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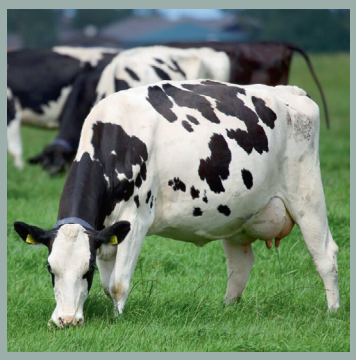


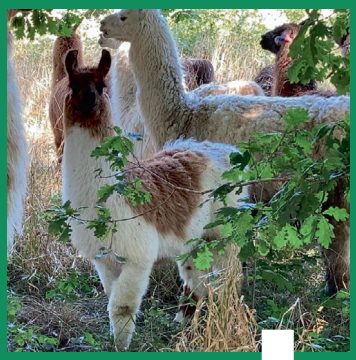

# animal science proceedings

## Proceedings of the 11th International Ruminant Reproduction Symposium (IRRS 2023)

28th May – 1st June 2023
Galway, Ireland

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May 2023
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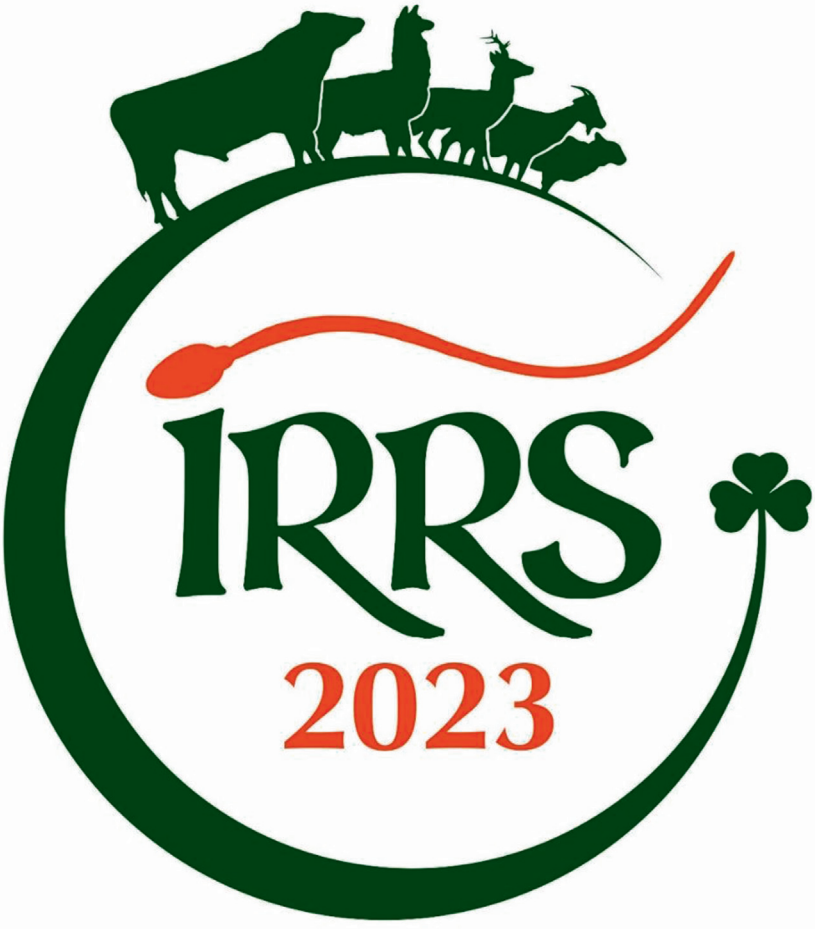


# THE 11th INTERNATIONAL RUMINANT REPRODUCTION SYMPOSIUM

28th May - 1st June Galway, Ireland

# *animal - science proceedings*

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The Proceedings of the 11th International Ruminant Reproduction Symposium constitutes summaries of abstracts to be presented at the Galway Bay Hotel, 29th May – 1st June 2023.



All of the work reported complied with the relevant country regulations on ethics and animal welfare.



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## Welcome

On behalf of the local organizing committee, it is my pleasure to welcome you to the 11th International Ruminant Reproduction Symposium (IRRS) held in Galway, Ireland, from May 28th -June 1st, 2023.

The IRRS is recognized as one of the most prestigious global conferences on reproduction in ruminant animals. Held every four years, leading scientists from around the world present cutting-edge talks on reproductive biology and technology in a variety of ruminant species, including cattle, sheep, goats, buffaloes, and camelids. Sessions are designed to bridge the gap between basic and applied science and cover the entire reproductive axis from follicle development and ovulation, to oocyte maturation and fertilisation, corpus luteum development and maternal recognition of pregnancy, early embryo development, implantation, placentation and foetal development, as well as state-of-the-art assisted reproductive technologies applied to reproduction in ruminants.

Revolving around 3.5 days of scientific sessions, the symposium also includes poster sessions and an exciting social programme, this year with a unique Irish/Gaelic flavour. The symposium itself is truly international, attracting delegates (academics and practitioners) from Europe, North and South America, Africa and Asia. As evidence of its international ethos, the geographical location of the symposium moves significantly every four years. Previous meetings have been in:

1980 - Leura, Australia
1986 - Ithaca, New York
1990 - Nice, France
1994 - Townsville, Australia
1998 - Colorado Springs, USA
2002 - Crieff, Scotland
2006 - Wellington, New Zealand
2010 - Anchorage, USA
2014 - Obihiro, Japan
2018 - Foz do IguaÓu, Brazil

Originally planned for 2022, like many things, this year's meeting was a victim of the consequences of the global Covid-19 pandemic. With the International Congress on Animal Reproduction (ICAR) meeting being pushed from 2020 to 2022, a decision was taken to move the IRRS to 2023 to avoid direct competition and facilitate those who wanted to attend both meetings.

We thank all of the invited speakers for accepting the invitation to come and speak at the symposium, for providing their manuscripts to a very tight deadline and for their willingness to cover their own travel expenses, as is the tradition of the meeting.

Financial support is crucial to the running of any conference. We are very grateful to our many sponsors for their generous support. We realise that such companies and organisations are likely bombarded with similar requests for sponsorship and we appreciate them taking the decision to come to our meeting in Galway. We gratefully acknowledge the support of Ceva Animal Health, Calier, IMV Technologies, DSM Nutrition, Society for the Study of Reproduction (SSR), Failte Ireland, Stroebech Media, IVF Bioscience, EggTech, Minitube, Watanabe Applied Technology, Duneval, Burleigh Dodds, Embryocloud, Genus, Vytelle and the Society for Reproduction and Fertility (SRF).

The Proceedings of the first nine symposia are now open access on the Bioscientifica Proceedings website (https://www.biosciproceedings.org/browse). The Proceedings of the 10th symposium, held in Foz do Iguaçu, Brazil, in 2018 were published in the open access journal, Animal Reproduction. They can be found at the following link: http://www.animal-reproduction.org/ed/5b89839c0e8825a567e4c89d. The manuscripts associated with the talks presented at the 2023 symposium will be published in the open access journal animal. We thank the guest editors and reviewers and the journal staff, particularly Maria Font, for their support.

Lastly, this symposium could not have been organised without the excellent collaboration of our conference partners, the British Society of Animal Science (BSAS). We thank CEO Maggie Mitchell and her team for her excellent guidance and collegiality throughout the process.

Pat Lonergan

Chair, Local Organising Committee

## Pioneer Awards

It has become a tradition at International Ruminant Reproduction Symposia (IRRS) to recognize the outstanding contributions of a select group of individuals to our understanding of ruminant reproduction. At this meeting, the local organising committee are delighted to acknowledge the lifetime achievements of two highly distinguished Irish scientists. The criteria which form the basis of the Pioneer Award include the following: 1) Development of new knowledge that opened areas of research in ruminant reproduction, 2) Development of new technologies that have enabled other investigators to make important contributions to ruminant reproductive biology, 3) A person who is recognised as an international scholar, 4) Known for his/her contributions towards mentoring younger scientists, and 5) An individual with a record of contributing and participating in the IRRS over the years. We are delighted to recognise both Maurice Boland and Jim Roche with the Pioneer Award at the 2023 IRRS meeting in recognition of their contributions, both together and independently, to our knowledge of the reproductive biology of cattle, particularly in the areas of ovarian follicle development, superovulation and embryo transfer.

**Maurice P. Boland**

Maurice Boland was born on a small mixed farm at Kilanerin, near Gorey, County Wexford in what is known as 'the sunny south-east' of Ireland. The youngest of 4 children, his father died when Maurice was less than one year old; his mother, Claire, subsequently re-married and the family grew to a total of eight children over the following years.

Maurice graduated from University College Dublin with a B.Agr.Sc. Degree in 1970. He immediately began a postgraduate research career under the supervision of Professor Ian Gordon (himself a pioneer in the area of reproduction in domestic animals), obtaining a M.Agr.Sc. Degree in 1971 and a Ph.D. in 1973. Much of his Ph.D. was concerned with developing embryo transfer in sheep as a means of breed propagation. As a post-doc, Maurice worked extensively on embryo transfer in cattle and was one of the first to achieve success with nonsurgical embryo transfer techniques. At that time in Ireland there was a particular interest in establishing twin pregnancies in beef cows by transferring an embryo to the contralateral uterine horn of a bred recipient, thereby increasing the efficiency of beef production. The establishment of a simple non-surgical technique for transferring embryos in cattle with relatively high levels of success led to the almost universal adoption of the technique by the embryo transfer industry.

Two periods of sabbatical leave followed. The first of these was as Visiting Professor at the University of California-Davis from 1979 to 1980 working with Gary Anderson, Bob Bondurant and others on various aspects of embryo development including embryo survival following short term storage at 4 degrees Celsius. The second period of leave was at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) at Prospect, Sydney from 1983 to 1984, working with Colin Nancarrow and Jim Murray. This period resulted in the publication of numerous papers ranging from studies on the ovarian response to PMSG and GnRH in ewes immunized against oestradiol 17-beta to the study of chromosomal abnormalities in embryos from superovulated Merino ewes. Maurice has acknowledged that these visits enhanced his view of the research questions he was interested in addressing and always encouraged staff to take such opportunities to see how other groups operated internationally.

Maurice returned invigorated from his stay in Sydney and embarked on major research effort in the area of superovulation and embryo studies in cattle. During this time he worked extensively with Jim Roche and together with successive postgraduate students they published numerous key landmark papers on follicle turnover and postpartum follicular dynamics in cattle.

Over his career, Maurice's main areas of interest have included 1) production and transfer of embryos produced both in vivo and in vitro, 2) factors affecting follicular growth and atresia in cattle and sheep, 3) fertility in the high-yielding dairy cow, and 4) the link between nutrition and reproduction. His impressive list of publications is testament to an extraordinary drive and work ethic and was acknowledged by the awarding of a DSc degree on Published Work in 1983, the most prestigious degree awarded by UCD. Through his career, he supervised approximately 40 Masters students and 30 PhD students in animal

reproduction. He served on the editorial boards of several international journals and has been an active member of both the Association of Embryo Technology in Europe (AETE) and International Embryo Technology Society (IETS), serving as a Board Member of both societies and as President of IETS.

Within UCD, Maurice rose swiftly through the academic ranks from his appointment as College Lecturer (1976), Senior Lecturer (1986), Associate Professor (1990) to his appointment as Chair and Professor of Animal Husbandry (1994). He was appointed Head of what was the Department of Animal Science and Production in the Faculty of Agriculture in 1992 and retained this position until 2001. At the time, he was the youngest staff member in the Department, a measure of the President's confidence in him. He subsequently took on the role of Associate Dean for Research for three years before being appointed Dean of the Faculty of Agri-Food and the Environment in 2004. Following a major re-structuring exercise in the University in 2005, which saw the disappearance of Faculties and Departments and their replacement with Colleges and Schools, he became the Head of the new UCD School of Agriculture, Food Science and Veterinary Medicine. Under Maurice's guidance, the largest School in UCD became one of the most productive in the university. In January 2008, Maurice was appointed as Principal of the College of Life Sciences. This was a significant achievement and a reflection of the esteem in which he is held within the university. After spending almost 40 years in academia, Maurice retired from UCD in 2013 and joined Alltech as Research Director where, for the next five years, he continued to use his research and academic knowledge to expand the role of research in the company.

Maurice is married to Geraldine ('Ger') and together they have 3 daughters, Lisa, Clara and Emma, Maurice and Ger now live in Kilteel, County Kildare, close to the Dublin border and are the proud grand-parents of eleven children. Now retired, Maurice's main interests include set dancing, cycling, bridge and relaxing with his grandkids.

In summary, Maurice Boland is an outstanding scientist, educator and mentor who has made significant contributions to our understanding of reproductive biology in domestic ruminants and is a worthy recipient of the 2023 IRRS Pioneer Award.

**James F. Roche**

James ('Jim') Roche was born and reared on a farm in Oulart, Co. Wexford, Ireland where his interest in agriculture and animal science was nurtured. Jim graduated from University College Dublin with a B.Agr.Sc. Degree in 1965. He then pursued a postgraduate research career under the supervision of Professor Ian Gordon, obtaining a M.Agr.Sc. Degree in 1966. Jim then went to the University of Illinois to complete a Ph.D. under the outstanding supervision of Phil Dzuik (another pioneer of reproductive biology in his own right). While in Illinois, Jim had the privilege of working alongside other significant names in the earlier years of reproductive biology including Fred Karsch, Gordon Niswender, Rees Midgley, Brian Cook, and Doug Foster, to name a few. It was an extremely vibrant laboratory at the time and gave Jim a firm foundation in reproductive biology research. Much of his Ph.D. was concerned with LH secretion in ewes in different physiological states.

After his time in the U.S., Jim returned to Ireland to a 2-year post doc position in the Agricultural Institute (now Teagasc) at Dunsinea, Dublin, following which he was appointed a Senior and, subsequently, Principal Research Officer with the Agricultural Institute Animal Management Department in Dunsany, Co. Meath. While with the Agricultural Institute, Jim commenced working on the use of prostaglandin and progesterone for synchronisation of oestrus in cattle; indeed, it was he who developed the early PRID coil device for progesterone synchronisation having met a person in engineering who helped with the idea of a metal coil surrounded by silastic material. This was a particularly scientifically prolific period in Jim's outstanding research career. He also developed an interest in studying follicle dynamics in cattle and in collaboration with his lifelong friend and colleague in science, Jim Ireland (Michigan State University), they used slaughter studies to develop the dominant follicle hypothesis in cattle based on follicular dynamics through the oestrous cycle. Included in this period was a 6-month sabbatical and Visiting Professorship at Michigan State in 1977. Jim was a very early career recipient of a D.Sc. (the highest degree awarded by the National University of Ireland), in 1980, in recognition of his research.

In 1980, a vacancy arose in the UCD Faculty of Veterinary Medicine. The anatomist/embryologist, Tommy McGeady, recognised Jim's outstanding early career in reproductive biology and convinced him to apply for this position. Despite the fact that, at the time, it was unheard of for a non-Vet to be employed as an academic in the Veterinary School, Jim joined UCD in 1981 as Professor and Head of Department of Animal Husbandry and Production in the Faculty of Veterinary Medicine. In UCD, Jim continued his illustrious scientific career focusing on follicle dynamics and oestrous synchronisation in cattle, reproductive seasonality in sheep while also dabbling in mare and pig reproduction and aspects of sperm preservation in sheep and horses. He successfully supervised ~17 PhD students and 12 Masters Students to completion and was a co-supervisor to a further 10 post-graduates. He was an exceptional PhD supervisor, mentor, colleague and friend. He led a very active research team in reproductive biology alongside his colleague Maurice Boland in the then UCD Faculty of Agriculture to bring about substantial disruptive changes in the areas of bovine follicle dynamics, oestrous synchronisation and embryo transfer. The Faculty of Veterinary Medicine recognised Jim's research by the award of an Honorary MVM in 1987; the Royal Irish Academy admitted Jim Roche to its membership in 1989 - the highest Academic Honour that can be awarded in Ireland.

From 1988, ultrasound scanning became a game changer in understanding and manipulating bovine reproduction and fertility. During this period, Jim, with input from Maurice and an Argentinian PhD student Jorge Savio, pioneered the use of ultrasound scanning of the ovaries and uterus of cattle. This allowed the team to go on to validate many of the hypotheses he had earlier developed around follicle dynamics. A debate ensued regarding the number of follicular waves per cycle, as Jim's team were publishing papers on 3 waves while the North American groups (Ginther in University of Wisconsin and Fortune in Cornell University) were publishing and describing 2 waves of follicle growth per oestrous cycle. Eventually clarity prevailed, much due to Jim's understanding and focus, and it became evident that high yielding dairy cows had a propensity for 2 follicular waves per cycle while heifers, beef cows and moderate yielding dairy cows had a propensity for 3 follicular waves per cycle. Indeed, progesterone concentrations (liver metabolism / clearance) and its feedback on GnRH and LH pulse frequency were the major driver of follicle wave numbers per cycle.

In 1990, Jim requested a sabbatical from the University to go to New Zealand, but the then President of UCD asked him instead to take on the senior role as Director of Development for the University. This led Jim into an administrative role from 1990 to 1992 and he got a curtailed 3-month leave to visit New Zealand as Visiting Professor at the Dairy Research Corporation in 1991. Jim returned to the Faculty of Veterinary Medicine from 1992 to 2000. He then took another career change from 2000 to 2002 when he took on the significant role as Director of Research for Teagasc (formerly the Agricultural Institute). This involved scientific management and prioritisation of the research programme of Teagasc, involving 120 permanent staff, 60 contract and 100 postgraduate Walsh Fellows and a research budget of 60m per year.

Jim returned to the Faculty of Veterinary Medicine in 2002 as Professor of Animal Husbandry and Production where he became Director of Research in the newly combined School of Agriculture, Food Science and Veterinary Medicine from 2005 until his retirement in 2008. During this period, he was successful along with Maurice in winning a Science Foundation Ireland Investigator grant on uterine biology and was also a key member of the team who successfully held a Science Foundation Ireland Reproductive Biology Research Cluster grant. Post retirement he continued to help and contribute to the Cluster.

Key highlights of Jim Roche's research career in ruminant reproduction include:

- Development of the 12-day progesterone PRID coil device for oestrous synchronisation in cattle
- Application of ultrasound scanning to characterise follicle wave dynamics in cattle during the oestrous cycle, post partum (both beef and dairy cows), during pre-puberty and during pregnancy.
- Understanding post-partum resumption of follicle growth, oestrous cyclicity and treatment options for ovarian dysfunction.
- Understanding of progesterone feedback on LH pulsatile secretion and how that regulates follicle dynamics
- Use of this knowledge of progesterone on follicle dynamics to modify and shorten progesterone synchronisation programmes to the 7- to 9-day treatments that are currently in use (this enhanced fertility by avoiding extended periods of follicle dominance and adverse effects on oocyte quality).

Jim now lives and enjoys retirement close to his daughter Aoife in Sligo. He enjoys time gardening and with his family (children and grand-children).

In summary, James Roche is an exceptional scientist, educator, mentor and leader who has made significant contributions to our understanding of ruminant reproductive biology in cattle and sheep, and is a worthy recipient of the IRRS pioneer award.

# Proceedings

## Proceedings of the 11th International Ruminant Reproduction Symposium 2023

### Abstracts

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# The 11th International Ruminant Reproduction Symposium (IRRS 2023)

## MALE FERTILITY

### 001
### Shearing rams in winter triggers an increase in testosterone basal concentrations and a greater response to a GnRH challenge, which are unrelated to scrotal temperature

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**Application:** Shearing rams can trigger their reproductive status.
**Introduction:** Shearing pregnant ewes triggers metabolic changes that increase the energy available. Shearing rams can increase the energy available and facilitate testicular thermoregulation. Therefore, the aim of this study was to compare the basal testosterone concentrations and the response to a challenge with GnRH of rams sheared or not sheared in winter. A complementary aim was to determine whether possible changes were related to changes in testis temperature.
**Materials and Methods:** Eleven adult Corriedale rams were sheared in August (winter in the South Hemisphere), and 11 remained non-sheared. Blood samples were collected 3 days before shearing, and 2, 4, 7, 9, 11, and 14 days later, at 08:00 h. Two days before shearing, and 3, 4, 6 9, 11, and 13 days after shearing thermographic images of the scrotum were taken. Fourteen days after shearing, all rams received 4.2 μg of buserelin, a GnRH analogue, and blood samples were collected immediately before the administration, 30, 90, 150, 210, and 300 min later, and thermographic images were recorded immediately before, 60, 120 and 240 min later. Testosterone concentration was measured with radioimmunoanalysis, and maximum surface temperature was determined in proximal, medial, and distal scrotal areas. The environmental minimum, medium and maximum temperatures during this period were 4.6 °C, 11.6 °C, and 24.4 °C. The data were analyzed with a mixed model including the treatments, time, and their interaction.
**Results:** Sheared rams had greater basal testosterone (5.78 nmol/L vs 3.25 nmol/L, SEM = 0.83, $P = 0.034$) and testosterone response after the GnRH challenge (22.50 nmol/L vs 16.26 nmol/L, SEM = 2.05, $P = 0.044$), with no interaction between treatment and time. Scrotal maximum temperatures during the days after shearing were similar in both groups (proximal area: 31.4 °C vs 31.1 °C, SEM = 0.3, medium area: 30.9 °C vs 30.4 °C, SEM = 0.3, distal area: 29.9 °C vs 29.4 °C, SEM = 0.4 in sheared and non-sheared rams, respectively), and after the GnRH challenge (proximal area: 31.7 °C vs 31.8 °C, SEM = 0.4; medium area: 30.8 °C vs 30.2 °C, pooled SEM = 0.6, distal area: 29.7 °C vs 29.1 °C, SEM = 0.7 in sheared and non-sheared rams, respectively).
**Conclusions:** Shearing rams triggered an increase in testosterone concentrations for at least two weeks and increased the sensitivity to a GnRH challenge. These responses were unrelated to changes in testes' temperature, so are probably explained by metabolic changes. Shearing can be used to enhance ram reproductive performance.
**Acknowledgements:** Funded by CSIC, Universidad de la República, Uruguay

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### 002
### Findings in pre-breeding soundness exams on 93 rams

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**Application:** The health and breeding soundness of the ram is crucial to the results of the breeding season and, thereby, farm economy and sustainability.

**Introduction:** Pre-breeding soundness examinations are easily performed, inexpensive, and a useful tool for assessing the ram's ability to mate and produce offspring. However, in Norway they are rarely used. The aim of the study was to discover the proportion of Norwegian breeding rams failing the physical demands of a pre-breeding soundness examination.
**Materials and Methods:** In October 2020, pre-breeding soundness exams were performed on 93 rams in twelve Norwegian farms. 40 rams were spring-born in 2020, whereas 53 were born in 2014–2019 (median age two years). 69 rams were of the breed Norwegian White Sheep. Other breeds were Suffolk, Texel, eight traditional Norwegian breeds and crossbreeds. For each ram, the general condition was assessed, body condition scored, scrotal circumference measured, penis exteriorised and examined visually, and the scrotum and scrotal contents palpated and examined visually (ultrasound). Semen evaluation was not performed. Based on general health, genital examination, and scrotal circumference, rams were categorised as satisfactory, questionable, or unsatisfactory.
**Results:** Physical examination revealed head wounds (20%), foot lesions (20%), dental problems (13%), chest wounds (13%), ocular (11%) and nasal (2%) discharge, and lameness (4%). Preputial lesions were registered on one spring-born ram and three adult rams. The mean (±SD) scrotal circumference was 36.9 (±3.2) cm for adult rams and 31.1 (±3.1) cm for spring-born rams. Soft, small, or soft and small testicles were noted in 20%, 15% and 10% of spring-born rams, and 6%, 2% and 2% of adult rams, respectively. A hard and uneven consistency of testicles and/or epididymis was noted on palpation in three adult rams, wherein ultrasound examination revealed epididymal/testicular abscesses or large areas of testicular degeneration, or both, in two, one and one rams, respectively. In one adult ram an epididymal abscess, not detected on palpation, was revealed by ultrasound. Testicular degeneration was noted in 21% of adult rams. The rams were categorised as satisfactory (68.8%), questionable (23.7%), and unsatisfactory (7.5%).
**Conclusions:** These results are typical for ram breeding soundness examinations worldwide. Because this study was performed early in the breeding season, some animals with small testicles and a low scrotal circumference may have passed a breeding soundness examination closer to the start of breeding. This study confirms the importance of performing ram breeding soundness evaluations – even if semen evaluation is not possible.

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**003**
## Sire conception rate in dairy and beef cows submitted to timed artificial insemination with sexed and unsexed semen

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**Application:** Sires used for artificial insemination can significantly influence reproductive efficiency in both dairy and beef cows.
**Introduction:** It is recognized that paternal genetics have a substantial role in placenta development, pregnancy establishment, and maintenance, as well as the risk of pregnancy loss. Moreover, sires with comparable initial conception rate differ significantly in the incidence of pregnancy loss. Therefore, the objectives of this study were to assess the effect of sires (dairy and beef) with sexed and unsexed semen on sire conception rate and pregnancy loss during different periods of pregnancy.
**Materials and Methods:** A total of 227 either dry or lactating cows (dairy; $n = 130$ and beef; $n = 97$) were subjected to a 9-day Ovsynch + controlled intravaginal drug release protocol. All cows were timed artificially inseminated using sexed or unsexed semen. Eight sires (four Holstein Friesian and four Angus) with different ejaculates were used, two sexed and two unsexed from each breed type (dairy and beef sires). Pregnancy was confirmed on days 35, 65 and 95 by transrectal ultrasonography and hand palpation to determine pregnancy/embryo loss for first (between days 35 and 65) and second (between days 66 and 95) periods of pregnancy. All data were analysed by general linear model procedure of Statistical Analysis System, 9.3. Chi-square test was used to determine significant differences of conception and pregnancy loss.
**Results:** Sexed semen dairy sires accounted for 61.9% conception rate on day 35 when compared with 56.0% for beef sires, whereas unsexed semen on the same day/period, dairy sires accounted for 62.0% conception rate when compared to 52.2% for beef sires ($P < 0.05$). Concurrently, sexed semen dairy sires accounted for 41.4% conception rate on day 95 when compared with 38.0% for beef sires ($P < 0.05$), whereas unsexed semen dairy sires accounted for 48.5% conception rate on the same day/period when compared with 37.0% for beef sires ($P < 0.05$). Sexed semen dairy sires had 33.3% incidence of pregnancy/embryo loss between days 35 and 65 when compared with 28.6% for beef sires, whereas, on the same period, unsexed semen beef sires had 29.2% incidence of pregnancy loss when compared with 18.2% for dairy sires ($P < 0.05$). Sexed semen dairy sires between days 66 and 95 had 7.7% incidence of pregnancy loss when compared with 5.0% for beef sires, whereas unsexed semen dairy sires had 8.3% incidence of pregnancy loss when compared with 0.0% for beef sires on the same period ($P < 0.05$).
**Conclusions:** These results are significant to quantify sexed and unsexed semen sires involvement in pregnancy success and maintenance, which is crucial to improving reproductive efficiency in dairy and beef cows.
**Acknowledgements:** The study was funded by Agricultural Research Council and Gauteng Department of Agriculture and Rural Development (Grant: P13000022-02).

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## 004
## Fertility of ram semen frozen in pellets or straws

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**Application:** Increase the quality and fertility of frozen ram semen used for artificial insemination (AI).
**Introduction:** A potential source of variation in the success of sheep AI programs is the quality of frozen semen used. Many factors contribute to the post-thaw quality of frozen ram semen, including whether semen is frozen as pellets on dry ice or in PVC straws over liquid nitrogen vapour. Contemporary methods of freezing ram semen in straws and pellets have not been directly compared. As such, this study was undertaken to assess the difference in post-thaw motility, morphology and fertility of ram semen frozen in 0.25 mL straws or as 0.2 mL pellets.
**Materials and Methods:** Six to eight ejaculates were collected from four merino rams ($N = 30$). Each ejaculate was diluted with tris-citrate-egg yolk-glycerol extender and split-processed for straw and pellet freezing using industry standard methods (Evans and Maxwell, 1987). Pellets and straws were thawed, incubated (37 °C, 6 h) and assessed for motility (0, 2, 4, 6 h) and morphology (0 h) using light microscopy. A subset of pellet and straw frozen semen from each ram was used for laparoscopic intrauterine insemination of oestrus synchronised ewes ($N = 536$). Pregnancy of inseminated ewes was determined by ultrasound ~50 days post-AI. Statistical analysis (R studio) of the effect of semen freezing method on *in vitro* semen quality and *in vivo* fertility was undertaken using independent t-tests and binomial logistical regression, respectively.
**Results:** Post-thaw motility (±SEM) of ram semen at 0 h (69.2 ± 0.53% vs 66.8 ± 0.50%, $P = 0.61$), 2 h (65.8 ± 0.36% vs 66.8 ± 0.34%, $P = 0.70$), 4 h (64.3 ± 0.39% vs 63.8 ± 0.27%, $P = 0.85$) and 6 h (64.4% ± 0.49% vs 63.2 ± 0.28%, $P = 0.74$) incubation was not significantly different between samples from straws or pellets, respectively. Sperm morphological abnormalities (±SEM) did not differ ($P = 0.96$) between semen frozen in straws or pellets (13.7 ± 0.23% and 13.8 ± 0.30%, respectively). Pregnancy and reproduction rates of ewes inseminated with semen frozen in straws (68.8 ± 0.03%; 103.6 ± 0.03%) did not differ ($P = 0.32$) to that of ewes inseminated with semen frozen in pellets (66.5 ± 0.03%; 101.2% ± 0.03%), respectively.
**Conclusions:** These findings demonstrate that the post-thaw motility, morphology and fertility of ram semen is similar whether frozen as 0.2 mL pellets or in 0.25 mL straws using current industry practices. Variation in the success of sheep AI programs is likely caused by other factors, the identification of which requires further research.
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## 005
## Testicular heat stress impairs Leydig and Sertoli cell functions in rams

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**Application:** Understanding how heat stress affects testicular function is critical for developing strategies to mitigate its deleterious effects.
**Introduction:** Gonadal heat stress (HS) decreases intratesticular testosterone concentrations and modulates transcriptional profiles of genes involved in testicular antioxidant and pro-apoptotic functions. However, little is known regarding its effects on transcripts regulating Leydig and Sertoli cell functions in ruminants. Our objective was to investigate effects of HS on genes critical for Leydig cell steroidogenesis and Sertoli blood-testis barrier (BTB) functions.
**Materials and Methods:** Twenty-five crossbred rams were subjected to testicular insulation (disposable diapers) for 0–48 h. Infrared thermography was used at diaper removal to confirm HS. Rams were castrated at 0 h (control), 24 h, 48 h, 7 d, or 14 d after the start of insulation (5 rams/time point), and testes tissue samples were snap frozen and stored at −80 °C for RT-q-PCR assay.
Results: Insulation increased testicular temperature by ~5 °C without affecting body temperature (~39 °C). Testicular HS decreased relative mRNA abundance of Low-density lipoprotein receptor (LDLR) and Steroidogenic acute regulatory protein (StAR) at 24 h (−0.5-fold; $P < 0.05$), whereas relative abundance of Peripheral-type benzodiazepine receptor (PBR) mRNA was reduced from 48 h to 7 d (−0.5-fold;

$P < 0.05$). Furthermore, HS decreased relative abundance of junctional adhesion molecules 1 and 3 (JAM-1, JAM-3) at 24 h (−0.4 and −0.25-fold, respectively, $P < 0.05$) mRNA. However, despite recovery of relative mRNA abundance of JAM-1 on day 7, JAM-2 remained downregulated until day 14 ($P < 0.05$). Additionally, relative abundance of zonula occludens-1 (ZO-1) and Claudin (CLDN) were downregulated at day 14 (−0.5-fold; $P < 0.05$).
Conclusions: As LDLR, StAR and PBR genes are critically involved in cholesterol uptake and intracellular transport in Leydig cells, their downregulation explains impaired steroidogenesis. Furthermore, downregulation of JAM-1, JAM-3, CLDN, and ZO-1, key constituents of Sertoli cell's tight junctions forming the BTB, reflect critical impairment of seminiferous epithelium function.
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## 006
## Testicular heat stress reduced testicular weight and spermatid concentration in rams

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**Application:** Understanding how heat stress affects testicular function is critical for developing strategies to mitigate its harmful effects.
**Introduction:** Testicular heat stress (HS) is a major disruptor of spermatogenesis, activating apoptotic pathways and potentially causing testicular cell death. Spermatids are one of the most impacted cell types. However, the time frame of cellular response to testicular HS is not well understood. We hypothesised that HS immediately impacts the testis and decreases the spermatid population.
**Materials and Methods:** Twenty-five crossbred rams (∼1 yo; ∼45 Kg; Fall – temperature variation of 12–22 °C) were subjected to testicular insulation (with disposable diapers) from 0 to 48 h and castrated at 0 (Control), 24 and 48 h, and at 7 and 14 d; $n = 5$/time point). Immediately after castration, testes were weighed and tissue samples were snap frozen and stored at −80 °C, or fixed in buffered 10% formalin for histological measurement of the diameter of the seminiferous tubule (STD). To enumerate spermatids, frozen samples were thawed, weighed individually (average weigh ∼0.8 g), and sonicated for 30 s in 1 ml of milliQ water. After dilution (1:15 v/v) in milliQ water, spermatids were counted in a Neubauer chamber, with data expressed as numbers of spermatids/mg of testicular tissue. After standard embedding, sectioning at 5 μm, and staining with hematoxylin and eosin, 200 tubules were viewed under light microscopy at 1 000× magnification, and STD determined using Opticam® software. Data were analyzed using ANOVA, followed by a Tukey test, and statistical significance was considered when $P < 0.05$.
**Results:** Insulation increased scrotal skin surface temperature from (mean + SEM) 30.23 ± 2.3 °C (Control) to 35.3 ± 1.1 °C and 34.9 ± 1.1 °C after 24 and 48 h, respectively ($P < 0.001$). However, rectal temperature was not affected (∼39 °C). Reductions in spermatid concentration were first identified at 24 h and remained lower throughout the study (Control, $48 \pm 4.2 \times 10^d$/mg vs. $20.5 \pm 3.5 \times 10^d$/mg [data averaged from 24 h to 14 d], $P < 0.01$). Testes weight was greater in Control vs. 14 d (8.15 ± 0.9 vs. 5.2 ± 0.7 g/kg of body weight, $P < 0.05$). Lastly, STD was smaller at 14 d compared to all other days combined (138.9 ± 1.13 vs. 163.7 ± 2.65 μm).
**Conclusions:** In conclusion, heat stress immediately decreased the spermatid population. However, reductions in testicular weight and STD were only significant on day 14, perhaps due to the greater resistance of early spermatogenic stages to HS.
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## 007
## Influence of ram on *in vitro* embryo production efficiency

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**Application:** Selection of the highest semen fertility rams to improve *in vitro* blastocyst yield.
**Introduction:** *In vitro* embryo production efficiency is influenced by several factors. The ram is one of the most important factors that affects the blastocyst yield, due to differences in fertility between male breeds. The objective of this study was to evaluate three rams on *in vitro* embryo production efficiency.
**Materials and Methods:** Ovaries were obtained from a slaughterhouse and oocytes were matured in TCM 199 medium (*In vitro* S. A., Mexico City, Mexico), supplemented with: 10% fetal bovine serum (Mayimex S. A., Mexico City, Mexico), 5 μg/mL FSH (Bioniche, Ontario,

Canada), 5 IU/mL hCG (Merck, New Jersey, United States), and 1 μg/mL 17-β estradiol (Sigma Aldrich, Mexico City, Mexico) for 24 h. The incubation conditions for the entire experiment were 5% $CO_2$, 38.5 °C, and saturated humidity. Fertilization was performed in commercial medium (*In vitro* S.A., Mexico City, Mexico) with fresh semen from three individual rams: Katahdin (*K*), Dorper (*D*), and Rideau Arcott (*R*), all of known fertility. Semen was collected with an artificial vagina 2 h before fertilization and only semen samples with >90% motility were used. Sperm capacitation and selection were performed using the Swim-up technique. Matured oocytes were assigned to one of three treatments: *K* = 314, *D* = 355, and *R* = 338 oocytes. Insemination was performed with $1 \times 10^f$ $mL^{-1}$ of sperm from each ram. After 18 h of incubation, the presumptive zygotes were cultured in cleavage and blastocyst media (Cook IVF, Brisbane, Australia) for 72 and 96 h, respectively, up to the blastocyst stage. The morula and blastocyst rate were determined and analyzed with GENMOD, while the blastocyst diameter was analyzed with GLM, both procedures from SAS 9.3.

