using ET in LB and LZ cows that could be classified as inferior embryo donors. The analysed data was obtained in Latvia in 1984-85. Thirty Holstein-Friesian cows of which 22 (73.3%) were 9-10 years old were removed from their herds due to reproductive problems (83%) or mastitis (17%) and used as embryo donors. Uterine biopsies were taken from 30 cows on Days 8-9 i.e.1-3 days before superovulatory treatment initiated. Results are presented as average ± standard error and Student's t-test was used to compare two independent samples. Histological investigation revealed different types of endometrial alterations in 91.7% of the cows. A different degree of endometrial oedema and hyperaemia were observed in 23 (76.7%) cows. Despite the fact that vascular fibrosis, hyalinosis concurrently with lymphoid-histiocytic infiltration in stratum compactum of the endometrium were established in 12 (40%) cows, superovulation was reached in 9 (75%) and the amount of corpora lutea (CL) was 6.3±1.42. Embryos of excellent or good quality (0.9±0.45) were obtained from 5 (41.7%) cows. Eighteen cows without strongly expressed endometrial pathologies had successful superovulatory response (CL 8.6±0.82) with 4.5±0.96 embryos of which 3.5±0.66 were viable. In cows with or without serious endometrial pathologies, the number of CL was equal ($P>0.05$), but the number of good quality embryos differed significantly ($P<0.05$). Also, in recipients receiving embryos from relatively healthy cows versus embryos from cows suffering from serious endometrial pathologies, the viability of transferred embryos significantly differed ($P<0.05$). In general, a successful superovulatory response occurred in 22/30 (73.3%) cows. No embryos were obtained from 9 (30.0%) cows. After 2 months, pregnancy was approved in 49% of recipient heifers. Lymphoid-histiocytic, plasmatic and mast cells infiltration in endometrial subepithelium and stroma as well as vascular hyalinosis and fibrosis had a significantly negative influence on embryo recovery. It is profitable to conduct a histological investigation of uterine tissue alongside with other analyses if a cow is considered as an inferior embryo donor due to its health and general condition.

A254E Embryology, developmental biology, and physiology of reproduction

# Artificial activation of ovine oocytes is required after ICSI with freeze-dried spermatozoa

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**Keywords**: freeze-drying, ICSI, spermatozoa.

Freeze-drying allows to store the biological samples in a dry state and represents an interesting alternative low-cost strategy of semen biobanking to save the endangered species. Here, we have established a dry sperm biobank from an endangered Italian sheep breed (Pagliarola) and tested its efficiency through ICSI. The motile spermatozoa from ram ejaculates collected with artificial vagina was selected by swim-up in TRIS-based medium (2.42g TRIS, 1.36g citric acid, 1.00g fructose, 100.000 U.I. penicillin G, 100mg streptomycin, in 67.20ml bidistilled water ($ddH_2O$); pH was adjusted to 6.7) for 20 minutes at 38.5°C. The motile spermatozoa were frozen in freeze-drying medium (10mM EGTA and 50mM NaCl in 10mM Tris–HCl buffer; pH was adjusted to 8.4) in a -80°C freezer for 75 minutes and subsequently lyophilized by the freeze-drying apparatus SP Scientific-VirTis, Freeze-dryer 2.0 BenchTop, 20 hours with a condenser temperature of -58°C and vacuum of 20 mTorr). The vials were sealed in glass vials under vacuum and stored in the dark at 4°C for 1-2 months. Just before the ICSI, the freeze-dried spermatozoa were rehydrated by adding 100µl $ddH_2O$. To evaluate the fertilizing capability of freeze-dried spermatozoa, 108 MII sheep oocytes were subjected to ICSI and allocated to two groups: 56 oocytes were activated by incubation with 5µM ionomycin (ICSI-FDS*a*); 52 were left un-activated (ICSI-FDS*na*). Forty-four oocytes injected with frozen spermatozoa (ICSI-FS) and left un-activated, served as control. Pronuclear formation (2PN) and blastocyst development were investigated at 14-16 hours and 7-8 days after ICSI, respectively. Differences were considered statistically significant for $p<0.05$ (*Chi-square* test). Data were analyzed using PRISM, software version 5.0; GraphPad. The freeze-dried spermatozoa were completely immotile after rehydration, however they maintained the capacity to fecund oocytes after ICSI. Two PN were found in 83.3% of ICSI-FDS*a,* 81.4% of ICSI-FS while only in 14.3% of ICSI-FDS*na* ($p<0.05$ ICSI-FDS*na* vs ICSI-FDS*a; *$p<0.01$ ICSI-FDS*na* vs ICSI-FS). Likewise, the ICSI freeze-dried spermatozoa yielded blastocysts only following artificial activation (ICSI-FDS*a*: 10.2%; ICSI-FS: 31%; ICSI-FDS*na*: 0%; $p<0.05$ ICSI-FDS*a* vs ICSI-FDS*na* and ICSI-FS; $p<0.0001$ ICSI-FDS*na* vs ICSI-FS). Our finding show that freeze-dried spermatozoa have lost the capacity to trigger oocyte activation but maintained their nuclear viability, whose developmental potential was fully released following artificial activation. Our results support the evidence that freeze-drying effective approach of spermatozoa storage to save endangered species. This work was supported by DRYNET H2020-MSCA-RISE-2016 and ERAOFART-H2020 -TWINN-2015.
The authors thank Dr. Luca Valbonetti, University of Teramo, Faculty of Veterinary Medicine, for the kind help in confocal microscopy analysis.

A255E Embryology, developmental biology, and physiology of reproduction

# AVEN and BCL-xL expression pattern and protein-protein interaction assessment through bovine early embryo development

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**Keywords**: oocyte, embryo, apoptosis.

Apoptosis in embryonic cells is important for embryo development; stabilizing cell numbers and playing a role in cell quality control. However, it is also associated with embryonic loss and cellular response to suboptimal developmental conditions and stress. AVEN, a novel P4-regulated protein, inhibits the mitochondrial apoptosis pathway by binding to and enhancing antiapoptotic BCL-xL activity. The objective of this study was evaluate the protein expression profile and protein-protein interaction of AVEN and BCL-xL during early embryo development in cattle. Briefly, cumulus oocyte complexes were recovered from abattoir derived ovaries and submitted to in vitro embryo production (IVP). Six replicate sets of samples were retrieved from IVP at 7 different developmental stages (Germinal vesicle (GV), Metaphase II (MII), zygote (16 h), 2-cell (44 h), 8-cell (72 h), compact morula (Day 5) and blastocyst (Day 7), -post insemination (pi)), and fixed in 4% paraformaldehyde. Samples were then processed for Proximity Ligation Assay (PLA) or whole-mount fluorescent immunocytochemistry: PLA detection of Aven-BCL-xL interaction was achieved using the DUOLink™ In Situ Red Starter Kit reagents according to the manufacturers (Sigma-Aldrich) instructions. Whole-mount immunofluorescence: Following washing and blocking, samples were incubated overnight with a single: monoclonal mouse (m) anti-AVEN (1:400, Abcam, ab77014), or polyclonal rabbit (r) anti-BCL-xL (1:400, Abcam, ab2568); dual: (mAven & rBCL-xL) and negative control: none, primary antibodies. Secondary antibodies Alexafluors- 594 goat anti-mouse and 488 goat anti rabbit were employed at a dilution of 1:400, for immunofluorescent labelling. Fluorescent labelling was observed under epifluorescent and confocal laser scanning -microscopy. The number of PLA Aven-BCL-xL interaction foci was counted per unit area of oocyte or embryo sample at each developmental stage (n= >14, per stage), using ImageJ software. Data was analysed using a One-Way ANOVA followed by post-hoc Tukey's test. There were significantly more foci in blastocysts compared to GV oocytes, 2-cell, 8-cell and morula -stage embryos ($P<0.05$, mean and SEM = 332,8 ± 56,04 vs. 158 ± 27,94, 162,7 ± 16,98, 160,1 ± 14,02 and 80,34 ± 13,67, respectively). The lowest number of interaction foci was detected in compact morulas (80,34 ± 13,67), this was significantly lower than MII oocytes (213 ± 22,12), zygotes (273,5 ± 41,68) and Blastocysts (332,8 ± 56,04). A distinct labelling pattern was observed during development; foci were evenly distributed in the cytoplasm of GV and MII oocytes, zygotes, 2-cell and 8-cell -embryos, but restricted mainly to peripheral cells in compact morulas and to the trophoblast cells of blastocysts. These results were corroborated by the co-localization pattern of AVEN and BCL-xL in the whole-mount samples. This is the first study to employ PLA for the analysis of protein to protein interaction in bovine oocytes and embryos and shows a very precisely regulated interaction of AVEN and BCL-xL during bovine embryo development.

A256E Embryology, developmental biology, and physiology of reproduction

# Caspases and TNFa activation in sperm storage tubules is correlated with hen's fertility

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**Keywords**: sperm storage tubes, Caspase, TNFa.

Blebbing is a basic event of apoptotic cell death mediated by caspase proteins. Used by cells to package cytoplasm portions, organelles or DNA, this biochemical cascade can also be used by secreting cells to release vesicles by Actin–Myosin II contraction (Mills et al., J. Cell Biol., 146(4):703–707, 1999). Blebbing does not occur in some cells lacking caspase 3 (Janicke et al., J. Biol Chem. 273:9357–9360, 1998; Zheng et al., Proc. Natl. Acad. Sci. USA. 95:13618–13623, 1998). Avian sperm storage tubules (SST) are apocrinal tubular glands located in the hen's utero-vaginal junction (UVJ) that store spermatozoa for long period (Fujii and Tamura, J. Fac. Fish. Anim. Husb. 5(1): 145-163, 1963). In this work, we investigated the correlation between capases activation in SST cells and hen's fertility. Hens from two divergent fertility lineages with high (DF+=21 days) and low (DF-=10 days) period of sperm storage (Beaumont C., J. An. Sci. 72:193-201, 1992.) were artificially inseminated (AI) (200 x $10^6$ sperm). Animals (3 hens per condition) were sacrificed without insemination (control), and at 24 hours, 1 wk, 2 wks and 3 wks after AI. Paraffined UVJ tissue was prepared for immunohistochemistry (IHC) against Tumor Necrosis Factor a (TNFa), Caspase-3 (CASP-3), and -8 (CASP-8). The same antibodies were used for Western blotting (WB) quantitative analysis. Statistical analysis was performed by Kruskal-Wallis test and Turkey post-test, the significance threshold was set at $p<0.001$. Protein quantitative analysis (WB) reveals that CASP-3 was 1.3-fold lower in control DF+ than in DF-. At 24 h after AI, CASP-3 was 1.7-fold higher in DF+ than in DF-. CASP-8 was 6.4-fold higher in control DF+ than in DF-, 5.3- and 10.4-fold lower after 1 wk and 2 wks of insemination, respectively, and 1.6-fold higher after 3 wks of insemination. TNFa was 3.4-fold lower in control DF+ than DF-, similarly to caspases, and 2.5- and 1.3- and 3.6- fold higher after 24 hours, 2 wks and 3 wks of insemination, respectively. TNFa, CASP-3, and -8 were observed (IHC) inside UVJ in all moments in both lineages. CASP-3 and CASP-8 were detected with high intensity in external UVJ epithelial surface, and in SST cells of control DF+ and DF- hens. After 24 hours of insemination, CASP-3 and CASP-8 were observed specifically in DF- SSTs and DPF+ SSTs, respectively. TNFa was strongly distributed in connective tissue in association with SST cells as well as in surface epithelia at all moments. These results suggest that caspase activation in SST cells can be correlated with hen's fertility. Rapid caspase activation (within 24 hours) correlated to TNF pathway after insemination could allow longer period of sperm storage.
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A257E Embryology, developmental biology, and physiology of reproduction

# Effect of non-esterified fatty acids during *in vitro* oocyte maturation on the development of bovine embryos after transfer

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**Keywords**: NEFA, oocyte, embryo transfer.

Metabolic disorders, as in negative energy balance (NEB) dairy cows, are associated with elevated non-esterified fatty acid (NEFA) concentrations, predominantly palmitic acid (PA), in the follicular fluid. These NEFAs are known to jeopardize oocyte *in vitro* maturation and elicit altered blastocyst quality and physiology. Lipotoxic conditions during final oocyte maturation also influence epigenetic reprogramming in the resultant day (D) 7 embryo and may thus affect subsequent development, potentially imprinting lasting marks during later stages of life. Therefore, we hypothesized that exposure of oocytes to high NEFA concentrations during IVM affects post-hatching development of D7 blastocysts after embryo transfer. Bovine oocytes were matured for 24h under 2 conditions: 1) physiological NEFA conditions (28µM stearic acid (SA), 21µM oleic acid (OA), 23µM PA (BAS) and 2) elevated PA concentration as present in follicular fluid during NEB (150µM) with physiological concentrations of SA (28µM) and OA (21µM) (HPA). Matured oocytes were routinely fertilized and cultured in SOF with serum until D7. Cleavage (D2) and blastocyst rate (D7) were compared among treatments using a binary logistic regression model. Eight blastocysts (normal and expanded, equally distributed per treatment and per replicate) were transferred per cow (n=8, 5 replicates). Four cows were attributed to HPA or BAS per replicate and were crossed over for the next replicate. Embryos were recovered at D14 and morphologically assessed (n=46). Glucose, lactate and pyruvate turn-over and interferon-tau (IFNT) secretion were measured in extra-embryonic tissue (EXT) after 24h culture (n=62). Morphological, metabolic and IFNT data were tested for normality with a Kolmogorov-Smirnov test and differences between treatment were analysed with a T-test. Data are presented as mean ± SEM. Developmental competence at D7 was not significantly different between treatments (blastocyst rate of 26 vs. 29.6% for HPA and BAS, resp.). Recovery rate at D14 was 30% and 36% for HPA and BAS, resp. ($P$>0.05). HPA during IVM significantly reduced embryo elongation (3.7±1.5 vs. 8.6±1.7mm, $P$=0.001) but did not affect diameter of embryonic disc compared to BAS. EXT from HPA group consumed similar amount of glucose but tended to produce less lactate compared to EXT from BAS group (1732±211 vs. 2428±355pmol/mm$^2$/h, $P$=0.073). IFNT secretion was significantly lower in HPA group (0.47±0.71pg/ml) compared to BAS group (3.79±1.16pg/ml, $P$=0.018). In conclusion, exposure to elevated PA during *in vitro* oocyte maturation affected post-hatching development at D14. Embryos were less elongated, were metabolically altered and produced less IFNT, a major signal of pregnancy recognition, than their physiological counterparts. This suggests that metabolic stress during oocyte maturation may have long-lasting effects on embryo development that may lead to higher pregnancy loss and reduced fertility in high yielding dairy cows. More research is ongoing to investigate underlying mechanisms through genome wide transcriptome pathway mapping.

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# Hypothermic storage (4?C) of ovine embryos with different medium and duration

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**Keywords**: ovine, embryo, hypothermic storage.