**Results:** No difference ($P > 0.05$) was found in the morula rate between *K*, *D*, and *R* (63.7 ± 2.7, 69.9 ± 2.4, and 73.3 ± 2.4%, respectively). However, a higher ($P < 0.05$) blastocyst rate was found when inseminating with semen from *K* and *R* (36.9 ± 2.7 and 39.1 ± 2.6%) compared to *D* (21.4 ± 2.1%). The blastocyst diameter was similar (p>0.05) among the three ram breeds (*K* = 234 ± 9.8, *D* = 230 ± 9.4, and *R* = 224 ± 10.7 μm).

**Conclusions:** In conclusion, only the *in vitro* blastocyst rate was higher when inseminating with semen from Katahdin and Rideau Arcott rams compared to Dorper, under the conditions of this study.

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## 008
## Administration of C6 – a kisspeptin analogue – to improve the reproductive performance in rams during the non-breeding season

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**Application:** To improve the reproductive performance of rams, including their fresh semen quality during the non-breeding season.

**Introduction:** Most sheep breeds have seasonal reproduction patterns, with changes in gonadotropins and testosterone concentration throughout the year. Kisspeptin (kp) is a neuropeptide that stimulates the secretion of gonadotropins. However, the greatest limitation of using kp to stimulate rams' reproductive performance is its short half-life (Chan et al., 2011). However, kp analogs like Compound-6 (C6) have a longer action. C6 administration induces an increase in testosterone concentration in lle-de-France rams (Beltramo & Decourt, 2018). The aim of the study was to compare the testosterone response to three doses of C6 and the associated changes in fresh semen quality in rams during non-breeding season.

**Materials and Methods:** The study was performed during November (spring: southern hemisphere) with adult Corriedale rams, allocated to four treatments. C6 was administered IM (08:00 am) on Days 0, 4, and 8 to rams of the three groups, differing in their doses (G2: 2 nmol/animal, *n* = 10, G5: 5 nmol/animal, *n* = 8 or G15: 15 nmol/animal, *n* = 9). The control group remained untreated (GCon, *n* = 9). Testosterone serum concentration was measured from blood samples daily collected (06:00 am; Day 6–Day 23). Semen was collected on Days 5, 2, 9, 16, and 23 by electroejaculation to assess fresh semen quality. Data were compared with a mixed model that included the treatments and the interaction between treatment and time as main effects.

**Results:** Treatments did not modify testosterone concentration, semen volume, sperm concentration and total sperm per ejaculate. Also, did not modify the percentage of motile sperm, with progressive motility and with normal morphology. The percentage of sperm with functional membrane was greater in GCon, G2, and G5 than in G15 rams (GCon: 69.8 ± 3.4, G2: 67.2 ± 3.5, G5: 70.2 ± 4.0, G15: 56.1 ± 3.7, $P < 0.05$). Also, treatments tended to affect semen mass motility (GCon: 2.3 ± 0.3, G2: 3.0 ± 0.2, G5: 3.0 ± 0.3, G15: 3.0 ± 0.3, $P = 0.06$).

**Conclusions:** Under these experimental conditions and design, there was no effect of C6 on testosterone secretion or fresh semen quality. It is possible that blood samples were not frequent enough to detect rapid changes in testosterone secretion, and those C6 injections were probably too far apart to induce a stable effect. However, further experiments are planned to evaluate if a different dosing regimen provides better results.

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## 009
## Scrotal circumference and body weight ratio as a potential sperm freezability biomarker in young bucks of *Cabra Blanca de Rasquera*

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**Application:** Obtain a useful tool to help in the early selection of young bucks suitable for semen freezing,
**Introduction:** In young males, very few studies have been done related to potential sperm freezability biomarkers in order to perform an early breeder selection for the creation and maintenance of sperm cryobanks. Therefore, the objective of the present study was to perform a retrospective assessment to relate the thawed sperm motility quality of 6 male donors of the *Cabra Blanca de Rasquera* breed, a Catalonian local breed in danger of extinction, at the age of 3 years old with previous measured parameters such as scrotal circumference (SC) and body weight (BW) registered at early age.
**Materials and Methods:** SC and BW measurements of the 6 males were performed every month starting at the age of 9 months from June to December and after this date, every 2 months until the following June. The frozen sperm samples selected ($n$ = 6 replicates/male) for this study belong to semen collections taken in autumn in Northern hemisphere when the males were 3 years old approximately. All the males were subjected to semen collection by artificial vagina performed routinely at a rate of 2 collections/day and 2 days per week. Then, all individual fresh ejaculates were washed in a solution composed of Tris (0.3M), citric acid anhydrous (94.7 mM), and D (+) glucose (27.75 mM) by centrifuging twice and diluted in the extender (15% powdered egg yolk and 5% of glycerol, final concentration). Sperm concentration was adjusted to $400 \times 10^f$ sperm/mL, equilibrated for 4 h at 5 °C and packed into 0.25 mL straws before freezing in liquid nitrogen vapour. After thawing, sperm kinematic parameters were assessed using computer-assisted sperm analysis (CASA) system, ISAS® (PROISER S.L., Valencia, Spain). Statistical analyses were performed using RStudio by the rcorr() function (in Hmisc package) to compute the significance levels for Pearson correlations.
**Results:** No significant correlations were found between thawed sperm motility parameters from adult males and SC measurements registered in early age. However, the SC/BW ratio at 9 months of age was positively correlated with total and progressive motility after sperm thawing ($r$ = 0.82 and $r$ = 0.88, $P$ < 0.05, respectively).
**Conclusions:** The relationship of SC and BW of bucks of 9 months of age could be useful as a predictive measurable indicator of sperm freezability in early age.

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## 010
## Evaluation of semen characteristics during pubescent maturation of Brahman bulls

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**Application:** During sexual maturity, identifying the time period when changes in bovine semen characteristics that hinder reproductive efficiency occurs will improve effectiveness of breeding procedures.
**Introduction:** Given that there are numerous factors that influence reproductive efficiency, the purpose of this study was to compare semen characteristics [motility, viability, chromatin structure, mitochondrial membrane potential (MMP), and/or reactive oxygen species (ROS)] at different points during sexual development of bulls.
**Materials and Methods:** Semen collected from Brahman bulls ($n$ = 23) at six-week intervals was classified based on motility and concentration as prepubertal (<10% and <50 million/mL), peripubertal (≥10% or ≥50 million/mL), pubertal (≥10% and ≥50 million/mL), or sexually mature (≥30% or ≥500 million/mL). Propidium Iodide and SYBR-14 were used to determine viability at collection, after thawing, and after a 3-h stress test. The proportion of sperm with damaged chromatin was identified via the Sperm Chromatin Structure Assay. Mitochondrial membrane potential was determined with JC-1, while the percentage of sperm with elevated ROS was determined by sufficient $H_2O_2$ to convert 2′,7′-dichlorodihydrofluorescein (H2DCFDA) to dichlorofluorescein (DCF) and sufficient superoxide ($^{\cdot}O_2$) to convert hydroethidine to ethidium. Statistical analysis (PROC GLIMMIX; SAS 9.4) included the fixed effect of time on maturation parameters. Maturity was considered significant at $P$ < 0.05.
**Results:** Scrotal circumference ($P$ < 0.0003) and motility [at collection ($P$ < 0.001) and post thaw ($P$ < 0.03)] increased as bulls matured. The proportion of viable sperm cells with high MMP was greater ($P$ < 0.01) in early peripubertal collections (low motility) compared to pubertal and mature collections. The proportion of viable sperm (fresh, post thaw, and post stress) did not differ ($P$ > 0.05) among collections. Nor

did stage of sexual maturity affect the proportion of sperm cells with damaged chromatin ($P > 0.05$), or the proportion of viable sperm cells with high ROS ($P > 0.05$).
**Conclusions:** Stage of sexual maturity did positively impact scrotal circumference and motility (at collection and post thaw), as well as negatively impact MMP. However, stage of maturity was not a factor regarding viability (at collection, post thaw, and post stress), chromatin structure, nor amount of ROS. Therefore, motility and MMP inversely change with sexual maturity, but the proportion of sperm with poor viability, chromatin structure, or elevated ROS did not improve with maturity.
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## 011
## Copy number variation of β-defensin genes and association with bull fertility

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**Application:** Our results may provide insight into the extent and nature of the CNV of β-defensin genes and lay the basis for their inclusion in a future dairy cattle breeding programme.
**Introduction:** β-defensin are cationic peptides traditionally viewed as antimicrobial molecules. However, some β-defensins roles have been uncovered in regulating important traits like fertility in mammals. For instance, DEFB126 has been shown to mediate the attachment of sperm to the oviductal epithelium in macaques and sperm transmigration in cattle. It has also been suggested that β-defensins such as DEFB126 and DEFB129 can be used as potential biomarkers in fertility. Genes encoding β-defensins show extensive copy number variation (CNV) involving both duplications and deletions in cattle and other mammals. However, in cattle, the extent and nature of β-defensin CNV, and its association with fertility, remains unclear.
**Materials and Methods:** We mapped publicly available sequencing data (Consortium of 1000 Bulls) from 100 bulls of different breeds into the genome (ARS-UCD1.2-bosTau9) assembly for the measurement of CNV of 33 β-defensin genes. From these 100 bulls, we chose four and acquired matching genomic DNA to act as positive controls to develop digital droplet PCR (ddPCR) assays to measure CN of β-defensins genes in Holstein-Friesian and Jersey bulls. To investigate whether loss of β-defensin is responsible for impaired field fertility in the Holstein-Friesian, we used bulls with a high ($n = 10$) and low fertility ($n = 10$) phenotype (mean fertility scores +6.5% and −6.6%, respectively), and we determined its statistical significance with Fisher's exact test.
**Results:** For 29 genes, the CN for these four positive control from WGS matched the CNs estimated by ddPCR, therefore ddPCR assays were taken as robust estimates of CN for these genes. According to Fisher's exact test used to determine whether deletion of β-defensins is associated with field fertility, we found that β-defensin gene CNV did not have a strong effect on the field fertility phenotype in Holstein-Friesian. In the evaluation of CNV of β-defensin using different methods, we determined that β-defensin on chromosome 27, in particular DEFB103, shows extensive CNV across breeds, including loss of the gene in Holstein-Friesian with both approaches and even complete loss in some bull. In the Jersey breeds, conversely, this gene showed extensive duplication.
**Conclusions:** However, we could not detect a strong relation regarding the β-defensin gene with fertility.
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## 012
## Experimental challenge with bovine respiratory syncytial virus (BRSV) alters testicular transcriptome and DNA methylation profile in 21-week old Holstein-Friesian bull calves

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**Application:** The results of this study indicate that exposure to bovine respiratory disease during early life could negatively impact testicular development of the bulls. Further work is warranted to examine the latent issues on sexual development and fertility.
**Introduction:** Bovine respiratory syncytial virus (BRSV) is responsible for 60–80% and 5–11% of morbidity and mortality, respectively, in calves globally. Bull calves infected with BRSV between 5 and 7 months of age, had a lower non-return rate when used for breeding at 14 months which was associated with a decrease in normal spermatozoa (Alm et al., 2009). The aim of this study was to examine the concurrent effects of BRSV infection on the testicular transcriptome and methylome at 21 weeks of age in Holstein-Friesian bull calves.
**Materials and Methods:** Holstein-Friesian bull calves ($n = 12$, mean age ± s.d. = 120.7 ± 14.15 days) were either challenged with BRSV ($n = 6$) (BRSV) or received a placebo of sterile phosphate buffered saline (PBS, $n = 6$) (CONT). On day 7 relative to the challenge, calves were

euthanised; testes were removed and weighed. Testicular parenchyma was collected and stored at −80 °C. RNA was sequenced and bioinformatics analysis was performed to detect differentially expressed genes (DEG) between CONT and BRSV calves. Reduced representation bisulfite sequencing analysis was conducted. Methylated CpGs with a minimum coverage of 5x in at least two samples per group were retained. Differentially methylated CpGs (DMCs) and differentially methylated regions (DMRs) were identified using DSS v2.14.0 software with an adjusted *P*-value <0.05 and a minimum methylation difference between groups of 10%.
**Results:** Treatment had no effect on paired testes weight (64.5 ± 4.75 g vs 61.3 ± 3.05 g; $P > 0.05$ for CONT vs BRSV calves, respectively). There were 73 DEG (FDR <0.1, Fold-change >1.5) between CONT and BRSV calves. These DEGs were associated with biological functions involved in connective tissue disorders and immunological disease. There were 610 DMCs and 36 DMRs identified between BRSV and CONT groups. Significant DNA methylation differences between the BRSV and CONT groups were identified for IGF1R. IGF1R is involved in testicular development.
**Conclusions:** Respiratory disease in young calves results in a systemic immune response that is detectable at a molecular level in the testes. Further investigation is required on whether the BSRV induced testicular transcriptome and methylome aberrations observed here have latent effects on testicular development bulls.
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## 013
## Fertility following germline transplantation in sterile *NANOS2* knockout surrogate bulls

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**Application:** In most beef cattle production systems, genetic improvement is largely limited by geographic location due to the logistical need for natural mating schemes. Through spermatogonial stem cell transplantation into germline-ablated recipient males, generation of surrogate sire bulls that produce sperm possessing the genome of higher genetic merit males would be possible. This reproductive tool would allow for improved dissemination of desirable genetics through natural breeding schemes and, therefore, more rapid improvement of production efficiency.
**Introduction:** Previously, we used CRISPR-Cas9 gene editing to generate male mice, pigs, and goats with inactivation of *NANOS2* and found that they were sterile due to ablation of endogenous germ cells but could be a host to produce donor-derived sperm following stem cell transplantation (Ciccarelli et al., 2020; Miao et al., 2019; Park et al., 2017). Applying this strategy to beef cattle, we aimed to generate sterile *NANOS2* knockout bulls and demonstrate fertility in these animals following transplantation.
**Materials and Methods:** Two germline-ablated Angus crossbred bulls were generated by CRISPR-Cas9 editing of the *NANOS2* gene and transplanted during early pre-pubertal development with spermatogenic stem cells from a Holstein donor male. At maturity, the bulls were collected via electroejaculation, and the ejaculates were analysed for sperm concentration, motility, and morphology. Embryos were produced through *in vitro* fertilisation to prove donor origin of the sperm through genotyping analysis. To demonstrate functional fertility, the surrogate bulls were introduced to three mature females for natural mating.
**Results:** After semen analysis, one bull was found to be producing ejaculates with an average sperm concentration of $1.5 \pm 0.7 \times 10^{h}$ cells/ml (mean ± SEM for $n = 5$ different collections) and 89.1 ± 3.6% progressive motility at 18 months of age which is in the normal range for bulls of similar breeds. Post-thaw survival of cryopreserved sperm was in the normal range and use for *in vitro* fertilisation resulted in embryo production. Genotyping analysis of the embryos indicated that the sperm were donor derived. Following natural mating, pregnancies were achieved in 100% of the females bred by the surrogate bull. Assessment of donor-derived sperm production is ongoing for the second transplanted *NANOS2* knockout bull.
**Conclusions:** Together, these findings significantly advance the development of surrogate sires as a potential breeding tool for the beef cattle industry to achieve large scale and widespread dissemination of select genetics to accelerate genetic improvement.
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## 014
## Treatment of ram semen post thaw improves kinematic parameters after incubation in normo-osmolar extenders

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**Application:** Treating frozen ram semen after thawing improves sperm quality.
**Introduction:** At insemination, thawed sperm shift from high osmotic media of the freezing extender (1200 mOsm) to the intrauterine fluids (285 mOsm), representing an osmolar stress as described previously (Hammerstedt et al., 1990). Incubating the thawed semen in normo-osmolar media (1:1) would provide an acclimatation of spermatozoa before insemination. This study aimed to assess sperm kinematic parameters after incubation in three media along 5 or 10 minutes.
**Materials and Methods:** Commercial frozen semen from eight Merino Dohne rams were used. Two straws/ram were thawed (37 °C/40 s) and pooled. Three aliquots were treated (37 °C) with three media (1+1 v/v): ultra-pasteurized skim milk (UHT), embryo holding media (HOLD; BoviHold®), and the same freezing extender (CTRL; Triladyl®). Aliquots were assessed in a CASA system (AndroVision®) at 5 and 10 minutes. Fixed effects were media, incubation-time and their interaction; ram effect was included at random. Results are presented as LSM ± SEM.
**Results:** Media had a significant effect, but not incubation-time or their interaction. UHT samples decreased in progressive and fast progressive motility compared to HOLD and CTRL (70.3, 77.9 and 80.4 %, $P = 0.01$; 55.6, 66.6 and 65.0 %, respectively, $P = 0.008$). Circular motility significantly increased in CTRL, UHT and HOLD (0.5, 1.0, and 1.7 ± 0.17%, respectively, $P = 0.0004$). About sperm velocities, circular was similar for CTRL and HOLD (133.6 and 140.4 μm/s, respectively), but decreased in UHT (114.8 ± 7.9 μm/s, $P = 0.002$); straight and average line were higher in HOLD (68.0 and 79.0 μm/s) compared to CTRL and UHT (56.0 and 67.4 μm/s; 53.8 and 63.5 μm/s, $P = 0.002$), and were similar between CTRL and UHT. Tracked distances were higher in HOLD compared to CRTL ($P = 0.01$), while UHT was similar to CTRL and HOLD (circular: 37.9, 35.8 and 34.3 μm; straight: 16.7, 15.1 and 12.3 μm; average: 20.6, 18.9 and 16.2 μm; HOLD, UHT and CTRL respectively). Similar to distances, beat cross frequency, wobbling, linearity and straightness were higher in HOLD, compared to CTRL. Results might be affected by the viscosity of the media, and/or due to a true benefit on kinematic of decreasing glycerol during incubation, that might impact fertility.
**Conclusions:** Ram sperm kinematics was affected by normo-osmolar media after thawing, five minutes incubation were enough to observe effects. HOLD treatment had better sperm kinematic parameters. If this finding improves fertility must be studied.
**Acknowledgements:** We acknowledge Nicolas Rubio (semen doses donation) and María Ines farm (sheep).

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## 015
## Treatment of ram semen post thaw with normo-osmolar extenders does not improve fertility after cervical or intrauterine artificial insemination

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**Application:** Treating frozen ram semen post thaw might improve fertility results after cervical or intrauterine insemination.
**Introduction:** Thawed sperm undergo osmotic stress (Hammerstedt et al., 1990) from high (about 1200 mOsm) to normo-osmolar environment when deposited into the female reproductive tract (about 285 mOsm). The use of normo-osmolar media to treat semen would mitigate such stress before insemination. The aim of this study was to evaluate fertility of ewes inseminated intracervical or intrauterine with thawed semen preincubated (1 + 1) in a normo-osmolar medium.
Materials and Methods: All procedures were approved by ethics committee (CEUA CENUR-LN, Prot. N°1372). Intracervical insemination was performed in 200 Merino Dohne ewes in natural oestrus (detected by androgenised wethers). Commercial frozen semen doses from four rams were thawed (37 °C/40 s), pooled, and incubated (37 °C/5 min) with embryo holding media (HOLD, 1 + 1 v/v, BoviHold®, Minitube), or with the same freezing extender (CTRL, 1 + 1 v/v, Triladyl®, Minitube). Ewes were randomly inseminated with HOLD treated semen or CTRL (0.5 ml/ewe, 90 million progressive sperm). Intrauterine insemination was performed in 160 Australian Merino ewes synchronized with progesterone sponges (60 mg of MAP, Progespon®, Syntex) for 12 days, plus eCG (300 IU, Novormon®, Syntex) at sponge withdrawal; the ewes were inseminated 60hrs using a laparoscope (Karl Storz®). Commercial semen from two rams was used, and incubated (1 + 1 v/v, 37°C/5 min) with HOLD or CTRL. Transabdominal pregnancy diagnosis (3.5 MHz, Aloka 500) was performed 55 days after insemination. Pregnancy results (%) were compared by the Chi-square Test.

**Results:** Fertility after cervical insemination was high (57%), without statistical differences between HOLD (54%) and CTRL (59%). Pregnancy rate after intrauterine insemination was also high (77.4%), without statistical differences between HOLD (77.3%) compared to CTRL (77.5%).
**Conclusions:** It is concluded that fertility was not improved by osmotic acclimatisation for 5 min, by any of the insemination routes (intracervical or intrauterine). Despite of the osmolar sperm acclimatisation before insemination, the high concentration of the semen dose probably fades any possible improvement of semen treatment post thaw (Salamon and Maxwell, 2000). Future experiments should consider the use of insemination doses with lower sperm numbers.
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## 016
## Unraveling bovine sperm membrane proteins

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**Application:** Artificial insemination is common practice in the cattle industry and, therefore, it allows the use of pre-selected semen. Either for sex selection or quality refinement, immunological methods are increasingly described as possible great allies. Membrane proteins have great potential to be applied in these technologies.
**Introduction:** Further studies on the sperm proteome are needed to develop immunological methods for sperm cells separation according to different intended objectives. Our aim was to determine and characterise the proteome of sperm extracts enriched in cell surface proteins using liquid chromatography with tandem mass spectrometry (LC-MS /MS).
**Materials and Methods:** Sperm surface proteins were isolated from a heterogeneous pool of commercialised bovine semen samples, using the Pierce™ Cell Surface Protein Biotinylation and Isolation Kit, and analysed by LC-MS/MS (EPIC-XS project 186, Horizon 2020). Proteins with less than two valid values in at least one group were not quantified. Functional annotation was performed by using the UniProt database and eggNOG-mapper v2.1.9. Transmembrane topology was predicted using the DeepTMHMM v.1.0.13. Also, PANTHER v17.0 software (http://www.pantherdb.org/, DOI: 10.5281/zenodo.6799722) was used to perform an overrepresentation test (GO-slim) against the *Bos taurus* database to determine the most prevalent gene ontology (GO) terms for biological process, molecular function, and cellular component and their fold enrichment (FE).
**Results:** A total of 17 673 peptide-to-spectrum matches were performed, resulting in 1 337 identified unique peptides, corresponding to 433 identified proteins. After the removal of contaminants, 130 proteins were reliably quantified. At least 40% were already described as present in the plasma membrane, 18% were predicted to have transmembrane domains, and 4% were related to the X-chromosome. Regarding gene ontology, after organizing the results by decreasing FE values ($P < 0.05$), the three most overrepresented biological processes were related to chaperone cofactor-dependent protein refolding (FE = 55.58), 'de novo' protein folding (FE = 53.16), and chaperone-mediated protein folding (FE = 42.16). As expected, two processes related to reproductive structure and system development were also significantly overrepresented, with an FE of 31.35. The three most overrepresented molecular functions were misfolded protein binding (FE = 59.93), ATP binding (FE = 41.95), and heat shock protein binding (FE = 29.11). Regarding cellular components, the most overrepresented were the acrosomal vesicle (FE = 31.35) and the cytoplasm (FE = 1.77).
**Conclusions:** These results contribute to the characterization of the bovine sperm (membrane) proteome and sperm function, which appears to be highly related to protein (re)folding and protein/ATP binding.
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## 017
## Effect of mycotoxins and their mixtures on bovine spermatozoa

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**Application:** Considering the possible harmful effect of mycotoxin mixtures in cattle feed.
**Introduction:** There is growing concern about the effects of mycotoxins on mammalian reproduction. Although the effects of single mycotoxins have been well documented, the impact of their mixtures on sperm quality is less well known.
**Materials and Methods:** Frozen–thawed semen from 5 bulls ($n$ = 2 straws from each bull) was *in vitro* cultured (2 h) with methanol (control), or with (i) a single mycotoxin (zearalenone, ochratoxin A, T-2 toxin or diacetoxyscirpenol) at different doses (0.1, 0.5, 1, 5, 10 ppm; 0.05, 0.1, 0.25, 1, 10 ppm; 0.01, 0.05, 0.1, 0.5 ppm, and 0.01, 0.05, 0.1, 0.5 ppm, respectively); (ii) binary mixtures (ochratoxin A/T-2 toxin, ochratoxin A/zearalenone, ochratoxin A/diacetoxyscirpenol, zearalenone/T-2 toxin, and zearalenone/diacetoxyscirpenol) at three concentration combinations (lowest, intermediate or highest concentration for each mycotoxin); or (iii) ternary mixtures (ochratoxin A/diacetoxyscirpenol/T-2 toxin, ochratoxin A/zearalenone/T-2 toxin, and zearalenone/diacetoxyscirpenol/T-2 toxin), each at intermediate concentration. Then the spermatozoa were examined for cellular features, including plasma-membrane and acrosome-membrane integrity, mitochondrial membrane potential and oxidation status by a flow cytometer. Differences between groups were statistically analysed by ANOVA, followed by Dunnett's method for multiple comparisons. The effect of interactions between mycotoxins was interpreted based on the P-values as follows: (1) a significant higher difference in the mixture vs. the single mycotoxin was interpreted as a synergistic interaction; (2) a significant lower difference between the mixture vs. the single mycotoxin was interpreted as an antagonistic interaction; (3) No significant difference between the mixture vs. the single mycotoxin was interpreted as an additive interaction.
**Results:** Exposure to single mycotoxins or binary mixtures did not affect the spermatozoa's cellular characteristics. However, exposure to the ternary mixtures ochratoxin A/diacetoxyscirpenol/T-2 toxin and ochratoxin A/zearalenone/T-2 toxin reduced ($P < 0.05$) mitochondrial membrane potential relative to the control. In addition, the ternary mixture of ochratoxin A/zearalenone/T-2 toxin impaired the oxidation status of the spermatozoa, reflected by an increased ($P < 0.05$) proportion of spermatozoa with reactive oxygen species relative to the control. The most frequent suggested interaction effect between mycotoxins was additive. A synergistic interaction between mycotoxins was found with respect to oxidation status of the spermatozoa.
**Conclusions:** The current study sheds light on the potential risk of exposing spermatozoa to a mixture of mycotoxins. Further examination of mycotoxin mixtures' effects on fertilization capacity of spermatozoa is warranted.
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## 018
## Variations in sperm kinematic parameters over time is associated with specific genomic regions in Retinta beef cattle

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**Application:** We aim to establish a baseline to help to elucidate the genomic mechanisms associated with variation of sperm motility parameters over time.
**Introduction:** Kinematic traits are a valuable set of characteristics associated with the fertilizing capacity of sperm. In mammals, sperm must keep moving over time in a progressive and viable way across the reproductive tract until they fertilize the egg. In cattle, these parameters are essential in artificial insemination programs, in which the use of cryopreserved sperm reduces sperm motility after thawing. Therefore, uncovering the putative mechanisms affecting the variation of the sperm kinematic over time could be significant in improving the selection of males who are potentially fertile. This study aimed to determine the genetic influence on sperm kinematic parameters over time in cattle.
**Materials and Methods:** We performed a longitudinal experiment over five h evaluating 106 sperm samples from 53 Retinta bulls (two replicates). Five kinematic traits (curvilinear velocity, average path velocity, straight-line velocity, amplitude lateral head displacement, and beat-cross frequency) were assessed. All of them were determined at six times every 60 min using a computer-assisted sperm analysis (CASA). After that, we determined individual pseudo-phenotypes (by trait) which accounted for the kinematic variation over time using an autoregressive linear mixed-effects model, including the time as a fixed effect and the individual as a random effect. In addition, individuals were genotyped using a high-density single nucleotide polymorphism (SNP) array (Axiom BOS 1, 670K). Finally, we performed a genome-wide association study implemented by a linear mixed model-based approach per trait using previously determined pseudo-phenotypes. The raw association *P*-values were adjusted for false discovery rate using the Sequential Goodness-of-Fit (SGoF) multi-test procedure at the chromosome level, with a threshold level of significance of 0.001.

**Results:** A total of 15 SNPs were found as significantly associated with the kinematic traits analysed (*P*-adjusted <0.001). These markers were located on five different chromosomes (BTA1, BTA5, BTA10, BTA11, BTAX), close to the genomic position of 8 genes previously associated with sperm physiology control.
**Conclusions:** This is the first report of genomic regions and SNPs associated with post-thawing sperm kinematic parameters over time in cattle. Most of the genomic regions detected were associated with genes related to sperm kinematic traits, early capacitation and cryopreservation damage. However, further studies including a larger number of individuals are still necessary to obtain more conclusive results.
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## 019
## Sperm Toll-like receptor 2 regulates sperm penetration to uterine glands thereby triggering uterine inflammatory cascade in cattle

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**Application:** The sperm Toll-like receptor 2 (TLR2) mechanism on sperm-triggered mild uterine inflammation may help to improve endometrial receptivity, which in turn improves fertility and embryo transfer efficiency.
**Introduction:** The arrival of sperm into the uterus induces the uterine innate immune response to eliminate dead and excess sperm to prepare the endometrium for implantation. Previously, we have revealed *ex-vivo* that sperm interact with uterine glands mainly *via* the endometrial-TLR2 signalling pathway to induce an acute inflammatory response against sperm (Akthar et al., 2020). Moreover, we have shown that sperm-TLR2 is involved in sperm penetration of oocytes during IVF (Ma et al., 2022). Therefore, this study aimed to test the hypothesis that sperm-TLR2 play a key role in sperm penetration into the uterine glands to induce uterine immune responses.
**Materials and Methods:** To activate TLR2, sperm were treated with specific agonist, Pam3Cys-Ser-(Lys)4. Computer-assisted-sperm-analysis was conducted to investigate sperm hyperactivation (VCL > 200 μm/s & ALH > 3 μm & LIN < 0.4). The % of mucus-penetrated sperm was assessed by layering sperm over estrous-uterine-mucus. The sperm-uterine interactions and immune responses were evaluated using sperm-endometrial *ex-vivo* model. The experiments were repeated five times and the data were presented as mean ± SEM. Statistical analysis (GraphPad Prism) was performed as a *t*-test for 2-groups and ANOVA for more than 2-groups. The effects were considered significant at $p < 0.05$.
**Results:** TLR2-activation increased the % of hyperactivated sperm (19.5 ± 2.4 vs 11.9 ± 2.2) and these TLR2-activated sperm penetrated more (%) the estrous-uterine-mucus (73.9 ± 7.27 vs 54.4 ± 3.90). The TLR2-activated sperm that penetrated the estrous-uterine-mucus showed a higher % of total (87.3 ± 2.99 vs 75.3 ± 4.13), progressive (71.7 ± 3.25 vs 51.0 ± 6.61) and hyperactivated (18.4 ± 1.68 vs 12.5 ± 1.42) motility. A higher number of TLR2-activated sperm (16.5 ± 1.10 vs 12.5 ± 0.55) entered the uterine glands with a clear endometrial immune response, seen as the upregulation ($p < 0.05$) of *TNFA*, *IL1B*, *IL8*, *PGES*, and *TLR2* expression.
**Conclusions:** The findings reveal that sperm-TLR2 activation enhances sperm hyperactivation and subsequent estrous-uterine-mucus penetration with higher motile activity, for entering the uterine glands to initiate the sperm-induced uterine inflammatory cascade. It seems that sperm-TLR2-activation system in the uterus is involved in the mechanisms to eliminate excess sperm from the uterus.
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## 020
## Optimization of cryopreservation protocol for wisent (*Bison bonasus*) epididymal spermatozoa

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**Application:** Creation of high-quality genetic resources for further use in assisted reproduction techniques.

**Introduction:** Wisent (*Bison bonasus*) is one of the endangered ruminant species which population is constantly growing, therefore its protection has been focused on preserving genetic variability, and currently some part of assisted reproduction techniques (ART) is implemented. Epididymal spermatozoa obtained post mortem constitute a source of valuable genetic material that would be lost irrevocably, which makes them a key element in the creation of a gene bank in the restitution program. The aim of this study was to establish an effective protocol for dealing with wisent epididymal sperm.
**Materials and Methods:** Wisent's spermatozoa were obtained post mortem from 14 individuals out of breeding season. Depending on the step, spermatozoa were frozen in Tris buffer and egg yolk-based extender ($n = 9$), with or without prior Percoll® density gradient centrifugation ($n = 4$) or in Tris buffer and egg yolk based extender and Andromed® ($n = 1$). Samples were assessed pre- and post-freezing for sperm motility (CASA), viability (eosin-nigrosin staining), morphology (Watson staining), and functionality (flow cytometry and zona binding assay).
**Results:** On average, $1086.5 \times 10^6$ (range from $245.1 \times 10^6$ to $3700 \times 10^6$) spermatozoa were obtained per one epididymis. Cryopreservation in Tris-based extender allowed to obtain on average: 28.6% (range from 4 to 74%) of viable and 12.8% (range from 0 to 40%) motile spermatozoa. The decrease in the percentage of sperm with intact sperm membrane, intact acrosome and the increase in the percentage of viable spermatozoa with: damaged chromatin, lipid peroxidation, and apoptotic was observed in comparison to pre-freezing values. Centrifugation in the Percoll® density gradient, despite significant losses in a total number of spermatozoa ranging from 43.9 to 86.7% improved the overall quality of the samples and each time allowed to obtain at least a few insemination doses suggested for cattle. In the comparison of the two extenders, sperm motility after thawing was higher in Tris and egg yolk extender (35%) than in Andromed® (15%). The percentage of sperm with an intact cell membrane was higher in the Andromed®. In the zona pellucida binding assay, the average number of bound spermatozoa was significantly higher in the Tris and egg yolk extender (66.3 ± 23.0) than in Andromed® (27.37 ± 6.3).
**Conclusions:** The obtained results of cryopreservation wisent spermatozoa are satisfactory from the point of view of preserving valuable gametes and their use in ART. However, further research is needed to determine the extender that will allow obtaining gametes of higher post-thaw quality.

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## 021
## Evaluation of prenatal transportation stress on semen characteristics of Brahman bulls

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**Application:** Understanding the mechanisms underlying hindered reproductive efficiency among prenatally stressed bulls will allow further investigation into mitigating harmful effects and increase efficiency.
**Introduction:** Prenatal stress reduces reproductive efficiency, but the associated mechanisms are not clear. The aim of this study was to compare sperm viability, reactive oxygen species (ROS), and chromatin structure between bulls that were or were not stressed in utero as these characteristics have explained some of the variation in male fertility.
**Materials and Methods:** Prenatal stress was attempted by transporting Brahman dams for 2 h on days 60, 80, 100, 120, and 140 (±5 d) of gestation in two replicates (Rep1; $n = 9$; Rep2; $n = 18$). Semen collected from sexually mature male offspring born to transported (PNS; $n = 13$, Rep1 = 4, Rep2 = 9) or non-transported (CON; $n = 14$, Rep1 = 5, Rep2 = 9) dams was analyzed to determine semen characteristics. Propidium Iodide and SYBR-14 were used to determine the proportion of spermatozoa that were nonviable or viable, respectfully, both prior to and after a 3-h stress test. The proportion of spermatozoa that contained sufficient $H_2O_2$ to convert 2′,7′-dichlorodihydrofluorescein ($H_2$DCFDA) to dichlorofluorescein (DCF) and sufficient superoxide ($^{\cdot}O_2$) to convert hydroethidine (HE) to ethidium were identified. Mitochondrial membrane potential (MMP) was determined with JC-1. The proportion of spermatozoa with damaged chromatin was identified using the Sperm Chromatin Structure Assay (SCSA) which utilizes acridine orange. Statistical analysis (PROC GLIMMIX; SAS 9.4) included the fixed effects of treatment, replicate, and the treatment × replicate interaction. Treatment was considered significant at $P < 0.05$. Mean separation was performed using LSmeans.
**Results:** Treatment × replicate interaction was not significant ($P > 0.05$). Proportion of viable ($P > 0.05$; PNS = 32 ± 5%; CON = 37 ± 5%) and nonviable ($P > 0.05$; PNS = 68 ± 5%; CON = 63 ± 5%) spermatozoa prior to and after (Viable: $P > 0.05$, PNS = 10 ± 1%, CON = 10 ± 1%; Nonviable: $P > 0.05$, PNS = 90 ± 1%; CON = 90 ± 1%) the stress test did not differ between treatments, nor did the proportion of viable spermatozoa with high ($P > 0.05$; PNS = 33 ± 5%, CON = 25 ± 5%) or low ($P > 0.05$; PNS = 68 ± 5%; CON = 76 ± 5%) mitochondrial membrane potential. Treatment did not impact proportions of viable spermatozoa with sufficient $H_2O_2$ to convert $H_2$DCFDA to DCF ($P > 0.05$; PNS = 30 ± 4%; CON = 37 ± 4%) nor spermatozoa with sufficient $^{\cdot}O_2$ to convert HE to ethidium ($P > 0.05$; PNS = 64 ± 5%; CON = 61 ± 5%). The proportion of spermatozoa with damaged chromatin did not differ ($P > 0.05$; PNS = 1.8 ± 0.2%, CON = 1.8 ± 0.2%) between treatments.

**Conclusions:** Prenatal stress did not impact the semen characteristics measured, thus previously reported differences in embryo development are likely due to epigenetic modifications.
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## 022
## Metabolomics markers associated with fertility in bull semen

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**Application:** Metabolomics may be a tool to predict bull fertility.
**Introduction:** Bull fertility is crucial in bovine industry because one ejaculate from an individual bull is used for artificial insemination (AI) in hundreds of cows. Thus, the potential increase in productivity using bulls with better genetics may enhance the profitability and sustainability of farms. Nevertheless, no reliable test is available for bull fertility prediction with high accuracy. Therefore, the objective of the study was to identify fertility-associated metabolites and pathways in bovine semen.
**Materials and Methods:** Cryopreserved semen samples ($n$ = 40) were used from Angus bulls with high fertility ($n$ = 5; 5 ejaculates per bull) and low fertility bulls ($n$ = 3; 5 ejaculates per bull). Fertility data were obtained from Sexing Technologies database, which had the results from FTAI in the partnering herds (high fertility bulls presented 9% more pregnancy per AI than low fertility bulls; $n$ = 2 000 AI). Samples were processed for metabolite extraction and subjected to high performance liquid chromatography-mass spectrometry (HPLC-MS) target analysis. Data were analyzed by the partial least squares discriminant analysis (PLS-DA), biomarkers and enrichment analysis using MetaboAnalyst 5.0.
**Results:** Seventy-two metabolites were identified in the semen (sperm post-thaw) of all bulls. The PLS-DA showed a clear clustering between groups (High vs. Low fertility). The first two components (1 and 2) explained 44.6% and 21.3% of the variance in the data set, respectively. Six significant metabolites in supervised analyses ($p < 0.05$ and VIP score > 1.5) were observed: Succinic acid, C24 Sphingomyelin, Lauroyl-L-carnitine, O-Decanoyl-L-carnitine, Phosphatidylserine (16:0/18:0) and histidine. The metabolites C24 Sphingomyelin, Lauroyl-L-carnitine, O-Decanoyl-L-carnitine, Phosphatidylserine (16:0/18:0) and histidine had greater concentrations in high fertility bulls. Furthermore, the metabolite succinic acid had lower concentrations in high fertility bulls. Biomarker analyses found differences between groups, with receiver operating characteristic-ROC curve = 0.996; $P < 0.01$). In addition, four metabolic pathways associated with differential metabolites were also explored: carnitine synthesis, oxidation of branched chain fatty acids, methylhistidine metabolism and arginine and proline metabolism.
**Conclusions:** The current study described the metabolome of bull semen using HPLC-MS and presented metabolites as potential biomarkers of bull fertility.
**Acknowledgements:** IonVet and ST repro Brasil.

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## 023
## Relocation of aquaporin-3 (AQP3) in ram sperm after the freeze-thawing process

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**Application:** Cryopreservation of sperm is highly important in sheep conservation and farming profitability. Therefore, acquiring a sound knowledge of molecular mechanisms underlying sperm cryoresistance may provide valuable biomarkers (e.g., aquaporins) for male fertility and sperm cryoresistance.
**Introduction:** The osmotic changes occurring during the freeze-thawing process could be related to variations in cryoresistance. Moreover, aquaporins (AQPs) could adapt their sperm membrane domain location due to osmotic changes. Freezing-thawing processes may exert changes in AQP3 location along the sperm membrane, but it is unknown whether this relocation occurs temporarily or permanently.
**Materials and Methods:** We have explored the AQP3 sperm membrane expression and location in seven rams after freeze-thawing and after incubating the freeze-thawing samples for 6 h in Tyrodés medium, by Western blotting (WB), inmunocytochemistry (ICC) and imaging flow cytometry (IFC) with commercial anti-AQP3 rabbit polyclonal antibody (ab125219).

**Results:** WB confirmed the presence of AQP3 in the sperm samples. The ICC assay showed AQP3 in different sperm regions, including the acrosome, post-acrosome, mid-piece, principal–piece, and end-piece, with a greater ($P < 0.05$) AQP3 location in the post-acrosome region after incubation. In addition, IFC revealed a greater ($P < 0.05$) location of AQP3 in the middle-piece and principal-piece after incubation.
**Conclusions:** In conclusion, incubation after the freeze-thawing process determines the relocation of AQP3 in different sperm areas.
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## 024
## Resulting sex ratio and pregnancy success utilizing different doses of sex-sorted heterospermic semen in beef cows submitted to fixed-time artificial insemination

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**Application:** Use of heterospermic sex-sorted semen in beef cows submitted to TAI.
**Introduction:** The sorting process of SuperConventional semen (70% Y-bearing and 30% X-bearing) can potentially increase pregnancy success as a result of a less damaging sorting process compared to the industry-standard sex-sorted semen (>90% accuracy for X or Y bearing). In addition, the use of heterospermic semen (mixture of semen from more than one male) has been reported to improve fertility in cattle and rabbits. Therefore, the objective of this study was to determine pregnancy success and sex ratio between three different dosages of SuperConventional heterospermic semen.
**Materials and Methods:** Three Angus bulls were utilized to generate the SuperConventional heterospermic semen with each sire contributing to equal amount of sperm cells. A total of 532 multiparous beef cows from 4 different locations were subjected to a standard estrus synchronization protocol and randomly assigned to be fixed time artificially inseminated with one of the following semen treatments: SC4, $4 \times 10^f$; SC6, $6 \times 10^f$ and SC8, $8 \times 10^f$ live sperm cells/straw. Estrus detection patches were applied to all cows at time of progesterone device removal and were evaluated for estrus expression at TAI. Pregnancy diagnoses was performed via transrectal ultrasonography between 30 and 45 d after TAI following by a second pregnancy diagnosis between day 65 and 80 for fetal sexing.
**Results:** Estrus expression at TAI was 39.6% and was similar ($P = 0.34$) between treatments. No interaction between pregnancy rate and location was observed ($P = 0.07$). Pregnancy rates between cows receiving SC4, SC6 or SC8 semen did not differ (29.7 ± 3.6% vs. 33.7 ± 3.4% vs 29.6 ± 3.5%, respectively; $P = 0.63$); however, a positive effect ($P < 0.01$) of estrus expression on pregnancy rate was observed. Cows in estrus inseminated with SC4, SC6 and SC8 had increased pregnancy rates (50.7 ± 5.0%, 50.7 ± 5.2%, 54.8 ± 5.5%, respectively) compared to cows that did not express estrus at TAI (13.0 ± 4.5%, 22.9 ± 4.1%, 14.9 ± 4.2%, respectively [CCL1]). Embryonic mortality between first and second pregnancy diagnosis was 7.6% and was not affected by treatment ($P = 0.90$) or location ($P = 0.98$). The resulting sex ratio was 76.2% male and 23.8% female and did not differ between treatments ($P = 0.91$).
**Conclusions:** Increased dosage of sex-sorted SuperConventional heterospermic semen did not improve pregnancy rates of cows submitted to TAI and the resulting sex ratio was close to 70%, as expected.
**Acknowledgements:** This work was supported by ST Genetics.

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## 025
## Characterization of extracellular vesicles from ovine semen and blood using nanoparticle tracking analysis

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**Application:** Extracellular vesicles are found in several fluids and have important roles as vehicles for exchange of proteins, nucleic acids and lipids among cells. They are also involved in semen cryotolerance in mammalians.
**Introduction:** The objective of this work was to isolate and characterize extracellular vesicles from ovine seminal and blood plasmas.
**Materials and Methods:** Five adult and reproductive sounded rams were submitted to two sections of blood (jugular venipuncture) and semen (artificial vagina) collection, one day apart, and both fluids were centrifuged for obtaining plasmas. Semen was evaluated regarding motility (70–95%) and concentration. Seminal and blood plasmas were firstly submitted to two consecutive centrifugations (300*g* and 2 000*g*, 10 minutes, room temperature) for removal of debris and cells. Fixed volumes (100 μL semen, 1 000 μL blood) of purified plasmas were processed for extracellular vesicles isolation through serial ultracentrifugation: 16 500*g*, 30 minutes, 4 °C, and 2 × 119 700*g*, 70 minutes, 4 °C. Vesicles were observed and quantified using Nanoparticle Tracking Analysis (Nanosight, NS300). The analysis was performed by

capturing five 30-second videos each. Data containing the average number of particles/frame and particle size mode was submitted to descriptive analysis.
**Results:** The average number of particles in 1 000 μL purified blood plasma was 8.08 ± 0.6 billion. Coefficients of variation ranged from 1.6 to 19.1 within individuals and, between individuals, was 19.9. Particle diameter was 173.1 ± 3.8 nm. Coefficients of variation were from 1.7 to 12.3 within individuals and 5 between individuals. Ejaculate volumes were 1.13 ± 0.12 mL and total number of spermatozoa per ejaculate was 1.85 ± 2.51 billion. The average number of particles in 100 μL purified seminal plasma was 89.5 ± 21.7 billion, and coefficients of variation were 10.5 to 68.6 within individuals and 62.4 between individuals. Particle diameter was 138.9 ± 6.5 nm, and coefficients of variation were 1.8–20.8 within individuals and 11.0 between individuals. Upon the analysed volume, the expected number of vesicles per ejaculate (256 ± 49 billion) and the number of vesicles per spermatozoa (152.5 ± 27.1, ranging from 26 to 286) were calculated.
**Conclusions:** The particles found in blood and semen were compatible, regarding size, with small extracellular vesicles. Extracellular vesicles were approximately 100 times more concentrated in seminal plasma than in blood plasma. Concentrations of extracellular vesicles in semen can be highly variable among individuals or individual collection sections. The ration between vesicle and spermatozoa varies from approximately 30 to 300 in the ram semen.
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## 026
## Characterisation of hyperactivation in sperm from bulls with divergent field fertility

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**Application:** A greater understanding of how hyperactivation in sperm differs between bulls with divergent fertility has potential to improve sperm quality testing.
**Introduction:** Many bulls pass stringent semen quality control checks but yield lower than expected pregnancy rates in the field. Recent work by our group demonstrated that frozen-thawed sperm from low-fertility bulls had a reduced capacity to hyperactivate, when stimulated with caffeine, compared to sperm from high-fertility bulls. To better understand the mechanism which could be responsible for the failure of sperm hyperactivation, we assessed a number of inducers with different mechanisms of action including an increase of intracellular pH (procaine), activation of calcium channels (caffeine), or release of intracellular calcium (thimerosal). We hypothesized that the ability of sperm from low fertility bulls to regulate extracellular calcium is compromised and leads to a reduced ability to undergo hyperactivation.
**Materials and Methods:** Frozen-thawed sperm (3 ejaculates/bull) from low ($n$ = 10) and high ($n$ = 10) fertility bulls used in artificial insemination were washed and resuspended in medium with or without calcium. Sperm were either untreated or treated with caffeine (20 mM), procaine (10 mM) or thimerosal (25 μm) and assessed for motility and kinematic parameters, including hyperactivation by computer-assisted sperm analysis. Sperm were stained for intracellular calcium (FLUO-4AM) and viability (Propidium Iodide) and assessed using flow cytometry (Cytoflex) at time 0, 15, 30 and 60 min. Acrosome integrity (PNA-A647) was assessed after 60 min by flow cytometry. All statistical analyses were performed using SPSS. After normality test, either Multivariate General Linear Model (GLM) or GLM for repeated measures using the Tukey post-hoc test was performed. Differences were considered as significant at $P < 0.05$.
**Results:** Caffeine, procaine and thimerosal, all induced hyperactivation in sperm compared to the control ($P < 0.05$) without affecting total motility. Compared to high-fertility bulls, sperm from low-fertility bulls exhibited a reduced ability to undergo hyperactivation when induced with caffeine ($P < 0.05$). This difference was not observed with procaine and thimerosal. In relation to calcium influx, there was only increased uptake of intracellular calcium in sperm resuspended in media with calcium when compared to medium without ($P < 0.05$); however, there was no difference with respect to fertility. Finally, none of treatments induced the acrosomal reaction.
**Conclusions:** The results indicate that hyperactivation in low-fertility bulls is compromised due to calcium channel dysfunction. Further studies are required to study the ability of these calcium channels to regulate hyperactivation in low-fertility bulls.
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# FOLLICLE/OOCYTE

## 027

## *In vitro* embryo development and extracellular vesicle signaling during bovine oocyte maturation in response to palmitic acid and tumour necrosis factor-α supplementation

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**Application:** To understand the impact of lipolysis and inflammation on cumulus-oocyte complexes (COCs) extracellular vesicles (EV) signaling and developmental capacity *in vitro*.
**Introduction:** Negative energy balance and systemic inflammation in the transition period of dairy cows are mirrored in the follicular environment, often resulting in impaired oocyte development. However, less is known about the impact on EV signaling and their miRNA cargo during *in vitro* maturation.
**Materials and Methods:** We supplemented palmitic acid (PA), tumor necrosis factor (TNF)-α, or both to the oocyte maturation medium and evaluated day 8 blastocyst development and quality, and the EV miRNA cargo in the spent maturation medium. Over 10 replicates, 2 517 abattoir-derived COCs were matured in serum-free maturation medium supplemented with 150 μM PA, 10 ng/mL TNF-α, PA +TNF-α (dissolved in ethanol and 0.75% bovine serum albumin), solvent control (ethanol and 0.75% bovine serum albumin only), or control (no supplementation). All groups were routinely fertilized and cultured *in vitro*. Extracellular vesicle isolation from the spent maturation medium was done using size exclusion chromatography and their presence was confirmed via transmission electron microscopy, nanoparticle tracking analysis, and western blotting. Extracellular vesicle miRNA sequencing was done on a NextSeq 500 device (Illumina). Development data were fitted in mixed-effects models, and blastocyst quality parameters were evaluated in mixed linear regression models using the replicate as random.
**Results:** As no differences in blastocyst development nor quality were found between the solvent and control groups ($P > 0.76$), further comparisons were done between the solvent and the experimental groups. Supplementation with PA, TNF-α, and PA+TNF-α decreased ($P < 0.02$) the blastocyst rate (31.5 ± 2.0, 25.2 ± 1.9, 29.7 ± 2.0%, respectively) compared to the solvent control (40.5 ± 2.2%). Palmitic acid, TNF-α, and PA+TNF-α during COC maturation produced blastocysts with lesser numbers of trophectoderm ($P < 0.0001$) and inner cell mass cells ($P < 0.001$) and a greater proportion of apoptotic cells ($P < 0.0001$) than solvent control. Extracellular vesicle-derived miRNA sequencing detected 668 features. Among groups, a total of 17 miRNA were differentially expressed ($P < 0.05$ and log2 fold-change >1) including known miRNA bta-miR: -660, -30a-5p, -30d, -30c, -2904, -12034, -184, -148a, -196a, -92b, and -423-5p. Nine differentially expressed miRNAs were found between PA and TNF-α, which targeted 270 differentially expressed gene ontology terms between groups.
**Conclusions:** Lipotoxicity and systemic inflammation impair oocyte development in which alterations in the EV cargo may play an important role. The analysis of COC EV-derived miRNA suggests different pathways of molecular actions involved in this impairment.
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## 028

## *In vitro* study to examine the effect of the foodborne mycotoxin aflatoxin B1 on bovine oocyte developmental competence and embryo kinetics

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**Application:** Foodborne toxins can potentially be found in food grains, fruit, nuts and other crops used for animal feed. The study demonstrates the risk of exposing bovine oocytes to relatively low concentrations of the mycotoxin aflatoxin B1 (AFB1), suggesting that better regulation of foodborne toxins is required.
**Introduction:** AFB1 is produced by the fungus Aspergillus flavus and considered the most toxic mycotoxin to mammals. About 0.02 μM AFB1 was found in the plasma of lactating cows that were fed with contaminated food. However, the accumulated AFB1 in the follicular fluid and its effect on the enclosed oocyte is unclear. The aims of the current work were to (1) study the effect of AFB1, at relatively low concentrations, on the oocyte, and (2) explore the potential mechanisms underlying AFB1-induced impairments.
**Materials and Methods:** Cumulus oocyte complexes (COCs; $n = 829$) were matured (22 h) with AFB1 (0.032, 0.32, 3.2, 32 μM) or DMSO (0.01% v/v; control). The oocytes were then fertilised (18 h) and the putative zygotes were individually cultured for 190 h in a Miri® time-lapse incubator to evaluate the kinetics and developmental competence of the embryos. In addition, 800 COCs were examined for oxygen consumption (Seahorse); denuded oocytes ($n = 407$) were examined for nuclear and cytoplasmic maturation. A subgroup of oocytes

($n$ = 221) was fertilized, and the putative zygotes were stained with JC1 dye and cultured for 12 h in a IncuCyte™S3 to examine mitochondrial membrane potential. Statistical analysis was conducted by one-way ANOVA followed by Dunnett multiple test.
**Results:** Exposing COCs to 32 μM AFB1 reduced the proportion of zygotes cleaving to 2-to 4-cell-stage embryos relative to controls ($P$ < 0.001); exposure to 3.2 or 32 μM AFB1 reduced the proportion of developed blastocysts ($P$ < 0.01); exposure to 3.2 μM AFB1 resulted in a 2.6 h delay to first cleavage and 2.0 h delay to second cleavage ($P$ < 0.01). Exposing COCs to 32 μM AFB1 impaired oocyte nuclear maturation, expressed as a lower proportion of MII-stage and higher proportion of germinal vesicle-stage oocytes ($P$ < 0.006). Exposure to 3.2 or 32 μM AFB1 impaired oocyte cytoplasmic maturation, expressed as a higher proportion of oocytes with immature cytoplasmic pattern ($P$ < 0.02). Mitochondrial membrane potential in embryos developed from 3.2 μM AFB1-treated COCs was lower at 22 h postfertilization but higher at 26 h postfertilization ($P$ < 0.05).
**Conclusions:** Exposing oocytes to AFB1, even at relatively low concentrations, impaired their developmental competence and kinetics of the *in vitro* cleaved embryo. These were associated with impaired nuclear and cytoplasmic maturation and mitochondrial membrane potential.

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## 029
## *In vitro* embryo production after vitrification of bovine mature and immature oocytes

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**Application:** Freezing and thawing of egg cells must be improved.
**Introduction:** Vitrification is a fast freezing method for oocytes. The aim of this pilot study was investigate the association between vitrification and the developmental capacity of immature and mature bovine oocytes *in vitro* as well as sperm binding to zona pellucida after fertilisation.
**Materials and Methods:** Throughout this study, *in vitro* embryo production started with immature oocytes collected from slaughterhouse ovaries. Oocytes were matured, fertilised and cultured according to standardised procedures. Embryos were assessed through cleavage (44 h after fertilisation) and development (day 8 after fertilisation). Immature oocytes ($n$ = 26) from 5 different batches were denuded and vitrified with an oocyte vitrification kit (Stroebech Media ApS, Hundested, Denmark) according to the manufacturer's recommendations with 3–5 oocytes on each device. Mature oocytes confirmed by the presence of a polar body ($n$ = 28) from 5 different batches were denuded and vitrified as above. The oocytes were kept in liquid nitrogen for a minimum of one week and then thawed using an oocyte warming kit from the same manufacturer. In parallel to the thawing, fresh oocytes were denuded to act as controls (immature: $n$ = 34 and mature: $n$ = 38) and divided into two batches. Day 8 after fertilisation the number of spermatozoa attached to zona pellucida in the immature groups was subjectively but blindly assessed after nuclear 4′,6-diamidino-2-phenylindole staining.
**Results:** The immature vitrified oocytes had a cleavage% of 27% and the controls had 41%. From those that had cleaved around 50% had progressed beyond 2-cell stage in both groups. Day 8 after fertilisation no blastocysts had developed in either immature group. The mean number of spermatozoa attached to the zona pellucida in the immature vitrified group had a mean of 3.5 spermatozoa/zona while the control had 7.9, suggesting a possible change in the zona after vitrification. The matured vitrified oocytes also had a cleavage% of 43% vs. the control with 86% cleaved. From those that had cleaved, 58% had progressed beyond 2-cell stage in the vitrified group and 87% in the control group. On day 8 after fertilisation there were 11% blastocysts in the vitrified group and 43% in the control group (counted from the total number of oocytes before fertilisation).
**Conclusions:** Both immature and mature vitrified oocytes *in vitro* could be fertilised and progress to the first cell divisions but in this study we could only find development all the way to the blastocyst stage from matured vitrified bovine oocytes.
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## 030
## Preliminary effects of pre-maturation of bovine oocytes with C-type natriuretic peptide and oestradiol on blastocyst yield *in vitro*

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**Application:** Bovine embryo *in vitro* production remains inefficient. One of the problems is the acquisition of competence by aspirated oocytes to develop to an embryo after *in vitro* maturation. Meiosis inhibitors, such as C-type natriuretic peptide used at the pre-maturation step to increase oocyte competence, may help to improve blastocyst outcome.
**Introduction:** In an ovary, meiotic arrest enables oocyte growth, along with gaining developmental competence. Oocytes collected by ovum pick up require maturation regardless of insufficient competence level. The proportion of oocytes that develop to the blastocysts stage following *in vitro* maturation (IVM) and *in vitro* embryo production remains low (Lonergan et al., 2006). The inclusion of a pre-maturation step, which inhibits resumption of meiotic maturation, but facilitates improvement of oocyte developmental competence may improve blastocyst yield (Xi et al., 2018).
**Materials and Methods:** Bovine ovaries were obtained from a local slaughterhouse. Cumulus Oocyte Complexes ($n$ = 729) were collected by aspiration. During the pre-maturation step oocytes were cultured in media supplemented with 200 nM (group 1; $n$ = 259) or 200 nM C-type natriuretic peptide and 100 nM 17β-oestradiol (group 2; $n$ = 289) for 6 h, followed by IVM for the next 24 h. The control group ($n$ = 181) was matured for 24 h without pre-maturation. After 10 h of fertilization presumptive zygotes were transferred to culture medium for 8 days. Cleavage (day 3), blastocyst (day 7) and hatched blastocyst (day 8) rates were recorded and compared. A chi-square test (Statistica 13.3.) was performed to analyse whether the pre-maturation step affects the rate of cleavage, blastocysts on day 7 and 8 or hatching. The effect was considered significant at $P \leq 0.05$.
**Results:** Inclusion of the pre-maturation step in group 2 did not improve cleavage, day 8 blastocyst or hatched blastocyst rate: 200 (69.2%), 82 (28.3%), 54 (18.6%) compared to control group: 130 (71.8%), 45 (24.9%), 31 (17.1%), respectively. However, the day 8 and hatched blastocyst ratio in group 1 was significantly lower ($P \leq 0.05$) than that of the control group: 44 (17.0%), 25 (9.7%), respectively.
**Conclusions:** Despite the use of the pre-maturation step with C-type natriuretic peptide and oestradiol, a higher outcome of blastocysts was not obtained. Further studies of other combinations of meiosis inhibitors are in progress.
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## 031
## Measuring cumulus expansion of bovine cumulus-oocyte complexes: comparing the reliability of three methods

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**Application:** To define the most repeatable method for non-invasive cumulus expansion measurement.
**Introduction:** Evaluating cumulus expansion is a commonly used approach to determine oocyte quality. Although several methods have been described to assess cumulus expansion non-invasively, all methods are subjective and no gold standard is declared. Therefore, three methods were compared to determine the most reliable evaluation for cumulus expansion measurements.
**Materials and Methods:** Bovine cumulus-oocyte complexes ($n$ = 232) were individually matured *in vitro* for 22 h in 20 µl droplets of tissue culture medium-199 in 5% $CO_2$ in humidified air at 38.5°C and their images were acquired at 0 h (immature) and 22 h (mature) of *in vitro* maturation. Three observers evaluated cumulus expansion from these images, whereby every observer applied three methods: (1) area (comparing the area of immature vs mature cumulus-oocyte complexes); (2) 3-distance (calculating the shortest, median and longest distance between zona pellucida and extreme of cumulus before and after maturation); and (3) scoring (scoring on a Likert scale ranging from 0 to 4, with 0 = no expansion and 4 = complete expansion). Observers performed all evaluations in duplicate. The reliability of each method was calculated in Python, using a two-way random effects model for inter-observer agreement and a one-way random effects model for intra-observer agreement. Consequently, intra-class correlation coefficients (ICC (95% confidence interval)) were calculated and interpreted as follows: <0.50, poor; 0.50–0.75, moderate; 0.75–0.90, good; >0.90, excellent agreement.
Results: Inter-observer agreement (ICC (95% CI) was good for the area method (0.90 (0.88–0.92)), moderate for the 3-distance method (0.56 (0.49–0.63)) and poor for the scoring method (0.23 (0.12–0.34)). Similarly, for intra-observer agreements, the area method resulted in a good to excellent agreement for observer 1, 2 and 3 (0.87 (0.84–0.9); 0.90 (0.87–0.92); 0.96 (0.95–0.97) respectively). Intra-observer agreement (ICC (95% CI) was moderate for the 3-distance method for observer 1, 2 and 3 (0.61 (0.53–0.69), 0.59 (0.5–0.67), and 0.64 (0.56–0.71), respectively). The scoring method resulted in a poor to moderate intra-observer agreement (0.69 (0.63–0.76), 0.11 (−0.01 to 0.24) and 0.51 (0.42–0.6)) for observer 1, 2 and 3 respectively.
**Conclusions:** Although scoring cumulus expansion on a Likert scale is the most cited method, this study showed that it was the most vulnerable to subjective interpretation. Application of the area method resulted in the most reliable evaluation of cumulus expansion, since this method achieved best inter- and intra-observer agreements.