Generally, sheep embryos are transferred immediately after collection, however developing of a culture media for successful cooling and short-term storage would have enormous theoretical and practical implications in sheep embryo transfer programs (Romao et al., 2016). Differences in species' cryosensitivity of embryos are responsible for different approaches in their cryopreservation. It was reported that, one of the major concerns in embryo cryopreservation is its lipid content that can hamper the process (Dattena et al., 2000), as it happens in species such as sheep or pigs. Embryo lipid content effect on chilling sensitivity is not totally elucidated at the moment. In cattle, Ideta et al., (2013) introduced a medium that bovine embryos can be held for up to 7 days at 4°C and pregnancy rate of 75% was obtained for embryos held in this medium and transferred to primed recipients. The objective of this study was to determine if the commercial holding media (SYNGRO, S) and hypothermic medium (199 plus 50% FBS supplemented with 25 mM HEPES) could maintain viability of ovine embryos during extended hypothermic storage at 4°C in vitro as assessed by transfer to synchronized recipients. Donor ewes from prolific breeds (n=26) were superovulated to recover embryos on day 6 and embryos with grade 1 to 2, morula and blastocyst-stage embryos were sorted in holding media (SYNGRO, S) at room temperature (22°C). The embryos were then allocated at random to different treatment groups within 2 h of collection. T1 embryos (n=80) were washed three times in hypothermic medium (199 plus 50% FBS supplemented with 25 mM HEPES) and loaded into a plastic straw (1/4 cm3 clear straw; 4-6 embryos/straw). T2 embryos (n=20) were stored in commercial holding media (SYNGRO, S) in a plastic straw (1/4 cm3 clear straw; 4-6 embryos/straw). Then a water jacket was prepared by placing a number of 10mm goblets into a 65mm goblet filled with room temperature tap water and with wet cotton or gauze to keep the small goblets from floating/tipping. The holding container was placed in a 4°C fridge and kept adding straws of embryos for at least 24h. Then, 65mm goblet bath was be placed in a Styrofoam box with ice packs for air transportation. Upon the arrival, embryos were placed in a 4°C fridge for 168h in T1 group and 48h in T2 group. For transfer, the straws were kept at 4°C until needed and were them emptied into a dish of fresh holding media at room temp and then loaded into IVF catheters as they are transferred twin into the recipients (number of recipients for T1 embryos=40; number of recipients for T2 embryos=10). Following hypothermic storage for 7 d (T1) and 2 d (T2), embryos were transferred into recipients by laparoscopy to the uterine horn ipsilateral to a corpus luteum on day 6, and survival was determined on day 50 by ultrasound. There was no pregnancy established with either T1 or T2 group of embryos. It was concluded that these two techniques were not found successful for enabling liquid nitrogen-free storage and air transportation of embryos.

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# Bovine endometrial cells are responsive to embryonic sex *in vitro*

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**Keywords**: embryo, sex, endometrial response, *in vitro* culture.

Bovine endometrium recognizes embryonic sex at early stages with multiple embryos in vivo (Gómez et al, J Proteome Res 12:1199-210). In this work, we investigated the influence of embryonic sex on carbohydrate, protein and gene expression of endometrial cells co-cultured in vitro (EC) with a single embryo. Day-6 in vitro-produced morulae were individually cultured for 48h on the epithelial side of ECs. EC consisted of epithelial cells grown in inserts plated on stromal cell cultured in wells with TCM-199. Day-8 expanded blastocysts were collected and sexed by amelogenin gene amplification. Samples of EC and conditioned media were cultured with one male embryo (ME), one female embryo (FE) and no embryo (negative control; C). Samples were collected from 4 uterus as follows: uterus-1, 2 ME, 1 FE, 1 C; uterus-2, 2ME, 1 FE, 1 C; Uterus-3, 2 ME, 0 FE, 1 C; Uterus-4, 0 ME, 2 FE, 1 C. Expression of genes (N=13) coding for growth factors, receptors for hormones that regulate estrus cycle, receptors that bind embryonic signals, and metabolism, were analyzed in epithelial and stromal cells. Concentrations of glucose, fructose, lactate, artemin protein and total protein were determined in conditioned medium from the epithelial side. Data were analyzed using GLM and REGWQ Test and Principal Component Analysis (PCA). The relative mRNA abundances for candidate genes were compared using ANOVA y All Pairwise Multiple Comparison (Student-Newman-Keuls Method). Embryos altered transcription only in epithelial cells, not in stromal ones. Thus, expressions induced by ME were lower ($P<0.01$) than FE and controls (C) in hexose transporters solute carrier family 2 member 1 (*SLC2A1*: M=1.00±0.12, FE=6.05±0.07, C=7.19±0.10) and member 5 (*SLC2A5*: M=1.00±0.13, FE=5.58±0.08, C=7.17±0.11), connective tissue growth factor (*CTGF*: ME=1.00±0.22, FE=3.13±0.15, C=2.85±0.09), interferon alpha and beta receptors subunit (*IFNAR1*: ME=1.00±0.18, FE=2.46±0.29, C=2.86±0.15; *IFNAR2*: ME=1.00±0.17, FE=2.30±0.10, C=2.24±0.29). Male embryos elicited lower expression of artemin (*ARTN*) than FE (ME=1.00±0.26, FE=4.53±0.15, $P<0.05$) and controls (ME=1.00±0.26, C=3.74±0.30; $P<0.01$). Female embryos reduced ($P<0.01$) *SLC2A1* and *SLC2A5*, and increased ($P<0.05$) *ARTN* expression with respect to controls (*SLC2A1:* FE=6.05±0.07, C=7.19±0.10; *SLC2A5:* FE=5.58±0.08, C=7.17±0.11; *ARTN*: FE=4.53±0.15, C=3.74±0.30). ARTN protein and gene expressions strongly correlated ($R>0.90$; $P<0.05$) in the group of ME or FE, but not in controls. Embryonic sex did not alter hexoses or lactate concentrations in EC-conditioned medium. The concentrations of carbohydrates and expressions of genes that showed sexual dimorphism covariated significantly (|0.429971|; PCA). In contrast total protein mainly covariated with expressions of estrogen and progesterone receptors. Isolated male and female embryos may differentially release signaling factors that induce sexually dimorphic responses in endometrial cells. MINECO (AGL2016-78597-R) and FEDER; AM: SENESCYT-Ecuador-II Fase 2013. COST Action 16119, In vitro 3-D total cell guidance and fitness (Cellfit). Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013-2017 (GRUPIN 14-114).

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# Transcriptomic response of bovine oviduct epithelial cells to the early embryo

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**Keywords**: BOEC, transcriptome, embryo.

Previous data from our group and others indicate that the early bovine embryo may be more than just a passive structure during its journey through the oviduct, and may in fact elicit a transcriptomic response from the oviduct epithelial cells. However, such an effect is likely to be very local in nature, making it challenging to detect *in vivo*. In order to examine the possible embryo effect on BOEC transcriptome and whether it is local or not, we used an *in vitro* model involving co-culture of early embryos with a monolayer of bovine oviduct epithelial cells (BOEC). Oviducts corresponding to the early luteal phase were collected from the slaughterhouse and BOEC were mechanically harvested from the isthmus and cultured in 500 µl of Tissue Culture Medium-199 supplemented with 10% fetal calf serum (FCS) in four-well culture plates in a humidified atmosphere at 5% $CO_2$ in air at 38.5°C during 6 days until confluence. A day before co-culture the medium was replaced with synthetic oviduct fluid (SOF) supplemented with 10% FCS. Embryos (n=50) at the 2- to 4-cell stage (Experiment 1) or at the 8-cell stage (Experiment 2) were cultured on BOEC in a polyester mesh to maintain the position of the embryos on top of the cells. After 48 h of co-culture, the cells directly beneath the embryos and those in the same well but located away from the embryos (i.e., not in direct contact) were recovered as well as cells from a control well without embryos. BOEC were snap frozen from 5 replicates and they were analyzed by qPCR to assess the expression of 12 candidate genes. These included oviduct genes previously shown to be affected by the presence of an embryo *in vivo* (Maillo et al. Biol Reprod. 2015. 92: 144) and *in vitro* (Schmaltz-Panneau *et al.* Anim Reprod Sci. 2014. 149(3-4):103-106). Statistical differences were assessed by ANOVA. Regardless of being in direct contact or not with 2- to 4-cell embryos, BOEC displayed a decreased abundance of *ARG3;* a gene implicated in the regulation of intracellular calcium and cytoskeleton organization; compared to control cells ($P<0.05$). Co-culture with 8-cell embryos also lead to an increased abundance of *ARG3* besides to others genes involved in BMP signaling pathway (*SMAD6*, *TDGF1*) and a decreased abundance of oxidative stress gene (*GPX4*) in BOEC whatever they have been in direct or indirect contact with embryos. While *SOCS3* a gene related to the inflammatory response was decreased in cells in direct contact with the embryos compared to both other groups ($P<0.05$). In conclusion, under our experimental conditions, the transcriptomic response of BOEC is embryo–stage dependent. For one gene, *SOCS3*, expression was only altered in BOEC in direct contact with the embryo. This may be due to a non-diffusible embryo-secreted factor.
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# Effect of nutritional level on the onset of puberty in the Sardi ewe lamb: relationships with FSH, GH and Leptin

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**Keywords**: ewe lamb, nutrition, puberty.

The aim of this study was to investigate the possibility of a compensatory growth following starving and re-feeding of ewe lambs, their puberty onset and the related endocrine events in Sardi sheep.Thirty lambs born in autumn were assigned to 3 groups (10 animals per group): HH fed a high-level diet, LL a low-level diet and LH a low-level diet during 2 months (period 1) followed with a high level diet during 4 months (period 2). The low-level diet consisted of straw (500g) and concentrate (200g) while in high-level diet the concentrate (1kg per animal per day) and straw were fed ad libitum. The assay started on July the 1st when the lambs were 7 months old and an average weight of 22 kg, and finished on December the 30th.The lambs were weighed at birth, at weaning and at 2 week intervals thereafter until the end of the experiment. Blood samples were taken three times per week for measuring progesterone, FSH, GH and leptin concentration. Plasma P4 level > 1 ng/ml is a reliable indicator for an active corpus luteum and is related to cyclic ovarian activity.The main results showed a significant difference between growth rates of lambs on low or high diet but the average daily gain remained low 87 ± 9 g/d. The mean live weight at the end of experiment of HH lambs was significantly higher compared to LL and LH lambs (37.4±0.5 vs 24.8±2.7 vs 33.3± 3.3 kg respectively, $p< 0.05$) with HH lambs being 12kg heavier than those of LL group. Similarly, LH lambs were significantly heavier than LL lambs (33.3± 3.3 vs 24.8±2.7 kg, $p<0.05$). There was a partial compensatory growth in fasted-reefed lambs.The onset of puberty was mainly related to the live-weight in all ewe lambs. Thus, the average live weight at puberty was 31.4 ±0.7 kg and 29.8 ± 1.2 kg in HH and LH groups, respectively. In the LL group, however, no ewe lamb reached the puberty at 400 days, but the average LW was then only 25 kg. Before first ovulation, an increase in FSH plasma level was observed (2.5±1.2 vs 0.20 ng/ml) in HH group and (3.4±2.2 vs 0.23 ng/ml) in LH group.The average plasma GH concentrations in the HH group seemed to be lower (20.7±1.03 ng/ml) than in the other groups (25.5±2.6 vs 33.4±3.6 ng/ml, respectively, in the LH and LL)./>No significant relationship was found between the age at puberty (320 ±18 vs 372 ± 9 days) and the plasma leptin levels (1.8±1.1 vs1.9±1.3 ng/ml ) in the HH and LH groups, respectively. These results confirm the importance of body development on the appearance of puberty, and raise the possibility of a compensatory growth in the lambs. Elsewhere, FSH concentrations were not significantly altered by feed restriction. The observed absence of relationship between the age at puberty and the plasma leptin levels may reflect the presence of an other metabolite, rather than leptin, to signal the nutritional status to the reproductive axis in lamb.

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# Elevated non-esterified fatty acid concentrations during bovine oviduct epithelial cell and zygote coculture hamper early embryo development

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**Keywords**: NEFAs, BOEC/zygote coculture, polarized cell culture system.

Maternal lipolytic disorders and the associated systemic rise of non-esterified fatty acids (NEFAs) have been suggested to affect oviduct physiology and functionality. An altered oviduct micro-environment may influence early embryo development, however its consequences remain largely unknown. Therefore, we hypothesize that elevated NEFAs in a polarized cell culture system hamper early embryo development. Furthermore, we state that effects will depend on the presence of bovine oviduct epithelial cells (BOECs) and the direction of NEFA exposure. In 4 repeats, early luteal BOECs were seeded at $1x10^6$ cells/mL in a polarized cell culture system. After reaching 100% confluency (day 7) monolayers were cocultured with 25 zygotes per insert in 100µL SOF with 10% FBS and 0.75% BSA for 96h. Hereto, bovine oocytes were matured and fertilized *in vitro* following standard procedures. During subsequent BOEC/zygote coculture in SOF, NEFA exposure (720µM containing 210µM oleic acid + 230µM palmitic acid + 280µM stearic acid) was implemented in 3 groups: 1) [APICAL NEFA] i.e. 720µM NEFA + 0.45% EtOH in the apical compartment, 2) [BASAL NEFA] i.e. 720µM NEFA + 0.45%EtOH in the basal compartment, 3) [A/B NEFA+] i.e. 720µM NEFA + 0.45%EtOH in both compartments. Treatments were compared to [SOLVENT+] i.e. 0.45% EtOH in both compartments with BOEC coculture, [A/B NEFA-] i.e. 720µM NEFA + 0.45%EtOH in both compartments but without BOEC coculture, and [SOLVENT-] i.e. 0.45%EtOH in both compartment without BOEC coculture. After 96h, all morulae were transferred to SOFmedium in a 96-well plate without BOEC. Embryo development was assessed using cleavage- (48h pi), morula- (120-126h pi), and blastocyst rates (192h pi). Data were analysed using binary logistic regression with Bonferroni correction in SPSS, and were considered statistically different when $P<0.05$. Total cleavage in A/B NEFA+ (51.63%) and A/B NEFA- (43.19%) differed significantly ($P=0.02$), and were lower compared to other treatments. From the cleaved oocytes APICAL NEFA showed an increased percentage of zygotes in 3-cell stage (17.61%; $P=0.032$). Morula rates were on average 28.05% out of total oocytes and 47% out of cleaved oocytes, and similar between all treatments ($P>0.05$). Blastocyst rates (out of total oocytes) were significantly higher in SOLVENT+ and SOLVENT- (26.11% and 22.67% resp) compared to NEFA treatments (12.59%; $P<0.001$). In all treatments, day 8 blastocysts were mostly in expanded stage (55.06%), except for APICAL NEFA which showed 48.14% young blastocysts. In conclusion, NEFAs negatively affect embryo developmental competence. During cleavage, but not at blastocyst level, these effects are limited to bidirectionally exposed groups, and the cocultivation with BOECs seemed to have beneficial effects. Data suggest that elevated NEFAs in the oviduct may attribute to the complex pathogenesis of sub- and infertility during lipolytic disorders, however, more research is required to further elaborate on potential compensatory effects mediated by the oviduct.

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# Functional activity of actin cytoskeleton in porcine oocytes during *in vitro* maturation

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**Keywords**: oocyte, cytoskeleton, IVM.