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## 032
## Transcriptome response of oocytes to seasonal heat stress in beef cows

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**Application:** Identify pathways and associated genes that may play an important role in the response of oocytes to elevated temperatures in cows.
**Introduction:** Reduced reproductive performance due to seasonal heat stress is a major problem in the beef and dairy industries. Examining the effects of heat stress on the molecular mechanism of bovine oocytes can help to better understand their alterations on a transcriptional level and to plan and mitigate those effects. We aimed to study the response of oocytes to seasonal thermal stress.
**Materials and Methods:** Multiparous angus dry beef cows ($n$ = 11) were kept together during the study and subjected to synchronisation and stimulation of follicular growth using a 5-day CIDR and follicle-stimulating hormone (FSH) protocol. Ovum pick-up (OPU) was conducted on all animals in the winter (January) and summer (August). Cumulus-oocyte-complexes (COCs) were isolated from the follicular fluid aspirated during the OPU procedure. Denuded oocytes were further isolated from the COCs, snap frozen, and stored at −80 °C until further use for library sequencing, RNA sequencing, bioinformatics and gene enrichment analysis. Additionally, rectal temperatures were recorded on the day of each OPU. Environmental data were collected daily three weeks before the day of each OPU using the Florida Automated Weather Network. Statistical analysis for average rectal temperature included overall mean (average rectal temperature), treatment effects (season), and the residual. Treatment effect was considered significant at $P \leq 0.05$. Data are presented as mean ± standard error of the mean. RNA was extracted from five biological replicates/pools of oocytes (each containing $n$ = 2) followed by library preparation and sequencing (NovaSeq; Illumina).
**Results:** As expected, environmental conditions were contrasting [average air temperature (11.5 °C vs 27.5 °C), average max air temperature (16.9 °C vs 33.7 °C), relative humidity (83.5% vs 82.3%), and temperature-humidity index (53.39 vs 79.16) for winter and summer, respectively]. Average rectal temperature was greater ($P$ = 0.03) in summer (39.2 ± 0.2 °C) than in the winter (38.8 ± 0.2 °C). Data analysis revealed an up-regulation of 446 transcripts and a down-regulation of 940 transcripts in oocytes collected during summer compared to winter (Fold Change ≥ 2; FDR $P$-value ≤ 0.05). Upregulated transcripts are involved in protein digestion and absorption, ATP-binding cassette transporter, oocyte meiosis, and progesterone-mediated oocyte maturation pathways. Conversely, down-regulated transcripts are involved in pathways related to extracellular matrix-receptor interaction, focal adhesion, and PI3K-Akt signaling.
**Conclusions:** In conclusion, exposure of cows to thermal stress can significantly alter the transcriptome of oocytes, which may negatively impact subsequent developmental competence.

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## 033
## Effect of oocyte recovery method on bovine embryo development using an individual *in vitro* culture system

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**Application:** Acquire data on molecular factors affecting oocyte competence to refine *in vitro* bovine embryo production.
**Introduction:** Developing suitable techniques for oocyte collection and maturation is necessary to efficiently study molecular markers of oocyte competence using slaughterhouse bovine ovaries. An individual *in vitro* culture system allows tracking the development of immature oocytes to the blastocyst stage. However, the conventional technique of follicle aspiration (FA) to retrieve oocytes precludes information on the follicle of origin, critical for molecular studies. We aimed to evaluate follicular dissection (FD) as an alternative technique to recover oocytes when investigating molecular dynamics of oocyte competence.
**Materials and Methods:** Slaughterhouse-derived genital tracts were collected and ovaries were selected if a corpus luteum was present in the contralateral ovary and no follicles ≥15 mm were present in the ovary used for oocyte collection. Selected ovaries were cut in half before processing them for FD or FA respectively. Cumulus-oocyte complexes (COCs; $n$ = 47) were liberated after FD by rupturing the follicles. Recovery of COCs ($n$ = 64) by FA was performed using a 10 ml syringe fitted with an 18-gauge needle. Only 2–8 mm follicles were considered and only COCs with ≥5 compact cumulus layers were selected for individual *in vitro* maturation-fertilization-culture as described previously (Azari-Dolatabad et al., 2019). Group culture (GC) of COCs ($n$ = 310) selected from a random pool of ovaries was per-

formed as a control for each replicate ($n = 4$). Effect of culture systems (individual or group) and oocyte recovery techniques on cleavage, day 7 and day 8 blastocyst rates were fitted in generalized mixed-effects models in which the cow was set as a random factor.
**Results:** Cleavage, day 7, and day 8 blastocyst rates were higher ($P < 0.03$) in GC (86.7 ± 4.6%, 29.0 ± 3.9%, 36.4 ± 3.8%, respectively) compared to FA (74.5 ± 9.0%, 12.2 ± 4.4%, 20.0 ± 5.4%, respectively), whereas they were not different from FD ($P = 0.4$; 78.2 ± 8.9%; 29.2 ± 7.4; 46.1 ± 8.0, respectively). Day 8 blastocyst rate was higher in FD compared to FA ($P = 0.012$), although cleavage and day 7 blastocyst rates were similar ($P = 0.89$).
**Conclusions:** Embryo development is not impaired by FD and improves day 8 blastocyst development when using an individual culture system. These results help to link molecular markers in the follicular environment to the developmental competence of individual oocytes.
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**034**
**Changes in DNA methylation profile associated with the age of oocyte donors influence the metabolic control of bovine blastocysts**

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**Application:** The crosstalk between epigenetics and mitochondrial function is an important aspect of embryonic adaptation, and therefore a good target for improvement of ART technologies.
**Introduction:** The literature recently documented that assisted reproduction procedures may impact gene expression of the embryos possibly leading to altered phenotypes in the offspring. In this study, we describe how modifications in the DNA methylation status of the embryos associated with the age of the oocyte donor may influence metabolism.
**Materials and Methods:** Holstein cows were subjected to ovarian stimulation and oocyte collection in a commercial context at either young (8–10 months old) or older age (16–18 months old). Oocytes were fertilized *in vitro* and collected at blastocyst stage (pools of 5/group, $n = 3$). Total DNA was extracted and submitted to Enzymatic Methyl Sequencing to verify the methylation status of nuclear DNA (nDNA) and mitochondiral DNA (mtDNA). For nDNA, differentially methylated regions (DMRs) considered a window size of 500 bp, and methylation difference was calculated in CpG sites (DMCs) between groups using Methylkit (adjusted-$P < 0.05$). For mtDNA, single cytosine sites were evaluated in all contexts and considered significant when methylation levels (# of methylated reads/# total reads) presented fold-change of 1.5 and adjusted-$P < 0.05$.
**Results:** Age-related analysis revealed that embryos obtained from younger cows were significantly hypermethylated compared to embryos from older ones. A total of 490 DMRs were identified in the nDNA of blastocysts obtained from younger individuals, and ∼52% of these DMRs were located in gene regions (promoters or exons). Besides, 26 DMRs were found in genes that are associated with mitochondrial function. Also, 1 074 DMCs were found in the blastocysts from younger compared to older cows (871 vs 203, respectively). According to gene ontology, the affected biological processes were related to regulation of transcription, pyruvate and lipid metabolism, cell differentiation and migration, all of these being important mechanisms for embryo survival and competency. In mitochondrial DNA, a total of 219 DMCs were identified, most of them also being hypermethylated in blastocysts from heifers compared to adults (143 vs 76, respectively) and located in gene regions that are important for cell homeostasis and maintenance of oxidative phosphorylation (COX1, COX3, CYTB, ND4, ND5 and ND6).
**Conclusions:** In summary, these results indicate that DNA methylation distribution in blastocysts is associated with the age of the oocyte donors. Moreover, embryos produced with oocytes of younger animals show modifications in both nuclear and mitochondrial DNA that could impact metabolic regulation.
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**035**
**Effect of a pre-treatment with C-type natriuretic peptide and estradiol on developmental competence of lamb oocytes**

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**Application:** Improve the developmental competence of juvenile sheep oocytes with increased blastocyst development rates by a pre-IVM incubation with C-type natriuretic peptide (CNP) and estradiol (E2).

**Introduction:** Spontaneous nuclear maturation of oocytes during *in vitro* oocyte maturation (IVM) has been shown to cause a desynchronisation of nuclear and cytoplasmic maturation and a reduction in developmental competence of oocytes. CNP has a role in maintenance of meiotic arrest, with E2 increasing CNP's inhibitory effects through upregulation of the natriuretic peptide receptor 2 (NPR2). The aim of this study was to determine the effect of prematuration (pre-IVM) in the presence of CNP and E2 on meiotic maturation and developmental competence of lamb oocytes.
**Materials and Methods:** Cumulus-oocyte complexes (COCs) were aspirated from abattoir sourced lamb ovaries. Experiment 1: COCs were incubated for 6 h in pre-IVM media containing CNP (0, 10, 100 and 200 ng/ml) or E2 (0, 1, 10 and 100 nM) and meiotic maturation was assessed. Experiment 2: COCs incubated 6-h pre-IVM in media containing 200 ng/ml CNP, 10 nM E2, 200 ng/ml CNP + 10 nM E2, or no CNP or E2 (control) on meiotic maturation was assessed. After 6-h pre-IVM, COCs were matured for a further 18 h in standard IVM medium followed by fertilisation and blastocyst development to day 6 (day 0 = day of IVF). A general linear model with pairwise comparison (SPSS version 25) was used to assess blastocyst rates (6 replicates).
**Results:** Experiment 1: At 6 h, both 200 ng/ml CNP and 10 nM E2 increased ($P < 0.05$) the percentage of oocytes in the germinal vesicle (GV) stage (CNP200: 8.7%; control: 3.9%; E2 10 nM: 11.9%; control 0%). Cleavage and blastocyst development rates were not affected by pre-IVM with any concentration of CNP. Experiment 2: inclusion of E2 and CNP increased the number of blastocysts developed as a percentage of oocytes cleaved (control: 40.6%; E2: 43.9%, CNP: 43.6%; E2 + CNP: 52.9%; $P < 0.05$) and efficiency (percentage of total oocytes) (control: 32.6%; E2: 32.2%; CNP: 35.7%; E2 + CNP: 44.0%; $P < 0.05$), compared with all other treatments.
**Conclusions:** Pre-IVM of lamb oocytes in media containing CNP and E2 improved oocyte developmental competence. This suggests that E2 could play a role in upregulating the CNP receptor NPR2, therefore maintaining CNP's inhibitory effects. Combination of CNP and E2 can be implemented to improve two-step IVM protocols in juvenile ovine oocytes.

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**036**
**Insulin promotes *in vitro* development of ovine preantral to antral follicles**

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**Application:** Producing competent oocytes *in vitro* from small ovarian follicles is a challenge in animal and human reproductive biotechnologies. Besides, such an *in vitro* system would be valuable to study the mechanisms underlying ovarian function.
**Introduction:** Ovarian folliculogenesis up to ovulation, relies on the coordinated development of the oocyte and surrounding cells, including proliferation and differentiation of granulosa cells under the influence of growth factors. Insulin plays an important role throughout follicle development, hence fertility problems commonly associated with metabolic disorders. *In vitro* growth of ovine follicles has been implemented in media containing serum and/or growth factors (growth hormone, activing. . .). Insulin is usually added at a concentration between 10 ng/mL and 10 μg/mL. We have used a defined medium containing insulin as the sole growth factor and investigated the effect of insulin concentration on follicular survival and development.
**Materials and Methods:** Preantral follicles ($n = 185$) between 160 and 240 μm were isolated from peri-pubertal ewe ovaries and incubated individually in a defined medium, which consisted of alpha MEM supplemented with glutamine, hypoxanthine, ascorbic acid, BSA, transferrin, selenium, linoleic acid and 10–50–100–1 000–6 250 ng/mL insulin, but no FSH. The follicles underwent morphological evaluation on days 0, 6, 13 and 20. At the end of culture, they were frozen for subsequent gene expression analysis using the Fluidigm technology. Estradiol concentration was measured in the medium.
**Results:** In the presence of 1 000 ng/mL or more insulin, follicle survival to day 20 was much improved (80% vs 50–57%) and follicles grew to a larger diameter (0.5 mm vs 0.35–0.4 mm). This was accompanied by a spectacular increase in the rate of antrum formation (above 64% vs 0–10%). Expression of 37 genes known to be involved in follicle development was analysed. Clustering segregated samples based on the low or high insulin concentration during culture. However, whether this is rather due to insulin per se or to the larger final follicle diameter can not be delineated. Expression of CYP19A1 encoding aromatase was at least 10-fold higher in granulosa cells from follicles cultured in high concentration of insulin. This, combined with a higher number of cells in these larger follicles, resulted in an increased secretion of estradiol.
**Conclusions:** In our defined medium, addition of 1 000 ng/mL insulin promotes survival and growth of ovine follicles, as well as antral formation.
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## 037
## Nuclear progesterone receptor expression and localisation in growing bovine oocytes

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**Application:** Identifying the factors that confer quality and competence on an oocyte will provide opportunities to manipulate the outcomes and efficiency of *in vitro* oocyte and follicle systems as well as *in vitro* embryo production in cattle.
**Introduction:** Blocking progesterone (P4) signaling during bovine *in vitro* oocyte maturation leads to decreased embryo development following subsequent *in vitro* fertilization and *in vitro* embryo culture, indicating a role for P4 and P4 receptor (PR) activity in oocyte developmental competence (Aparicio et al., 2011). Thus, the objective of this study was to characterize the expression and localization of nuclear PR (nPR) during the growth phase of bovine oocytes.
**Materials and Methods:** Bovine cumulus-oocyte complexes (COC) were recovered from ovaries collected at a local abattoir by ovarian slicing. The COCs were denuded, oocyte diameters were measured and oocytes were subsequently allocated to the following diameter groups 60–100 μm, 100–110 μm and 120–130 μm. The samples were fixed in 4% paraformaldehyde for whole-mount immunofluorescence analysis or snap-frozen for Western blotting. In a separate study, the diameter of 153 bovine oocytes were measured (<50 up to >120 μm) and individually snap-frozen for single-cell RNAseq. A total of 240 immunolabeled oocytes were visualized using a confocal microscope (CLSM). The number of nPR receptors and the volume of each oocyte was determined using IMARIS software. The nPR expression in oocytes 60–100 μm ($n$ = 100), 100–110 μm ($n$ = 70) and 120–130 μm ($n$ = 50) was resolved by SDS-PAGE, and immunoblotting using nPR antibody (Epredia #MS-298). The sum of pixel intensities within an identified band was quantified by iBright software. The raw counts from the RNAseq dataset for nPR gene (PGR) was normalized and reported as counts per million. Comparison between groups were analyzed using Kurskal-Wallis. In addition, the relationship of nPR foci number and oocyte volume was evaluated by Poisson regression.
**Results:** The isoform A of nPR (nPR-A) was detected in oocytes from 60 to 130 μm and generally localized in the cytoplasm of all oocytes. The number of nPR-A foci was positively correlated with oocyte diameter ($P < 0.001$). In addition, growing oocytes (70 μm diameter) presented greater numbers of nPR foci/$\mu m^3$ compared with oocytes 120–130 μm (182 ± 19.08 vs 48.8 ± 84.70, $P < 0.05$). PGR mRNA and nPR-A protein expression were not significantly different between groups.
**Conclusions:** nPR expression is continuous during bovine oocyte growth, suggesting a role for P4 and PR signalling during this process. Further nPR functional analysis will indicate the contribution of P4/PR signalling during oocyte growth and maturation.
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## 038
## Combined effect of thermal stress and cadmium on *in vitro* maturation of ovine oocytes

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**Application:** Effects of combined environmental stressors on mammalian female reproduction.
**Introduction:** The impact of a broad spectrum of environmental stressors, such as heavy metals, on female reproductive health and fertility in mammals is an alarming problem. Cadmium (Cd) is one of the most toxic heavy metals and it is known to adversely affect oocyte quality even at low concentrations (Martino et al., 2017). Information on the interaction of stressors, such as Cd toxicity and global warming on the mammalian female gametes, is still lacking. This study aimed at exploring the effects of combined exposure to heat stress (HS) and low Cd concentration on *in vitro* maturation (IVM) and developmental competence of ovine oocytes.
**Materials and Methods:** Oocytes were collected from ovaries of slaughtered juvenile Sarda ewes, submitted to IVM for 24 h at 38.5 °C without ($n$ = 114, control group, CTR) or with 100 mM $CdCl_2$ ($n$ = 107, Cd group) and at 41.5 °C without ($n$ = 109, HS group) or with $CdCl_2$ ($n$ = 126, HS-Cd group). Following IVM, (i) cumulus cell (CC) expansion and prevalence of apoptosis (Tunel labelling); (ii) oocyte meiotic maturation (Hoechst-33342 staining) and (iii) oocyte intracellular reactive oxygen species (ROS) levels were measured. Moreover, the pronuclear formation and embryo cleavage rates were assessed after oocyte parthenogenetic activation (PA, 10 μM Ionomycin-2 mM,6-DMAP). Data on CCs, nuclear maturation, activation and cleavage were analyzed by Chi-square test; ROS levels by parametric analysis of variance (ANOVA) using Stata/IC 11.2.
**Results:** The percentage of oocytes showing CCs full expansion was lower ($P < 0.05$) in HS-Cd group (11.4%) compared to HS (22.3%), Cd (44.4%) and CTR (53.5%) groups. The CCs apoptotic rate was higher ($P < 0.05$) in HS (74.4%) and HS-Cd (73.5%) groups than CTR (85.5%) and Cd (81.9%) groups. Levels of ROS increased ($P < 0.05$) in HS-Cd group compared to the other groups. Nuclear maturation rate decreased

($P < 0.05$) in HS (79.8%) and HS-Cd (73.8%) oocytes respect to CTR ones (91.2%). After PA, HS-Cd group showed lower percentage of total activated oocytes (71.8%) compared to CTR (88.8%) and lower cleaved embryos compared to the other groups.
**Conclusions:** Our findings indicated that warmer temperature may potentiate the negative impact of low Cd concentration on CCs and oocytes. Further studies are ongoing to better understand joint toxicity mechanisms of HS and Cd exposure on the quality of female mammalian gametes.
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## 039
## Comparison of molecular cargo of follicular fluid exosomes from large dominant and small subordinate follicles in cows

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**Application:** Taken in account a beneficial effect of follicular fluid exosomes (ffExo) in reproduction biotechnologies in bovine, out study will enlighten potential factors relying their role on oocyte quality.
**Introduction:** A beneficial effect of ffExo supplementation during IVM on oocyte quality and embryo development was reported in several animal species including cattle (Asaadi et al., 2021; Singina et al., 2022). However, this effect was not observed with ffExo extracted from large dominant follicles (da Silveira et al., 2017). We aimed to compare protein and lipid cargo between ffExo preparations from small follicles (SF) and large dominant follicles (LF).
**Materials and Methods:** Follicular fluid (FF) was aspirated from small antral follicles (3–6 mm) and dominant follicles (>8 mm) of 12 slaughtered cows ovaries. Fractions enriched in ffExo were obtained by 4 serial centrifugations followed by ultracentrifugation at 100 000*g*. Transmission electron microscopy (TEM) was performed on fixed ffExo. Presence of exosome markers was analyzed by Western blot. Peptide/protein and lipid profiles of ffExo samples ($n = 24$) were acquired by MALDI-TOF mass spectrometer RapifleX Tissuetyper (Bruker Daltonics) in the 200–1 200 $m/z$ range for lipids and 2 000–30 000 $m/z$ range for proteins, in 9 technical replicates each. Spectral processing and statistical analyses were performed using home R software based on MALDIquant & MALDIquantForeign packages. Peaks annotation were obtained from Top-Down proteomics database and Lipidmaps.
**Results:** Exosomes from SF and LF were similar in size (mean diameters 61.4 nm and 59.1 nm, respectively); however, abundance of exosome specific marker CD81 was significantly higher in SF-ffExo preparations ($p < 0.05$). Peptido-protein MALDI fingerprints of ffExo revealed 261 peaks. Among them, abundances of 6 proteoforms were up-regulated and 11 were down-regulated in LF compared to SF exosomes ($p < 0.05$, fold change >1.5). 15 out of 17 differential peaks were annotated and contain protein proteolytic fragments or small proteins known involved in cumulus cells functions. Lipid profiles in negative and positive acquisition modes gathered in total 666 $m/z$ features correspondent to different lipid isoforms. 34 features were significantly up-regulated and 222 down-regulated in ffExo from LF compared to SF (Wilcoxon test, $p < 0.01$, fold change >2). According to annotation, lysophospholipids LPC and LPE were more abundant in LF exosomes, whereas different phosphatidylcholines and sphingomyelins were more abundant in small follicles.
**Conclusions:** Preparations of follicular fluid exosomes from large dominant or small follicles differed more by their lipid composition than by proteins. Significant difference in membrane lipids might explain different affinity of ffExo to target cells including oocyte.
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**040**

## Excessive FSH doses during superovulation of small ovarian reserve heifers induce premature cumulus expansion and diminish oocyte developmental potential

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**Application:** To understand the role of excessive FSH action during superovulation on egg quality.
**Introduction:** Our studies using the small ovarian reserve heifer (SORH) model show excessive doses of Folltropin-V, a commercial FSH-enriched porcine pituitary preparation (cpFSH), do not increase number of ovulatory-size follicles during ovarian stimulation but result in follicular hyperstimulation dysgenesis (FHD) in nearly all ovulatory-size follicles. FHD is characterized by severe abnormalities in multiple cell-signaling pathways in granulosa, cumulus cells, and oocytes critical for folliculogenesis, steroidogenesis, luteinization, cell survival, ovulation, and oocyte maturation and quality. These observations support the hypothesis that excessive cpFSH doses during ovarian stimulation impair cumulus function.
**Materials and Methods:** We used the SORH model to test our hypothesis. Animals received twice daily injections of either 70 IU (industry standard) or 210 IU (3X industry standard) cpFSH for 4 days ($n$ = 10–18 heifers per dose). In Exp 1, ovaries were removed 12 h after the last cpFSH injection, follicles ($\geq$10 mm) were excised, and COC aspirated. Heifers were subjected to oocyte pick-up (OPU) to collect COCs 12 h after the last FSH injection (no LH stimulus given) in Exp 2 and 3, or 24 h after an hCG injection in Exp 4. COCs were classified as expanded or compact. A subset of COCs from heifers 12 h after the last 210 IU cpFSH injection was subjected to IVF ($n$ = 37 COC from 7 heifers) to examine cleavage and blastocyst rates.
**Results:** Ovarian stimulation with excessive vs industry-standard cpFSH doses increased the proportion of ovulatory-size follicles per heifer with expanded COCs in Exp 1 (72 ± 3% vs 0%, $P < 0.001$), Exp 2 (22 ± 5% vs 0%, $P < 0.0001$) and Exp 3 (32 ± 5% vs 3 ± 2%, $P < 0.0001$). However, following the ovulatory hCG stimulus in Exp 4, excessive cpFSH reduced the proportion of expanded COCs (24 ± 4% vs 45 ± 8%, $P < 0.05$). Cleavage and blastocyst rates following IVF of COCs from abattoir ovaries are routinely 80% and 30%, respectively, in our laboratory. However, cleavage and blastocyst rates following IVF of expanded COCs from excessive cpFSH were 5% (2 of 37 COCs) and 0%, respectively.
**Conclusions:** Results indicate that excessive doses of cpFSH during ovarian stimulation disrupt cumulus function and impair oocyte quality, thereby enhancing wastage and risk of poor ART outcomes.
**Acknowledgements:** This study was supported by the USDA-NIH Dual Purpose Program Grant no. 2017-67015-26084, the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health under Award Number T32HD087166, and MSU AgBioResearch.

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**041**

## Single-cell analysis of DNA methylation in growing bovine oocytes

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**Application:** The elucidation of bovine-specific knowledge can provide critical insights regarding factors that affect the epigenetic quality of oocytes in larger mammals, as the chromatin state established in the oocyte likely have a major impact on embryo development as well as postnatal.
**Introduction:** The epigenetic modifications occurring during oogenesis play an important role in gamete competence and embryo development. DNA methylation is erased in primordial germ cells and re-established in oocytes at their growth phase. Information about this wave of DNA methylation is limited for cattle. Therefore, the aim of the present study was to characterise DNA methylation patterns in bovine oocytes during their growth phase; focusing on DNA methylation onset.
**Materials and Methods:** Bovine ovaries sourced at a local abattoir were sliced to obtain oocytes ranging from 60 to >120 μm in diameter. Samples were either fixed in 4% paraformaldehyde (PAF) and processed for immunocytochemistry or snap-frozen for single-cell bisulfite conversion sequencing (scBS-seq). Ovarian cortex samples were also fixed in 4% PAF and embedded in paraffin for sectioning. Statistical analysis was performed using Kruskal-Wallis test.
**Results:** Following scBS-seq data quality control, global CpG methylation was quantified in 149 oocytes demonstrating 13% CpG methylation in oocytes <70 μm, which increased to 42% in fully-grown ones, suggesting that methylation establishment is underway in early antral follicles. To determine if DNA methylation is initiated in oocytes from pre-antral follicles, ovarian cortex tissue sections were processed for immunofluorescent labelling of 5-methylcytosine (5mC) and confocal laser scanning microscopy (CLSM). The images analysed

with ImageJ showed less ($P < 0.01$) labelling in oocytes from pre-antral follicles (2.3 ± 0.3) compared to antral follicles (7.7 ± 1.2), suggesting a baseline methylation level until the secondary follicle. To complement the scBS-seq analysis, 5-hydroxymethylcytosine (5hmC) and 5mC wholemount immunofluorescence was performed on oocytes measuring <100, 100–110, and >110 μm in diameter. The fluorescence intensity of 5hmC was significantly greater ($P = 0.01$) in oocytes <100 μm (15.1 ± 0.9) compared with the other groups (12.1 ± 1.2). In contrast, 5mC labelling was less in ($P < 0.01$) oocytes <100 μm (16.1 ± 0.9) compared with the larger diameter groups (22.4 ± 0.9).
**Conclusions:** This is the first study to evaluate the onset of CpG methylation in growing bovine oocytes, suggesting that DNA methylation is maintained at a basal level until the secondary follicle stage after which it increases until a fully grown stage.
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## 042
## Combined vitrification and *in vitro* culture systems of ovarian tissue in the bovine animal model

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**Application:** Cryopreservation and ovarian tissue culture techniques in a biodiversity preservation program.
**Introduction:** Worldwide, almost 40% of cattle breeds are vulnerable or at serious risk of extinction. Ovarian tissue cryopreservation and in situ ovarian tissue culture are valuable options for genetic salvage of threatened animal breeds and for human fertility preservation. However, ovarian tissue cryopreservation and ovarian tissue culture are still inefficient and experimental, particularly in large mammals. The present study aimed to combine the vitrification technique with different tissue culture conditions to optimise in situ culture of bovine follicles.
**Materials and Methods:** Fragments of ovarian cortex isolated from ovaries collected at a slaughterhouse were divided into three groups. Group 1 was immediately placed in culture. Group 2 was vitrified-warmed and cultured, while Group 3 was only equilibrated, warmed, and cultured. Fragments from each Group were cultured in 24-well dishes with Waymouth's medium in two systems: on culture inserts (0.4 μm pore size; Yang et al., 2017) or on agarose inserts (Gohbara et al., 2010). Spent media were collected on 2, 4, and 6 days of culture to evaluate interleukin 1β and interleukin 6. At the end of each culture period, ovarian fragments were fixed and processed for histology and immunohistochemical detection of apoptotic (activated caspase-3) and proliferation (Ki-67 and MCM-7) markers. Histology/immunohistochemistry data were analysed using chi-square test, interleukin concentration by analysis of variance.
**Results:** A higher percentage ($P < 0.05$) of grade one (morphologically intact) follicles was observed in fragments on agarose compared to those on culture inserts on 2nd and 4th days. No significant differences in grade one follicles were observed on the 6th day except in Group 2. Conversely, we found a higher ($P < 0.05$) shift of primordial follicles to transitional follicles in fragments on culture inserts, consistent with Ki-67 and MCM-7 positive follicle proportion. The opposite was observed with activated caspase-3 positive follicles. No significant variation in the values of stromal cell density and interleukin 6 was found, whereas the concentration of interleukin 1β was higher in fragments on agarose.
**Conclusions:** In situ culture of bovine vitrified-warmed ovarian cortex fragments on agarose inserts maintained follicle morphology, low follicle activation and apoptosis rate compared to culture inserts.
**Acknowledgements:** This work was supported by Regione Lombardia PSR INNOVA n. 201801061529 and PSR R-INNOVA n. 202102146691, Polish National Agency for Academic Exchange under Grant No. PPI/APM/2019/1/00044/U/00001, Università degli Studi di Milano "Piano di Sostegno alla Ricerca 2021 (Linea 2 Azione A)".

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## 043
## A transcriptomic approach towards the improvement of physiological systems for the *in vitro* culture of isolated bovine primordial follicles

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**Application:** Advancement of culture strategies targeting pre-antral follicle development to pursue female fertility preservation.
**Introduction:** The ability to grow undifferentiated oocytes *in vitro* from primordial follicles would increase the supply of fully grown oocytes destined for downstream applications in the livestock industry and fertility preservation programs. To date, the production of living offspring using *in vitro* follicle growth from the primordial follicle reserve has only been achieved in mice (Eppig and O'Brien, 1996), proving the principle of the potential value of follicle culture as a source of fully grown oocytes. While, in large mammals, *in vitro* follicle growth systems to produce mature oocytes from primordial follicles are still experimental (Araújo et al., 2014) due to high follicle mortality following isolation from the surrounding tissue. Herein we study the transcriptome profiles of isolated bovine primordial follicles to identify programmed cell death mechanisms triggered after a short period in culture.
**Materials and Methods:** Bovine ovaries were collected from the abattoir and transported on ice to the laboratory in saline. Primordial follicles were mechanically isolated and cultured in a defined system (Dey et al., 2023). Follicle viability was assessed at the collection and after 16 and 24 h of culture. Freshly isolated and 16-h cultured primordial follicles were pooled for RNA extraction and library preparation. RNA sequencing was then performed on Illumina NextSeq2000, generating 50bp paired-end reads. Raw data were trimmed with TrimGalore to remove artificial constructs and low-quality bases. Trimmed data were mapped to the *Bos taurus* ARS-UCD1.3 transcriptome, and reads were quantified with Salmon. Differentially expressed genes were then obtained with DESeq2.
**Results:** After 16 h of culture, a significant reduction in follicle viability was observed, while no significant differences were observed between 16 and 24 h ($P < 0.0001$ and $P = 0.9753$, respectively, two-way ANOVA followed by Tukey's test). PCA plot of global transcriptomic results showed evident clustering of the samples. A total of 1 430 genes were differentially expressed with FDR < 0.05.
**Conclusions:** Here, we report for the first time the transcriptome profiling of isolated bovine primordial follicles at the time of collection and after a short period of culture following the triggering of cell death. We hypothesize that by contrasting the transcriptome profiles of cultured primordial follicles against freshly isolated ones, activated cell death signaling networks could be delineated and subsequently inhibited to improve current culture systems.
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## 044
## Gene expression profiling using next generation sequencing of primordial, primary and secondary bovine follicles

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**Application:** Improve the exploitation of the ovarian reserve with the development of a culture system from primordial to secondary follicles.
**Introduction:** Folliculogenesis is a highly regulated process, and from the primordial follicle's formation, several pathways intervene to control different fates of the follicle: it can remain dormant, undergo cell death or activation (Dri et al., 2021). Two of the most studied activation pathways are PI3K/AKT/mTOR and Hippo. From the pre-granulosa cells, various stimuli can regulate the PI3K/AKT signaling and interact with the oocyte for the activation and subsequent growth of the follicle (De Felici and Klinger, 2021; Zhang et al., 2014; Zhao et al., 2018). However, most mechanisms guiding the primordial to secondary follicle stage, are still not well-identified and deciphering the intricate networks involved in the earlier stages of folliculogenesis is paramount to orchestrating the follicular growth in a culture system. The present study aims to delineate the mechanisms involved in follicle differentiation from the primordial to the secondary stage through analysis of the transcription profile of isolated primordial, primary, and secondary follicles.
**Materials and Methods:** Heifer ovaries were collected at the abattoir, kept on ice and processed to mechanically isolate follicles at different stages of preantral development (Dey et al., 2023). An average of 177 primordial follicles ($N = 3$), 47 primary follicles ($N = 4$), and 53 sec-

ondary follicles ($N = 3$) were isolated, RNA extracted, libraries prepared and sequenced on Illumina NextSeq2000 generating 50 bp paired-end reads. TrimGalore was used to trim artificial constructs and low-quality bases. Trimmed data were mapped against Bos taurus ARS-UCD 1.3 transcriptome with Salmon, and differentially expressed genes were obtained with DESeq2.
**Results:** PCA analysis showed clear clustering of the samples. Sixty-eight genes were differentially expressed on comparing primary versus primordial follicles and 1 301 genes between secondary and primary follicles with FDR < 0.05.
**Conclusions:** Our preliminary data report the transcriptome profiles of isolated bovine primordial, primary, and secondary follicles for the first time. Identifying key regulators of follicular differentiation will help ameliorate current *in vitro* culture systems.
**Acknowledgements:** Work supported by MSCA-ITN-ETN 2019 EUROVA n. 860960, PON MUR and MUR-PRIN2020 n.20209L8BN4 "InfinitEGG".