Actin is an abundant protein with well established roles in fundamental processes ranging from cell migration to membrane transport (Sun Q. &Schatten H. Reprod. 131, 193–205, 2006). Brilliant cresyl blue (BCB) staining has been used for selection of the functional status of oocytes. $BCB^+$ oocytes (oocytes that have finished growth phase in vivo) had significantly higher development competence than $BCB^-$ oocytes (oocytes that have not finished growth phase in vivo, Ishizaki C. et al., Theriogenology, 72(1): 72–80, 2009). The aim of the present study was to compare the functional activity of the actin cytoskeleton [the intensity of fluorescence of rhodamine-phalloidin (IFRF) conjugated with actin filaments] in dynamics of meiosis of $BCB^-$ and $BCB^+$ porcine oocytes. Before IVM compact cumulus oocyte complexes (COCs) were incubated in BCB solution(13 μM) for 60 min. at 38.5°C in 5% $CO_2$. Then oocytes were divided into $BCB^-$ (colorless cytoplasm) and $BCB^+$ (colored) oocytes. COCs were cultured in maturation medium (NCSU 23) supplemented with 10% follicle fluid (FF), 0.1 mg/ml cysteine,10 IU/ml eCG and 10 IU/ml hCG at 38.5°C in a humidified atmosphere containing 5% $CO_2$. FF was collected from follicles with 3 - 6 mm in diameter. COCs cultured in maturation medium with pieces of follicle's wall (600-900 μm in length, Abeydeera L, et al., Biol Reprod. 58:213-218.1998). After 22 h of culture COCs and pieces of wall were washed and transferred into the same maturation medium but without hormonal supplements for next 22 h of culture. For assessment of chromatin and IFRF fixed oocytes were incubated sequentially in rhodamine-phalloidin ( RF, R415 Invitrogen, Moscow, Russia), 1 IU/ml, for 30 min to label actin. Then oocytes were incubated in 4',6-diamidino-2-phenylindole, 10 |μg/ml, for 10 min to label chromatin. Oocytes were examined using confocal laser scanning system Leica TCS SP5 with inverted fluorescent microscope. Diode 405 nm, argon 488 nm and helium-neon 543 nm laser lines were used for fluorochrome excitation. IFRF were expressed in arbitrary units. All chemicals used in this study, except for RF, were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test**.** Chromatin status and IFRF of 159 $BCB^+$ and 101 $BCB^-$ oocytes (total 260 oocytes, in 3 replicates, 10-26 oocytes/group) were evaluated before and after 22 and 44 h of cultivation. Significant differences in IFRF of $BCB^+$ (33.2 ± 2.2) and $BCB^-$ oocytes (42.6 ± 2.1) were identified before cultivation ($P < 0.05$). There were no differences between the IFRF in $BCB^+$ and $BCB^-$ oocytes on metaphase-I stage(49.1 ± 6.9 and 51.2 ± 4.8, respectively). The decrease of IFRF in BCB+ oocytes was found after 44 h of IVM (49.1 ± 6.9 and 35.8 ± 6.3 ,respectively, $P < 0.01$ ). There were no differences between the IFRF in $BCB^-$ oocytes on metaphase-I (51.2 ± 4.8) and methaphase II (49.1 ± 6.9 ) stages. Overall our data clearly showed that actin cytoskeleton actively involves in maturation of porcine oocytes in vitro. Features of the functional activity of actin cytoskeleton in $BCB^+$ and $BCB^-$ oocytes during IVM have been identified.

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# Modifying the fastness to age-related alterations of *in vitro* maturing bovine oocytes by luteotrophic factors and granulosa cells

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**Keywords**: progesterone, prolactin, oocyte aging.

The quality of mammalian oocytes determines their ability to embryonic development. Once the oocyte matures to the metaphase-II (M-II) stage, it undergoes accelerated senescence processes leading to an impairment of its quality. The objective of this work was to study effects of prolactin (PRL), progesterone (P4), and granulosa cells during the completion of in vitro maturation of bovine oocytes on their subsequent resistance to age-related functional alterations. Bovine cumulus-enclosed oocytes (CEOs) were cultured for 12 or 24 h in the first IVM medium (TCM 199 supplemented with 10% fetal calf serum (FCS), 10 μg/ml FSH, and 10 μg/ml LH) at 38.5°C and 5% $CO_2$. After the 12 h-culture, the CEOs were transferred to the second IVM medium (TCM 199 supplemented with 10% FCS) and matured for next 12 h in the absence and in the presence of granulosa cells (GCs) preliminary cultured under the same conditions for 12 h. The following additives to the second IVM medium were applied: (1) no additives (Control), (2) 25 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia), (3) 50 ng/ml PRL, and (4) 50 ng/ml P4. Then CEOs were cultured for additional 24 h in the aging medium (TCM 199 containing 10% FCS). At the end of culture, the state of the oocyte nuclear material was evaluated by the Tarkowski's method. Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA). The data for the nuclear status (n=4, 80-91 oocytes per treatment) and apoptosis (n=5-6, 91-121 oocytes per treatment) were analyzed by ANOVA. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). During 24 h aging of CEOs matured in the first IVM medium without transfer, the rates of M-II oocytes with destructive changes of chromosomes (decondensation, adherence, clumping) and apoptotic oocytes rose ($P < 0.001$) from 28.6 ± 2.1% and 10.1 ± 1.4% (prior to aging) to 67.1 ± 2.0% and 24.3 ± 0.4%, respectively. Similar age-related increases in these rates were observed in case of CEOs matured during two-step culture. In the absence of GCs, P4 decreased the frequency of chromosome abnormalities in aged oocytes from 68.5 ± 1.9 (Control) to 51.2 ± 2.9% ($P < 0.01$), whereas PRL did not. Maturation of CEOs in the presence of GCs and PRL resulted in a reduction (at least $P < 0.01$) in the rate of oocytes with abnormal chromosome modifications following aging (from 67.9 ± 2.3% (Control) to 50.6 ± 3.9% (25 ng/ml of PRL) and 46.5 ± 5.0% (50 ng/ml of PRL)). By contrast, the addition of GCs to the second IVM medium abolished the positive effect of P4 ($P < 0.001$). Furthermore, P4 caused a decline ($P < 0.05$) in the rate of aged CEOs with apoptotic signs matured in the absence of GCs (from 24.3 ± 0.4% (Control) to 17.6 ± 1.6%). Thus, during the completion of bovine oocyte maturation, PRL and P4 can raise the subsequent resistance of aging ova to age-related changes in their quality, with GCs being able to modulate the hormonal effects. The study was supported by the Russian Science Foundation (project 16-16-10069).

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# Effect of different estrous sheep serum batches on sperm capacitation and *in vitro* embryo development

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**Keywords**: sperm capacitation, *in vitro* fertilization, estrous sheep serum.

Nowadays, the estrous sheep serum (ESS) is the additive used to capacitate ram spermatozoa in *in vitro* fertilization systems. This non-defined substance is present during the co-incubation of spermatozoa and oocytes. The main problem of using non-defined substances is the need to test their validity and the effect of these substances on future embryo (García-Álvarez et al., Theriogenology 84:948-956 2015). The aim of this work was to assess the effect to capacitate thawed ram spermatozoa with different batches of estrous sheep serum on tyrosine phosphorylation, key event in capacitation, and on embryo yield. A pool of thawed semen from three rams was used to carry out the analysis of tyrosine phosphorylation by western blot and an *in vitro* fertilization trial. After discontinuous density gradient on Percoll, spermatozoa were incubated for 15 min in synthetic oviductal fluid (SOF) with 10% of three ESS batches (ESS1, ESS2 and ESS3). A negative control (SOF without ESS) was also used (NCap). The intensity signal of different bands was analysed with C-Digit® Blot Scanner from LI-COR and relativized to Tubulin. For the *in vitro* fertilization 395 oocytes were used and was performed according to García-Álvarez et al. (García-Álvarez et al., Theriogenology 84:948-956 2015). General lineal models were used with the following dependent variables: signal intensity, cleavage rate at 48 h post insemination (cleavage rate) and percentage of blastocyst at 9 days (embryo rate), and fixed variables: treatment (NCap, ESS1, ESS2 and ESS3) and replicate. There were no differences in the band intensity between treatment. However, cleavage and embryo rates were different for NCap and ESS1, ESS2 and ESS3 (0±9, 41±5, 46±6, 56±5, and 0±7, 27±5, 29±5, 31±5, respectively) but were similar between the ESS batches. In conclusion, the ESS batches do not influence sperm capacitation, cleavage and embryo rates although the presence of ESS is necessary to fertilize oocytes. Nevertheless, more studies of the quality of these embryos are necessary.

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# Improvement of *in vitro* produced bovine embryo quality using Charcoal:Dextran Stripped Fetal Bovine Serum on culture media

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**Keywords**: CDS FBS, HI FBS, bovine embryo.

This study investigated the effect of Charcoal:Dextran Stripped fetal bovine serum (CDS FBS) and heat-inactivated FBS (HI FBS) in embryo culture medium (SOF-BE1 medium supplemented with 10% of serum) on their ability to support *in vitro* development of bovine embryos. Charcoal:Dextran treatment of FBS removes lipophilic chemicals, certain steroid hormones and certain growth factors. The developmental ability and quality of bovine embryos were determined by assessing their cell number, lipid content, mitochondrial activity, gene expression, and cryo-tolerance. The differences in embryo development (350 oocytes per each group were cultured in six replicates), integrated optical intensity, and expression levels of the various genes between experimental groups were analyzed by one-way ANOVA. The percentages of embryos that underwent cleavage and formed a blastocyst were significantly ($P < 0.05$) higher in medium containing CDS FBS than in medium containing HI FBS (42.84 ± 0.78% vs. 36.85 ± 0.89%, respectively). The total number of cells per day 8 blastocyst was ($P > 0.05$) higher in the CDS FBS group (208.40 ± 14.77) than in the HI FBS group (195.11 ± 19.15), however, this difference was non-significant. Furthermore, the beneficial effects of CDS FBS on embryos were associated with a significantly increased mitochondrial activity, as identified by MitoTracker Green, and reduced intracellular lipid content, as identified by Nile red staining, which increased their cryo-tolerance. The post-thaw survival rate of blastocysts was significantly ($P < 0.05$) higher in the CDS FBS than in the HI FBS group (85.33 ± 4.84% vs. 68.67 ± 1.20%). Quantitative real-time PCR showed that the mRNA levels of acyl-CoA synthetase long-chain family member 3, acyl-coenzyme A dehydrogenase long-chain, hydroxymethylglutaryl-CoA reductase, and insulin-like growth factor 2 receptor were significantly increased upon culture with CDS FBS. Moreover, the mRNA levels of sirtuin 1, superoxide dismutase 2, and anti-apoptotic associated gene B-cell lymphoma 2 in frozen-thawed blastocysts were significantly ($P < 0.05$) higher in the CDS FBS group than in the HI FBS group, however, the mRNA level of the pro-apoptotic gene BCL2-associated X protein was significantly reduced. Taken together, these data suggest that supplementation of medium with CDS FBS improves in vitro bovine embryo developmental competence and cryo-tolerance. This work was partly supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ01107703) and IPET (Grant no. 315017-5, 117029-3).

A267E Embryology, developmental biology, and physiology of reproduction

# Novel approach for the measuring mitochondrial function in bovine oocytes and embryos

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**Keywords**: oxygen consumption, oocytes, metabolism.

Oxygen consumption is an established marker for cellular energy metabolism and an indicator of mitochondrial function. In reproductive biology, it has been correlated to a range of outcomes including oocyte viability and maturation, embryo development, implantation potential and pregnancy rate. However, measuring oxygen consumption is technically challenging, requiring specialist equipment. The recent availability of Seahorse Bioanalysers has transformed the study of cellular metabolism in a range of systems, however to date, this technology has not been applied to oocytes and embryos. We have therefore examined whether Seahorse XFp is capable of measuring oxygen consumption of small groups of oocytes and embryos. Bovine oocytes were collected from abattoir-derived ovaries and cultured overnight in maturation media (nutrient and hormone-supplemented M199). Oocytes were either allowed to mature or treated with cycloheximide to maintain their immature state. 2-cell embryos generated by IVF were selected after culture for ~24 hours in synthetic oviduct fluid (SOF). Media used was made up in-house as has been reported previously (Guerif et al., PloS One, Volume 8, e67834, 2013). Oocytes or embryos were loaded into Seahorse XFp bioanalyser plates in groups of 6 and oxygen consumption rate (OCR) was measured non-invasively. To further probe the constituents of oxidative function in bovine oocytes, mitochondrial uncoupler FCCP, and electron transport chain inhibitors oligomycin and Antimycin A/rotenone were serially injected (Sigma Aldrich). Assays were repeated on three independent occasions. Data was analysed using one way ANOVA with Tukey's post-hoc. Using this approach, we were able to generate reproducible OCR values for bovine oocytes and embryos. 2-cell embryos were significantly different ($P<0.01$) to germinal vesicle (GV) stage and metaphase-II (met-II) stage oocytes – 0.62 ± 0.15 pmol/min/embryo, compared to 2.36 ± 0.22 and 1.83 ± 0.31 pmol/min/oocyte respectively (mean ± SEM). Crucially, fertilisation rates for oocytes having undertaken the assay were not significantly different to controls ($P>0.05$). The response to mitochondrial inhibitors, shown in real-time, indicated that the approximately 60% of oocyte OCR was coupled to ATP synthesis, 20% was non-mitochondrial with the remaining being proton leak. Furthermore, oocytes have the capacity to increase OCR by approximately 60% spare capacity. These data demonstrate the use of the Agilent Seahorse XFp as a technique for the direct assessment of mitochondrial function in bovine oocytes and embryos. Importantly, we demonstrate that oocytes are competent to undergo fertilisation after this assay, indicating the non-invasive nature of the test. Compared to previously applied assays for oxygen consumption, Seahorse is fast, simple and automated, allowing investigations of higher throughput. With increasing recognition of the critical role mitochondria play in supporting healthy reproduction; this tool facilitates investigation into mitochondrial function which has extensive scope for applications within reproductive biology.

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# Bovine embryos release extracellular vesicles into the medium during group culture *in vitro*

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**Keywords**: embryo-embryo communication, extracellular vesicles, embryo culture.

Efficient communication between cells and tissues is paramount in many physiological process, including embryo development. Inside the genital tract, embryos usually communicate with the mother and vice versa through autocrine, paracrine and endocrine signaling. From our previous research (Wydooghe et al. Reprod. Fertil. Dev, 26, 115, 2013) it has been clearly demonstrated that, in the absence of maternal genital tract, preimplantation embryos cultured in group are able to promote their own development *in vitro* by the production of autocrine embryotropins. Recent studies indicate that among autocrine secreted factors extracellular vesicles play a prominent role in communication. Extracellular vesicles are membrane bound vesicles that are found in biological fluids and in culture media conditioned by embryos or cells. They carry and transfer regulatory molecules, such as microRNAs, mRNAs, lipids and proteins. Here we show that preimplantation bovine embryos cultured in group can release extracellular vesicles into the medium, as novel way of embryo communication. The aim of the current study is to standardize a protocol for isolation and quantification of extracellular vesicles from culture medium conditioned by bovine embryos. Since BSA(Sigma A9647) may contain extracellular vesicles, for optimization of this protocol, bovine presumed zygotes (n = 1140, 4 replicates) produced *in vitro* were allocated to two culture media (SOF with insulin, transferrin and selenium supplemented with either 0.4% BSA (Sigma A9647) or with 0.1 % PVP(Sigma P5288)) and were cultured until Day 8. Media conditioned by embryos were pooled at day 8 until 1ml was obtained, and subjected to density gradient ultracentrifugation (Van Deun et al., J. Extracell. Vesicles. 3, 2014) to extract extracellular vesicles. Extracted suspension with extracellular vesicles was analyzed with Nano particle tracking for quantification. For identification, negative staining electron microscopy was performed, and specific antibodies CD9 (CST), CD63(Serotec) and TSG101(Abcam) were tested for further confirmation of extracellular vesicles presence in the extracted suspension. Blastocyst development rate on day 8, was analyzed by using Student t test (Statistical Analysis System (SAS) for Windows) had showed no significant difference between both media's (40±3.43 % vs 38.64±2.88 %; in SOF+BSA vs SOF+PVP respectively). Results obtained from extracellular vesicles quantification and identification analysis provided evidence that bovine embryos can release extracellular vesicles with a size ranging from 40~200nm into the culture medium. The concentration of extracellular vesicles extracted from 1ml of conditioned medium was $9.18 \times 10^7$ +/- $4.52 \times 10^7$ particles/ml. Further experiments will be performed to extract a higher concentration of extracellular vesicles from a limited amount of medium conditioned by embryos, by following different isolation techniques, such as size exclusion chromatography and ultra-centrifugation.

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# The effect of diet on fatty acid composition of elongated bovine conceptuses

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**Keywords**: dairy cattle, CLA, milk fat depression.