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## 045
## Microplastics are taken up by the bovine cumulus-oocyte-complex and dose-dependently reduce the metaphase-II rate of oocytes

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**Application:** To investigate the potential hazards of microplastics on female reproduction.
**Introduction:** Microplastics have raised increasing concerns due to their widespread presence in the environment. The detection of plastic particles in human blood implies that microplastics are able to transfer through biological barriers and reach organs (Leslie et al., 2022). Furthermore, after oral uptake of microplastics they accumulate in the mice ovary (Liu et al., 2022). However, the impact of microplastics on oocyte and embryo development in mammals remains largely unknown, as the focus has been on aquatic animals. The first rodent studies in mice are alarming, suggesting an effect of microplastics on oocyte maturation. In this study the potential impact of microplastics on bovine oocytes was investigated, which is a superior model to study the human oocyte, given the large reproductive similarities between human and cow (Sirard, 2017).
**Materials and Methods:** Cumulus-oocyte-complexes were collected from 2 to 8 mm follicles of bovine slaughterhouse ovaries. Experiment 1. Cumulus-oocyte-complexes matured *in vitro* for 23 h in our standard maturation condition without (control condition), or with 10 μg/mL green-fluorescent polystyrene microplastics of 50 nm or 200 nm (Polysciences, Europe GmbH). After maturation, cumulus-oocyte-complexes were fixed (4% PFA) and stained with Hoechst (DNA) and phalloidin (actin) and analysed by OLYMPUS IXplore SpinSR microscopy, to examine the uptake of microplastics. Experiment 2. Cumulus-oocyte-complexes were 23 h *in vitro* matured in the presence of 0 (control), 1, 3, 10 or 30 μg/mL of 50 nm polystyrene microplastics ($n = 3$ runs, $\geq$100/group) fixed (4% PFA) and stained with DAPI and by fluorescent microscopy scored for their nuclear maturation stage; germinal vesicle, metaphase-I or metaphase-II. Chi-square was used for statistical analysis.
**Results:** Confocal microscopy revealed that microplastics of 200 nm are taken up by cumulus cells, while microplastics of 50 nm are taken up by cumulus cells and oocytes. There was a decrease ($P < 0.05$) in the percentage of oocytes in metaphase-II after exposure to 50 nm microplastics at concentrations of 3 μg/mL (62.9%) and 10 μg/mL (69.4%), compared with the control condition (84.5%), whereas the concentrations of 1 μg/mL (78.5%) and 30 μg/mL (73.1%) were not different.
**Conclusions:** Microplastics are taken up by the cumulus-oocyte-complex, which forms a potential threat given the recently observed plastics circulating in blood. The metaphase-II rate of maturing oocytes was reduced after exposure to 50 nm microplastics at levels of 3 and 10 μg/mL. Future studies will investigate the impact of microplastics on oocyte developmental competence.

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## 046
## Mathematical modelling as a tool to describe development of bovine ovarian follicles

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**Application:** Bovine follicle growth and multiple ovulations are described in terms of hormone levels. This may lead to future improvements in bovine reproductive efficiency.
**Introduction:** Two methods of mathematical modelling of follicle development are described. The first is based on experimental – biological results and the second on in-silico studies.
**Materials and Methods:** (1) In the first study, whole follicles were cultured in 5 different media (Control, IFN, TNF, IFN+TNF, ZVAD), to investigate atresia in ovarian follicles. 137 whole follicles were studied and their mass was recorded. Image processing was used to establish the relationship between the diameters and masses of follicles. A related technique was used in (McEvoy et al., 2022) to model granulosa volume as a function of the number and diameters of follicles. This permitted the use of experimental results from other datasets originating from public repositories. Gene expression levels were measured using microarrays and retrieved from the databases (E-GEOD-42535 and E-GEOD-9439589). (2) A mathematical model of multiple ovulations (Soboleva et al., 2000) based on the behaviour of follicles within the ovulatory wave was generated. Thousands of simulations were run (using the random uniform distribution) within a specified set of initial estradiol (E2) values in serum to obtain the ratio of multiple and single ovulations at a given value of maximum level of E2 in serum.
**Results:** (1) A regression equation was calculated in open-source software R (Diameter = 3.9603 + 8.3907 mass - 1.7679 mass$^b$) with $R^b = 0.4669$ ($p < 0.05$). PCA plots were created, which showed the separation between the granulosa cells from atretic and healthy follicles, based on gene expression values. This separation was also recorded for samples of granulosa cells cultured at different treatments (Control and FSH group were separated from FSH+TNFalpha and TNFalpha group). (2) Simulations were run with different maximum values of E2. This gave a graph showing the proportion of multiple and single ovulations at different maximum values of estradiol.
**Conclusions:** (1) It can be determined whether a follicle is healthy or atretic, based on its gene expression values. (2) The simulation graph generated showed there is a certain threshold of maximum E2 value, above which mainly single ovulations occur and below which mainly double ovulations occur. This agrees with an observed higher percentage of multiple ovulations at lower levels of E2, observed in high producing cows (Wiltbank et al., 2006).
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## 047
## Changes in proteome of membrane fractions from bovine oocyte to zygote

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**Application:** To understand the mechanisms behind the recognition and fusion of gametes membranes.
**Introduction:** The identity of the key proteins that directly mediate the membrane binding and fusion of gametes is largely unknown. Here, we propose to apply data screening strategies to identify new molecular candidates involved in the recognition of gametes and pre-

sent at the time of fertilization. The experimental design was based on two principles; (i) The receptors responsible for the recognition of gametes in the oocyte membrane appear exposed in the ovulated egg and disappear once a single sperm has fertilized the egg (zygote), to facilitate the prevention of polyspermy. (ii) These interactions are usually found to be highly transient.
**Materials and Methods:** A comparison of the proteomics profile from membrane fractions of oocytes and zygotes will be analysed. For this purpose, 400 *in vitro* matured bovine oocytes and 400 zygotes were produced in 3 replicates. Briefly, cumulus-oocyte-complexes were selected for *in vitro* maturation. At the end of the maturation period, oocytes were split into two groups, (i) vortexed to remove cumulus cells, washed in PBS, snap frozen and stored at −80 °C, or ii) prepared for *in vitro* fertilisation (IVF). Bull spermatozoa were selected by swim-up and added at a final concentration of $1 \times 10^f$ spermatozoa/mL. After 22 h of coculture, zygotes were gently stripped of cumulus cells by vortexing, washed twice in PBS, snap frozen and stored at −80 °C. For cell membrane purification, cells were washed in PBS, suspended in a hypotonic lysis buffer, incubated in an ice bath, disrupted using a dounce homogenizer with a tight-fitting pestle, and centrifuged to collect the cell membranes. Later, samples were further processed and analysed by mass spectrometry by nLC coupled to an ion trap mass spectrometer equipped with a Captive source. Three different runs were carried out per sample. Once proteins were identified, gene ontology analysis was carried out filtering on membrane proteins.
**Results:** From all identified proteins (216 oocyte proteins, 295 zygote proteins), a total of 109 and 188 were identified exclusively in the oocyte and zygote samples respectively, and 107 were common to both groups, from which gene ontology analysis revealed 49 and 33 proteins overexpressed in oocytes and zygotes, respectively.
**Conclusions:** The proteomic analysis show turnover of the membrane proteins from the oocytes to zygotes. Further detailed study of the proteins exclusively detected in the oocyte membrane may undelved new candidates involved in gamete binding and fusion.
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**048**
## A meta-analysis suggests that the culture environment affects mRNA translation in bovine oocytes

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**Application:** Refining culture environment to support mRNA translation may improve egg quality.
**Introduction:** Upon removal from antral follicles, fully-grown oocytes resume meiosis and fertilize *in vitro*. However, embryo development is lower compared to *in vivo* matured oocytes. Such loss of performance is likely caused by two main factors: (1) skipping final differentiation, also known as 'oocyte capacitation', occurring during follicular dominance and (2) inability of fully recreating a microenvironment that supports oocyte maturation. Given that resumption and completion of meiosis I is largely driven by post-transcriptional mechanisms and that the epidermal growth factor (EGF) network partially regulates maternal mRNA translation in mice, we conducted a meta-analysis in the attempt of better elucidating how the culture environment affects translation in bovine oocytes.
**Materials and Methods:** Isolation of polysome-associated mRNAs requires high amount of starting material. Therefore, we exploited deposited datasets to gain information on (1) mRNAs polysome association in immature (GV) and mature (MII) bovine oocytes (GSE56603); (2) extent of amplification of polyadenylated mRNAs in GV and MII bovine oocytes (GSE61717); (3) mRNAs polysome association in MII mouse oocytes upon activation of the EGF network (GSE46640). A comparison between the datasets was conducted to identify translation patterns that are affected by maturation and by EGF-like growth factors. Since there was no suitable dataset on bovine oocytes to inform on the latter, a mouse dataset was used. GEO-retrieved datasets were re-analyzed using R-Studio. Differential expression was determined using edgeR (Bioconductor – Software packages). AdjP < 0.05 and LogFC > 2 were considered.
**Results:** Twenty-seven transcripts were differentially associated to the polysomes in MII compared to GV bovine oocytes, and only one was common to the 320 transcripts overexpressed in response to EGF network. Therefore, we included a second bovine dataset (GSE61717), which preferentially identifies polyadenylated, and therefore translated, mRNAs. However, also in this case the overlap between maturation-induced and EGF network-induced differences was minimal. To test if the failure to detect overlap was due to inter-specificity, we compared intraspecifically the polysome-associated and polyadenylated transcripts. Notably, while the overlap was still limited for MII oocytes, all the mRNAs preferentially associated to the polysome in GV were also overexpressed in the polyadenylated dataset at the same stage, indicating that the two experimental approaches yield comparable results for immature oocytes, but this homogeneity is somehow lost with *in vitro* maturation (IVM).
**Conclusions:** This meta-analysis represents indirect evidence that IVM may lower egg quality by disrupting the oocyte translational program.
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# EMBRYO/PREGNANCY

## 049
## Pain management in non-surgical embryo recovery in Santa Inês ewes: effects on animal welfare

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**Application:** Pain management during non-surgical embryo recovery (NSER) can promote better welfare conditions in sheep.
**Introduction:** Non-surgical embryo recovery, although less invasive than surgical techniques, still triggers responses that affect animal welfare (i.e., increased heart rate and cortisol concentrations), probably explained by cervical manipulation. Thus, this study evaluated the effectiveness of meloxicam with dipyrone on the welfare of ewes subjected to non-surgical embryo recovery.
**Materials and Methods:** A total of 29 multiparous Santa Inês ewes received a standard oestrus synchronisation treatment and a superovulatory protocol. Non-surgical embryo recovery was performed after a standard hormonal protocol for cervical dilation (Leite et al., 2018). The animals were either administered Meloxicam and Dipyrone ($n$ = 15), which received both meloxicam before (1 mg/kg, i.v.) and 24 h after cervical transposition (1 mg/kg, i.m.), and dipyrone (50 mg/kg, i.m.) before, 12 h, and 24 h after cervical transposition, or not (control; $n$ = 14) which were treated with saline solution. Heart and respiratory rates, cortisol, glucose, total proteins, albumin, and globulin blood concentration were recorded before sedation, after sedation, after cervical transposition, immediately after collection, and 0.5, 1.5, 3, 6, 12, 24, and 48 h after embryo collection. Data were compared using a mixed model (SAS on Demand for Academics), including treatment, time, and their interaction as main effects. For all tests, $P < 0.05$ was considered significant, and $0.051 > P \leq 0.1$ were considered as tendencies.
**Results:** Glycaemia had a significant interaction between treatments and time ($P < 0.0001$), tending to be greater in the control group after sedation ($P = 0.052$), and being greater at 3 h ($P < 0.0001$) and 6 h ($P = 0.03$) after embryo collection. In ewes in the Meloxicam and Dipyrone group, blood glucose increased from before sedation to after sedation ($P = 0.02$), from after sedation to after cervical transposition ($P < 0.0001$), returning to baseline values at 6 h after embryo collection ($P < 0.05$); and in the control group increased from before sedation to after sedation ($P < 0.0001$), peaking at 1.5 h after embryo collection and 3 h after embryo collection, returning to baseline at 12 h after embryo collection ($P < 0.05$). Cortisol values tended ($P = 0.1$) to be greater and serum total proteins and globulins values were greater ($P < 0.0001$) in control ewes. The other variables were not affected ($P > 0.1$) by treatments, varying only with time.
**Conclusions:** The combination of drugs used for supporting NSER recovery in sheep induced transient changes indicating stress and possibly pain. Although the treatment applied reduces pain, it apparently only had minimal effects on reducing the negative responses triggered by NSER.
**Acknowledgements:** Faperj and CNPq.

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## 050
## MircoRNA-665 derived from bovine conditioned media is a potential non-invasive biomarker for preimplantation embryos

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**Application:** MicroRNA-665 is a potential non-invasive biomarker for preimplantation embryo developmental competence.
**Introduction:** In recent years, there has been a surge of interest in using embryonic microRNAs (miRNAs) as non-invasive biomarkers. However, these signalling molecules have only been profiled in media conditioned with blastocyst-stage embryos. We previously identified 363 miRNAs derived from bovine embryo-conditioned media or extracellular vesicles isolated from embryo-conditioned media (Lin et al., 2019; Pavani et al., 2022). The current study aimed to test the functionality of miR-665.
**Materials and Methods:** To study the functionality effect of miR-665, *in vitro* produced bovine presumptive zygotes ($n$ = 1925, 18 replicates, Holstein) were allocated in five treatments groups with supplementation of bta-miR-665 (mimics, inhibitor, mimics negative-control, inhibitor negative control) with concentration of 1 μM/25 μl to the SOF medium (containing insulin, transferrin, and selenium supplemented with 0.4% BSA (Sigma A9647)) along with the control group. Embryo cleavage was assessed on Day 2. At Day 8, the blastocyst rate was determined, and the effect of the miRNA on the expression of selected target genes was determined using RT-qPCR ($n$8181, 3

replicates) and Western Blotting ($n$ = 135, 3 replicates), as well as differential-apoptotic staining ($n$ = 203, 3 replicates). Data were analysed using the student $T$-test and ANOVA, and logistic and linear regression models were fitted, with the replicates set as a random effect.
**Results:** Bta-miR-665 was highly expressed in blastocyst culture medium (Log2Cq≈4.1 ± 0.4, $P < 0.01$) compared with non-blastocyst/degenerated culture medium. Supplementation of bta-miR-665 mimics to the culture medium significantly ($P < 0.05$) increased the blastocyst rate (42.12 ± 7.13%) (on Day 8) compared to control, mimic negative control (35.17 ± 6.08%, 33.75 ± 5.04%, respectively). On contrary, supplementation of bta-miR-665 inhibitor reduce the blastocyst rate (28.63 ± 5.87%) compared to inhibitor control and control (33.40 ± 4.90%, 35.17 ± 6.08%, respectively). In terms of embryo quality, inner cell mass (ICM) ratio was higher in miR-665 mimic (51.83 ± 3.23%) compared to control (43.31 ± 0.81%). As anticipated supplementing miR-655inhibitors had drastically declined ICM ratio (21.25 ± 0.35) compared to control (43.31 ± 0.8)($P < 0.005$). Expression of genes prolonging cellular DNA repair (CDK2/4,ERK1/2), regulating cell division (ERK,TGF-, STMN2) and blocking apoptosis (TNF-a,JNK/P38,PI3K/AKT) was significantly influenced at mRNA and protein level ($P < 0.05$).
**Conclusions:** Bta-miR-665 promotes the ability of zygotes to develop into blastocysts, decreases apoptosis of the embryonic inner cell mass (ICM) and increases embryo quality.
**Acknowledgements:** The work was funded by Ghent University GOA030-18 BOF.

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## 051
## Non-surgical embryo recovery in superovulated and synchronous estrus-induced goats

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**Application:** Non-surgical embryo recovery (NSER) can be performed in goats subjected to synchronous estrus induction only (SYNC) or followed by superovulation treatment (SOV) during the non-breeding season.
**Introduction:** Although undoubtedly efficient, embryo recovery by laparotomy is not typically performed in non-SOV or low-responder donors, and those females might be subjected to NSER. Thus, the objective of the present study was to test NSER success in both SOV and SYNC goats.
**Materials and Methods:** All goats received a 0.33 g progesterone device (CIDR) for 6 days. In G-SYNC ($n$ = 10), goats were injected with 37.5 μg d-cloprostenol and 200 IU of eCG at 24 h before the device removal. In G-SOV ($n$ = 10), goats received 37.5 μg d-cloprostenol both at device insertion (D0) and 24 h before its removal, plus 200 mg of pFSH administered twice daily in six decreasing doses (25-25-15-15-10-10%), starting 48 h before device removal. Females were naturally mated every 12 h while in oestrus. In addition, both groups received three doses of 2.2 mg/kg flunixin meglumine (24 h intervals) starting 84 h after onset of oestrus. Ovarian ultrasonography was performed one day before NSER and all goats received 37.5 μg d-cloprostenol on NSER day 6 am. Qualitative (%) data were compared by Fisher Exact test and quantitative data were subjected to ANOVA (mean ± SEM), both at a 5% minimum significance level.
**Results:** Oestrous response (90 vs 100%), duration of oestrus (38.3 ± 4.0 vs 32.8 ± 2.8 h), number of breedings (2.6 ± 0.3 vs 2.4 ± 0.3), percentage of goats ovulating (100 vs 90%) and interval from device removal to ovulation (67.6 ± 7.8 vs 81.6 ± 8.7 h), media fluid recovery efficiency (96.9 ± 1.0 vs 98.3 ± 0.6%) and ova/embryo recovery rate (64.1 ± 22.0 vs 74.1 ± 25.8%) were similar ($P > 0.05$) between G-SOV and G-SYNC, respectively. Corpus luteum count (10.8 ± 0.5 vs 1.6 ± 0.3) and the number of ova/embryos recovered (6.3 ± 1.9 vs 1.0 ± 0.2) were greater ($P < 0.05$) in G-SOV, compared to G-SYNC goats, respectively. The number of unfertilized ova (5.3 ± 1.9 vs 0.2 ± 0.1) was superior ($P < 0.05$) and viable embryos (0.7 ± 0.7 vs 0.6 ± 0.3) were similar ($P > 0.05$) in the comparison between G-SOV and G-SYNC goats, respectively.
**Conclusions:** The parameters obtained from superovulated and synchronized females allowed us to conclude that the NSER technique is ideal to be used in donor goats regardless of ovarian stimulation.
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## 052
## Morphokinetic changes and apoptotic cell death in vitrified and non-vitrified *in vitro*-produced ovine embryos

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**Application:** This abstract addresses morphokinetic changes and the extent of apoptotic cell death in vitrified and non-vitrified *in vitro*-derived ovine blastocysts.
**Introduction:** Vitrification is commonly used worldwide for long-term embryo storage. However, both the exposure to sub-zero temperatures and potentially cytotoxic levels of cryoprotectants can have debilitating effects on embryonic cells post-vitrification.
**Materials and Methods:** Ovaries were obtained after slaughter from cycling (October-March) Polish Longwool ewes (aged 1–3 years and with a satisfactory body condition score) and were transported to the laboratory within 1–3 h of collection in phosphate-buffered saline (PBS) at 30–35 °C, and then washed three times in PBS (30–35 °C) before oocyte retrieval. Cumulus-oocyte complexes were collected after ovarian scarification and *in vitro* maturation was performed in TCM 199 medium supplemented with Earle's Balanced Salts, 10% of foetal bovine serum, and 5 μg/mL of ovine luteinising hormone/follicle-stimulating hormone at 38 °C for 24 h. After maturation, the oocytes were co-incubated with thawed ram semen for 19 h, and presumptive zygotes were transferred to a 16-well dish containing culture medium. Embryo development was monitored with the aid of the Primo Vision Time-Lapse system (Vitrolife, Göteburg, Sweden). Thirty-one blastocysts at the early blastulation stage were vitrified using the Cryotop system. Embryo survival was confirmed by the ability of the blastocoel to re-expand after warming, amounting to 80.6% (25/31) of vitrified embryos. Both the vitrified and non-vitrified (control; $n = 28$) blastocysts were examined for detection of apoptosis using the Terminal dUTP Nick End-Labeling (TUNEL) assay and blastomeres were counted at the time when all embryos attained the expanded blastocyst stage.
**Results:** Blastocyst formation occurred earlier in non-vitrified than in vitrified and warmed ovine embryos ($P < 0.05$). The average number of blastocyst collapses was greater, but the number of weak contractions was less for vitrified than non-vitrified ovine blastocysts ($P < 0.05$). The mean duration of weak contractions was less by ~1 h 20 min for vitrified blastocysts ($P < 0.01$) compared with their non-vitrified counterparts. The mean number of blastomeres was greater ($P<0.05$) while the number of TUNEL-positive cells and apoptotic index were less ($P < 0.05$) in non-vitrified compared with vitrified blastocysts.
**Conclusions:** Vitrification of ovine embryos was associated with delayed blastocyst formation, greater numbers of apoptotic cells, significant reduction in the number of blastomeres, and higher/lower incidence of blastocyst collapse/weak contractions. Whether these vitrification-induced alterations have an impact on ensuing blastocyst competence and developmental potential after transfer to the synchronized recipients remains to be determined.

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## 053
## Alkaline phosphatase: an important regulator of ovine conceptus development

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**Application:** Improved understanding of the transport of minerals in the pregnant uterus provides a platform for the improvement of conception rates and conceptus development in ruminants.
**Introduction:** Phosphate is essential for conceptus (embryo/fetus and placental membranes) development, yet little is known regarding the mechanisms regulating phosphate homeostasis in pregnancy. Tissue-nonspecific alkaline phosphatase (TNSALP; encoded by ALPL) regulates phosphate homeostasis postnatally; however, the role(s) of TNSALP in growth and development of conceptuses is poorly understood.
**Materials and Methods:** The expression of ALPL mRNA, immunolocalization of TNSALP protein, and quantification of TNSALP enzymatic activity was performed on ovine endometria and conceptus tissue from gestational days (GD) 9, 12, 17, 30, 50, 70, 90, 110, and 125. To assess the effects of TNSALP on conceptus development, wildtype (WT) ewes and ewes with heterozygous (Het) or homozygous (Hom) loss of function mutations in TNSALP (c.1077 C>G; generated by CRISPR/Cas9) were bred with fertile rams. Ewes were euthanized and hysterectomized on GD97 and conceptuses analyzed.
**Results:** Expression of ALPL mRNA was greatest between GD30 and 70, and then decreased in both endometria and placentomes with advancing stages of gestation. TNSALP enzymatic activity decreased in endometria during the peri-implantation period and increased between GD30 and 90 in endometria and placentomes. TNSALP protein localized to uterine epithelial and stromal cells, blood vessels, myometrium, caruncles, and cotyledons. In early gestation, apical localization of TNSALP in uterine epithelial cells suggests a role in the regulation of secretion and/or transport of phosphate into the uterine lumen for utilization by the conceptus. Het and Hom ewes had lighter uterine weights compared to WT ewes ($P < 0.05$), suggesting impaired uterine capacity. Morphologically, placentomes from Het ewes were abnormally large and irregularly shaped, suggesting differential nutrient transport capacity. Further, there were more small cotyledons

present in Hom compared to Het pregnancies ($P < 0.05$). Het fetuses were heavier, longer, and associated with longer placentae with more cotyledons than WT and Hom fetuses ($P < 0.05$). Het and Hom placentomes were histologically abnormal and there were associated alterations in expression of mRNAs with roles in apoptosis, cell proliferation, and extracellular matrix composition (*BAX, CASP3, KI67, VIM, SPP1*). Further, the expression of mRNAs involved in angiogenesis was altered in both endometria (*ANGPT1, CD31, TIE2, VEGFA*) and placentomes (*CD31, eNOS, TIE2, VEGFA, HIF2α*) in Het and Hom pregnancies.
**Conclusions:** These findings suggest previously undescribed roles for TNSALP beyond phosphate transport in the regulation of ruminant conceptus development.
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## 054
## Effect of the antioxidant Mito-Tempo on bovine IVP embryos with respect to early embryonic development and cryo-resistance

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**Application:** Improvement of embryo quality under *in vitro* conditions using antioxidants.
**Introduction:** Despite many efforts, bovine embryos produced *in vitro* are characterised by lower development rates, lower pregnancy rates, and reduced cryogenic viabilities compared to *ex vivo* embryos. Recently it has been reported that the addition of the mitochondrial active antioxidant Mito-Tempo to the maturation medium exerts a positive effect on levels of reactive oxygen species (ROS), developmental rates, and cryogenic fitness of subsequent bovine embryos. Therefore, the present study aimed to analyze beneficial effects of Mito-Tempo supplementation to the culture medium on early embryonic development and cryogenic viability of bovine blastocysts.
**Materials and Methods:** For the study, cumulus oocyte complexes were obtained from slaughterhouse ovaries collected by slicing ($n = 3150$). Maturation (TCM199, 39 °C, 5% $CO_2$, 5% $O_2$) and *in vitro* fertilization (Fert.-TALP, 39 °C, 5% $CO_2$, 5% $O_2$) of oocytes was done via routine procedures and media using frozen thawed semen for *in vitro* fertilization ($2 \times 10^6$/ml). Presumptive zygotes were cultured for up to 8 days in SOFaa + 0.3% BSA (39 °C, 5% $CO_2$, 20% $O_2$) supplemented with 1 μM Mito-Tempo ($n = 1577$) or without Mito-Tempo ($n = 1573$). On day 7 of culture, quantification of intracellular ROS levels was performed on blastocysts from both experimental groups using fluorescence staining (DCFDA, Sigma, 5 μM) and a comparative analysis was performed using an image analysis tool (ImageJ). In addition, day 7 blastocysts of both groups were vitrified using "BO-VitriCool™"-media emerging the Cryotop®-vitrification system. Warming of these blastocysts (Bo-VitriWarm™-media) was followed by subsequent culture for 72 h to determine viability rates, re-expansion rates and hatching rates. The statistical analysis was performed with the help of the program done with GraphPad Prism® (ANOVA) with $p$-values < 0.05 considered to indicate significantly different values.
**Results:** There was no significant effect ($P > 0.05$) of Mito-Tempo supplementation during *in vitro* culture on blastocyst rate as well as hatching rate. Similarly, no effect became obvious with respect to intracellular ROS-level of subsequent blastocysts between the experimental groups. However, vitrified-thawed blastocysts of the group supplemented with Mito-Tempo during *in vitro* culture realized significantly higher ($P < 0.05$) re-expansion rates compared to control embryos at 32, 48, 56 and 72 h post warming. In agreement, hatching rates of blastocysts cultured with Mito-Tempo were significantly higher compared to control embryos at 48, 56 and 72 h after warming.
**Conclusions:** These results confirm our hypothesis that the antioxidant Mito-Tempo exerts a positive effect on reduction of cryo-induced damage after vitrification.

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## 055
## A single-cell transcriptomic atlas of the developing bovine placenta

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**Application:** Reproductive success in cattle is critical for profitability and sustainability of beef and dairy enterprises. Pregnancy establishment and loss can be influenced by many factors including placental development.
**Introduction:** Placental formation begins with implantation of the elongated bovine conceptus to the uterine epithelium. Soon after, binucleate trophoblast cells (BNC) begin to differentiate from uninucleate trophoblast cells (UNC) by day 19–20 and can migrate and fuse with maternal endometrial epithelial cells to form an interrupted fetal-maternal syncytium. Secreted products from BNC are involved with maintenance of progesterone secretion and placental cotyledon formation. By day 30, fetal cotyledons begin to develop and interdigitate

with maternal caruncles to form placentomes which facilitate fetal-maternal exchange. Although the morphology of the bovine placenta is well known, little is known about the transcriptome of specific cell populations across placental development nor how BNC differentiate.
**Materials and Methods:** Single-cell RNA-sequencing was performed on days 17 ($n$ = 3), 24 ($n$ = 3), 30 ($n$ = 4), and 50 ($n$ = 3) of gestation using the 10X Genomics platform. Quality control and clustering analyses were performed with Seurat v4.0 and integrations with Harmony v0.1.0. Cell trajectory analyses were performed with Monocle3 and Slingshot v2.5.2, transcription factor binding site analyses with ChEA3 leveraging the ENCODE database, and ligand-receptor analyses with CellChat v1.6.1.
**Results:** Cell-type specific transcriptomes were profiled from immune, epithelial, mesenchymal, and trophoblast populations in the developing bovine placenta. Differences in populations across development were identified primarily in mesenchyme and trophoblast populations including the appearance of BNC at day 24 expressing bovine placental lactogen (chorionic somatomammotropin hormone 2; *CSH2*) and pregnancy-associated glycoproteins (*PAG*s). Ligand-receptor analyses identified predicted cell-cell communication pathways related to placental structure formation (collagen, laminin, and fibronectin), vasculature development, and other pathways such as galectin and secreted phosphoprotein 1 that are conserved across mammalian species. Trophoblast populations were then collated and re-clustered to investigate subpopulations. Cell trajectory analyses modeled UNC differentiation to BNC, and upstream transcription factor binding enrichment analyses identified GATA binding proteins 2 and 3 (*GATA2, GATA3*) and transcription factors AP-2 alpha and gamma (*TFAP2A*, *TFAP2C)* among other transcription factors that may be involved in this process.
**Conclusions:** This study lays the foundation for creation of a gene expression atlas of cell types in the bovine placenta during development and understanding trophoblast lineages, which fills a significant gap in our knowledge regarding transcriptional differences between cell populations and genes that may govern cell fate during placental development in the ruminant.
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## 056
## Case report: Abnormal sex ratio in a buffalo embryo transfer program

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**Application:** It is a priority in the buffalo industry to increase the use of embryo transfer in buffaloes. This report reports an unusual variation in the sex ratio of calves born after an *in vitro* fertilization-embryo transfer program (IVF-ET).
**Introduction:** In domestic farm animal species, the ovum pick-up (OPU) technique allows the collection of competent oocytes to produce *in vitro*-derived embryos. It is expected that the ratio of males/females born is 1/1, but it could be altered during the culture and handling of gametes and embryos. A complex group of mechanisms has been proposed (gene expression, epigenetic, metabolic) (Gutiérrez-Adán et al., 2006). Additionally, the physiology of the embryo *in vitro*-produced male embryos has a higher metabolic rate, grow faster than females, and they also have differential gene transcription of genes located in the Y-, X-, or in autosomal-chromosomes (Cameron and Linklater, 2007).
**Materials and Methods:** Buffalo oocytes were recovered during 2021 from Argentinian selected donors. Oocytes were matured *in vitro* for 18–20 h in TCM-199 + 10% FCS supplemented with FSH and 0.5 LH, at 38,5 °C and 5% $CO_2$. Frozen semen from four different bulls was used for IVF. After thawing, fertilization was performed in SOF-IVF media supplemented with heparin and phenylalanine. Presumptive zygotes were cultured in modified SOF supplemented with MEM amino acids for six days. Grade 1 blastocysts was vitrified using an open-pulled-straw system. Recipients (heifers $n$ = 16 and uniparous cows $n$ = 26) were synchronized using the CIDR-Synch protocol on Day 17, of the protocol. Each female received one embryo, nonsurgically in the ipsilateral horn to the functional corpus luteum. Pregnancies were evaluated by ultrasonography 30 days after transfer and confirmed by rectal palpation at day 60. Sex ratios were recorded at birth 300–310 days later.
**Results:** Of the 42 recipients synchronized, 36 (86%) had a good quality corpus luteum, 14 females were diagnosed pregnant at day 60, and 14 males were born after normal gestation.
**Conclusions:** It has been reported in cattle that polyunsaturated fatty acids may influence offspring sex ratio (Marei et al., 2018). An Iranian team report no differences in the sex ratio after vitrification for the specie (Mahmoud et al., 2015). It has been reported that there is more evidence that male embryos are more vulnerable than females (Burgoyne et al., 1995). It is hypothesized that in this case two situations converge: the rapid development and resistance of male embryos to vitrification protocols. This study demonstrated that it is not blastocyst culture itself or vitrification alone that significantly alters the sex ratio.