Conceptus elongation is crucial for establishment of pregnancy in ruminants. During the elongation step, endometrium secreted lipids are required as a source of energy and for the remodeling and proliferation of cellular membranes (Ribeiro et al., Reprod 2016;152:R115-R126). The present study investigated the effects of diets designed to cause 15 % reduction in milk fat synthesis (milk fat depression) on fatty acid (FA) composition of elongated bovine conceptuses. The day-14 conceptuses were collected from 22 Nordic Red multiparous dairy cows during two sequential indoor housing periods. Treatments comprised a grass silage based basal diet (CO; 10 cows), basal diet supplemented with 12 g/d of rumen protected *trans*-10, *cis*-12 CLA (Lutrell Pure, BASF, Germany) (CLA; 8 cows), and grass silage based diet containing high-starch concentrate components and supplemented with 26.7 g/kg diet dry matter of sunflower oil and 13.3 g/kg diet dry matter of fish oil (MFD; 4 cows). CLA supplement was mixed in total mixed ration and administered in two equal proportions per day. Oil supplements replaced concentrate ingredients. Cows were randomly allocated to the treatments immediately after parturitions. The diets were total mixed rations with 55:45 forage:concentrate ratio on dry matter basis. In total of 45, 35 and 13 conceptuses having visible embryonic discs were used after recovery from the superovulated CO, CLA and MFD donors 130 days after parturitions, respectively. Lipids were extracted separately from each cryopreserved conceptus using a mixture of hexane and 2-propanol (3:2, vol/vol). FA were transesterified to methyl esters using methanolic sodium methoxide and analyzed with a gas chromatograph equipped with a flame ionization detector (Shingfield et al., Anim Sci 2003;77:165–179). Data were analyzed using linear mixed models with MIXED procedure in SAS 9.4. The proportions of the most abundant FA in conceptuses, *cis*-9 18:1 (30-32 g/100g FA, %), 16:0 (25-26 %), and 18:0 (12-14 %), did not differ between treatments ($P > 0.10$). In addition, total proportions of *cis* unsaturated FA (53-54 %) and saturated FA (43-45 %) and ratio of *cis* unsaturated FA to saturated FA was not different among treatments ($P > 0.10$). CLA had no effect on the conceptus' FA composition compared with CO. However, MFD induced higher ($P < 0.01$) 22:6n-3, and lower ($P < 0.05$) 22:4n-6 and 22:5n-6 proportions compared with CLA and CO. The proportion of *cis*-12 18:1 and *trans*-9, *cis*-12 18:2, which are biohydrogenation products of 18:2n-6 found in rich amounts in sunflower oil, tended to be higher ($P < 0.10$) in MFD compared with other treatments. In conclusion, although CLA and MDF caused changes in lipogenesis in the mammary gland and milk fat depression, a substantial effect on the FA composition of conceptuses was not observed. However, unsaturated FA deriving from the MFD diet and metabolized in the rumen biohydrogenation processes had a specific impact on conceptus FA profiles during elongation stage. Acknowledgements: This research was supported by the Ministry of Agriculture and Forestry (Grant No. 1834/312/2014).

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# Expression profile of genes involved in sex determination in cattle

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**Keywords**: sex determination, cattle, gonad.

Sex determination is the process by which a bipotential gonad develops into a testis or ovary depending on the genetic background of the individual. There is a paucity of information about this process in large mammalian species. Bovine *SOX9* (a critical gene for sex determination in mice and humans) has lost the two transactivation motifs that are essential for sex determination in mammals; therefore, an alternative sex determination pathway could be responsible for sex determination in cattle. In order to clarify the genes involved in gonad differentiation in cattle we performed quantitative analysis of RNA expression in the genital ridges of bovine foetuses collected at the abattoir. The age in days from conception was estimated based on the crown-rump length (CRL). We collected 14 female foetuses ranging in CRL from 18 mm (Day 38 of development) to 57 mm (Day 59) and 19 male foetuses ranging from 13 mm (Day 33) to 48 mm (Day 56). The sex of the embryos was determined by PCR with primers for bovine amelogenin. RNA was extracted and converted to cDNA using a reverse transcription kit (Applied Biosystems, Carlsbad, CA). All qPCR reactions were carried out using a PCR mix (GoTaq® qPCR Master Mix, Promega Corporation, Madison, USA) containing the primers selected for the bovine genes *SF1* and *WT1* (related with gonadal formation)*; FOG2*, *GATA4*, *SOX9*, *SRY*, *DMRT1* (involved in sex determination and testis development); *WNT4*, *FOXL2* (participating in ovary formation and maintenance, respectively); and *ZRSRY2*, *SOX8*, *SOX10* (candidates genes potentially involved in testis determination in cattle). The reference gene used was H2AFZ. In addition, the location of the primordial germ cells (PGC) was evaluated by immunohistochemistry to identify testis and ovary formation differences between the sexes after SRY peaks. Genital ridges were fixed and stored in Bouin's solution for immunohistochemistry of cell marker OCT4. We found that *SRY* expression peaked at a CRL of 18 mm (Day 38). We detected expression of *SOX8* and *SOX10* in male foetuses after the SRY peak (earlier than observed in mice and humans), and *ZRSR2Y* (a splicing factor related to RNA processing and RNA splicing) expression along all the stages analysed showing an increasing pattern from Day 33 to Day 56. Regarding immunohistochemistry, we identified that PGC follow two distinct patterns in males and females. Before SRY peak, PGC localize along the genital ridges of both sexes. After SRY peak, testis cords begin to be distinguishable at a CRL of 25 mm (Day 42) in males, with one to three PGC within each of the developing tubules. In the case of females, PGC tend to distribute along the periphery of the developing ovary at a CRL of 36 mm. Overall, these results indicate that sex determination in bovine genital ridges present characteristic features with SOX8 and SOX10 showing early expression after SRY peak, and ZRSR2Y as a splicing factor that could be involved in sex determination.

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# Nuclei number and DNA fragmentation in pig embryos derived from IVF, *in vivo*-IVC and *in vivo*-derived blastocysts evaluated by TUNEL assay

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**Keywords**: pig, embryos, quality.

Embryo quality is assessed on the basis of the rate of development, morphology, number of cell nuclei and the degree of apoptosis of the embryonic DNA. Detection of apoptosis in embryos is possible due to the occurrence of DNA fragmentation, the degree of which can be determined using the TUNEL assay. The aim of the study was to compare the quality of pig embryos obtained after IVF (IVP), *in vivo*-IVC (*in vivo* zygote and *in vitro* culture) with *in vivo*-derived blastocysts (control) by TUNEL assay. IVP embryos were obtained from *in vitro*-matured, *in vitro* fertilized oocytes. The putative zygotes were cultured in NCSU-23 medium at 39°C and 5% $CO_2$ in the air up to the blastocyst stage. *In vivo*-IVC zygotes were obtained surgically from superovulated and inseminated donor gilts and cultured in NCSU-23 medium up to the blastocyst stage. *In vivo*-derived blastocysts were obtained surgically on the sixth day after insemination from superovulated and inseminated donor gilts by flushing the uterus with PBS-BSA solution. The IVP, *in vivo*-IVC and *in vivo*-derived blastocysts were subjected to TUNEL assay according to the manufacture protocol (TUNEL reagent In Situ Cell Detection Kit, Roche Diagnostic, Germany). The analysis was carried out under an epifluorescence microscope using the following filters: 520 nm (TUNEL) and 358-461 (DAPI). Statistical analysis was performed using the t-test. It was observed that the mean number of cell nuclei was statistically significantly higher in *in vivo* embryos compared to *in vivo*-IVC and IVP embryos (106.47; 39.20 and 38.73; respectively, $P<0.01$). In turn, the mean number of apoptotic nuclei was significantly higher in embryos derived *in vivo*-IVC compared to IVP and *in vivo* embryos (2.56; 1.63 and 0.06 respectively, $P<0.01$). The TUNEL index was 4.20% for IVP, 0.06% for *in vivo*-derived blastocysts and 6.53% for *in vivo*-IVC blastocysts. The study showed that quality of IVP and *in vivo*-IVC embryos was lower compared to the quality of embryos derived *in vivo*. The quality of the embryos thus obtained is mainly affected by the *in vitro* culture conditions.

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# Investigating the impact of hyperglycaemia on bovine oviduct epithelial cell physiology and secretions *in vitro*

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**Keywords**: oviduct, hyperglycaemia, epithelium.

A key role of the oviduct, or Fallopian tube, is the creation of the environment where fundamental developmental processes take place, including gamete activation, fertilisation and early embryo development. Previous studies have partially determined the composition of oviduct fluid. However, the impact of maternal physiology on the oviduct environment is unknown. The aim of this study was to investigate the impact of a hyperglycaemic challenge on the physiology of oviduct epithelial cells in vitro as well as the biochemical and physical properties of oviduct-derived fluid, using an air:liquid model of the oviduct. Bovine oviduct epithelial cells, harvested from slaughterhouse-derived tissues (mainly stage II reproductive tracts), were cultured in DMEM-F12, at 39oC and 5%CO2 for 6 days. Cell identity was confirmed using confocal and optical microscopy. The cells were grown to confluence on a permeable membrane, allowing selective transportation of nutrients between apical and basal chamber. TransEpithelial Electrical Resistance (TEER) measurements, >700Ohm.cm2 were used to indicate the barrier properties of the epithelial monolayer(n=4). This was independently confirmed by observing that fluorescein was unable to cross the monolayer(n=3), when the cells were determined confluent by TEER measurements. Once confluence was achieved, the apical medium was discarded and cells cultured in an air:liquid interphase, mimicking the in vivo environment. After 24h, a thin film of fluid accumulated, which was collected for biochemical analysis. In Experiment 1, physiological (7.3mM) and hyperglycaemic (8.5mM,11mM) concentrations of glucose were added together with 20ng/ml of insulin to the basal compartment for 24h. In Experiment 2, cells were exposed to the same conditions but for 7 days. Data were analysed using Kruskal-Wallis test with Dunn's post-hoc. Chemicals and consumables were used as previously (Simintiras et al, Reproduction, 153,23–33,2017). Our data revealed that acute hyperglycaemia in the basolateral compartment did not change the luminal concentrations of glucose, pyruvate or lactate. However, the presence of insulin reduced glucose in the lumen when cells were exposed to hyperglycaemia. By contact, 7-day basolateral exposure to hyperglycaemia in the absence of insulin increased luminal concentrations of glucose (1.09mM for normoglycaemia compared to 8.9mM for chronic hyperglycaemia). Notably, the presence of insulin reduced the volumes of oviduct-derived fluid (6.24µl in hyperglycaemia compared to 75.5µl for normoglycaemia ($P<0.05$). Using an in vitro oviduct model we have shown that long term exposure to hyperglycaemia induces glucose transport in oviduct secretions and that insulin appears to reduce fluid flow across the oviduct monolayer. Future work will focus on investigating differences in gene expression in response to hyperglycaemia, as well as a detailed evaluation of how insulin affects ion transport. Furthermore, we will determine the impact of hyperglycaemic-conditioned oviduct fluid on gamete maturation and early embryo development.

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# Effect of oxygen tension on the development of *in vitro* embryos from Iberian red deer (*Cervus elaphus hispanicus*)

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**Keywords**: oxygen tension, iberian red deer, *in vitro* fertilization.

Culture conditions during *in vitro* oocyte maturation and fertilization, such as exposure to different oxygen concentrations, have been shown to affect in the developmental ability and the generation of reactive oxygen species (ROS). Low oxygen concentrations may significantly improve the developmental potential of cleavage stage embryos, thus resulting in a positive effect on subsequent blastocyst formation in different species (Leoni et al., Reprod Domest Anim 42(3):299–304. 2007). With the aim to improve the *in vitro* production of embryos in the Iberian red deer (*Cervus elaphus hispanicus*), we evaluated the influence of two oxygen tensions (5 and 21%) during *in vitro* maturation (M) and fertilization (F) on developed blastocysts. The *in vitro* embryo production was performed as García-Álvarez et al., Theriogenology 75:65-72, 2011 and Berg et al., Anim Reprod Sci 70:85-98. 2002. Similarly, we analysed differences in gene expression of the resulting expanded blastocysts. To assess embryo production, a total of 588 COCs were divided into four experimental groups that were evaluated according to the oxygen tension used (M5F5, M5F21, M21F5 and M21F21). Relative poly(A) mRNA abundance of *GAPDH*, *G6PH*, *HPRT*, *SOD2*, *BAX*, *SHC1*, *AKR1B*, *PLAC8*, *GJA1* and *SOX2* was analyzed using quantitative real-time RT-PCR (qRT-PCR). General linear models were used with the independent variable being percentage of total blastocysts at 9 days (embryo rate=96 blastocysts); and the fixed variables being treatment (M5F5, M5F21, M21F5 and M21F21) and replicate (n=7). Additionally, relative mRNA abundance differences in blastocysts were analyzed by one way ANOVA. Results showed that regardless of the oxygen concentration, blastocyst rates did not differ ($P \geq 0.05$) (M5F5=21.98±6.26; M5F21=12.46±5.78; M21F5=18.21±2.90; M21F21=20.54±5.75). With regard to gene expression, *SOD2* was up-regulated ($P<0.05$) in oocytes matured in low oxygen, independently of the tension used during fertilization, whereas *SOX2* was down-regulated ($P<0.05$) in oocytes that were also matured in low oxygen but fertilized in high oxygen tension ($P<0.05$). Likewise, *AKR1B* and *PLAC8* were up-regulated ($P<0.05$) when oocytes were matured and fertilized under high tensions. To our knowledge, this is the first study that demonstrates that Iberian red deer embryos can be produced *in vitro* using different oxygen tensions. Although the four groups compared do not reflect significant differences in terms of embryo production, the use of different oxygen tensions during *in vitro* maturation and fertilization significantly alters the expression of genes related to oxidative stress (*SOD2*), implantation (*AKR1B1* and *PLAC8*) and transcription factors involved in the regulation of embryonic development and determination of cell fate (*SOX2*). In conclusion, both oxygen tensions (5 and 21%) result in similar embryonic development and therefore are feasible for *in vitro* production of Iberian red deer embryos, but more studies are necessary to determine blastocyst quality. Supported by MINECO (AGL2013-48421-R).

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# Regulatory actions of progesterone and luteotrophic hormones on bovine oocyte apoptosis during the terminal phase of *in vitro* maturation

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**Keywords**: progesterone, luteotrophic hormones, oocyte apoptosis.

The currently available evidence points out the cumulus-derived progesterone (P4) as a key antiapoptotic signal involved in maintaining the bovine oocyte viability during in vitro maturation (O'Shea et al., Biol Reprod, 89:146, 2013). However, effects of exogenous P4 on the oocyte quality are not quite clear. The aim of the present research was to compare actions of P4 and two luteotropic hormones, prolactin (PRL) and LH, on apoptosis of bovine oocytes during the second phase of in vitro maturation (from M-I to M-II). Bovine cumulus-oocyte complexes (COCs) were cultured for 12 h in TCM 199 containing 10% fetal calf serum (FCS), 10 μg/ml of porcine FSH, and 10 μg/ml of ovine LH at 38.5°C and 5% $CO_2$. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Then COCs were transferred to the following culture systems: (1) TCM 199 containing 10% FCS (Control 1) and (2) a monolayer of granulosa cells (GCs) precultured for 12 h in TCM 199 containing 10% FCS (Control 2). Just before the oocyte transfer, the medium of experimental groups was supplemented with either 50 ng/ml of P4 or 50 ng/ml of bovine PRL (Research Center for Endocrinology, Moscow, Russia) or 10 μg/ml of ovine LH. At the end of culture, the nuclear status of oocytes was assessed by staining with DAPI. Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA). The content of P4 and estradiol-17β (E2) in culture media was determined by ELISA. All data (n=5, 87-99 oocytes per treatment) were analyzed by ANOVA, with percentage data being arcsine transformed. After 24 h of culture, the rate of M-II oocytes was similar in the compared groups and reached 80.3-89.2%. The addition of P4 to the control medium of both systems resulted in the reduction ($P<0.05$) of the apoptosis frequency in matured oocytes from 11.7±1.2 to 5.9±1.7% (System 1) and from 13.8±1.6 to 7.2±0.9% (System 2). In the absence of GCs, PRL and LH did not affect oocyte apoptosis. When COCs were cocultured with GCs, the apoptosis rates increased ($P<0.05$) from 9.4±1.6 (without GCs) to 16.3±1.8% for the PRL groups and from 13.0±1.5 (without GCs) to 17.6±2.2% for the LH groups. Meanwhile, in the presence of GCs, these rates were higher than that for the P4 group ($P<0.01$). At the end of oocyte culture in both systems, the content of P4 in the medium was 1.2-1.3 times lower ($P<0.05$) in groups treated with PRL or LH than in the group treated with P4, whereas the content of E2 did not differ between groups tested. Furthermore, concentrations of P4 and E2 were increased 1.2-1.3 times (at least $P<0.05$) in the presence of GCs regardless of the hormonal treatment. Our findings indicate that exogenous P4 can exert granulosa-independent antiapoptotic action on bovine oocytes during the second phase of in vitro maturation. At the same time granulosa cells are able to cause proapoptotic effects of PRL and LH on the oocytes that complete maturation. This research was supported by the Russian Science Foundation (project 16-16-10069).

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# Effect of prolactin and dithiothreitol during prolonged culture of aging oocytes on the development potential of parthenogenetic bovine embryos

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**Keywords**: oocyte aging. parthenogenetic embryos.

The evaluation of factors responsible for the protection of the oocytes attained the metaphase-II stage from aging is important for successful in vitro embryo development. The aim of the present research was to study dose-dependent effects of two potential regulators of oocyte quality, prolactin (PRL) and dithiothreitol (DTT), during the prolonged culture of bovine oocytes on their developmental potential after artificial activation. Slaughterhouse-derived cumulus-oocyte complexes (COCs) were matured for 22 h in TCM-199 supplemented with 10 % fetal calf serum (FCS), 0.2 mM sodium pyruvate, 10 μg $mL^{-1}$ porcine FSH, and 10 μg $mL^{-1}$ ovine LH. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After 22 h maturation, the part of COCs were transferred to the fresh medium consisting of TCM 199 supplemented with 10 % FCS and cultured for 10 h in the absence (Control) or in presence of bovine PRL (20 and 50 ng $mL^{-1}$; Research Center for Endocrinology, Moscow, Russia) or DTT (2.5, 5 and 10 μM). After maturation (22 h) or the prolonged culture (10 h), oocytes were activated by culturing in 5 μM ionomycin solution during for 5 min followed by 4 h in 2 mM 6-dimethylaminopurine. Activated oocytes were cultured in CR1aa medium (Rosenkrans, First, J Anim Sci 1994, 72:434-7) until Day 5 and then transferred to the same medium supplemented with 5 % FCS and cultured up to Day 7. All the cultures were performed in at 38.5°C and 5% $CO_2$ in humidified air. At Days 2 and 7 after activation, the cleavage and blastocyst rates were determined. In addition, obtained blastocysts were fixed with 4% paraformaldehyde, and the total cell number was determined by DAPI staining. The data from 4 replicates (111-122 oocytes per treatment) were analyzed by ANOVA. For oocytes activated just after IVM, the cleavage and blastocyst rates, and total blastocyst cell number were 74.1±3.5 and 20.6±2.8 %, and 54,0±1,8, respectively. The prolonged culture of matured COCs in the aging medium (10 h) (Control) had no effect on the cleavage rate (74.7±2.9 %) and the total number of cells in embryos (49.1±2.0), but caused the blastocyst yield to decline to 9.8±1.2 % ($p<0.05$). At the same time, the addition of both PRL (50 ng $mL^{-1}$) and DTT (5 μM) to the aging medium raised the blastocyst rate to 18.0±3.1 and 18.8±2.5 % ($p<0.05$), respectively. Cleavage rates of aging oocytes after their activation and total cell number in blastocyst produced from aging oocytes were unaffected by both PRL and DTT (except DTT 10 μM group). In the case of DTT 10 μM, the blastocyst rate and total blastocyst cell number (9.6±1.3 % and 35.4±1.1, respectively) was lower than in the DTT 5 μM group ($P<0.05$). Thus, PRL and DTT are able to maintain competence for parthenogenetic development of bovine COC during their prolonged in vitro culture.

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A276E Embryology, developmental biology, and physiology of reproduction

# Initial characterization of bovine embryos developing at the air-liquid interphase on oviductal epithelial cells

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**Keywords**: Air-liquid interphase, oviductal epithelial cells, embryo development.

Air-liquid interphase cultures of bovine oviductal epithelial cells (ALI-BOEC) have recently been reported to support embryo development in co-culture up to the blastocyst stage without the addition of embryo culture medium. To initially characterize such ALI-BOEC produced embryos, we assessed the expression of 41 target genes in 8-cell embryos and blastocysts. For comparison, we analyzed embryos produced in standard IVP media (IVP-S) and in a commercial serum-free media suit (IVP-SF; IVF Bioscience, Falmouth, UK). A total of fifteen 8-cell embryos (randomly divided into 3 pools of 5 embryos) and six single blastocysts were collected under each culture condition. Gene expression was analyzed by means of a 48.48 Dynamic Array™ on a Biomark HD instrument. To identify genes differentially expressed in 8-cell embryos and blastocysts, we applied one-way ANOVA with Tukey post-hoc test in SPSS. Furthermore, we re-analyzed previously published transcriptomics data from *in vivo* embryos (GSE12327). The cleavage rate in ALI-BOEC co-culture (70.71%) was comparable to the standard IVP procedure (74.75%), and lower than the cleavage rate reached with the commercial media suit (90.79%). However, the blastocyst rate in ALI-BOEC co-culture (9.1%) was much lower than in either IVP-S (33.1%) or the commercial IVP-SF system (54.7%). Re-analysis of the *in vivo* data set revealed that sixteen of the chosen target genes were significantly regulated between the 8-cell and blastocyst stage embryos *in vivo*. The *in vitro* embryos showed expression patterns similar to the *in vivo* embryos. The culture conditions lead to differential gene expression in both 8-cell embryos (CDH1, NOS2, OVGP1, APEX1, REX1, PLAGL, BAX, SREBP1, SMPD2) and blastocysts (CCL26, CDH1, NID2, IFNAR1, SLC2A5, SREBP1, SERPINE1, LDLR, CYP51A1), respectively. Five of the genes differentially expressed in blastocysts from different culture conditions (LDLR, CDH1, NID2, SLC2A5 and CYP51A1) were previously reported to also be differentially expressed between *in vivo* and *in vitro* blastocysts. Embryos produced in the ALI-BOEC co-culture system followed the *in vivo* expression pattern for all five genes. The present study confirmed that the ALI-BOEC co-culture system is much less efficient in supporting blastocyst formation than conventional IVP procedures. Given the lack of a direct comparison to *in vivo* embryos, interpretation of the biological relevance of the differentially expressed genes warrants caution. However, our results indicate that blastocysts produced on ALI-BOEC may have an improved *in vivo*-like gene expression signature. The establishment of a sequential culture system of oviductal and uterine epithelial cells including a hormonal stimulation protocol might further increase the efficiency of the co-culture both quantitatively and qualitatively. Acknowledgments: This study was partly funded by the Schweizerischer Nationalfonds (SNF). Data analyzed in this paper were generated in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich.

A277E Embryology, developmental biology, and physiology of reproduction

## Maternal metabolic disorders and early embryonic loss: pathways to bridge the gap between bovine embryo quality and endometrial receptivity

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**Keywords**: nutrient sensing, blastomere differentiation, BEEC responses.

The pre-implantation embryo is very sensitive to perturbations in its micro-environment and, therefore, a tight regulation of the embryonic milieu is essential. Such an environment is not assured in females suffering metabolic disorders. Our previous data show that altered nutrient abundance in the embryonic micro-environment results in suboptimal embryonic cell differentiation patterns. Here, we hypothesize that suboptimal nutrient conditions during embryo culture can affect the blastocyst's capacity to participate in the first maternal-embryonic interactions. Earliest preimplantation phases of embryo development were studied as 'window' for nutrient sensitive manipulations. Embryos (4 repeats; 890 zygotes) were cultured during the first 4 days after fertilization (p.i.) in distinct nutrient conditions: [control] based on serum-free SOF medium; [HIGHGLUC] with 3.5mM glucose; [LOWAA] with 10% lower amino acid concentrations as presented in control. At morula stage, embryos were transferred to monolayers of bovine luminal epithelial endometrial cells (BEEC; subculture 1), in SOF medium + 5% serum, till D8 p.i.. In D8 blastocysts, mRNA expression of 12 genes involved in nutrient sensing, pluripotency and differentiation was analyzed by qRT-PCR. Differently expressed genes (DEG) were identified using (mixed model) ANOVA. Using NGS, transcriptomes of BEEC (4 repeats) exposed to distinct groups of embryos were sequenced and data were normalized by EdgeR. Blastocysts originating from HIGHGLUC morulae displayed a tendency for increased transcript levels of *PDK1* (P=0.075), a key gene in nutrient sensing regulation. Also a down-regulated expression of the pluripotency marker, *OCT4* (P=0.002), was observed compared to controls. Transcriptome reaction of BEEC exposed to the HIGHGLUC embryos was rather limited. Only 27 DEG genes were identified, of which 20 down- and 7 up-regulated in BEEC exposed to HIGHGLUC embryos compared to control embryos (Padj<0.1). Enriched genes involved endoplasmic reticulum activities, whereas cell-cell signalling pathways were down-regulated. Blastocysts from LOWAA conditions showed tendencies (P≤0.1) for decreased transcript levels of *SIRT1*, *mTOR*, *GLUT1* and *LDHA*, all involved in mTOR pathways. Also a down-regulated mRNA expression was observed for *OCT4* (P<0.0001) and *SOX2* (P<0.1), both genes involved in pluripotency, and for *ITGB5* (P<0.05) and *CTNN1* (P≤0.1), two blastomere differentiation markers. BEEC exposed to LOWAA embryos revealed 120 DEG compared to BEEC exposed to controls (Padj<0.1). Here, 63 of the 120 DEG were down- and 57 were up-regulated in the LOWAA condition. Up-regulated genes involved transcription regulation and down-regulated genes concerned inhibition of both Notch and immune responses. Overall, suboptimal metabolite conditions during the first 4 days of embryo culture can impact on resultant blastocyst cell proliferation and differentiation pathways. Furthermore, BEEC genes were differently regulated when placed in contact with the three distinct groups of embryos.

A295E Cloning, transgenesis, and stem cells

# Subfertility and zona pellucida alterations in ZP4 KO rabbits produced by CRISPR

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**Keywords**: CRISPR, zona pellucida, rabbit.

Mammalian zona pellucida, the glycoprotein layer that surrounds oocytes and embryos up to the blastocyst stage, may be composed by 4 different glycoproteins. One of these proteins, ZP4, is present in the zona pellucida of rabbits, cattle and women, among others, but it is absent in the only species where Knock-out (KO) models were readily available: the laboratory mouse. For this reason, the function of ZP4 remains elusive. CRISPR technology greatly simplifies the generation of KO models in livestock species such as rabbits. In this experiment, we have generated ZP4 KO rabbits, i.e. rabbits lacking ZP4 protein, by CRISPR technology and have compared their reproductive performance to that of heterozygous (Hz) and wild type (wt) rabbits. Delivery rates following natural breeding with males of proven fertility were analysed in 5 animals per experimental group (wt, Hz and KO). Pregnancy was clearly impaired in KO animals, with only one female producing a litter of 4 pups, resulting in a significant reduction in litter size compared to wt or Hz groups (pups delivered: wt 9.2±0.6; Hz 10.6±0.5; KO 0.8±0.8; mean±s.e.m., Kruskal-Wallis ($P<0.05$)). Aiming to elucidate the possible causes of subfertility, ovulation and cleavage rates were assessed following natural mating. Surprisingly, neither ovulation (oocytes ovulated: wt 11.7±1; Hz 15±2.9; KO 13.3±2.9), nor cleavage rates (% of cleavage: wt 81.7±0.1; Hz 95.5±0.1; KO 87.3±0.1) showed significant differences between groups. However, clear morphological differences were noted on the zona pellucida from oocytes ovulated by KO rabbits compared to those produced by Hz or wt rabbits. Zona pellucida thickness was significantly reduced in KO compared to Hz or wt (thickness μm: wt 15.2±1.5; Hz 15.3±1.4; KO 10.9±0.7, ANOVA ($p<0.05$)). Besides, KO rabbits produced irregular zonae pellucida, i.e. not perfectly spherical as in Hz of wt animals, and noticeable less elastic and easier to deform. These results suggest that the impaired fertility in ZP4 KO rabbits is not due to reduced ovulation or cleavage, and that ZP4 may act as a crosslinker of other ZP proteins, conferring mechanical properties to the zona pellucida which are important for embryo survival. This study is supported by the projects AGL2014-58739-R and RYC-2012-10193 (to PBA), AGL2015-70159-P (to MA) and AGL2015-65572-C2-1-R (to PGR and PL). ILT and NFB are supported by FPI grants.

A296E Cloning, transgenesis, and stem cells

# Does scriptaid-dependent epigenomic modulation of peripheral blood-derived fibroblast-like cells affect the *ex vivo* developmental abilities of caprine-porcine nuclear-transferred embryos to reach blastocyst stage?

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**Keywords**: SCPT-dependent epigenomic modulation, adult goat peripheral blood-derived fibroblast-like cell, inter-species (caprine-porcine) NT embryo.

The present research was carried out to ascertain whether inter-family and inter-genus (caprine→porcine) nuclear-transferred (NT) embryos can acquire and retain the competences to complete their extracorporeal development to the blastocyst stage. To generate inter-species (caprine→porcine) cloned embryos, enucleated *in vitro*-matured pig oocytes were subzonally microinjected and subsequently electrofused with adult goat peripheral blood-derived fibroblast-like cells (AGPB-FLCs) that either had been epigenetically transformed by exposure to 350 nM scriptaid (SCPT) during their 24-h contact inhibition (Group I) or had not been exposed to SCPT (Group II). Efficiently electroactivated caprine→porcine nuclear-ooplasmic hybrids were cultured to the morula and blastocyst stages for 7 to 8 days. Among 231 inter-species NT embryos assigned to Group I, 172 (74.5%)[a] underwent cleavage divisions. The percentages of embryos that progressed to the morula and blastocyst stages were 65/231 (28.1%)[a] and 26/231 (11.3%)[a], respectively. In Group II, out of 217 hybrid NT embryos, 147 (67.7%)[a] were able to divide *ex vivo* ([a,a] $P \geq 0.05$; $\chi^2$ test), but 36 (16.6%)[b] and 0 (0.0%)[b] developed to the morula and blastocyst stages, respectively ([a,b] $P < 0.01$; $\chi^2$ test). Summing up, inter-species (caprine→porcine) NT embryos that had been reconstructed with porcine enucleated oocytes and SCPT-treated AGPB-FLC nuclei exhibited developmental capabilities to reach the blastocyst stage. In contrast, their counterparts originating from porcine enucleated oocytes and SCPT-untreated AGPB-FLC nuclei were not developmentally competent to progress to the blastocyst stage. Additionally, due to desirable enhancement of donor cell nuclear reprogrammability, the strategy of SCPT-mediated epigenomic modulation of AGPB-FLCs resulted in not only remarkable improving morula formation rate of hybrid (caprine→porcine) NT embryos, but also acquiring and maintaining capacities to complete the *in vitro* development to the blastocyst stage.

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A297E Cloning, transgenesis, and stem cells

# Effect of Estradiol and Progesterone on ovine Amniotic Epithelial Cells

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**Keywords**: oAEC, estradiol, progesterone.