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**057**

## Effect of *in vivo* derived and *in vitro* produced bovine conceptuses on the endometrial transcriptome using a synchronized conceptus-endometrial co-culture system

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**Application:** Near Day 16 of pregnancy in cattle the conceptus elongates and secretes interferon-tau, disrupting the endometrial luteolytic mechanism to sustain luteal progesterone and support gestation. Thus, interferon-tau is the maternal recognition of pregnancy signal and greatly affects the endometrial transcriptome.
**Introduction:** During a previous study, using a conceptus-endometrial co-culture system, we identified Day 15 conceptus-induced endometrial transcripts independent of interferon-tau and dependent on conceptus origin (*in vivo*-derived or *in vitro*-produced). The data supports the notion that bovine conceptuses secrete factors other than interferon-tau that modify the maternal environment, which may be negatively impacted by *in vitro*-produced conceptuses. However, during the previous study, the developmental stage of the conceptus was not synchronized with the endometrium, limiting discovery of transcripts. In this study, we utilized a synchronized conceptus-endometrial co-culture system to identify novel endometrial transcripts modified by *in vivo*-derived and/or *in vitro*-produced bovine conceptuses.
**Materials and Methods:** Angus-Holstein heifers ($n = 41$) underwent estrus synchronization and were divided into one of three groups: bred by artificial insemination on Day 0 to produce elongated *in vivo*-derived conceptuses; received age and sire-matched *in vitro*-produced embryos on Day 7 to produce elongated *in vitro* produced conceptuses, or were not bred nor received embryos, remaining cyclic to produce synchronized endometrium. On Day 16, uteri were harvested and synchronized endometrial explants from cyclic animals and conceptuses from pregnant animals were used to develop the following treatments in 1 mL of RPMI medium: endometrium alone (Control; 8 mm explant; $n = 13$); endometrium cultured with an *in vivo* derived conceptus ($n = 15$); and endometrium culture with an *in vitro* produced conceptus ($n = 13$). After 12 h, endometrial RNA was isolated and analyzed by RNA-Sequencing.
**Results:** More than three-times as many transcripts were identified compared to the previous study. Overall, 1509 and 1438 transcripts were modified in endometrium treated with an *in vivo*-derived or *in vitro*-produced conceptus, respectively ($P < 0.001$; FDR < 0.05). After comparing the data, 966 transcripts were shared between both conceptus types whereas 543 and 472 were unique to the *in vivo*-derived and *in vitro*-produced conceptuses, respectively. Gene ontology analysis of endometrial transcripts upregulated exclusively by *in vitro*-produced conceptuses (297) identified biological processes related to excessive inflammation, enhanced immune cell recruitment, cell-mediated killing, and reduced protein secretion (FDR < 0.05).
**Conclusions:** In conclusion, compared to *in vivo*-derived bovine conceptuses, *in vitro*-produced conceptuses induce an exacerbated endometrial immune response and altered secretome which likely contributes to reproductive failure.
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**058**

## Effect of freeze-thawing on mitochondrial respiration characteristics in bovine expanded blastocysts derived from contrasting developmental environments

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**Application:** Identification of metabolic key points after freeze-thawing of *in vitro* derived bovine embryos.
**Introduction:** Although *in vitro* production (IVP) of bovine embryos has steadily increased during the last decades, transfer of cryopreserved IVP embryos to recipients remain at rather low levels (Viana, 2021). This is attributed to lower freeze-ability compared to ex-vivo derived counterparts caused by suboptimal *in vitro* culture conditions (Rizos et al, 2003). Consequently, the present study aimed to (I.) investigate the effect of embryo cryopreservation on mitochondrial respiration features and (II.) investigate the impact of contrasting developmental environments on mitochondrial respiration after freeze-thawing of embryos.

**Materials and Methods:** Expanded bovine IVP Day 7-blastocysts (VITRO-FRESH) were generated by routine procedures using slaughterhouse ovaries (SOFaa + 5% serum, 5% $CO_2$ & 5% $O_2$, 38.8 °C). Subsequently, Day 7-embryos were randomly allocated to VITRO-FROZEN and VITRO-FRESH groups. VITRO-FROZEN embryos were cryopreserved by a standard slow freezing procedure (embryo freezing media, IVM, Nr. 019447) followed by freeze-thawing whereas the VITRO-FRESH group served as control. Metabolic measurements compared VITRO-FRESH and VITRO-FROZEN embryos re-expanded within 4h after freeze-thawing (pools of 10 embryos, 6 replicates) by an extracellular FLUX analyser using a Cell-Mito Stress Test Kit (Seahorse XFp, Agilent). To analyze the developmental environment effect, ex-vivo derived bovine expanded blastocysts (VIVO-FROZEN) were compared against their *in vitro* derived counterpart (pools of 7 embryos, 6 replicates). Mitochondrial respiration was characterized by non-mitochondrial- and mitochondrial respiration, mitochondrial reserve capacity and ATP-coupling efficiency (Wave Software, Agilent). After confirmation of normal distribution (Komolov-Smirnov test) and variance homogeneity (F-test), results of contrasting groups were compared (unpaired t-test, Graph Pad Prism) with *P*-values <0.05 considered to be significant.
**Results:** The results of the study revealed that VITRO-FROZEN embryos consumed significantly lower amounts of oxygen (89.1% vs 98.2%, $P < 0.05$) and had lower mitochondrial reserve capacities after thawing (244.3% vs 285.9%, $P < 0.05$) compared to VITRO-FRESH embryos. Adversely, non-mitochondrial oxygen consumption (17.8% vs 7.8%, $P < 0.05$) and ATP-coupling efficiency (71.1% vs 61.5%, $P < 0.05$) were significantly higher in VITRO-FROZEN compared to VITRO-FRESH embryos. Secondly, mitochondrial respiration rates did not differ between in VIVO-FROZEN and VITRO-FROZEN embryos after thawing (76% vs 85.8%) with any detectable difference with respect to ATP-coupling efficiency (78.3% vs 73.8%). However, VIVO-FROZEN embryos had significantly higher mitochondrial reserve capacities compared to VITRO-FROZEN embryos (340.2% vs 241.5%, $P < 0.05$).
**Conclusions:** Collectively, our study proved that cryopreservation of bovine embryos by slow freezing impairs mitochondrial respiratory properties with *in vivo*-derived embryos bearing higher mitochondrial reserve capacities after freeze-thawing compared to IVP embryos.

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## 059
## Effect of bta-miR-483-3p of extracellular vesicles from the oviductal fluid of pregnant cows on *in vitro* early embryo development

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**Application:** This study strengthens the understanding of embryo-maternal communication mechanisms through miRNAs to improve assisted reproductive technologies.
**Introduction:** MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and signalling pathways related to proliferation and differentiation in early embryo development through post-transcriptional mechanisms. A previous study showed that bta-mir-483-3p was exclusively detected in extracellular vesicles from the oviductal fluid of pregnant cows compared to non-pregnant (Mazzarella et al., 2021). Therefore, we aimed to determine whether bta-miR-483-3p is uptaken by bovine *in vitro*-produced embryos through passive transfection (gymnosis) and to evaluate the effect of miRNA supplementation on early embryo development and quality.
**Materials and Methods:** Presumptive zygotes were cultured in SOF + 3% BSA (control; C) or supplemented with 1 μM miR-483-3p mimic (miRCURY LNA miRNA Mimics; Qiagen; 483-3p); or 1 μM fluorescently labelled control mimics (miRCURY LNA miRNA Mimic 5′FAM, No 339173, Qiagen; CMimic). Embryos at ≥16-cell and blastocyst at day 7 after fertilization (BD7) were snap-frozen in LN2 (3 pools $n = 10$/group) to examine the expression pattern of miR-483-3p by RT-qPCR using miRCURY LNA miRNA PCR Assay. To confirm the miRNA uptake, BD7 ($n = 10$/group) were fixed, stained with Hoechst 33342, and observed under a widefield fluorescence microscope. In addition, bioinformatics analyses were performed with miRWalk 3.0 and Metascape tools. Data were tested for normality and transformed by arcsine square root before One Way ANOVA.
**Results:** Fluorescence staining and RT-qPCR showed that miR-483-3p can be taken up in embryos by gymnosis. Cleavage and blastocyst (BD7 and BD8) rates (%) did not differ between groups (C: 85.4 ± 1.7, 30.6 ± 2.8, 36.0 ± 2.0; CMimic: 86.8 ± 1.6, 29.2 ± 2.9, 36.0 ± 2.2; 483-3p: 85.0 ± 0.3, 27.5 ± 1.1, 36.2 ± 0.7, respectively). Although *in vitro* embryo production was not affected, bioinformatics analysis suggests that bta-miR-483-3p affects signalling pathways such as Hippo, Wnt, FOXO, Notch, and RAS. These pathways are related to maintaining pluripotency, lineage segregation, proliferation, apoptosis, and formation and hatching of blastocysts. Therefore, we suggest that bta-miR-483-3p and pluripotency signalling pathways could be modulated by mother's influence during bovine early embryonic development.
**Conclusions:** Our results demonstrate that miR-483-3p is uptake by gymnosis but does not affect cleavage and blastocyst rates. However, bta-miR-483-3p could modulate early maternal-embryonic communication in the oviduct due to its possible interaction with key signalling pathways. Our findings highlight the importance of advancing knowledge on the oviductal function and miRNAs regulation of biological pathways throughout early embryo development.

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## 060
## bta-miR-133b secreted by extracellular vesicles from the oviduct of pregnant cows could modulate signalling pathways during early embryo development

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**Application:** This study enhances the understanding of embryo-maternal communication mechanisms to improve IVP systems and promote the commercial and economic growth of the cattle industry.
**Introduction:** Extracellular vesicles (EVs) are present in reproductive fluids and play an important role in cell-to-cell communication through their cargoes, especially miRNAs which regulate gene expression and signalling pathways related to cell metabolism, proliferation and differentiation during early embryo development. In a previous study, we showed that bta-miR-133b was present exclusively in EVs from oviduct fluid in pregnant cows (Mazzarella et al., 2021). Thus, we aimed to verify that miR-133b is uptaken in bovine IVP embryos by passive transfection (gymnosis) and to evaluate the effect of miR-133b supplementation on embryo development and quality.
**Materials and Methods:** Presumptive zygotes were cultured in SOF + 3% BSA (Control; C) or supplemented with 1 μM miR-133b (miRCURY LNA miRNA Mimics, Qiagen; 133b); or 1 μM control mimic (miRCURY LNA miRNA Mimic 5′FAM, N° 339173, Qiagen; CMimic). To confirm miRNA uptake, Day 7 blastocysts (BD7, $n$ = 10/group) were fixed, stained with Hoechst 33342 and observed by a widefield fluorescence microscope. Besides, BD7 were snap-frozen in $LN_2$ (3 pools: $n$ = 10/group) to examine the expression pattern of miR-133b by RT-qPCR using miRCURY LNA miRNA PCR Assay. Bioinformatic analyses were performed with miRWalk 3.0 and Metascape tools. Data were tested for normality and transformed by arcsine square root before One-Way ANOVA.
**Results:** Cleavage and blastocyst (Day 7 and 8) rates (%) were not affected (C: 85.4 ± 1.7, 30.6 ± 2.8, 36.0 ± 2.0; CMimic: 86.8 ± 1.6, 29.2 ± 2.9, 36.0 ± 2.2; 133b: 86.8 ± 1.8, 29.0 ± 2.3, 37.0 ± 1.4, respectively), while fluorescence staining showed that miR-133b can be uptaken by gymnosis and was also confirmed by RT-qPCR. Although embryo development was not affected, miR-133b is predicted to modulate signalling pathways that regulate pluripotency, including Wnt, TGF-β, and JAK-STAT. Additionally, miR-133b target genes are part of Hippo and Ras/MAPK pathways reported to modulate cell fate determination, differentiation, proliferation, and apoptosis during embryo development. These results suggest that the mother can regulate embryo development to the blastocyst stage through miRNAs. Gene ontology analysis corroborates KEGG results as it indicates the enrichment of cellular growth, reproductive and metabolic processes.
**Conclusions:** Our results determine miR-133b as a novel regulator of pluripotency signalling related to embryo development and quality and, highlight the importance of providing a comprehensive understanding of the interaction between miRNAs, oviduct and signalling pathways throughout early embryogenesis.
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## 061
## Ovarian follicle flushing as a means of increasing the yield of oocytes and *in vitro* produced embryos in advanced cattle breeding

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**Application:** Ovarian follicle flushing can improve the efficiency of cattle embryo production within genetic improvement programmes.
**Introduction:** Maximising the yield of high-quality oocytes is key to the success of transvaginal follicular aspiration (Ovum Pick-Up; OPU) and *in vitro* embryo production (IVP). Follicle flushing (FF) is used widely in human OPU but not in cattle. A novel double-lumen needle (OxIVF) was designed in which the flushing fluid flows perpendicular to that of aspiration. Here we report on three studies that assessed the merits of this needle, and FF strategies for oocyte recovery, on IVP success.
**Materials and Methods:** Study 1 recovered oocytes from 189 abattoir-derived ovaries to compare FF with a standard 16G × 455 mm double lumen needle and the OxIVF needle (matched dimensions but flushes perpendicular to the needle shaft 7mm from the tip). Subsequently, 12 Holstein heifers underwent two stimulated cycles of OPU in a cross-over design (i.e., six flushed with OxIVF, six with the standard needle (Cycle 1); then swapped between needles (Cycle 2)). Net flow rates were 15 mL/minute for flush and aspiration in follicles ≥7 mm in diameter. In Study 3, 11 Holstein heifers underwent two stimulated cycles of OPU in a similar cross-over design, however both treatments used the OxIVF needle. One treatment flushed ≥7 mm follicles only, the other flushed follicles ≥7 mm followed by aspiration of 5–6 mm follicles. There then followed three cycles of conventional (single-lumen needle) follicle aspiration. Oocytes in both cross-over studies underwent standard IVP. Proportions were analysed by logistic regression; donor formed the random effect. Data are presented as means ± SEM.
**Results:** Oocyte recovery in the Study 1 was proportionately greater ($P = 0.034$) for the OxIVF than the Control needle (0.741 ± 0.0209 vs 0.670 ± 0.0223), as was Grade I oocytes ($P < 0.001$) (0.273 ± 0.0271 vs 0.122 ± 0.0216). In Study 2, oocyte recovery was proportionately greater ($P = 0.045$) for the OxIVF than the Control needle (0.891 ± 0.0298 vs 0.796 ± 0.0347). By Day 6 of culture, embryo yields were greater ($P = 0.017$) for the OxIVF than Control needle (0.872 ± 0.0438 vs 0.676 ± 0.0673). In Study 3, oocyte recovery was 0.821 ± 0.0506 vs 0.742 ± 0.0491 vs 0.662 ± 0.0348 for FF vs FF plus aspiration vs aspiration alone ($P = 0.033$). By Day 6 of culture, embryo yields were similar for FF and FF plus aspiration (0.672 ± 0.0879 vs 0.693 ± 0.0763).
**Conclusions:** Ovarian FF leads to high yields of quality oocytes contributing to IVP success in stimulated cycles of OPU in cattle.
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## 062
## Dose- and time-dependent effects of interferon tau on bovine endometrial gene expression

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**Application:** The improved understanding of the fundamentals of pregnancy establishment during the pre-implantation period, when a significant proportion of embryo loss occurs, could help to minimise early embryonic death and thus improve pregnancy rate in dairy cattle.
**Introduction:** Conceptus-derived interferon tau (IFNT) is responsible for maternal recognition of pregnancy (MRP) in cattle by blocking luteolytic pulses of prostaglandin F2alpha, thereby maintaining progesterone output by corpus luteum. We have demonstrated that short conceptuses fail to induce a large number of interferon-stimulated genes (ISGs) in the bovine endometrium that are altered by both IFNT and age-matched long conceptuses, suggesting insufficient IFNT production is a major contributing factor for lower survival of such conceptuses (Sánchez et al., 2019). The threshold level of IFNT required to establish pregnancy in cattle remains unknown. However, the transfer of conceptuses up to Day 16 of the oestrous cycle can establish pregnancy (Betteridge et al., 1980) suggesting that the signalling effect is quite acute. Thus, we aimed to test the hypothesis that there is a dose- and time-dependent relationship between IFNT and the endometrial expression of key genes involved in the signalling cascade leading to MRP, which might be associated with successful pregnancy establishment in cattle.
**Materials and Methods:** Bovine endometrial explants collected at the late luteal stage of the oestrous cycle were cultured in RPMI medium without (control) or with IFNT (1, 10, 100 ng/mL) for 6h. In parallel, endometrial explants were cultured in medium containing 100 ng/mL IFNT for different time periods (15 min, 30 min, 1 h, 3 h, 6 h). Endometrial explants from the same uterus ($n = 8$) were used for both dose- and time- dependent experiments in order to minimise variation. Gene expression was analysed by RT-qPCR. The treatment effect was considered significant at $P < 0.05$.
**Results:** The ISGs (*ISG15, OAS1, MX1* and *MX2*) were significantly ($P < 0.05$) up-regulated in the endometrial explants by 1 ng/mL IFNT, and the intensity of such changes were increased with higher concentrations (10 and 100 ng/mL) ($P < 0.05$). IFNT at 100 ng/mL significantly ($P < 0.05$) stimulated *ISG15, OAS1, MX1* and *MX2* in endometrial explants when cultured for 1, 3, or 6 h, but not shorter (15 min and 30 min, $P > 0.05$). The analysis of other target genes is currently on-going.
**Conclusions:** These results suggest that IFNT acts on the uterus in both a dose- and time- dependent manner in cattle that might be associated with successful pregnancy establishment.
**Acknowledgements:** The work was funded by the Irish Research Council (GOIPD/2021/46).

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## 063

## A potential role of bovine platelets in transfer of interferon tau signaling to neutrophils and endometrial epithelia; involvement in the immune cascade toward embryo receptivity

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**Application:** Platelet-conditioned media regulate endometrial immune micro-environment for successful pregnancy in bovine.
**Introduction:** Platelets are anucleate cells but they host a broad spectrum of transcripts, including a variety of messenger RNAs, non-coding RNAs, microRNAs and circular RNAs. Their RNAs are complemented by translational machinery, which allows translation of nascent transcripts into proteins after stimulation (Davizon-Castillo et al., 2020). Besides hemostasis, platelets are considered as an essential regulator for modulation of different immune responses. Recently, platelets become an area of interest in reproductive medicine to improve fertility in humans and animals. The present study was designed to determine whether platelets could sensitize interferon tau (IFNT) signalling and interact with neutrophils (PMN) and bovine endometrial epithelial cells (BUEC).
**Materials and Methods:** Platelets were isolated from whole blood of multiparous Holstein dairy cows ($n = 4$) on days 10–12 of estrous cycle, exposed to IFNT (0, 0.01, 0.1, 1, 10 ng/ml) for 0.5 or 3 h; and the changes in gene expression were examined by RT-PCR. Conditioned media from platelet (PL-CM) cultured with or without IFNT (1 ng/ml) were collected after 30 min. Subsequently, PMN and BUEC were exposed to PL-CM for 4 and 24 h, respectively, and then the immune-related gene expression was assessed. In addition, lipoxin A4, an endogenous anti-inflammatory lipid mediator, was assessed by ELISA. Each experiment was repeated in triplicate and the obtained data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The difference between platelet gene expression at different time points (30 min and 3 h) in the same group were compared using paired samples t-test. Results were considered to be statistically significant at $P < 0.05$.
**Results:** IFNT at 1ng/ml enhanced the IFN-stimulated genes; Interferon-stimulated gene 15 (ISG15) and 2'-5'-Oligoadenylate Synthase 1 (OAS1), TGFB, type I IFN receptors (IFNAR1, IFNAR2) and TLR4 expression on PL after 30 min. IL1B was suppressed after 3 h with all studied concentrations. PL-CM had anti-inflammatory effects on neutrophils by suppressing the expression of pro-inflammatory cytokines (TNF and IL1B) and inducing lipoxin A4 production. Importantly, PL-IFNT-CM enhanced the expression of TGFB (anti-inflammatory cytokine) in both PMN and BUEC.
**Conclusions:** Overall, the present study provides the initial evidence for platelet response towards IFNT signalling. Notably, the ability of platelets to communicate with immune and uterine epithelial cells, by inducing an anti-inflammatory action, may provide a novel insight for maternal recognition of pregnancy in bovine.
**Acknowledgements:** Supported by a Grant-in-Aid for Scientific Research (22F21401) from JSPS and JRA.

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## 064

## Oviductal magnetic spheroid is an effective *in vitro* culture system to study the estrous cycle stage effect in the oviduct

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**Application:** Obtain an efficient *in vitro* model to study the oviduct physiology and its interaction with the embryo.
**Introduction:** The oviductal cells, especially the epithelial ones, have been inefficiently cultured under two-dimensional (2D) conditions. Successfully, 3D systems have promoted cellular functionality and polarity maintenance. The magnetic 3D culture system (Greiner Bio-One CELLSTAR®) has been applied by our group to form the oviductal magnetic spheroids (OMS). As known, estradiol (E2) increases while pro-

gesterone (P4) decreases oviductal cell ciliation and secretory activity. Therefore, we hypothesized that OMS is hormone responsive. Hence, we aimed to evaluate the OMS's response before luteal (L), pre-ovulatory (Pre-Ov), and post-ovulatory (Post-Ov) treatments simulation.
**Materials and Methods:** In an attempt to reduce interference, bovine oviduct epithelial cells (BOEC) and stromal cells (BOSC) were collected at two estrous cycle stages: follicular (high E2 milieu, $n = 6$ animals) and luteal (high P4 milieu, $n = 5$ cows). After expansion (2D cultured), cells were magnetized by centrifugation with the nanoshuttle™-PL and seeded in a 96-well plate at 7:3 proportion (BOEC:BOSC), 10 000 cells/well. A magnetic force induced cell aggregation, forming the OMS within three days. For the next 7 days, OMS was treated to simulate the luteal phase (P4, 100 ng/mL), followed by 3-days Pre-Ov (E2, 300 pg/mL), and finally 4-days Post-Ov (no hormone). Acetylated-alpha tubulin (acTUB, cell ciliation marker) and OVGP1 (specific secreted oviductal protein) were analyzed per immunoassay in all OMS. Comparisons were performed between L (D7), Pre-Ov (D10), and Post-Ov (D14) in cells either collected at follicular or luteal stages. The raw intensity density was normalized by the spheroid area using ImageJ (version 1.53t). Data were analyzed in the GraphPad Software (version 8), using ANOVA or t-test, with $P < 0.05$ as the significance level.
**Results:** In general, great variation was observed between individuals. No difference was observed in the acTUB levels. The OVGP1 levels were similar in OMS submitted to L treatment ($P > 0.05$) when comparing stages of cell collection. But when OMS were submitted to Pre-Ov and Post-Ov treatments, higher OVGP1 levels were observed in cells collected in the luteal ($P < 0.01$) than in the follicular stage. Comparing the treatments in OMS from cells collected at the luteal stage, a progressive increase was observed from L < Pre-Ov < Post-Ov in OVGP1 levels ($P = 0.004$), with no difference in follicular stage ($P = 0.1113$).
**Conclusions:** The OMS is responsive to hormone treatment in a more physiological pattern if cells are collected at the luteal stage.
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**065**

## Simultaneous CRISPR-on activation of *TFAP2C* and *SMARCA4* improves developmental rates and increases trophoblast cells in bovine embryos

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**Application:** To produce trophoblast-enriched embryos to improve maternal-fetal recognition and implantation rates of *in vitro*-derived embryos.
**Introduction:** Assisted reproductive techniques are widely used to produce bovine embryos for commercial or research purposes. In cattle, aberrant differentiation of trophoblast cells during embryo development leads to failures in pregnancy establishment and placentation. In this work, we sought to induce the simultaneous activation of *TFAP2C* and *SMARCA4* expression by using CRISPR technology in bovine embryos to generate trophoblast-enriched blastocysts.
**Materials and Methods:** Four non-overlapping single guide RNAs (sgRNAs) targeting *TFAP2C* and *SMARCA4* were synthesized. Presumptive IVF zygotes were microinjected with a 9-µm pipette with a mix containing 100 ng/µl dCas9VP160 mRNA, 50 ng/µl of a mix of the 8 sgRNAs and 2 µl 10% Polyvinylpyrrolidone (PVP) (TF-SM group). A group without sgRNAs (SHAM) and a non-injected group (IVF) served as controls. Embryos were cultured in synthetic oviductal fluid medium supplemented with 2.5% fetal calf serum at 38.5 °C in humidified gas mixture (5% $CO_2$, 5% $O_2$, 90% $N_2$) and developmental rates were determined (Fisher's exact test). Samples were collected in pools of 10 embryos and 5 blastocysts at days 2, 4 and 7 of development to perform RTqPCR analysis (*t* test). Data were normalized to the IVF control. The expression of trophectoderm-specific marker CDX2 was analyzed in injected and SHAM blastocysts by immunostaining. Samples were scanned using an inverted confocal microscope and number of cells was determined by the *ImageJ* Software (*t* test). At least 3 biological replicates were included.
**Results:** Embryo development was improved ($P \leq 0.05$) in TF-SM ($n = 238$) relative to the SHAM control ($n = 230$) at cleavage (89.9 vs 83%), morulae (68.9 vs 48.6%) and blastocyst (44.5 vs 30.8%) stages. *TFAP2C* transcript abundance was significantly increased at days 2 (7-fold) and 4 (2.3-fold) in the TF-SM group relative to IVF control. However, *SMARCA4* and *CDX2* only showed significant differences at day 4 (2.3-fold and 2.2-fold, respectively) and *GATA3* transcripts exhibit 1.8-fold difference ($P \leq 0.05$) at day 2, between TF-SM and IVF control. No differences at the expression levels were observed at day 7. The number of $CDX2^+$ cells ($n = 35$ for both groups) in TF-SM group was statistically higher.
**Conclusions:** The simultaneous activation of *TFAP2C* and *SMARCA4* increased the transcript levels of the analyzed genes until day 4 post-microinjection. A significant improvement in blastocyst development and in the proportion of trophectoderm cells were achieved compared to the SHAM control.

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## 066
## A novel single embryo micro-magnetic resonance spectroscopy device to improve cattle ART outcomes

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**Application:** Assisted reproduction techniques (ART) efficiency remains relatively low despite advancements in *in vitro* produced (IVP) embryos for calf production. We propose a non-invasive method of embryo selection based on metabolic markers to predict cryopreservation and developmental potential.
**Introduction:** This study aimed to non-invasively assess embryo metabolism using micromagnetic resonance spectroscopy (micro-MRS). The technology under development at Annaida Technologies uses micro-MRS to access endogenous target compounds directly inside the sample body. Thanks to its non-invasive resolving power, it can be used to analyse samples at the nanolitre scale, typical of pre-implantation embryos and oocytes. In less than 1h, we could non-invasively identify distinct spectral peaks, mainly originating from lipids inside the samples. These signals can serve as biomarkers, setting up a novel non-invasive MRS-based embryo screening tool.
**Materials and Methods:** Samples were shipped cryopreserved to our lab from collaborators and were thawed according to their instructions. Naturally arrested IVP bovine embryos (produced at UZH Zurich, Switzerland) were grouped in early arrested (8-cell stage) and late arrested (morula or blastocyst). Delipidated porcine embryos were produced at LMU (Munich, Germany). Rabbit MII oocytes were purchased from Embryotools (Barcelona, Spain). A single sample was loaded in our micro-MRS device with pre-equilibrated culture medium. As previously described (Sivelli et al., 2022), the micro-MRS measurements were performed for 50min per sample. Statistical analysis was performed using GraphPad Software V9.0. Statistical significance was set at $P < 0.05$.
**Results:** When assessing bovine embryos, we identified up to 6 markers, of which five were significantly higher expressed ($P < 0.05$) in late-arrested versus early-arrested embryos ($n = 32$ embryos). The only marker that was not significantly different was the Poly marker ($P = 0.77$). To further investigate lipid-biomarkers, we analysed porcine embryos that were delipidated to improve cryo-preservation. Our results indicate that embryo delipidation is a process with an uncertain efficacy, readily captured by MRS through the Sat lipid biomarker. The observed signals' spread correlates with the embryo's ability to cryopreserve. 80% of the observed embryos were correctly identified in alive and dead samples post-thawing through Sat lipid marker ($n = 18$) ($P < 0.05$). Finally, a readily detectable Sat marker was confirmed in a few rabbit oocytes. These represent the first model where a single mammalian cell could be analysed with micro-MRS beyond its water content.
**Conclusions:** Our main findings involve lipid bio-markers across different mammalian species showing that our device can be informative about embryo viability and cryotolerance.
**Acknowledgements:** EU Horizon 2020 research and innovation program supported this work under grant agreement No 681002.

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## 067
## Changes in the expression of epigenetic modification regulatory enzymes associated with bovine adenomyosis

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**Application:** Understanding bovine adenomyosis to develop therapeutics.
**Introduction:** Uterine disorders, such as adenomyosis can interfere with reproductive success in mammals including cattle. It is characterized by endometrial epithelial cells and stromal fibroblasts abnormally found in the myometrium. Recently, epigenetic dysregulations have been proposed as one of the mechanisms leading to development of adenomyosis (Zhai et al., 2020). Therefore, we evaluated expression of a family of enzymes, which catalyze epigenetic modification in uterus of cows with adenomyosis.
**Materials and Methods:** Uterine tissue was collected from cows ($n = 107$) slaughtered at a local abattoir and endometrium was separated from the myometrium. Corpus-luteum morphology was used to select animals in the mid-luteal stage of estrous cycle. Histology of uterine sections was evaluated to divide animals in 5 groups ($n = 4$–6 in groups 0-II), stage 0: healthy endometrium with no changes; stage IA: minimal endometrial invasion with single glandular ducts below endometrium-myometrium junction, stage IB: proliferation of EGs within the perivascular connective tissue in myometrium and presence of foci within mucosal surface layer, stage II: transgression (greater degree of proliferation and penetration) of EGs to the myometrium, stage III: EGs within inner muscle layer of the uterus, stage IV: EG nests within the glandular serous uterine wall in myometrium up to the tunica serosa. We used real-time PCR to evaluate uterine gene expression of

deoxyribonucleic acid methyltransferases (DNMT): *DNMT1*, *DNMT3A*, *DNMT3B*, and histone deacetylases: *HDAC1*, and *HDAC2*. Changes in gene expression between healthy and adenomyotic cows were analysed using one-way ANOVA followed by a Tukey multiple-comparison test with significance set at $P < 0.05$. An insufficient number of samples in stage III-IV were available for inclusion in the study.
**Results:** A significant increase in the endometrial expression of *DNMT3B* was observed in cows with stage IB adenomyosis, but a decrease in expression was observed at stage II. The expression of both histone deacetylases *HDAC1* and *HDAC2* was decreased in stage II compared with the healthy endometrium. Expression of *HDAC2* was also lower in stage IB compared with the healthy endometrium. In the myometrium, *DNMT3A* and *DNMT3B* had higher expression in stage IA and IB compared with stage 0. In the case of histone deacetylases, *HDAC1* expression was increased during stage II, whereas *HDAC2* was significantly higher in stage IA.
**Conclusions:** Epigenetic modifications are possibly responsible for some of the molecular changes driving adenomyosis in cows. This knowledge will provide additional information to understand the pathological mechanism and identify therapeutic targets.
**Acknowledgements:** This study was supported by statutory funds from IAR&FR, Olsztyn, Poland.

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## 068
## Bovine Oviductal Organoids: 3-D biomimetic culture system to study the maternal cellular and extracellular response to thermal stress

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**Application:** Establish a physiologically-relevant *in vitro* model to study the impact of heat stress (HS) on the physiology of the oviduct and identify potential key players in the embryo-maternal dialog.
**Introduction:** A major consequence of global climate change is heat stress (HS) and its direct implications on reproductive function. Largely considered as a passive conduit, the mammalian oviduct comprises a highly specific and dynamic microenvironment considered as an optimal milieu influencing fertilization and early embryo development. Here we aimed to investigate the response of bovine oviductal organoids to thermal stress to increase our understanding of the maternal signals transmitted via extracellular vesicles (EVs) affecting embryo survival amid suboptimal conditions.
**Materials and Methods:** For this study, epithelium was obtained from bovine oviducts resected from reproductive tracts in the diestrus stage of the estrous cycle. Organoids were established, grown (14 days) and passaged (P2), with HS experiments being performed on low passage cells (P3) upon transfer into a xeno-free extracellular matrix. Following exposure to HS at 42 °C for 24 h, organoids and their EVs released into the conditioned medium were collected for RNA isolation. RNA was extracted from organoids and EVs with RNA library preparations and RNAseq (NextSeq500; Illumina) being performed by Novogene Co, Ltd.
**Results:** Transcriptomic results revealed 12 774 expressed genes with 11 725 genes being mutually expressed in oviductal organoids cultured under HS and thermoneutral conditions. Differential expression analysis indicated 2 570 differentially expressed genes (1 222 up- and 1 348 downregulated) under HS conditions. Genes activated in response to HS include *COX1, ACTB, CST6, TPT1*, and *HSPB1*, which are involved in pathways related to endocrine resistance, cellular senescence and notch signaling. Alternatively, small-RNAseq analysis revealed the detection of 251 EV-coupled miRNAs with 193 mutually expressed and 18 considered as DE-miRNAs (12 up- and 6 downregulated) within the EVs derived from oviductal organoids subjected to HS compared to thermoneutral conditions. EV-miRNAs enriched in response to HS include bta-miR-150, bta-miR-92a, bta-miR223 and bta-miR-202, found to potentially target genes involved in cellular senescence, p53 signaling and TGF-beta signaling pathways. Sequence motif analysis revealed the presence of two specialized sequence motifs in 8 out of the 12 upregulated miRNAs in EVs released in response to thermal stress, potentially bonded by RNA binding proteins RBM6, HuR and ZC3H14.
**Conclusions:** In conclusion, our findings indicate the transcriptome and EV-coupled miRNA mediated response of oviductal organoids to HS and revealed endocrine resistance and cellular senescence to be the major pathways affected by thermal stress in oviductal microenvironment.