This study was designed to clarify Estradiol (E2) and Progesterone (P4) steroid effects on ovine Amniotic Epithelial Cells (oAECs) that has a conserved plasticity and highly self-renewable capacity (Parolini et al., Stem Cells, 26(2), 300–311, 2008; Barboni et al., Stem Cell Rev Rep, 10:725–741, 2014). Based on their conserved immunomodulatory properties, oAECs are suitable for allo and xeno-transplantation (Barboni et al., Cell Transplant, 21(11), 2377–2395, 2012; Muttini et al., Res Vet Sci, 94(1),158–169, 2013). To date, no information is present on the effects of prolonged steroid exposition on AECs. oAECs were cultured as previously reported (Barboni et al., Cell Transplant. 21(11), 2377–2395, 2012) and treated with 12.5µM and 25µM of E2 or P4 (Sigma-Aldrich, Milan, Italy), alone and in both combinations, for three passages. Untreated cells were marked control (CTR). At 70% confluency, cells were detached for doubling time (DT) evaluation. Cells at fourth passage were differentiated for 21 days in osteogenic media (DM) (Mattioli et al., Cell Biol Int 36(1):7-19, 2012) without steroid. Alizarin Red and Alcian-Blue (Sigma-Aldrich, Milano, Italy) stainings were performed. RNA and cDNA were obtained as previously reported (Barboni et al., Cell Transplant. 21(11), 2377–2395, 2012). Real Time for *NANOG*, *SOX2* ,*OCT4* stemness genes expression were performed by SensiFast SYBR (Bioline, Aurogene, Rome, Italy) using specific primers (Mattioli et al., Cell Biol Int. 36(1):7-19, 2012).The protocol was: 5 min at 95°C, 30 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec. Comparative Ct $2^{-\Delta\Delta C(t)}$ normalization to *GAPDH* was applied. IHC analyses were carried out for Cytokeratin 8 and αSMA expression as previously reported (Barboni et al. PLoS ONE 7(2): e30974, 2012). Data expressed as mean (±SD), compared by one-way ANOVA followed by Tukey's test (GraphPad Prism 5). Significant values for $P < 0.05$. Steroids treated ovine AECs proliferate with significant differences between concentrations. While P4 treated cells showed cuboidal shape and Cytokeratin expression until third passage, CTR and E2 treated cells showed a rapid downregulation of Cytokeratin and increased αSMA expression. oAECs with E2+P4 showed both cell type morphology. Steroids modified stemness genes based on the concentration. 12.5 µM E2, 25µM P4 and 25µM of both E2+P4 treatments maintained higher *OCT4*, *NANOG* and *SOX2* expressions in treated cells despite their progressive downregulation in the CTR. Moreover, compared to CTR, after Alizarin staining, steroid pretreated cells suffered morphological changes under DM acquiring Alcian Blue-positive chondrogenic-like morphology. AECs stemness properties and plasticity can be modified by prolonged steroidal treatment. These data improve our knowledge, opening new prospective on oAEC use in stem cell-based therapy.
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A298E Cloning, transgenesis, and stem cells

# Intra-family and inter-genus (caprine-bovine) cloned embryos do not fail to complete their *in vitro* development to blastocyst stage

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**Keywords**: intra-family and inter-genus (caprine-bovine) cloned embryo, intra-species (caprine) cloned embryo, *ex vivo* developmental capacity.

The current study was undertaken to comparatively analyze the *ex vivo* developmental outcomes of inter-species (caprine→bovine) nuclear transfer (NT)-derived embryos (Group I) and intra-species (caprine) NT-derived embryos (Group II). In Group I, to create inter-species clonal cytoplasmic hybrids (cybrids), enucleated extracorporeally matured heifer/cow oocytes were reconstituted with the cell nuclei of adult goat peripheral blood-retrieved fibroblast-like cells (AGPB-FLCs) that had undergone the *in vitro* synchronization of mitotic cycle at the G1/G0 phases by contact inhibition. In Group II, to produce intra-species clonal cybrids, enucleated metaphase II-stage doe oocytes were reconstituted with the cell nuclei of contact-inhibited AGPB-FLCs. The inter- or intra-species clonal cybrids that had been successfully electrofused and then were subjected to calcium ionomycin- and 6-dimethylaminopurine (6-DMAP)-mediated activation were classified for *in vitro* culture. In Group I, from among 212 cultured inter-species NT-derived embryos, 168 (79.2%)[a] were cleaved. The proportions of embryos that developed to morula and blastocyst stages were 69/212 (32.5%)[a] and 41/212 (19.3%)[a], respectively. In Group II, out of 203 cultured intra-species NT-derived embryos, 172 (84.7%)[a] were able to divide, but 75 (36.9%)[a] and 48 (23.6%)[a] reached the morula and blastocyst stages, respectively ([a,a] $P \geq 0.05$; $\chi^2$ test). To summarize, the *ex vivo* developmental capacities of inter-species (caprine→bovine) cloned embryos to progress to the morula and blastocyst stages did not differ considerably from those indicated among intra-species (caprine) cloned embryos. This seems to result from close taxonomic distance and phylogenetic consanguinity between donor specimens of somatic cells (*Capra aegagrus hircus*) and donor specimens of nuclear recipient oocytes (*Bos primigenius taurus*). Such symptomatic relationships undoubtedly encompass intra-family (*Bovidae*) and inter-genus (*Capra-Bos*) model of inter-species cloning of domestic goats by somatic cell nuclear transfer (SCNT).
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A325E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

# Spindle configuration of *in vitro* matured bovine oocytes vitrified and warmed in media supplemented with a biopolymer produced by an Antarctic bacterium

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**Keywords**: vitrification, chromosome, microtubule.

Biological molecules isolated from organisms that live under subfreezing conditions could be used to protect oocytes from cryoinjuries suffered during cryopreservation. Bacterial exopolysaccharides (EPS) constitute a common class of molecules that interact with ice in nature either by triggering ice nucleation or by inhibition of ice nucleation and growth. The aim of this work was to evaluate the spindle configuration of in vitro matured bovine oocytes vitrified/warmed in media supplemented with exopolysaccharide (M1) produced by *Pseudomonas* sp ID1 (Carrión et al., Carbohydr Polym 117:1028. 2015). After 22 h of *in vitro* maturation, a total of 546 oocytes form prepubertal (3 replicates) and 405 oocytes from adult cows (4 replicates) were vitrified/warmed in media supplemented with various concentrations of EPS M1 (0, 0.001, 0.01, 0.1 and 1 mg/ml). After warming, oocytes were allowed to recover for 2 additional hours in IVM medium. Fresh, non-vitrified oocytes were used as a control. At 24 h of IVM, oocytes from all treatments were fixed and immunostained with the Alexa-fluor 488 antibody and DAPI. Microtubule and chromosome distribution was analyzed by immunocytochemistry under a fluorescent microscope. ANOVA was performed to analyze differences in meiotic spindle configuration ($P < 0.05$). When cow oocytes were vitrified, similar percentages of normal spindle configuration were observed when compared to fresh control oocytes, except for the 0.1 mg/ml EPS M1 group that showed significantly lower rates compared to the fresh control group. Significantly higher rates of prepubertal oocytes exhibiting a normal spindle configuration were recorded in the non-vitrified group compared to all vitrified/warmed groups, regardless of the EPS M1 supplementation. However, the addition of EPS M1 to the vitrification/warming media decreased the ratio of decondensation or absence of chromosomes and microtubules in prepubertal oocytes. Although percentages of normal spindle configuration after vitrification were lower for prepubertal than for cow oocytes, no significant differences were observed when oocytes were vitrified with 0.001, 0.1 and 1 mg/ml EPS M1. In conclusion, supplementation with EPS M1 concentrations during vitrification and warming did not induce adverse changes in the spindle of bovine oocytes, regardless of the concentration used. Although a more severe damage on spindle configuration could be observed after vitrification of prepubertal oocytes, EPS supplementation during vitrification and warming seems to have a greater benefit during vitrification of prepubertal than adult bovine oocytes. Further experiments are required to investigate if *in vitro*-matured oocytes vitrified/warmed in presence EPS M1 can improve their development competence after being vitrified/warmed. This study was supported by the Spanish Ministry of Science and Innovation (Project AGL2016-79802-P and grant CTQ2014-59632-R).

A326E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

# Comparison of lipid profiles and gene expression in granulosa and cumulus cells in bovine

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**Keywords**: *cumulus, granulosa, lipid metabolism.*

Cumulus cells (CC) derive from granulosa cells (GC) during follicular growth and antrum formation and are coupled with an oocyte. Fatty acid (FA) synthesis and oxidation in GC impact cell proliferation and steroidogenesis (Elis et al, 2015 Theriogenology. 2015, 83(5):840-53) whereas in CC these processes are crucial for oocyte maturation (Sanchez-Lazo et al, 2014, Mol Endocrinol. 2014 28(9):1502-21). Both GC and CC contribute to oogenesis and reflect oocyte quality. The objective of our study was to compare intracellular lipids and lipid related transcripts between these compartments. Lipid profiles obtained using MALDI-TOF mass spectrometry were compared between GC from individual follicles (n=12) and pools of CC (n=12) aspirated from 4-5 mm follicles of slaughtered cows. Freshly isolated cells were analysed using UltrafleXtreme MALDI-TOF/TOF instrument (Bruker) in positive (+) and negative (-) reflector mode, with 2,5-dihydroxyacetophenone matrix. Peaks were detected in 100 -1000 m/z range and values of the normalized peak heights (NPH) were quantified using Progenesis MALDI™ (Nonlinear Dynamics). Student's t-test was applied to NPH values for hunting lipid content variations between GC and CC. Peaks were annotated using MS/MS fragmentation confronted to lipid databases. Lipid fingerprints from CC and GC gathered 462 peaks in (+) and 486 peaks in (-) modes, with coefficients of variation = 27% for CC and 18% for GC. 143 species were significantly upregulated in CC ($P<0.01$, fold change >2.0). Among them, 2 lyso-phosphatidylcholines (LPC 20:4 and 20:3) 12 phosphatidylcholines (PC), and 12 sphingomyelins (SM) were identified. Among 44 molecular forms which were more abundant in GC, we identified LPC (14:0), 4 PC, ceramide (22:1), SM (15:1), phosphatidylethanolamines (28:0 and 38:7) and phosphatidylserine (29:0). 4 peaks were annotated as triglycerides. Gene expression in pools of CC (n=4) and GC (n=4) was analyzed using a customized 60K bovine microarray (Agilent technology, 61326 probes). Differential analysis revealed 2009 differentially expressed genes (DEG) which were up-regulated in CC and 694 in GC ($P<0.05$, Benjamini-Hochberg correction). DEG showed significant enrichment in the pathways related to carbon metabolism, glycolysis /gluconeogenesis, ATP-binding cassette transporters, amino acid and O-glycan biosynthesis, thyroid hormone, PI3K-Akt signaling, p53 and PPAR signaling pathway (corrected $P<0.05$). Among the DEG related to lipid metabolism and regulated by PPARs, genes *ACOX2, LPL, SCD, PPARG, FABP3, FADS2, ACADL, SLC27A2* were up-regulated in CC and *CPT1A, CPT1B, SCD5, PLTP* were more expressed in GC. Ten sphingolipid metabolism genes were over-expressed in CC. In conclusion, numerous genes related to lipid metabolism were differently expressed in CC and GC. This corroborates differences in GC and CC lipids and may reflect different involvement of GC and CC in glyceroneogenesis, lipogenesis, oxidation and steroid production.
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A327E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

# Reproductive Pixel Grey-Intensity Score with Image-J and Freezability in Angora Bucks

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**Keywords**: Angora goat, pixel gray intensity, ultrasound.

We aim to evaluate male reproductive tract ultrasonography and its relation with sperm quality and freezability in Angora bucks. A total of five Angora bucks were selected with age range (2-3 years). The reproductive monitoring of testicular and accessory glands was assessed with ultrasound (US) (Esaote® MyLab One, Italy). Scrotal circumference, total testicular volume, epididymal area, vesicular gland area and bulbourethral gland area were calculated. Semen collection through the Trans-rectal massage (TM) and collection time were recorded. Semen is frozen with Tris egg-yolk diluent and post-thaw Delta (Δ) motility, Δ progressivity and kinetic parameters with The Hamilton-Thorn computer-aided semen analyzer, version 10 Ivos (HTR analyzer, Hamilton-Thorn Research, Beverly, MA, USA), validated for buck semen analysis. Mean differences between PGI values and post-thaw motility were evaluated by paired Student's t-test. All the US images collected were analyzed for their pixel gray intensities (PGI) using Image-J software and classified depending on the difference between the average group value. Regression analysis was carried out among all the parameters collected. Post-thaw Delta (Δ) Medians of motility, progressivity, VAP, VSL, VCL, Lateral Amplitude, Straightness and Linearity were -23.22, -20.22, -0.04, -0.92, -10.21, 0.13, 1.13, and 0.36 respectively. High levels of relationship ($P<0.05$) were identified among ejaculate volume, PGI and area selected of the epididymis. Besides, a high relationship was identified between Total testicular volume and pixel gray intensity. Delta score and collection time was also positively correlated. We concluded that extreme PGI levels seem to be related to the worst sperm quality in terms of motility. Ultrasound monitoring represents an innovative technology, which may give a high impulse to the field application of the BSE in small ruminants. PGI of the reproductive tract result as useful diagnostic tools for sperm quality assessment and genetic material use.

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# Cell-signalling metabolites predominate among small molecules differently released by male and female bovine embryos cultured *in vitro*

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**Keywords**: bovine, embryo, sex.

The cow uterus recognizes embryonic sex, and male and female early bovine embryos show dimorphic transcription that impacts metabolism. Most metabolites are small molecules that may exert a role within early embryo maternal interactions. Individual release of metabolites was examined in a 24h single culture medium from Day-6 male and female morulae that developed to Day-7 expanded blastocysts. Embryos were produced in vitro from slaughterhouse oocytes, fertilized with a single bull and cultured in SOFaaci+6g/L BSA. Prior to metabolomics analysis, embryos had their sex identified (amelogenin gene amplification). Embryos (N=10 males and N=10 females) and N=6 blank samples (i.e. SOFaaci+6g/L BSA incubated with no embryos) were collected from 3 replicates. Metabolome was analysed by UHPLC-TOF-MS in spent culture medium as described allowing identification of 5 sex biomarkers (Gómez et al, J Chromatogr A 2016; 1474:138–144). The remaining output data were submitted to Principal Component Analysis (PCA) to detect outliers, Kolmogorov-Smirnov test to evaluate normality and Levene's test to assess the equality of the variances. Thereafter, analysis of variance by one-way ANOVA was performed to detect the different peak area averages ($P<0.05$). We found 1,720 metabolite signals showed significant differences between male and female embryos. Potential metabolites were tentatively identified by matching the m/z to those published in the Human Metabolome Database within a mass accuracy window of 10 ppm. In addition, Molecular Formula Generator algorithm of MassHunter software (Agilent) was used to support the tentative identification considering their isotopic distribution. N=13 metabolites were differentially identified. LysoPC(15:0) was the only metabolite found at higher concentration in females (fold change [FC] male to female = 0.766). FC of metabolites more abundant in male (12) varied from 1.069 to 1.604. Chemical taxonomy grouped metabolites as amino-acids and related compounds (DL-2 aminooctanoic acid, arginine, 5-hydroxy-L-tryptophan, and palmitoylglycine); lipids (2-hexenoylcarnitine; Lauroyl diethanolamide; 5,6 dihydroxyprostaglandin F1a; LysoPC(15:0); DG(14:0/14:1(9Z)/0:0) and triterpenoid); endogenous amine ((S)-N-Methylsalsolinol/(R)-N-Methylsalsolinol); n-acyl-alpha-hexosamine (N-acetyl-alpha-D-galactosamine 1-phosphate); and dUMP, a product of pyrimidine metabolism. Among the compounds originally contained in CM, female embryos significantly depleted more arginine than males and blank controls ($P<0.001$). Male and female embryos induce different concentrations of metabolites with potential signalling effects that may facilitate sex recognition in the uterus. The increased abundance of metabolites released from males is consistent with the higher metabolic activity attributed to such blastocysts. MINECO-project AGL2016-78597. Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013-2017 (GRUPIN 14-114) and FEDER. The authors are members of the COST Action 16119, In vitro 3-D total cell guidance and fitness (Cellfit).