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## 069
## Relationship between placental IGF2, IGF2R, and PHLDA2 and weight of bovine fetuses produced *in vivo* and *in vitro*

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**Application:** Understanding the relationship between expression of imprinted genes in the placenta and fetal weight.
**Introduction:** Calves derived from *in vitro* produced (IVP) embryos are on average heavier than those produced by artificial insemination. Misregulation of imprinted genes is correlated with inappropriate fetal growth, but how these genes control fetal-placental dynamics is not fully understood. This study evaluates the relationship of IGF2, IGF2R, and PHLDA2 in placenta and muscle with the weight and sex of bovine fetuses derived from IVP and artificial insemination.
**Materials and Methods:** At day 56 ($n$ = 41) and 105 ($n$ = 42) of pregnancy, IVP fetuses were surgically retrieved, weighed, sexed and samples of muscle, cotyledon, and intercotyledon were frozen. Same age ($n$ = 14 and 12, respectively) conceptuses produced by artificial insemination served as controls. Fetuses weighing >97 percentile of control weight were categorized as large offspring syndrome. The transcript amount of *IGF2*, *IGF2R* and *PHLDA2* were measured by qRT-PCR. Data were analyzed by analysis of variance using general linear model and Pearson correlation (SAS software v9.4).
**Results:** Thirty eight percent (17/42; female = 9/12; male = 8/30) of D105 IVP fetuses were considered as large offspring weighing on average 636.82 ± 165.88 compared to the rest of the IVP fetuses (471.5 ± 54.85 g). No correlations were detected between the normalized transcript amount of placental or muscle *IGF2*, *IGF2R* and *PHLDA2* and fetal weight on day 56. However, expression of *IGF2* was higher in muscle of female fetuses than in males ($P$ = 0.04). In the case of D105 IVP fetuses, there was a negative correlation between weight and the expression of *IGF2R* in cotyledon and muscle ($r$ = −0.52, $P$ = 0.0004, and $r$ = −0.69, $P$ < 0.0001, respectively) but not in intercotyledon. The expression of this gene in cotyledon was negatively correlated with weight in males (−0.54, $P$ = 0.0021) but not females. The levels of *IGF2R* in muscle were lower in females than males ($P$ = 0.0094), while expression of PHLDA2 in cotyledon and intercotyledon was higher ($P$ < 0.04). D105 IVP fetuses with large offspring syndrome had a 29 and 42% reduction in cotyledon and muscle *IGF2R*, respectively when compared to the IVP normal fetuses ($P$ < 0.008).
**Conclusions:** A negative correlation was observed between weight and cotyledon and muscle *IGF2R* but no correlations were detected for *IGF2* or *PHLDA2*. Ongoing work will analyze the imprinted status and protein of these genes.
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**070**
## Embryo mortality on re-check diagnosis in lactating dairy cows using sample-based pregnancy tests

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**Application:** Pregnancy losses produces a negative impact for reproductive and economic performance in dairy operations. They can occur for a variety of reasons and at different stages of gestation. In dairy cattle, embryo mortality and pregnancy loss cost over a billion dollars annually and decrease the overall efficiency of the operation.
**Introduction:** Giant trophoblast cells of the trophectoderm are the cells that produce pregnancy associated glycoproteins, these cells migrate and fuse with the uterine epithelium around day 19 to 21 of gestation delivering PAG's into the maternal circulation (Wallace et al., 2015). Pregnancy associated glycoproteins have provided the foundation for all blood and milk-based pregnancy testing. Doing early pregnancy diagnosis in a herd can be disappointing for producers because some cows diagnosed as pregnant will later be found non-pregnant due to embryo mortality or early pregnancy loss occurring during the first 30–45 days of gestation (Pohler et al., 2016). The aim of this study is to determine what happens with the developing pregnancy when a cow has a recheck diagnosis on Day 28–35 after AI, and how different risk factors may affect this.
**Materials and Methods:** A total of 13 520 pregnancy tests results were reviewed, only re-check and pregnant results were used in this study. Recheck and pregnant cows were divided in two groups: cows that maintained gestation and cows that did not maintain gestation.
**Results:** Test results (S-N) were significantly different between the two groups: recheck ($P$ < 0.001) and pregnant cows ($P$ < 0.001). There was no difference in pregnant cows in the same two groups ($P$ < 0.1). Cows that had a re-check result and maintained gestation had a 20% incidence of disease vs 34.4% of the cows that did not maintain gestation. In the pregnant group, 9.7% of the cows that Maintain Gestation had a disease (clinical mastitis, diarrhoea, metritis, etc.) vs 27.7% of the cows that did not maintain gestation. In all cows of each group, re-check and pregnant, 34 and 24% of the animals had a disease respectively. Cows with increased embryonic mortality or pregnancy loss had increased disease during gestation.
**Conclusions:** In summary, cows that fall into the recheck zone of the sample-based pregnancy test had a significantly higher chance of losing the pregnancy which are increased if the cow has some type of disease during this period of gestation.
**Acknowledgements:** Full Circle Jerseys, IDEXX Inc.

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## 071
## Superovulatory response and embryo production in Bos indicus and Bos taurus beef donors superstimulated with constant or decreasing doses of FSH

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**Application:** The implementation of accurate treatment protocols for superovulation is critical to the success of embryo transfer programs (Bo and Mapletoft, 2020).
**Introduction:** The objective of this study was to compare superovulatory response and embryo production in beef donor cows treated with constant or decreasing doses of a porcine pituitary extract (pFSH, Pluset, Calier, Spain).
**Materials and Methods:** Cycling Bonsmara and Angus cows ($n$ = 11), Brangus cows ($n$ = 16) and heifers ($n$ = 11), with a body condition score between 3 and 3.5 (scale 1 to 5) were superstimulated twice, in a cross-over design. On Day 0, all donors received a progesterone (P4) device with 1.2 g of P4 (Pluselar, Calier) and 5 mg of oestradiol-17β plus 50 mg P4 (Lab. Rio de Janeiro) intramuscularly. Superstimulatory treatments were initiated on Day 4 and the total dosage of pFSH was based on the manufacturer's recommendation for each breed and category (500–600 IU Bonsmara and Angus cows, 360–440 IU Brangus cows or 280 IU Brangus heifers) and each donor received the same dosage in both replicates. In the decreasing pFSH treatment (pFSH-D), the dosage was distributed in 8 twice-daily decreasing-dose intramuscular injections for 4 days (20%, 20%, 15%, 15%, 10%, 10%, 5% and 5% of the total dosage, respectively). In the constant pFSH group (pFSH-C), the dosage of pFSH was divided into 8 equal-dosages administered twice-daily for 4 days. All donors received two injections of 150 μg of D(+) cloprostenol (Veteglan, Calier) on Day 6. The P4 devices were removed on Day 7 and all donors received 20 μg Buserelin (Pluserelina, Calier) on Day 8 and were inseminated 12 and 24 h later. Ova/embryos were collected on Day 15 and evaluated according to IETS. Data were analyzed using the GLMM procedure for a Poisson distribution. Fixed variables were treatment, breed, category and their interactions. Cow (id) was a random variable.
**Results:** The number of corpora lutea and ova embryos collected were greater ($P < 0.05$) in cows in the pFSH-D group (14.3 ± 1.1 and 9.7 ± 1.1) than in the pFSH-C group (12.1 ± 1.1 and 8.1 ± 1.2). Although no differences were detected in the number of transferable embryos, the number of Grade 1 embryos tended ($P < 0.08$) to be greater in the pFSH-D than in the pFSH-C group (4.9 ± 0.9 vs 4.1 ± 0.7, respectively). No effects of category or breed were detected.
**Conclusions:** Superovulatory treatments with decreasing doses of pFSH seem to be preferable than treatments with constant pFSH doses in beef cows.
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## 072
## Lipid profile of bovine blastocysts under *in vitro* culture with C-type natriuretic peptide supplementation

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**Application:** Modifications of the lipid profile can improve the cryotolerability of the bovine embryo and consequently its survival after techniques such vitrification.
**Introduction:** The lipid composition of the bovine embryo directly reflects on the development and cryotolerance. The use of the C-type natriuretic peptide (CNP) in *in vitro* culture indicated a change in the bovine embryonic lipid profile (Costa et al., 2020). As the cryopreservation of *in vitro* production (IVP) embryos is one of the main obstacles for a wider dissemination and use of the technique, new approaches that potentially improve their cryotolerance are pursued. With this study we aimed to change the lipid profile of IVP bovine blastocysts with the addition of CNP from the beginning of *in vitro* culture.
**Materials and Methods:** A total of 50 blastocysts/group derived from 5 replicates were used (control group - no addition of CNP; CNP-400 - addition of 400 nM CNP). The CNP concentration was chosen based on previous pilot experiment. Blastocysts from Day 7 of culture were selected by morphological quality (excellent or good) and stage of development (blastocyst to expanded blastocyst). Then, the collected

structures were analyzed using the Multiple Reaction Monitoring (MRM)-profiling technique. Lipid extraction was performed according to Bligh and Dyer (1959) and adapted to the small sample volume. The mass spectrometry analysis, and lipid profile data was performed based on Lima et al. (2018). MetaboAnalyst 4.0 was used for multivariate statistics by Principal Component Analysis. Ions were evaluated by Student's *t* test. Fold-change (FC) values were also calculated and considered significant when FC > ±1.5 and $P < 0.05$.
**Results:** In comparison with control embryos, embryos cultured with 400 nM of CNP had the lowest relative abundance of four lipids ions: Phosphatidylserine (28:0); Cholesteryl ester (20:0); Cholesteryl ester (18:0); and Phosphatidylglycerol (36:0) ($P < 0.05$). Samples contained profiles that were related to glycerophospholipids, the main structural lipid components of cell membranes, which are the most injured in cryopreservation processes; and cholesteryl ester, which play an important role in the synthesis of second messengers during embryonic development.
**Conclusions:** Based on the results, it was observed a modification in the lipid profile of embryos supplemented with CNP during the entire culture period was observed. However, further studies are needed to show the biological impact of this profile change and the functional capabilities derived from it.
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## 073
## Modulation of target transcripts after the addition of C-type natriuretic peptide in *in vitro* culture of bovine embryos

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**Application:** On target-transcripts abundance modulation can alter embryonic behaviour in specific stress situations, as well as modulate metabolic responses.
**Introduction:** *In vitro* produced embryos have low resistance to cryopreservation, which is apparently associated with lipids, as well as the composition and morphology of the organelles present in embryonic cells. The use on the C-type natriuretic peptide (CNP) is already well established in oocyte maturation, but in *in vitro* embryo cultured there are limited reports (Costa et al., 2020). With this study, our aim was to screen the effects of *in vitro* culture with 400 nM CNP on the transcripts' abundance and indirectly relate it to the embryonic metabolism.
**Materials and Methods:** The Biomark HD platform was used to relatively quantify the mRNA of interest. This experiment was replicated five times and hatched blastocysts (D8 and D9; $n = 5$/group were collected from control (without addition of CNP), and C-400 (400 nM CNP) groups. The CNP concentration was chosen by data from a previous pilot experiment. Gene expression analyzes of bovine blastocysts were performed independently using the probe TaqMan with specific assays for *Bos taurus*. The pre-amplification and qPCR methodology was performed according to Fontes et al. (2020). For statistical analysis, it was calculated the $\Delta$Cq values relative to the geometric mean of the best reference genes – i.e., GAPDH, and ACTB – among the 96-gene set. Fold-change (FC) was calculated using the $2-\Delta$Cq method. All analyzes were performed using SigmaStat 4.0 and MetaboAnalyst 5.0. The evaluation of the transcripts was initially performed with the univariate statistical analysis method and in a second moment, we analyzed the data by multivariate methods. Differences with probabilities less than $P < 0.05$, and FC >1.5 were considered significant.
**Results:** Transcription abundance tended to reduce in the CNP group AGPAT9 ($P = 0.094$). However, it was upregulated in CNP group for BID, CASP3, SOX2, HSPA5 transcripts, and downregulated for BDNF, NLRP5, AGPAT9, ELOVL1, ELOVL4, IGFBP4 and FDX1 transcripts (FC >1.5). The transcript abundances related to lipid metabolism, predictor of embryo quality, fatty acid biosynthesis and associated with cell proliferation and cell metabolism.
**Conclusions:** The results suggest that it was possible to observe a reduction of the target transcripts abundance in embryos cultured with 400 nM of CNP. However, more studies are needed to prove the interference of CNP in the production and functional performance of bovine embryos.
**Acknowledgements:** The work was funded by CAPES (Finance Code 001), FAPESP (2019/10732-5), CNPq (301912/2019-0) and INCTeLEITE (465725/2014).

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**074**

## Superovulatory response and embryo production in beef donors superstimulated 2 or 3 days after ultrasound-guided follicle aspiration

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**Application:** An effective synchronisation of follicle wave emergence is critical to the success of a superstimulation treatment.
**Introduction:** Transvaginal ultrasound-guided follicular aspiration (also known as OPU) is the preferred technique to obtain oocytes for *in vitro* embryo production (IVP) and it is also effective for follicular wave synchronization for superstimulation. Therefore, it is possible to use this technique to combine IVP and superovulation in the same donor (Surjus et al., 2014). An experiment was designed to evaluate superovulatory response and embryo production in beef donors treated with pFSH (Pluset, Calier) 2 or 3 days after OPU.
**Materials and Methods:** Brangus and Braford cows ($n$ = 16) and heifers ($n$ = 10) were superovulated three times in a cross-over design. Donors in D2 and D3 groups were subjected to OPU (all follicles >3 mm were aspirated), whereas cows in the Control group underwent follicle aspiration of the two largest follicles only. All cows received a progesterone (P4) device (1.2 g P4; Pluselar, Calier) right after follicle aspiration. Superstimulatory treatments were initiated either 2 (Control and D2 groups), or 3 (D3 group) days later (Day 0). All donors received pFSH (440 UI cows and 320 IU heifers) in twice-daily decreasing doses for 5 days. On Day 2, donors received two injections of 150 μg D(+) cloprostenol (Veteglan, Calier) 12 h apart. The P4 device was removed on Day 3 and donors received 20 μg Buserelin (Pluserelin, Calier) on Day 4 and were inseminated 12 and 24 h later. Ova/embryos were collected on Day 11 and evaluated according to IETS. Data were analysed using the GLMM procedure for a poisson distribution. The fixed variables were treatment, category and their interactions. Cow was a random variable.
**Results:** Although the number of corpora lutea and ova/embryos collected did not differ among groups (18.0 ± 1.9 and 11.1 ± 2.1 vs 18.0 ± 2.0 and 10.6 ± 1.6 vs 16.4 ± 1.6 for Control, D2 and D3 groups, respectively), the number fertilized oocytes and Grade 1 embryos were greater ($P$ < 0.05) in the D3 group (7.7 ± 1.1 and 5.1 ± 0.9) than in the Control (6.8 ± 1.4 and 3.4 ± 0.8) and D2 (6.7 ± 1.2 and 3.6 ± 0.9) groups. No effect of category was detected.
**Conclusions:** It is possible to combine the OPU procedure with pFSH treatment for *in vitro* and *in vivo* embryo production from the same donor and based on the greater production of Grade 1 embryos, the interval of 3 days would be preferable between OPU and pFSH.
**Acknowledgements:** Laboratorios Calier.

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**075**

## Associations between uterine luminal fluid composition at late diestrus and subsequent success of preimplantation conceptus development in lactating dairy cows

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**Application:** Identification of biomarkers in the uterine luminal fluid (ULF) associated with subsequent success of conceptus development.
**Introduction:** The survival of preimplantation conceptus depends on endometrial physiology and composition of ULF (Ribeiro et al., 2016). Our objectives were to investigate potential differences in ULF composition at late diestrus between lactating cows that successfully developed or failed to develop an elongating conceptus in the subsequent estrous cycle.
**Materials and Methods:** Cows received a transcervical uterine flushing with 30 mL of PBS on day 14 of the cycle (59 ± 3 days postpartum). After flushing, cows received an injection of PGF2, followed by an injection of GnRH and timed AI three days later. On d 15 after AI, the uterus was flushed again for diagnosis of pregnancy based on detection of IFN-τ in the ULF. Eleven cows diagnosed pregnant (P) were paired with 11 cows that failed to become pregnant (NP) for retrospective comparison of ULF composition of the first flushing. Analyses

of composition included the investigation of 168 primary metabolites (GC-MS), 501 complex lipids (LC-MS), and 69 oxylipins (LC-MS). Data were analyzed by ANOVA using MetaboAnalyst and SAS. Statistical models included the effect of fertility category and parity.
**Results:** No differences in primary metabolites (saccharides, free fatty acids, amino acids and derivatives) were identified. The total amount of oxylipins in ULF did not differ between P and NP cows (186.6 vs 185.0 ± 75.5 nM/L) but the concentration of four individual oxylipins - 19,20-DiHDPE, 13S-HODE, 12,13-EpOME, and 15,16-DiHODE - were 80 to 107% greater ($P \leq 0.043$) in P than in NP cows. As for complex lipids, the total concentration in ULF tended ($P = 0.065$) to be lower in P compared with NP cows (37.5 vs 57.1 ± 8.0 μg/mL), which was mostly explained by differences ($P = 0.048$) in concentrations of cholesterol and cholesteryl esters (5.5 vs 10.4 ± 1.7 μg/mL). As percentage of total lipids, no classes of lipids differed between groups. For individual molecules, 34 were differently abundant in ULF of P vs NP. Ten of those, mostly glycerophospholipids, were 75 to 123% less abundant in P cows. The remaining 24 lipids, mostly triacylglycerols, were 74 to 100% more abundant in P cows.
**Conclusions:** Composition of lipids but not of primary metabolites in ULF at late diestrus were associated with subsequent success of preimplantation conceptus development through day 15 of pregnancy in lactating dairy cows.
**Acknowledgements:** Financial support was provided by the Natural Sciences and Engineering Research Council of Canada.

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## 076
## DNA content in embryonic EVs is independent of the apoptotic rate in the embryo

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**Application:** This study allows the application of a non-invasive method for embryonic biopsy improving genetic diagnosis in assisted reproductive technologies in bovines.
**Introduction:** Free DNA in the culture media of *in vitro* produced embryos provides an opportunity for early genotyping avoiding the invasive nature of embryo biopsy. The method is more accurate if the DNA contained in extracellular vesicles (EVs) is used for the analysis rather than total medium. However, in human embryos the amount of DNA in culture medium is negatively correlated with embryonic competence (Pallinger et al., 2017), suggesting that the DNA is detected in apoptotic bodies. The high rate of apoptosis induces developmental arrest being negative for the applicability of this method for embryo genotyping. This study aimed to evaluate the effect of embryonic apoptosis on DNA content of EVs.
**Materials and Methods:** Bovine embryos were produced by parthenogenesis using *in vitro* matured oocytes. Day 5 morulae were cultured individually in EVs depleted medium. Blastocyst and their CM were collected individually. EVs were separated by ultrafiltration (Amicon-100 kDa) and analysed by nanoparticle tracking analysis to determine size, total concentration and concentration of EVs containing DNA using a DNA specific stain (BioTracker 488). Blastocysts were fixed, permeabilized and stained to determined total cell number and number of cells suffering apoptosis. Images from embryos were taken in a multichannel fluorescence microscope and cell count was performed using Image J software. The relation among total number of EVs, ratio of DNA+/total-EVs and apoptotic rate (AR) in the embryos was analysed.
**Results:** The mean total cell count and apoptosis rate in blastocysts were 52.67+/−7.5 and 14.9+/−12.13% respectively. Isolated nanoparticles were positive for EVs markers (CD9, CD63 and CD81) and had a classical EV morphology determined by transmission electron microscopy. The mean size and concentration of nanoparticles without DNA stain were 153.9+/−4.9 nm and 2.9e+08+/−7.6e+07 particles/mL respectively. After DNA staining mean size of particles increased significantly in all samples (average 516.9+/−81.3 nm). This was probably due to an effect of the fluorescence during NTA video acquisition because this size range of nanoparticles was not detected without DNA staining. In average 4.2+/−2.5% of total nanoparticles were positive to DNA. No correlation between DNA+/total-EVs and AR was obtained using person correlation test.
**Conclusions:** During the *in vitro* culture, bovine embryos release EVs containing DNA fragments regardless of the apoptotic rate, thus, embryonic EVs could be used as a non-invasive biopsy for genetic analysis.
**Acknowledgements:** Funded by FONDECYT 1210334, ANID, Chile.

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**077**

## The impact of progesterone concentrations during the follicular growth on the transcriptome profile of early embryos recovered from superovulated Holstein heifers

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**Application:** Understand if progesterone concentrations during the follicular growth is associated with gene expression patterns of 7d old embryos and could be used in embryo transfer programs to increase the quality of embryos recovered from superovulated heifers.
**Introduction:** Greater progesterone concentrations during the follicular growth was associated with greater quality of embryos produced by superovulated cows (Rivera et al., 2011) and heifers (Marques et al., 2022). Although morphological classification of bovine embryos have substantial value in embryo transfer programs, the precision and accuracy of this classification may be affected by the subjectivity of this method. Detailed information concerning gene expression patterns linking the association between progesterone during the follicular growth and embryo quality remains to be researched. The objective of this study was to evaluate the impact of different progesterone concentrations during the follicular growth on the transcriptome profile of embryos recovered from superovulated heifers.
**Materials and Methods:** A total of 63 Holstein heifers were randomly assigned into two experimental groups: low progesterone ($n$ = 31) and high progesterone ($n$ = 32). Animals received a pre-synchronization protocol followed by a protocol of superovulation that included the allocated progesterone treatment (High = new progesterone intravaginal implant (CIDR); Low = second use CIDR and extra injections of $PGF_{2\alpha}$ on days -11 (a.m.; p.m.), -5 (p.m.) and -4 (a.m.; p.m.) of the protocol). Embryo collection was performed 7d post artificial insemination and embryos were evaluated for stage of embryonic development and grades of quality. Embryos graded as good/excellent quality (High = 27; Low = 27) were randomly allocated in 3 replicates per treatment group, balanced for stage of embryonic development. Embryo RNA was extracted from each replicate and six libraries were prepared and sequenced (Illumina). Principal component analysis and Ingenuity Pathways Analysis were performed.
**Results:** A total of 1 429 gene transcripts were identified to be differentially expressed between treatment groups. The expression of 563 gene transcripts were significantly increased, whereas 866 were significantly decreased in high progesterone embryos compared with low progesterone. Approximately 1% ($n$ = 13) of the differentially expressed genes were pseudo genes and 11.2% ($n$ = 160) represented novel transcripts. Upregulated genes exhibiting significant differential expression by 2.7–6.1 $\log_2$ fold-change included: CCR3, MPO, IGFBP1, and RSAD2 ($P < 0.05$). Downregulated genes (1.9–9.0 $\log_2$ fold-change) included: RETN, ESR1, CNFN and TNFRSF17 ($P < 0.05$).
**Conclusions:** Different progesterone concentrations during the follicular growth is associated with gene expression patterns of early embryos.
**Acknowledgements:** This study was supported by the Resilient Dairy Genome Project and the Natural Sciences and Engineering Research Council.

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**078**

## Is the endometrial environment before embryo transfer critical for pregnancy establishment? novel evidence from endometrial variables associated with pregnancy outcome

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**Application:** Reduce early pregnancy loss in beef cows caused by a dysfunctional endometrium.
**Introduction:** In beef cattle, 70% of the pregnancy losses occur in the first 3 weeks of gestation. Low estrus expression and poor endometrial receptivity are major contributors for losses. We hypothesized that cows which remain pregnant after sequential rounds of embryo

transfer have a greater estrus duration, endometrial thickness, and endometrial responsiveness to interferon-tau than cows that do not remain pregnant.
**Materials and Methods:** Fifty *Bos indicus*-influenced (3/8 of brahman genetics) primiparous cows were fitted with a collar-mounted accelerometer (SCR Engineers, Netanya, Israel) to detect estrus in response to synchronization in two subsequent experimental replicates. Only cows that showed estrus (day 0) remained in the study. Estrus duration was determined by the accelerometer software. On days −1 and 4, the endometrial thickness at the uterine body was measured by ultrasonography. On day 4, luminal epithelial cells were collected from the uterine body using a cytology brush, cultured, and treated with 0 or 10 ng/mL recombinant bovine interferon-tau for 24 h. The expression of Interferon-stimulated gene 15 (ISG15) in response to interferon-tau was measured by qPCR. On day 7, two embryos were transferred/cow. Pregnancies were diagnosed on day 32 and day 45, and then pregnancies were terminated.
**Results:** Overall, 51/100 (51%) cows showed estrus and 34/51 (66.7%) remained pregnant to embryo transfer. Cows that were detected pregnant on day 32 in both replicates were considered fertile ($n = 9$), and cows that failed to remain pregnant in both replicates, infertile ($n = 3$). Estrus duration was shorter on pregnant (10 ± 0.8 h) vs. non-pregnant cows (14.1 ± 1.12 h; $P < 0.05$). Endometrium was thicker on day −1 (1.39 ± 0.04) than on day 4 (1.14 ± 0.03; $P < 0.05$), regardless of pregnant status. The endometrial thickness on day 4 was greater in fertile (1.18 ± 0.03) than infertile (1.0 ± 0.06; $P < 0.05$) cows. Although pregnant and non-pregnant cows had similar basal expression of ISG15 (0.0035 ± 0.002), treatment with interferon-tau increased ISG15 expression more in pregnant (0.034 ± 0.0018) than non-pregnant cows (0.024 ± 0.002; interferon-tau*pregnancy interaction: $P < 0.05$).
**Conclusions:** In summary, endometrial thickness and responsiveness to interferon-tau on day 4 were positively associated with pregnancy success. We concluded that endometrial function, measured before embryo transfer, influences the pregnancy outcome. Further research is warranted to confirm and expand these findings.
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**079**
## Oleic acid dose-dependently compensates for the negative effect of stearic acid on early bovine embryos

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**Application:** Metabolic stress, characterized by high free fatty acid levels, is related with reduced female fertility.
**Introduction:** In previous studies we observed a dose-dependent adverse effect of saturated stearic acid on oocyte developmental competence, while monounsaturated oleic acid was harmless. Cumulus cells were able to protect oocytes with stearoyl-CoA desaturase-1 that converts stearic into oleic acid (Aardema et al., 2017). Since cumulus cells are lost after ovulation, here we investigated the impact of stearic acid and stearoyl-CoA desaturase-1 expression in embryos.
**Materials and Methods:** Cumulus-oocyte-complexes, collected from 2–8 mm follicles of bovine slaughterhouse ovaries matured *in vitro* for 23h (4 runs, $n = 400$/run) and fertilized. Presumed zygotes were cultured in synthetic oviductal fluid (control) and with; 25 or 50 μM oleic acid, 25 or 50 μM stearic acid or a combination of 25 or 50 μM oleic and 25 or 50 μM stearic acid during the first five days of embryo culture (oviductal period). At day 8 the number of blastocysts was recorded. Stearoyl-CoA desaturase-1 mRNA expression of oocytes, zygotes, day 5 and 8 embryos was assessed by RT-qPCR. Protein expression of stearoyl-CoA desaturase-1 in day 5 and 8 embryos was analysed by confocal microscopy by using an inverted Nikon A1R. Fixed in 4% paraformaldehyde, was followed by overnight immunostaining at 4 °C with primary antibody, stearoyl-CoA desaturase-1, and a 1 h incubation at room temperature with a second antibody, goat anti-rabbit AlexaTM fluor 647. SPSS 27.0 was used for statistical analysis using the general linear model.
**Results:** The blastocyst rates after exposure of embryos to 25 or 50 μM stearic acid from day one to five were significantly lower, respectively 18.9 ± 1.6% and 2.6 ± 4.6%, in comparison to the control treatment 28.7 ± 6.3% ($P < 0.05$). Interestingly, embryos exposed to oleic acid or a combination of stearic and oleic acid demonstrated blastocyst rates comparable to the control group, except for the group with 50 μM stearic and 25 μM oleic acid (16.4 ± 5.8%, $P < 0.05$). Both mRNA and protein of stearoyl-CoA desaturase-1 were detected in all groups with the highest expression present in day 8 blastocysts.
**Conclusions:** These data demonstrate that oleic acid is able to compensate for the adverse effect of stearic acid during embryo development, when oleic acid concentrations are equal or higher than those of stearic acid. Furthermore, the higher expression level of stearoyl-CoA desaturase-1 at day 8 suggests that blastocysts may protect themselves against saturated fatty acids by desaturating stearic acid, which will be further investigated.