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# *In vitro* assessment of acrosomal status of boar sperm bound to beads conjugated to ZP proteins

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**Keywords**: gamete interaction, ZP conjugated-beads, 3D model.

The oocyte's zona pellucida (ZP), composed by 3 or 4 glycoproteins, mediates the interaction with sperm. An *in vitro* 3D model mimicking the oocyte's shape based on studies showing the relevance of the processing of ZP2 at N terminal position in sperm-oocyte interaction (LADEN) (Avella, Sci Transl Med 8:336ra60, 2016) is being used to study the molecular mechanisms involved on gamete interaction in pigs (Hamze, Animal Reprod 13: 647, 2016). The model consists of magnetic beads (His Mag Sepharose™ Excel) conjugated with porcine ZP2, ZP3 and ZP4 recombinant proteins. The objective was to study the binding kinetics and acrosomal status of boar spermatozoa bound to beads conjugated with ZP2, ZP3 or ZP4. ZP2 and ZP4 models were produced as previously described (Hamze, Animal Reprod 13: 647, 2016) and ZP3 was identified by electrophoresis and western blot with anti-ZPC polyclonal antibody showing a molecular weight of 55kDa. Once produced, 45-50 ZP proteins conjugated-beads were incubated in TALP medium (500 ul) with 200.000 boar spermatozoa/ml for 30, 60 and 120 min. At each time, an aliquot of beads was washed (PBS), fixed (0.5% glutaraldehyde, 30 min) and stained for 15 min with bisbenzimide (0.01mM) and Peanut agglutinin (PNA, 4µg/µL) to evaluate the number and acrosomal status of sperm bound to the beads . Three replicates with a blind analysis were done. We calculated the rate of beads with at least one sperm bound (BSB), the mean number of sperm per bead (S/B) and the acrosomal reaction of bound sperm. Results were analysed by one-way ANOVA considering statistical differences when *P*-value <0.05. After 30 min of coincubation, the BSB was higher for ZP3 and ZP4 (71.54 ± 3.67%, n=158 and 75.56 ± 3.53%, n=155) than for ZP2 (56.52 ± 4.01%, n= 154) and the S/B was higher for ZP4 (3.87 ± 0.31) than ZP2 (2.58 ± 0.18) and ZP3 (2.69 ± 0.17). No differences were observed at 60 min for BSB but S/B was higher for ZP2 (6.66 ± 0.43) than ZP3 (4.87 ± 0.31) and ZP4 (4.23 ±0.24). Finally, at 120 min both BSB and S/B were higher for ZP2 (93.5 ± 2.0%, 9.00 ± 0.45) and ZP3 (93.6 ± 2.0%, 8.54 ± 0.49) than ZP4 (81.0 ± 3.2%, 6.68 ±0.61). After 30 min of incubation the ZP2 model induced a higher acrosome reaction since 77.50 ± 3.76% of the bound sperm had reacted whereas for ZP3 and ZP4 models rates were 69.84 ± 3.84 and 65.04 ± 3.43, respectively. No differences were found at 60 and 120 min probably due to the high capacitation ability of TALP medium. In conclusion, ZP2 conjugated beads bound a higher number of reacted spermatozoa at 30 min. Regarding binding kinetics, differences between groups were observed through time, being ZP2 and ZP3 conjugated beads the models with a higher S/B at 120 min. The full development of this 3D model will permit in the future a better and deeper understanding of gametes interaction in pigs and the reduction of female gametes in gametes interaction studies. Supported by MINECO and FEDER (AGL2015-70159-P) and Fundación Seneca-Agencia de Ciencia y Tecnología de la Región de Murcia (18931/JLI/13), "Jóvenes Líderes en Investigación".

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# Efficiency of Preimplantation Genetic Diagnosis (PGD) of bovine IVP embryos using blastocoele fluid or embryonic cells

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**Keywords**: embryo sexing, PGD, blastocoele fluid.

Preimplantation Genetic Diagnosis (PGD) involves collecting a few cells from a preimplantation embryo, which will then be used for genetic testing. Palini et al. were able to diagnose the sex human embryos using only the blastocoele fluid as a source of DNA [Palini, Reprod Biomed Online, 26(6):603-10, 2013]. Recently, we demonstrated that the DNA in the blastocoele fluid of bovine IVP embryos can be amplified by PCR to diagnose the sex of the embryos [Herrera, Proceedings in 42° Veterinär-Humanmedizinische Gemeinschaftstagung, Abstract 51, 2017]. The aim of our work was to compare the efficiency of PGD of bovine IVP embryos using blastocoele fluid or embryonic cells as a source of DNA for sexing the embryos by PCR. Bovine embryos were produced in vitro and all expanded blastocysts were randomly assigned to one of three experimental groups: 1) Collapsed Embryos (CE): blastocoele fluid was collected from blastocysts, 2) Biopsied Embryos (BE): 1 to 5 cells were collected form blastocysts by aspiration and 3) Intact Embryos (IE): blastocysts were left intact. In 1) and 2) blastocyst stage embryos were placed under an inverted microscope equipped with a micromanipulation system for the collection of blastocoele fluid or embryonic cells, as decribed previously for equine embryos [Herrera, Theriogenology 81(5):758-63, 2014; Herrera Theriogenology 83(3):415-20, 2015]. Collapsed, biopsied or intact blastocysts were vitrified and warmed using the vitrification method described by Vajta et al. (1999), except a hemistraw instead of an Open Pulled Straw (OPS) was used as a carrier. After warming, embryos were cultured in vitro and observed for 48 h to detect reexpansion and hatching. The DNA from the blastocoele fluid or from the embryonic cells was amplified by PCR as decribed previously [Herrera, Proceedings in 42° Veterinär - Humanmedizinische Gemeinschaftstagung, Abstract 51, 2017]. The survival rates after warming and in vitro culture for 48 h and the efficiency of amplification after PCR were compared by ANOVA and Fisher's exact test between the experimental groups. The post-warming survival rates of blastocysts did not differ significantly between CE, BE or IE (93.1%, 96.8% and 95.6% respectively) ($P>0.05$). The hatching rates after warming and 48 h of IVC, did not differ between BE or IE (75% vs. 47.8%), was significantly higher for CE when compared to IE (79.3% vs. 47.8%) and did not differ between CE and BE. The amplification rates after PCR was significantly higher for blastocoele fluid samples (41/41, 100%) that for biopsied cells (30/34, 80.3%) ($P<0.05$). The present results demonstrate that blastocoele fluid can be collected from IVP blastocysts and used as a source of DNA for PCR, without impairing the viability of the embryo. In our hands, the use of blastocoele fluid was more efficient than cells after PCR.

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# Bovine embryo lipid metabolism is affected by perfluorononanoic acid (PFNA) exposure during oocyte maturation *in vitro*

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**Keywords**: cow, reproductive toxicology, *in vitro* embryo production.

The final maturation of the oocyte, fertilization and the early embryo development are sensitive processes that can be affected by chemicals. In reproductive toxicology studies, bovine *in vitro* production (IVP) of embryos provides a controlled setting where testing of chemicals is possible without the use of laboratory animals. Bovine IVP is a good model for humans. PFNA is used in consumer products such as water and stain/oil repellent products. The substance is highly persistent and found in both wildlife and nature, but also in human follicle fluid (0.2-2.1 ng/ml (Petro et al., Sci Total Environ 496, 282–288, 2014)). Studies regarding PFNAs potential toxicological effects, especially developmental toxicity, are limited. The aim of this study was to explore the bovine IVP model (Abraham et al., Acta Vet Scand 54:36, 2012) and examine effects of PFNA exposure on oocytes during *in vitro* maturation by evaluation of blastocyst mitochondrial and lipid status since these are suspected to be involved in the mode of action via oxidative stress and peroxisome proliferation-activated receptor α. Abattoir-derived bovine ovaries were used to collect cumulus oocyte complexes (n = 440). The oocytes were matured *in vitro* under PFNA exposure (100 ng/ml to include a safety margin) or non-exposed controls. *In vitro* fertilization and culture were done according to standard protocols. Embryo development was assessed by cleavage rate and blastocyst development and morphology. Day 8 blastocysts were stained for visualization of active mitochondria (MitoTracker® Orange CMTMRos, ThermoFisher Scientific, Waltham, USA) and fixed in paraformaldehyde. Additional staining was done with nuclear stain (Deep Red Anthraquinone 5, BioNordika, Stockholm, Sweden) and neutral lipid stain (HCS LipidTOX™ Green Neutral Lipid Stain, ThermoFisher Scientific, Waltham, USA). For analysis of the embryos confocal laser scanning microscope was used (LSM 510, Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The embryos were manually evaluated. Mitochondrial staining was evaluated with regards to distribution (even distribution to uneven distribution with areas devoid of mitochondria or distinct aggregations of mitochondria). Neutral lipid staining was evaluated with regards to dominating size of lipid droplets. Statistical analyses were performed by linear mixed models and generalized linear mixed models, with replicate as random factor and observations on day 7 and 8 as repeated measures. There was no significant difference ($P>0.05$) between treated and control group regarding, cleavage rate, blastocyst development day 7 and 8, quality grade of blastocysts, stage of blastocysts, number of nuclei or mitochondrial distribution scoring. However, there was a significant difference in distribution of lipid droplet size where the treated group had an increased amount of large lipid droplets ($P = 0.048$). To conclude, the bovine IVP model suggests a disturbance in lipid metabolism but the exact working mechanism of PFNA must be further explored. Funded by Formas 2015-476.

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# Bull spermatozoa have better membrane integrity and mitochondrial membrane potential when cryopreserved with a liposome-based extender

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**Keywords**: semen, single layer centrifugation, liposome-based extender.

Functional, robust spermatozoa are needed for assisted reproduction biotechnologies. Good quality spermatozoa can be separated from the rest of the ejaculate using colloid centrifugation, especially Single Layer Centrifugation (SLC) (Morrell et al., Animal Reproduction 13, 340-345; 2016). To avoid including material of animal origin, such as egg yolk, commercial extenders have been developed containing soy lecithin or liposomes. The aim of this study was to evaluate the effects of SLC on bull sperm quality when spermatozoa were frozen in these extenders. Semen was collected from 12 bulls at a commercial bull station (Viking Genetics, Skara, Sweden). Immediately after collection, each semen sample was split into control and SLC samples. Controls were extended to provide a sperm concentration of $69 \times 10^6$ spermatozoa/mL, in AndroMed® (Minitube, Tiefenbach, Germany - soy lecithin based extender - control A) or OptiXcell® (IMV Technologies, L´Aigle, France - liposomes based extender - control O). SLC samples were extended to $50 \times 10^6$ /mL in Tris-egg yolk prior to centrifugation through the colloid Bovicoll; after SLC the sperm pellet was resuspended in AndroMed® (SLC A) or OptiXcell® (SLC O) to the same concentration as controls. All samples were frozen in 0.25 mL plastic straws. After 3-10 days storage in liquid nitrogen, the straws were thawed at 37°C for 12 s for sperm quality evaluation. Analyses of membrane integrity (MI) and mitochondrial membrane potential (MMP) were made by flow cytometer. Means were analysed using the proc mixed procedure for linear mixed models. Correlations were calculated using Pearson´s correlation test; Scheffe's adjustment was used for multiple-post ANOVA comparisons. Results are presented as LSMeans ± Standard error of means (SEM); the differences were considered significant at $P<0.05$. The samples cryopreserved with OptiXcell® showed better MI and MMP ($P<0.05$) than those cryopreserved with AndroMed®, both in control groups (control O versus control A, MI: 48.5+3.2 vs. 38.8+3.2; MMP: 55.8+3.0 vs 34.1+3.0) and SLC groups (SLC O versus SLC A, MI: 45.5+3.2 vs. 30.8+3.2; MMP: 66.4+3.0 vs. 41.4+3.0). Within extender, no differences were observed between control and SLC for MI ($P>0.05$) (control A versus SLC A, 38.8+3.2 vs. 30.8+3.2; control O versus SLC O: 48.5+3.2 vs. 55.8+3.0) or MMP (control A versus SLC A: 34.1+3.0 vs. 41.4+3.0; control O versus SLC O: 55.8+3.0 vs. 66.4+3.0). In conclusion, bull spermatozoa, selected or not by SLC, have increased MI and MMP when the liposome-based extender was used. Acknowledgement: I Lima-Verde received a fellowship from Brazilian Council of Research (CNPq-Brazil). The project was funded by a project grant from the Swedish Farmers´ Foundation (SLF; H13300339) awarded to JM Morrell and A Johannisson.

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# Comparison of the survival rates of ovarian tissue after slow freezing and vitrification by assessing histological structure and estradiol production during in-vitro culture

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**Keywords**: cryopreservation, ovarian tissue.

Our study was designed to identify a cryopreservation technique, which ensures better surviving of ovarian tissue. The aim is to compare effectiveness of slow freezing (SF) and vitrification (VIT), followed by in-vitro culture and histological analysis. All reagents were purchased from Sigma Aldrich SRL (Milan, Italy), unless other specified. Cortical tissue was isolated from pubertal ovine ovaries, transported from the local slaughterhouse. Slivers (1*5*5 mm) were randomly allocated into six groups (n=5 in each): 1-non frozen control, 2-SF protocol, 3-VIT protocol, 4-non frozen control for in-vitro culture (IVC), 5-SF protocol for IVC, 6-VIT protocol for IVC. The cryoprotectants used in SF protocol were 1.5M ethylene glycol (EG) and 0.1M sucrose (SUC). Vials with samples were thawed in a water bath at 37ºC and then washed in phosphate buffered saline containing 0.75M EG and 0.25M SUC. In VIT protocol cryoprotectants were 2.5M dimethyl sulfoxide, 2.5M EG and 0.5M SUC. Warming performedn at 37ºC in McCoy's 5a medium contained 0.5M SUC and then washed in the medium with 0.25M SUC. For the histological analysis pieces of tissue were fixed in 4% paraformaldehyde, then dehydrated in series of ethanol and embedded in paraffin. The samples were sectioned (5 μm) and stained with hematoxylin and eosin. Follicles in the tissue were assessed by criteria established in our laboratory (Martelli et al., J Mol Endocrinol, 2006) and classified into three quality groups: intact, partially damaged and degenerated. Cortical strips were cultured in McCoy's 5a medium for 6 days at 37ºC and 5% CO2 with medium changed every 2 days. Then culture medium was analysed for the content of estradiol (E2) by ELISA assay (DRG, Marburg, Germany). The proportion of normal follicles showed significant difference between SF (total number of follicles counted=177) and VIT groups (total number of follicles=223): 27,96% vs 19,36% (P<0,001, $\chi^2$ test). After the in-vitro culture, 84 and 69 follicles in total were counted for the SF and VIT groups, respectively. In this case, a higher percentage of intact follicles after slow freezing also has been shown: 21,87% vs 16,52% (0<0,001, $\chi^2$ test). The mean E2 concentrations for days 1,3 and 6 of in-vitro culture after SF protocol were 3,1 pg/ml; 11,4 pg/ml and 12,1 pg/ml, which were 20% lower, than values for non-frozen control (3,7 pg/ml; 13,6 pg/ml and 14,6 pg/ml). However, the difference of E2 concentration from the non-frozen control was even greater for the VIT group, where the values were more than 50% lower: 1,8 pg/ml; 5,9 pg/ml and 7,6 pg/ml. An increase of E2 concentrations during the in-vitro culture was observed, which proved tissue recovering after cryopreservation. Slow freezing is ensuring better morphological structure of ovarian cortex than vitrification. More specifically, a higher number of morphologically healthy follicles could be seen and a better production of estradiol during in-vitro culture of ovarian slivers was present.

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# Annual control chart of bull semen freezability

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**Keywords**: freezability, bull semen, CASA.

Optimization of the center bull production is depending on the application of a fine control chart creation. The use of CASA system improved the precision and accuracy on the early identification of production problems. Aim of the present work was the modelling of a control chart that considered the monthly variations during a whole year in terms of semen freezability and number of straws produced. A total of 536 ejaculates were immediately evaluated, diluted with Andromed® and frozen using an automatized freezer (Digitcoo5300 ZV, IMV). Thereafter the straws were plugged in liquid nitrogen. Fresh and post-thawed motilities where evaluated. Computer assisted sperm analysis (CASA) parameters were analyzed considering the monthly variations of individual kinetic characteristics as average pathway velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL) and hyperactive % (HYP). The variations were scored in terms of number of SD from the average value for each parameter. Delta motility (difference between post-thawed and fresh semen motility) was higher during the summer and autumn period and in Holstein bulls (up to -30%). Collected and elaborated data were analyzed through ANOVA for repeated measures using month and season as independent variables while breed, semen quality parameters and number of straws as dependent variables. The number of produced straws were significantly changed between the spring and the summer months indicating the lower resistance of the bulls to the hot environment. CASA parameters as VAP, VCL, VSL and HYP reinforce the indication that heat stress can influence up to three months the freezability of bull semen.

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# Effect of conceptus size on embryo-maternal communication during early pregnancy in cattle

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**Keywords**: uterine-explant, conceptus, transcriptome.

Conceptus elongation is an essential prerequisite to maternal recognition of pregnancy and implantation in cattle. During elongation, the trophoblast cells secrete interferon-tau (IFNT), which prevents the upregulation of oxytocin receptors in the endometrium required for prostaglandin-induced luteolysis. Large variation exists in the length of conceptuses recovered on the same day of gestation, which may reflect an inherent lack of developmental competency. For example, larger conceptuses produce more IFNT, but the underlying factors that regulate conceptus-maternal crosstalk between advanced (large) or retarded (small) conceptuses and the endometrium are unknown. Thus, the aim of this study was to interrogate the response of the endometrium to Day 15 conceptuses of different sizes by examining the global transcriptome profiles of uterine explants exposed to large vs small conceptuses. First, 10 grade 1 *in vitro* produced blastocysts were transferred into synchronized recipient heifers on Day 7 for further development. The resulting conceptuses were recovered on Day 15 by *post-mortem* uterine flushing. Seven large (mean length ± SEM 25.4 ± 5.7 mm) and six small conceptuses (1.8 ± 0.3 mm) were individually placed on top of endometrial explants that had been collected from uteri during the late luteal phase of the estrous cycle, and co-cultured for 6 h in one mL of RPMI media. Additional explants were cultured with media containing 100 ng/mL of recombinant ovine IFNT (IFNT; n=6) to identify endometrial responses dependent and independent of IFNT or in media alone (Control; n=6). Total RNA was isolated from explant cultures and analysed by RNA-Seq. Exposure of endometrium to IFNT, a large conceptus or a small conceptus altered ($P < 0.05$) expression of 491, 498 and 230 transcripts, respectively, compared with control endometrium. Further, 223 differentially expressed transcripts were common between conceptus-treated and IFNT-treated explants, and classical interferon-stimulated genes (e.g., RSAD2 and ISG15) were amongst the most upregulated transcripts compared to control endometrium. In addition, 369 transcripts were uniquely altered in explants exposed to large conceptuses and IFNT. Of these transcripts, 101 and 100 were specific to large conceptuses and IFNT-treated endometrium, respectively, while 168 were common to both groups. Only 6 of 108 conceptus-induced differentially expressed genes were shared between small- and large conceptuses. Interestingly, 101 transcripts were exclusively regulated by large conceptuses; of these, *PCSK1*, *TNNI3K*, *MPV17L*, and *IL17* were the most upregulated and *TEPP*, *CACNA1L*, *AQP1*, and *HIP1* the most downregulated. This study provides new knowledge of differences in gene expression in endometrial tissue induced by large and small conceptuses. The results provide a better understanding of the underlying molecular pathways involved in embryo survival and maternal recognition of pregnancy in cattle. Funded by Irish Department of Agriculture, Food and The Marine through the Research Stimulus Fund (Grant number: 13/S/528).

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# Development competence of bovine oocytes selected by brilliant cresyl blue before vitrification

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**Keywords**: oocyte, vitrification, cow.

Numerous factors influence *oocyte cryoresistance*, including diameter of follicle, meiosis stage, functional status of oocytes etc. (Papis K., Slovak J. Anim. Sci., 48, 2015 (4): 163-171). It was shown that bovine oocytes selected by brilliant cresyl blue (BCB) had different developmental competence. Native $BCB^+$ oocytes (oocytes that have finished growth phase in vivo) had significantly higher development competence than oocytes that have not finished growth phase in vivo ($BCB^-$ oocytes, Heleil B. et al., J. Reprod. & Infertility 1 (1):01-07, 2010).In previous studies we demonstrated that pretreatment of bovine oocytes with follicular fluid from follicle 3 mm before vitrification improves nuclear and cytoplasmic maturation and development of vitrified bovine oocytes (Kuzmina T.I. et al., *Reprod Biomed online,* 20(3): S38–S39, 2010). The aim of the present study was to evaluate the development competence of devitrified $BCB^+$ and $BCB^-$ oocytes. Before vitrification cumulus oocyte complexes (COCs) were incubated in BCB solution (26 μM) for 90 min. Oocytes were divided into $BCB^-$ (colorless cytoplasm) and $BCB^+$ (colored) and then COCs were incubated 40 min in follicle fluid (d of follicles ≤ 3 mm). Vitrification was performed by equilibration of oocytes in CPA (Cryoprotective Additive) - 1: 0.7 M dimethylsulphoxide (Me2SO) + 0.9 M ethylene glycol (EG) (30 sec); CPA-2: 1.4 M Me2SO + 1.8 M EG (30 sec); CPA-3: 2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose (20 sec) and loading into straws. After thawing COCs washed by step-wise dilution in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199 alone. COCs were cultured in TCM 199 + 10% (v/v) heat-treated FCS + 50 ng/ml PRL with $10^6$/ml granulosa cells. COCs were cultured in this medium 15 h. Then medium were supplemented by 10 IU/ml hCG. The time of cultivation for $BCB^+$ and $BCB^-$ oocytes were 24 h. After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols. Cleavage and development rates were examined on days 2 and 7 after fertilization, respectively. All chemicals used in this study were purchased from Sigma – Aldrich (Moscow, Russia). A total of 604 COCs were vitrified**,** 399 COCs were treated by BCB. Cleavage was significantly higher in $BCB^+$ oocyte in compared to $BCB^-$ oocytes [51% (103/201) vs. 31 %( 61/198), respectively, $P<0.05$, $\chi^2$ test]. Blastocyst development rate was significantly higher in $BCB^+$ vs. BCB- oocytes [9% (18/201) vs. 1% (2/198), respectively, $P<0.05$, $\chi^2$ test]. In the control group (oocytes have not treated by BCB) the cleavage and blastocyst development rates were significantly low in comparison to the $BCB^+$oocytes [39% (80/205) vs 51% (103/201) and 3(6/205) vs 9(18/201), respectively, $P<0.05$, $\chi^2$ test]. We have not find differences in cleavage and blastocyst development rate between control group of oocytes and group of $BCB^-$ oocytes. In conclusion, BCB test is an effective method for selection of more competent bovine GV- oocytes for vitrification.

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# Simplifying the oviductal cell adhesion test for bovine sperm quality assessment

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**Keywords**: Oviductal adhesion test, sperm quality, endangered breed.

Classic procedures for semen evaluation in breeding soundness evaluation require time and high cost. The evaluation techniques with CASA has limits due to the lack of tools standardization in different laboratories and to data analysis methods. Recent studies cast doubts on the actual predictive ability of this method regarding semen fertility at field level. Therefore, many studies have focused on finding a simple and objective test which can give the maximum correlation between in vitro and in vivo results. In this study, we aim to develop a repeatable protocol of sperm adhesion test using oviductal explant (AOC) and comparing the results with CASA parameters and field fertility (ERCR). In this test, the interaction between spermatozoa and oviduct was assessed by incubation in co-culture oviductal explants, in order to calculate the number of spermatozoa adhered per unit area or adhesion index (AI) and create a correlation between this index and field fertility. Oviductal explant cells on the glass slide were exposed to 5uL of diluted semen, containing approximately 35000 motile sperms. Slides were incubated for the 20 mins. Subsequently, reading of the slide by means of optical microscope (Olympus CX41) and without any staining with magnification of 400 X was performed. A mobile field in which the explant cells of oviductal rectilinearly ran through the major diameter of the field was considered and only motile sperm still adhering to one side of explant cell were counted. Three optic fields (OF) for each slide and the average of the three counts are evaluated. Class 1 (ERCR> 1.00): high fertility; Class 2 (ERCR -1 / + 1): medium fertility; Class 3 (ERCR <1.00): low fertility. Five Frisona breed bulls within each different class have been selected randomly and for each bull three doses of semen belonging to the production lots that have contributed to the definition ERCR were considered. Three doses of semen were considered and for each sample were counted sperm immobilized on three microscopic fields. AI was different ($P<0.05$) among the ERCRs with 26.22±2.34, 17.9±1.44 and 12.64±1.68 sperms/OF for Class 1, Class 2 and Class 3 respectively. After the developmental phase, AOC was applied to a group of endangered Burlina breed bulls (N:8) where the effective field results are difficult to obtain due to the small size of population. Burlina bulls resulted with an AI lower then Class 1 with 15.21±1.14 sperms/OF. AOC test provides a prediction on bull semen fertility. Counting the sperms adhered in three microscopic fields after a co-incubation in PBS can give useful information on the field fertility level. AOC gives additional information to the standard of semen evaluation methods applied to endangered breeds.

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# Effects of polyvinyl alcohol on fresh and post-thawed physiological motion characteristics of Angora goat semen

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**Keywords**: Angora goat, freezability, polyvinyl alcohol.

Small concentrations of the synthetic polymer polyvinyl alcohol (PVA) were found to inhibit the formation of ice in water/cryoprotectant solutions. Therefore, we aim to define a methodology to evaluate the freezability of Angora goat semen diluted with 3 different molecular weighted polyvinyl alcohol (PVA; 9, 18 and 100 kDa) with computer-assisted sperm analysis (CASA) before freezing in which each sperm head trajectory is reconstructed. In total, 30 ejaculates from seven mature Angora bucks (2 years old) were collected twice a week by artificial vagina. Immediately after collection, sperm samples were diluted with three different PVA co-polymers PVA 9, 18 and 100 kDa in with five different concentration 0,001 %, 0,01%, 0,1 %, 1% and %2 added to Tris-egg yolk diluent with 7% glycerol in three experimental groups respectively. After dilution semen was loaded into 0,25 ml French straws and cooled down to + 4C in three hours, frozen in a programmable freezing machine (Digitcool 5300, IMV, France). After thawing, following sperm motion characteristics were evaluated: Progressive motility and kinetic parameters with Hamilton-Thorn CASA, validated for buck semen analysis. Data collected and elaborated were analyzed using through ANOVA with PVA type and concentration as independent variable while CASA parameters as dependent variables. General post-thaw average of motility, progressivity, average pathway velocity (VAP), Straight line velocity (VSL), curvilinear velocity (VCL) and Lateral Amplitude were 55.76, 29.26, 121.84, 97.72 and 8.55 respectively. The mean differences (Δ) of motile percentages between the pre and post-thawed semen were 9.07, -9.73 and 14.58 and Δprogressive motility percentages were -24.71, -22 and -8.90 for three groups of PVA as 9 kDa, 18 kDa, and 100 kDa respectively. Lowest progressivity loss gained with group PVA 100 kDa along with ΔVAP, ΔVSL, ΔVCL and concordantly with ΔTotal rapid percentage. However, post-thaw motion trajectory/characteristics were better in PVA 18 kDa group average considering the lowest loss of ΔBeat cross frequency, ΔStraightness, and Δ Linearity. Δtotal static percentage was highest with PVA 9 kDa group ($P<0,05$). In conclusion, the addition of PVA 100 kDa was beneficial for sperm kinetic parameters. PVA 18 kDa group shown more proper motion characteristics. PVA addition to semen extender can decrease the glycerol concentration and thus can decrease the deleterious toxic effect. Synthetic PVA-derived ice blocking agents can be produced much less expensively than antifreeze proteins, offering new opportunities to improve the sperm cryopreservation.

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# Ultrastructure of porcine embryos after cryopreservation

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**Keywords**: pig, embryo, cryopreservation.

In this study we examined the effect of cryopreservation on the ultrastructure of porcine embryos. It is considered that low survival of pig embryos after cryopreservation is related to a high content of lipid droplets (LDs) in their cytoplasm. In porcine zygotes these organelles occupy up to 60% of their volume (Romek et al., Reprod Domest Anim 44, 24-32, 2009). Cryopreservation can cause damages in the structure of LDs and mitochondria, and microsurgical LD removal enhances survival after cryopreservation (Kawakami M., Animal Reproduction Science 106, 402-411, 2008). Therefore, in the present study we focused on the morphology of mitochondria and LDs, which play a crucial role in embryo metabolism. Under a transmission electron microscope (TEM) we analyzed *in vivo* and *in vitro* derived embryos at three developmental stages: zygote, morula and blastocyst. Polish Large White gilts were artificially inseminated and embryos were collected surgically after flushing oviducts (zygotes) or uteri (morulae and late blastocysts). Additionally, part of the zygotes were cultured up to the morula and late blastocyst stage in the NCSU–23 medium supplemented with 4 mg/ml BSA at 39° C in atmosphere containing 5% $CO_2$ in air. *In vivo* and *in vitro* derived control embryos were fixed immediately after acquisition with 2.5% glutaraldehyde in 0.67 M cacodylate buffer at 4 ° C for 24 h, post-fixed in 1% osmium tetroxide, dehydrated in graded series of ethanol and embedded in PolyBed 812 epoxy resin (Polysciences Inc., Warrington, USA). The embryos were then cut into ultra-thin sections, contrasted with uranyl acetate and lead citrate and examined under the TEM. Experimental groups of embryos were vitrified using the Open Pulled Straw technique, thawed and then processed for TEM. All chemicals, unless otherwise stated, were from Sigma-Aldrich Co. (St. Louis, USA). In non-cryopreserved embryos differences in morphology of mitochondria between developmental stages were evident. In zygotes they were round shaped, contained a small number of cristae and a dark matrix. In morulae and blastocysts mitochondria were elongated, contained more cristae and a bright matrix. LDs were not disturbed. However, *in vivo* and *in vitro* embryos showed damages in ultrastructure after cryopreservation. Mitochondria contained bright vesicles and disturbed inner membranes, while in LDs we observed long cracks, often reaching the surface of organelles. Moreover, vitrification caused changes in LD surface, which was less homogeneous and contained bright areas with irregular edges. Mitochondrial functions including ATP production and calcium homeostasis can be disturbed due to the inner mitochondrial membranes disruption. Furthermore, LD cracks break the continuity of the lipid monolayer on the surface of LDs, which plays a crucial role in regulation of embryo lipid metabolism including lipolysis. Therefore, damages in embryo ultrastructure following cryopreservation may impair its developmental potential, leading to embryo death .

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