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## 080
## Effect of *in vivo*-derived and *in vitro*-produced bovine conceptuses on metabolites within the conceptus-endometrial microenvironment

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**Application:** *In vitro*-produced embryos are lower quality and establish less pregnancies compared to *in vivo* embryos. Understanding origin-related differences can help improve *in vitro*-production systems.
**Introduction:** The endometrium secretes factors, including metabolites, that support conceptus development. Using a conceptus-endometrial co-culture system, bovine endometrial transcriptome was found to respond differently to *in vitro*-produced compared to *in vivo*-derived conceptuses. However, conceptus origin effect on endometrial metabolite production is unknown. Thus, we used the co-culture system to identify endometrial metabolites dependent on conceptus origin.
**Materials and Methods:** Angus-Holstein heifers ($n = 41$) underwent estrus-synchronization and were divided into groups: (1) artificial insemination to produce *in vivo*-derived conceptuses, (2) embryo transfer to produce *in vitro*-produced conceptuses, or (3) remained cyclic to produce synchronized endometrium. On Day 16, uteri were harvested and endometrial-explants from cyclic animals and conceptuses from pregnant animals were used to develop treatments in 1mL RPMI medium: (1) endometrium alone (Control; 8 mm; $n = 9$), (2) endometrium cultured with an *in vivo*-derived conceptus ($5 \times 2$ mm; $n = 10$), (3) endometrium cultured with an *in vitro*-produced conceptus ($5 \times 2$ mm; $n = 10$), (4) *in vivo*-derived conceptus alone ($5 \times 2$ mm; $n = 7$) and (5) *in vitro*-produced conceptus alone ($5 \times 2$ mm; $n = 7$). Medium was also cultured without conceptus or endometrial tissue ($n = 7$). After 12 h, media was collected for metabolomic analysis using ultra high-performance liquid-chromatography high-resolution mass-spectrometry. Known metabolite peak intensities were acquired using EI-MAVEN through an in-house metabolite library and statistical analysis performed using excel. Treatment comparison metabolite fold-change was calculated using group average intensity and compared using Student's t-tests for statistical significance. MetaboAnalyst was used to identify KEGG pathways associated with significant metabolites.
**Results:** Compared to *in vivo*-derived conceptuses, medium cultured with *in vitro*-produced conceptuses had greater levels of eight metabolites including l-phenylalanine, l-tyrosine and 2-oxoglutarate ($P \leq 0.05$). Associated KEGG pathways included phenylalanine, tyrosine and tryptophan biosynthesis and phenylalanine metabolism ($P \leq 0.05$, FDR $\leq 0.05$). Elevated intrauterine levels of phenylalanine are detrimental to fetal development. Regarding co-cultures, medium from *in vitro*-produced conceptus-endometrial co-cultures had greater and less levels of five and one metabolites, respectively, compared to *in vivo*-derived conceptus-endometrial co-cultures ($P \leq 0.05$). Cytidine, malate, fumarate, ornithine, and histidine were greater while trehalose/sucrose was less. Fumarate accumulation can modify the epigenome and lead to protein dysfunction through succination. No significant KEGG pathways were found.
**Conclusions:** Compared to *in vivo*-derived bovine conceptuses, *in vitro*-produced conceptuses are associated with an altered metabolite microenvironment that may negatively affect establishment of pregnancy.
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## 081
## HH3 embryos arrest their development prior to maternal recognition of pregnancy

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**Application:** All homozygous embryos for the allele Holstein Haplotype 3 (HH3) die before birth. The developmental period at which mortality occurs is unknown and this information is critical to calculate the economic losses caused to the inadvertent cross between HH3 carriers.
**Introduction:** HH3 lethality is caused by a mutation in SMC2 gene. The objective of this study has been to assess the developmental competence of HH3 double carrier embryos (DC) up to maternal recognition of pregnancy.
**Materials and Methods:** *In vivo* embryos were collected at Day 14 from 5 superovulated single-carrier (SC) cows inseminated with a SC bull. Conceptuses were genotyped and subjected to immunostaining to detect specific lineages. To assess the developmental competence at earlier stages of development, embryos lacking SMC2 (KO, functionally equivalent to DC) were generated *in vitro* applying CRISPR technology. *In vitro* matured oocytes were microinjected with Cas9 encoding mRNA and guide RNA against SMC2 (group C+G, partially composed by KO embryos) or with Cas9 alone (group C, injection control formed by non-carrier WT embryos). *In vitro* development was assessed up to Day 8 (conventional culture) and Day 12 (post-hatching culture). D8 and D12 embryos were genotyped and analyzed by immunostaining.
**Results:** *In vivo* D14 conceptuses did not deviate significantly from Mendelian inheritance (6:9:2 for WT:SC:DC), however conceptus length was significantly reduced in DC embryos (14.0 ± 4.1 vs 5.6 ± 2.6 vs 0.4 ± 0.0 mm for WT, SC and DC respectively, mean ± s.e.m. *t*-test

$P < 0.05$). DC embryos showed complete hypoblast migration but lacked embryonic disc (no SOX2+ cells). In contrast, embryonic discs were detected in most WT and SC embryos (6/6 for WT, 9/10 for SC). *SMC2* KO embryos generated *in vitro* (functionally equivalent to DC embryos) were unable to survive to D12 *in vitro*: 9 out of 41 structures recovered at D12 were KO and all of them have collapsed by D12 of culture (*t*-test $P < 0.05$). At D8, blastocyst rates were similar between the groups C+G (containing KO embryos) and C. However, KO embryos (9/39 analyzed in C+G group) showed an already impaired development evidenced by a reduced total (DAPI), trophectoderm (CDX2+) and inner cell mass number (SOX2+) compared to WT embryos (DAPI: 112.8 ± 11.8 vs 48.1 ± 2.8; CDX2+: 90.4 ± 11 vs 32.3 ± 1.6; SOX2+: 29.6 ± 7.4 vs 7.4 ± 1.9 for WT and KO, respectively, ANOVA $P > 0.05$).
**Conclusions:** HH3 DC embryos show impaired development well before maternal recognition of pregnancy.
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## 082
## Effect of dietary by-pass linseed oil on embryo, luteal and uterine gene expression linked to prostaglandin synthesis during maternal recognition of pregnancy in Sarda ewes

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**Application:** Improvement of embryo maternal relationships using strategic dietary supplementation of by-pass linseed oil (LO) during early stage of pregnancy in ewes.
**Introduction:** Previous studies have suggested the luteoprotective effect of polyunsaturated fatty acids omega 3 (PUFASω3) during early stage of pregnancy. However, the results obtained using by-pass LO (rich in α-linolenic acid/ALA/PUFASω3) are not conclusive. The use of gene expression (GE) could offer additional information about this topic. Therefore, the present study assessed the effect of dietary supplementation of by-pass LO on embryo and luteal and uterine GE linked to prostaglandin synthesis on Days 14 and 16 of pregnancy in Sarda ewes.
**Materials and Methods:** Control ewes (CT; $n = 8$) were fed a control diet without LO, while LO ewes ($n = 8$) were fed a diet supplemented with LO (10.8 g ALA/ewe/day). After 10 days of adaptation period, both diets were offered for 32 days (−16 to +15 days after expected mating; Day 0). Estrous synchronization was induced in all the ewes. Then ewes were mated. Four ewes from each treatment were slaughtered at Days 14 (CT = 4; LO = 4) and 16 (CT = 4; LO = 4). Corpora lutea, uterus and embryos were obtained for mRNA GE analyses. Blood plasma samples were obtained on Days -26 and 0 to determine concentration of PUFASω3 and ω6 (total fatty acid methyl-esters/FAME). Tissue specific GE was performed by RNA isolation, reverse transcription and gene-specific Real Time-PCR. Interferon-tau (IFNt) and lysophosphatidic acids receptors 1, 2 and 3 (LPAR1/R2/R3) GE were measured in embryos; estrogen-alpha (ERalpha), interferon-Type1 (IFNAR1), oxytocin (OXTR) and prostaglandin (PGR) receptors, peroxisome-proliferator-activated-receptor-delta (PPARD), cyclooxygenase-2 (COX2), prostaglandin-F-synthetase (PGFS) and prostaglandin-endoperoxide-synthetase (PTGS2) genes were assessed in uterine tissues; COX2 and PTGS5 were measured in luteal tissue. Data were analyzed by GLM-ANOVA, using treatment, day and interaction as fixed factors. Statistical differences were established at $P < 0.05$.
**Results:** Plasmatic concentrations of PUFASω3 were greater in LO group on Day 0 (CT = 4.93 ± 0.50 vs LO = 12.49 ± 0.73% of FAME; $P < 0.001$). Uterine ERalpha was lesser in LO group on Day 16 ($P < 0.05$). From day 14 to 16, PTGS2 decreased in CT group, but not in the LO. Luteal PGTS5 increased from 14 to 16 in both treatments ($P < 0.01$). Embryo INFt, LPAR1, LPAR2 and LPAR3 were greater on Day 14 compared with Day 16 ($P < 0.05$).
**Conclusions:** LO's bioavailability was evidenced at systemic PUFASω3 level. Also, the decrease of ERalpha and maintenance of PGTS2 GE in LO ewes suggest a luteotropic effect of LO.
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## 083
## Bovine embryo development *in vitro* following culture in the presence of cell culture supernatant derived from oviductal epithelial cells treated with interleukin-1 beta

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**Application:** Early embryonic mortality in cattle has a negative effect on production efficiency. Strategies that improve early embryo survival can promote greater profitability of cattle production systems.
**Introduction:** Early embryo development is regulated by various maternally derived factors, including cytokines. Prior work has demonstrated that the cytokine interleukin-1β is upregulated following uterine administration of seminal plasma and that interleukin-1β can increase the abundance of embryotrophic cytokines, such as colony stimulating factor-2 and interleukin-6, in the endometrium. The objective of the present study was to determine whether supernatant derived from oviductal epithelial cells treated with interleukin-1β could improve bovine embryo development *in vitro*.
**Materials and Methods:** Oviductal epithelial cells were harvested from abattoir-derived reproductive tracts obtained from cows that were between days 0–5 of the oestrous cycle and cultured as described previously (Hasan et al., 2020). At 90% confluence, cells were treated with 0, 10 or 100 ng/ml recombinant bovine interleukin-1β. Supernatant was harvested at 24 h and then stored at −20 °C for subsequent use. Embryos were produced *in vitro* using abattoir-derived oocytes ($n$ = 943) as described previously (Ortega et al., 2017). After fertilization, presumptive zygotes were randomly placed in groups of 15 into 25 μl microdrops of synthetic oviductal fluid supplemented with one of nine treatments: no supplement, 10 ng/ml recombinant bovine interleukin-1β, 10% (v/v) culture supernatant from untreated cells, and 1, 5 or 10% (v/v) supernatant from cells treated with either 10 or 100 ng/ml recombinant bovine interleukin-1β. All supernatant groups included the required amount of supernatant from untreated cells such that the total amount of supernatant in each group was 10% (v/v). Cleavage was assessed at day 3 after fertilization and blastocyst development at day 7. Blastocyst stage embryos ($n$ = 144) were subjected to staining to determine total cell number as described previously (Ortega et al., 2017). The study included eight replicates. Data were analysed by analysis of variance using a general linear model.
**Results:** Cleavage rate at day 3 and blastocyst development at day 7 after fertilization were not affected by treatment. Moreover, treatment did not affect the cell number of blastocyst stage embryos harvested at day 7.
**Conclusions:** Overall, bovine embryo development *in vitro* was not affected by culture in the presence of 10 ng/ml recombinant interleukin-1β alone nor by supplementation of culture medium with various amounts of supernatant derived from oviductal epithelial cells treated with either 10 or 100 ng/ml recombinant interleukin-1β.

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## 084
## Superstimulation prior to the ovum pick-up with a single dose of recombinant FSH improves *in vitro* embryo production in Holstein heifers

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**Application:** Treatment with a single dose of r-FSH 3 days before ovum pick-up (OPU) increase the efficiency of *in vitro* embryo production (IVEP) in Holstein heifers.
**Introduction:** Studies were carried out to evaluate the effect of treatment with long-acting recombinant FSH (r-FSH; CEVA Sante Animale) on the efficiency of OPU/IVEP in Holstein heifers. The effects of treatment on follicular diameter, viable oocyte rate, recovery rate, cleavage rate and blastocyst rate were evaluated.
**Materials and Methods:** Ninety Holstein heifers were used. On a random day of the estrous cycle, heifers received an intravaginal progesterone device (PRID) and 2 mg of estradiol benzoate plus PGF2α IM (Day 0). Heifers in the control group ($n$ = 30) received no further treatment, whereas heifers in the r-FSH group received a single dose of 50 μg ($n$ = 30) or 100 μg ($n$ = 30) of rFSH on Day 4. On Day 7 the PRID was removed and OPU was conducted. The same operator aspirated all donors, and the same batch of sexed-semen was used for IVEP.
**Results:** There was no difference between groups in the total numbers (mean ± sem) of follicles aspirated per OPU session (control = 13.2 ± 1.4 vs 50 μg=11.9 ± 1.2 vs 100 μg = 11.6 ± 0.9; $P$ = 0.90). However, treatment with r-FSH regardless of dosage (50 or 100 μg) increased the number of medium follicles (6–10 mm) and decreased the number of small follicles (<6 mm) compared to control at OPU. Furthermore, heifers treated with 100 μg of r-FSH had a greater number of large follicles (<10 mm) than control. Heifers treated with 100 μg of rFSH had higher ($P$ = 0.004) viable oocytes rate (number of COCs cultured/number of total COCs retrieved) than control (control = 54.0%$^{b}$ vs 50 μg = 62.0%$^{ab}$ vs 100 μg = 71,0%$^{a}$; $P$ = 0.004), without compromising the recovery rate (number of COCs recovered per total number of follicles aspirated; control = 79.7% vs 50 μg = 72.7% vs 100 μg = 75,5%; $P$ = 0.57). Furthermore, heifers treated with 100 μg of rFSH had a higher cleavage (control = 30.9%$^{b}$ vs 50 μg = 41.4%$^{ab}$ vs 100 μg = 52.3%$^{a}$; $P$ = 0.02) and blastocysts rates (control = 7.1%$^{b}$ vs 50 μg = 16.2%$^{ab}$ vs 100 μg = 17.4%$^{a}$; $P$ = 0.05). The number of embryos produced per OPU was similar between groups (control = 0.97 ± 0.22 vs 50 μg = 1.67 ± 0.40 vs 100 μg = 1.37 ± 0.28; $P$ = 0.29).
**Conclusions:** Treatment with a single dose of r-FSH increased the number of medium and large follicles at the time of aspiration, without compromising the recovery rate. In addition, it increased the viable oocytes, cleavage and blastocysts rates, improving the efficiency of OPU/IVEP in Holstein heifers.

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**085**

## Effects of ascorbate on blastocyst development following bisection or quadrisection of bovine morula stage embryos produced *in vitro*

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**Application:** *In vitro* embryo production can be applied effectively in cattle production systems to propagate offspring from genetically superior animals. Micromanipulation techniques, such as embryo bisection or quadrisection, could further maximise the potential of *in vitro* embryo production to enhance the rate of genetic gain.
**Introduction:** Embryo splitting techniques can induce cell damage and apoptosis leading to developmental arrest. Therefore, the objective was to determine whether ascorbate could increase the number of blastocysts produced following either bisection or quadrisection.
**Materials and Methods:** Embryos were produced *in vitro* as described previously (Ortega et al., 2017). At day 6 after fertilization, morula stage embryos ($n$ = 460) were randomly assigned in a 2 × 3 factorial design to no manipulation, bisection or quadrisection with or without 0.1 mM ascorbate included in both the embryo manipulation medium (Dulbecco's phosphate-buffered saline with $MgCl_2$ and $CaCl_2$) and culture medium (synthetic oviductal fluid). Bisection and quadrisection were performed using a manual micromanipulator with attached ophthalmic surgical blade. Embryos assigned to the no manipulation group were handled in a similar manner but were not subjected to splitting. After manipulation, embryos were cultured individually using the well of the well format. Blastocyst development was recorded on day 8 and the ratio of blastocysts produced per morula was calculated. Blastocysts ($n$ = 178) were harvested and subjected to differential staining as described previously (Ortega et al., 2017). The experiment was replicated six times. Data was analysed by analysis of variance using a general linear model.
**Results:** The number of blastocysts produced per morula was not affected by ascorbate. Bisection and quadrisection resulted in a greater ($P$ < 0.001) number of blastocysts per morula compared to no manipulation (1.57 ± 0.1 and 1.47 ± 0.1 vs 0.95 ± 0.1). Ascorbate reduced ($P$ < 0.01) blastocyst total cell number (75.6 ± 2.7 vs 87.5 ± 2.8) and number of cells in the trophectoderm (43.1 ± 2.0 vs 53.5 ± 2.1). Blastocysts derived from bisected and quadrisected morula had a lesser ($P$ > 0.001) total cell number, number of cells in the inner cell mass cells, and number of cells in the trophectoderm compared to non-manipulated morula. There was no interaction between ascorbate and method of manipulation affecting any variable analysed.
**Conclusions:** Bisection and quadrisection effectively increased the number of blastocysts produced per manipulated morula. However, development following manipulation was not improved by ascorbate. Future work needs to evaluate the capacity of bisected and quadrisected embryos to establish pregnancy following transfer.

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**086**

## Superstimulation prior to ovum pick-up with a single dose of recombinant FSH improves *in vitro* embryo production in lactating Holstein cows

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**Application:** A single-dose FSH for superstimulation facilitates donor management and increases the efficiency of ovum pick-up (OPU) for *in vitro* embryo production (IVEP) in cattle.
**Introduction:** Traditional superstimulatory treatments consist of twice-daily intramuscular injections of p-FSH for IVEP. The need of frequent treatment to induce ovarian superstimulation is due to the short half-life of FSH (5 h) in cattle. The treatment with long-acting recombinant FSH (r-FSH; CEVA Sante Animale) might reduce the number of treatment and improve the efficiency of OPU/IVEP protocol in lactating Holstein donors.
**Materials and Methods:** Cows received an intravaginal progesterone device (PRID) and 2 mg of EB plus PGF2α IM (Day 0). Cows in control ($n$ = 31) received no further treatment, whereas cows in the r-FSH groups received a single dose of 100 ($n$ = 31) or 150 μg ($n$ = 33) of r-FSH on Day 4. Cows in the p-FSH group (Folltropin, Vetoquinol; $n$ = 27) received a total dosage of 200 mg of p-FSH on Days 4 and 5 in four

decreasing doses 12 h apart (57, 57, 43, and 43 mg). On Day 7 the PRID was removed and OPU was performed. The same batch of sexed-semen was used for IVEP.
**Results:** There was no difference between groups ($P = 0.21$) in total numbers of follicles aspirated (mean of 12.6 ± 0.98). However, r-FSH (regardless of dosage) and p-FSH treatments increased ($P < 0.0001$) number of large follicles (<10 mm) and decreased number of small follicles (<6 mm) at OPU than control. Donors that received 100 (71.1%) and 150 μg (74.7%) of r-FSH showed a similar recovery rate (number of COCs recovered per total number of follicles aspirated) than control (81.9%). However, donors treated with p-FSH (68.0%) presented a lower oocyte recovery rate ($P < 0.05$) than the control. Furthermore, blastocysts rate (number blastocysts/number oocytes cultured) was higher in donors treated with 150 μg of r-FSH (27.4%) than in donors received 100 μg of r-FSH (14.1%; $P = 0.01$). The dose of 150 μg of r-FSH (2.58 ± 0.39) showed a higher number of blastocysts per OPU ($P = 0.07$) than 100 μg of r-FSH (1.58 ± 0.30) and p-FSH (1.46 ± 0.25). The P/ET of *in vitro* produced embryos from Holstein donors treated with a single dose of 100 μg of r-FSH 3 days before OPU was evaluated. Embryos were transferred into recipients (lactating Holstein cows). There was an increase in P/ET of recipients that received embryos from donors treated with r-FSH [control = 27.1% (39/144) vs r-FSH = 35.1% (60/171); $P = 0.06$].
**Conclusions:** Treatment with r-FSH facilitates donor management and increases the efficiency of OPU/IVEP in lactating Holstein cows.
**Acknowledgements:** Agrindus; Samvet; CEVA Sante Animale.

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**087**
## The impact of sequential stimulation and neuregulin 1 supplementation during IVM on post-IVF embryo production in cattle

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**Application:** To improve post-IVM/IVF embryo production in cattle.
**Introduction:** Gradual activation of the maturation cascade has been proposed to enhance nuclear-cytoplasmic synchrony and *cumulus*-oocyte communication, motivating the investigation of pre-IVM culture steps with agents capable to slow nuclear maturation. *In vivo*, before ovulation, the cumulus-oocyte complex (COC) is exposed to increased FSH levels, after which the maturation cascade is triggered by EGF-like factors secreted by granulosa cells in response to LH. Supplementation of the IVM medium with neuregulin 1 (NRG1), a modulatory EGF-*like* factor, improved oocyte developmental competence in cattle (Dellaqua et al., 2023). Herein, aiming to further develop our previously proposed two-step culture system for oocyte maturation, named "the follicular system" (Soares et al. 2017), we assessed the impact of the sequential exposure to FSH and amphiregulin (AREG) during IVM, with or without NRG1 supplementation during pre-IVM, on post-IVF embryo development.
b Bovine COCs were aspirated from 2–8 mm follicles of abattoir ovaries, pooled in groups of 25, and subjected to pre-IVM for 9 h in TCM199 containing 500 ng/mL 17β-estradiol, 50 ng/mL progesterone, $10^{-d}$ IU/mL FSH and 100 nM NPPC for Control and S (sequential IVM preceded by pre-IVM without NRG1) groups, further supplemented with 1 ng/mL NRG1 in the pre-NRG1+S group (sequential IVM preceded by pre-IVM with NRG1). Subsequently, COCs allocated to the control group were subjected to IVM in TCM199 containing $10^{-b}$ IU/mL rhFSH, 50 ng/mL 17β-estradiol, 150 ng/mL progesterone, 100 ng/mL AREG and 1 ng/mL NRG1 for 24 h. COCs allocated to S and pre-NRG1+S groups were first subjected to a 6 h culture in a medium identical to the control except for the exclusion of AREG and, subsequently, to a 18 h culture step in the complete IVM control medium. *In vitro* fertilization (IVF) was performed for 18 h and presumptive zygotes were cultured for seven days at 38.5 °C under 5% $CO_2$, 5% $O_2$, and 90% $N_2$ in humidified air. Production of total blastocysts and viable embryos (expanded and hatched blastocysts) in relation to total oocytes was compared between groups. Treatment effects were tested by ANOVA and groups compared with the Fisher's Protected test ($n = 3$).
**Results:** The combination of sequential stimulation during IVM with NRG1 supplementation during pre-IVM increased post-IVF rates of total (Control: 34.34 ± 1.66%; S: 40.26 ± 1.94%; pre-NRG1+S: 45.94 ± 3.04%; $P = 0.032$) and viable blastocysts (Control: 30.53 ± 1.47%; S: 29.71 ± 2.36%; pre-NRG1+S: 38.56 ± 1.91%; $P = 0.036$).
**Conclusions:** NRG1 supplementation during pre-IVM combined with sequential stimulation with FSH followed by AREG treatment during IVM may improve post-IVF embryo production in cattle.
**Acknowledgements:** FAPESP (2019/14588-6), CAPES (001).

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## 088
## The intricacies of conceptus-maternal interactions proposed by parthenogenetic beef cattle pregnancies

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**Application:** Reduce occurrences of late embryonic mortality, gain a better understanding of sire genomic contribution to early pregnancy, and evaluate novel molecular adhesion processes that may exist in bovine pregnancies.
**Introduction:** Molecular mechanisms governing conceptus elongation and active placentation remain relatively elusive, but previous studies suggest the paternal genome has a vital role in placental development. Osteopontin, an extracellular matrix adhesion molecule, was previously postulated to have promotive effects on elongation and attachment during early pregnancy in ovine and swine; few studies exist in bovine. Parthenogenetic embryos (lacking paternal genetic contribution) were previously created and transferred. Parthenogenetic conceptuses appeared to elongate and showed primitive attachment phenotypic profiles compared to conventional pregnancies. Parthenogenetic pregnancies showed decreased pregnancy-associated glycoprotein profiles in maternal circulation, suggesting an increased likelihood of pregnancy failure. Low circulating pregnancy-associated glycoprotein pregnancies have shown increased prostaglandin $E_2$ circulating concentrations. The objectives of this study were to compare transcriptomic profiles in parthenogenetic and conventional pregnancies during the placental attachment period for further insight into conceptus attachment/pregnancy maintenance, evaluate pregnancy-associated glycoprotein expression, and characterize osteopontin expression in bovine pregnancies.
**Materials and Methods:** The transcriptome of parthenogenetic ($n = 3$) and conventional ($n = 3$) *Bos taurus* pregnancies at gestational day 31 was analyzed with FDR $\leq 0.05$ considered statistically significant. Conceptuses and endometrium from parthenogenetic and conventional pregnancies were collected, and qPCR was performed with osteopontin, *SPP1*, as the gene of interest. Statistical analysis was performed with Proc Mixed on SAS with significance being $P \leq 0.05$. Immunolocalization of osteopontin and pregnancy-associated glycoproteins was performed using a polyclonal rabbit anti-osteopontin antibody against E-cadherin and dual staining for anti-pregnancy-associated glycoprotein rabbit polyclonal and mouse monoclonal antibodies.
**Results:** Osteopontin was increased in the parthenogenetic pregnancies' endometrium ($P < 0.01$) and was validated using qPCR ($P < 0.05$). Osteopontin was visualized in the glandular epithelium and luminal epithelium of uteri as well as the elongated trophectoderm and extraembryonic endoderm of conceptuses from both groups. The transcriptomic analysis revealed a potential shift in the prostaglandin biosynthesis pathway towards increased active prostaglandin E2 production in parthenogenetic pregnancies; these conceptus trophectoderm cells expressing pregnancy-associated glycoproteins were located at the apical boarder of the uterine luminal epithelium, but these cells did not migrate into the luminal epithelium.
**Conclusions:** The sire genome's role in embryogenesis remains remarkably unknown, but this data suggests a vital paternal role in mediating conceptus elongation, advanced placental development, and successful fetal-maternal attachment in bovine.
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## 089
## Replacing fetal calf serum by yeast extract during *in vitro* embryo culture

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**Application:** Bovine fetal calf serum (BFS) is broadly used as a medium supplement during *in vitro* embryo production.
**Introduction:** An intrinsic sanitary risk is associated with the use of animal sources of macromolecules. The aim of the present study was to evaluate the replacement of FCS by yeast extract (YE) during *in vitro* embryo culture.
**Materials and Methods:** Bovine cumulus oocyte complexes ($n = 1\,377$) obtained from slaughterhouse ovaries (Bos indicus) were *in vitro* matured during 22 h and submitted to *in vitro* fertilization during 20 h, using the standard protocols of the commercial laboratory Norte Embryo, located in Alta Floresta, Brazil. The presumptive zygotes were randomly distributed into five experimental groups, all submitted to *in vitro* culture (IVC) in SOF media added with BSA and supplemented with: G1, 3% FCS (positive control, $n = 282$); G2, no supplement (negative control, $n = 285$); G3, 0.1 mg/mL YE ($n = 271$) during all IVC; G4, 0.1 mg/mL YE only up to day 3 of IVC ($n = 267$); and G5, 0.1 mg/mL YE

up to day 3 and 3% FCS from days 3 to 9 of IVC ($n$ = 272). Cleavage rate was evaluated at day 3, blastocyst rate at day 7 and hatching rate at day 9. Data were analyzed using the Proc Glimmix of the SAS software, considering the effects of treatment, replica, and interactions. There was no effect ($P$ > 0.05) of replica for any of the endpoints analyzed.
**Results:** Cleavage rates were similar among groups (81.9% ± 1.1; 76.1% ± 1.3, 84.1% ± 1.0, 80.9% ± 0.8, and 82.4% ± 0.8 for G1, G2, G3, G4 and G5, respectively; $P$ = 0.6424). Greater ($P$ = 0.0016) blastocyst rates were obtained when YE was used up to day 3 of IVC and FCS from day 3 to day 7 (G5: 47.4% ± 0.8 vs. G1: 43.6% ± 0.9, G2: 29.8% ± 0.7, G3: 32.8% ± 0.6 and G4: 30.0% ± 0.9). The hatching rates in groups supplemented with FCS (G1: 74.0% ± 0.8) and with YE up to day 3 without (G4: 70.0% ± 0.8) or with (G5: 76.0% ± 0.8) FCS from days 3 to 9 were similar ($P$ > 0.05), but greater ($P$ = 0.0073) than those obtained in groups without any supplementation (G2: 48.2% ± 0.5) or supplemented with YE during all IVC (G3: 61.8% ± 0.7).
**Conclusions:** The present results suggest that YE supplementation had no negative impact on cleavage or on blastocyst rates. However, the potential beneficial effects of YE supplementation on blastocyst and hatching rates may depend on the period of IVC in which YE is used.
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**090**
## Pregnancy rate after tolfenamic acid treatment in bovine embryo recipients

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**Application:** Assessment of a nonsteroidal anti-inflammatory drug (NSAID) treatment at embryo transfer in recipient cows.
**Introduction:** Reproductive tract manipulation in recipient cows may induce endometrial prostaglandin release causing luteolysis and decreased progesterone levels, potentially contributing to early embryonic loss. A previous study showed that tolfenamic acid, an NSAID, improved pregnancy rate and embryo survival in mice, when administered prior to embryo transfer (Schlapp et al., 2015). The objective of this study was to assess the treatment of an NSAID in recipient cows, receiving *in vivo* produced embryos.
**Materials and Methods:** A total of 703 multiparous and nulliparous Angus and Hereford recipient cows were included after estrous synchronization with two doses of prostaglandin F2alpha analogue, separated 11 to 13 days. Estrous (Day 0) was detected with the aid of estrous patches. Fresh or frozen-thawed embryos were transferred on Day 6 to 8. *In vivo* produced embryos were graded according to IETS benchmarking and were transferred to the recipients in 17 replicates. At the time of embryo transfer, recipients were assigned to two groups: untreated control group ($n$ = 350), and NSAID treated group ($n$ = 353) receiving an intramuscular dose of 2 mg/kg (1 ml/20 kg BW) of tolfenamic acid (Tolfedine®CS, Vetoquinol S.A., France). Pregnancy was confirmed by ultrasonography between Day 37 to 47. Statistical analysis was performed by using logistic regression in GLMM with the treatment day of embryo transfer, recipient type (multiparous vs nulliparous), body condition score, corpus luteum size, difficulty in performing the embryo transfer, and embryo stage and grade were included as fixed variables.
**Results:** Mean overall pregnancy rates for the tolfenamic acid treated cows, versus untreated, was 56.7% and 54.9% respectively ($P$ = 0.48), while specific pregnancy rates were respectively 53.4% versus 49.8% for frozen embryos, 61.1% versus 52.9% for Grade 3 embryos, 75.0% versus 55.6% for blastocyst stage embryos, 57.0% versus 48.2% for nulliparous cows, and 57.0% versus 49.4% for embryo transfer on Day 8. Although previous numerical trends are noteworthy, none achieved statistically significance ($P$ > 0.05).
**Conclusions:** Despite numerical trends displaying promising results with the use of tolfenamic acid at the time of embryo transfer in recipient cows, we suggest that further studies are required to achieve a robust conclusion on whether, or not, to use this NSAID in recipient cows for both *in vivo* produced and *in vitro* derived embryos.
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**091**
**Development and characterisation of a long-term *in vitro* bovine endometrial organoid model**

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**Application:** Establishment of a three-dimensional *in vitro* model to maintain physiological characteristics of the bovine endometrium that allows the study of embryo-maternal interaction.
**Introduction:** Organoids are three-dimensional structures that show several advantages such as long-term culture of primary cells maintaining molecular, functional and epigenetic characteristics of the original tissue (Lancaster and Hunch, 2019). Organoids are increasingly. used for pharmacological assays, gene therapies and disease pathogenesis. Uterine and endometrial organoids are described in several species (Jamaluddin et al., 2022). These organoids respond to ovarian hormones and mimic the changes that occur in the different phases of the reproductive cycle in mice and humans (Turco et al., 2017). This study aimed to test the hypothesis that bovine endometrial organoids (EOs) maintain the tissue physiological response under ovarian hormone and Interferon tau (IFN-tau) stimulus.
**Materials and Methods:** Uteri from six cattle in follicular phase of the estrous cycle were collected. Endometrial biopsies were obtained from the first third of the uterine horn. The glandular and stromal fraction was used to establish the EOs which was exposed to defined culture media for expansion for 18 days. EOs were primed with Oestradiol for 48 h and finally differentiated with a cocktail of progesterone, cyclic adenosine monophosphate and IFN-tau for 4 days. After 22 days, EOs were analysed for essential markers for uterine functions such as strogen, citokeratin and vimentin and quantitative real-time PCR for genes cathepsin-L (CTS6), Cystatin-M (CTSL), ubiquitin like modifier (ISG15), MX Dynamin Like GTPase-1-2 (MX1-MX2), 2′-5′-Oligoadenylate Synthetase-1 (OAS1Y), Mucin-1 (MUC1), Prostaglandin E Synthase (PTGES), Wnt Family Member-7A (WINT7A), Oxytocin and Estrogen Receptors (OXTR, ESR1), Interferon Alpha and Beta Receptor Subunit-1-2 (IFNAR1-IFNAR2). Non-stimulated OEs were used as negative controls for all experiments. Statistical analysis of gene expression was performed using Wilcoxon non-parametric test.
**Results:** EOs retains structural features of endometrial tissue and expresses receptors for uterine function. The gene expression showed a mimic endometrial response of EOs upon exposure to the molecular cocktail such as an increase in mRNA level of CTS6, CTSL, ISG15, MX1, MX2, OAS1Y, MUC1, PTGES genes compared to the control. No differences were observed between treatment and control in WINT7A, OXTR and ESR1 genes. In contrast, a decrease in mRNA level of interferon receptors, IFNAR1 and IFNAR2 compared to the control was observed ($P < 0.05$).
**Conclusions:** We succeeded in generated bovine EOs *in vitro* than maintain endometrial characteristics after long-term culture, responding to hormonal stimuli.
**Acknowledgements:** Work funded by Fondecyt 3220291 and 1210334 from ANID, Chile.

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## FEMALE FERTILITY

**092**
**Plasma cell-free DNA concentration increases during luteolysis in beef cows**

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**Application:** This is the first experiment conducted at our lab aiming to use the detection of cell-free DNA (cfDNA) as a tool for early pregnancy diagnosis.
**Introduction:** Previous evidence has suggested that cfDNA can be used as a biomarker and a liquid biopsy for cancer diagnosis (Fleischhacker and Schmidt, 2007). cfDNA results from cell death and can help measure the processes of apoptosis in an animal. During structural luteolysis, apoptosis of the cells of the corpus luteum (CL) occurs, so a new estrous cycle begins. We hypothesized that cfDNA concentrations would increase when inducing luteolysis by administering a prostaglandin F2 alpha (PGF2α) analog to the cycling cow.
**Materials and Methods:** Multiparous Angus cows ($n = 15$) were synchronized using the 7-Day CoSynch + CIDR protocol. Ten days after detected estrus, cows were randomly assigned to a control treatment ($n = 5$) or were administered PGF2α ($n = 10$). Twice a day, gray mode and color-Doppler ultrasonography were used to record videos of the CL and to calculate area (CL-A) and luteal blood perfusion (LBP%).

Additionally, one blood sample was collected to evaluate plasma progesterone (P4) and cfDNA concentrations for 4 consecutive days. Data analysis was performed using repeated measures analysis of variance, and a Tukey's Test was used as a post hoc test to verify mean differences between days and treatment groups. The experimental design was a complete randomized design.
**Results:** Induction of luteolysis was demonstrated by a decrease in P4 concentrations ($P < .0001$, PGF = 1.38 ± 0.39 ng/mL, Con = 5.6 ± 0.56 ng/mL), and CL-A (PGF = 2.28 ± 0.17 $cm^b$, Con = 3.02 ± 0.25 $cm^b$) 12 h after the PGF2$\alpha$ injection. Also, a reduction of LBP% ($P = 0.0088$, PGF = 9.49 ± 2.4%, Con = 23.14 ± 4.4%) was observed in the PGF group 36 h after PGF2$\alpha$ injection. cfDNA concentration was greater in the PGF treatment compared with the control treatment 48 h after luteolysis induction (PGF = 11.91 ± 1.30 ng/mL, Con = 7.40 ± 1.84 ng/mL, $P = 0.05$). There was no difference in cfDNA concentration between treatments at 0, 24 and 72 h.
**Conclusions:** cfDNA concentration significantly increased 48 h after luteolysis induction. The present results lead us to presume that cfDNA concentration could be used as a luteolysis biomarker in plasma, although further research would be necessary to evaluate its role in estrous resynchronization programs.
**Acknowledgements:** Special thanks to Colombia's Ministry of Science, technology, and innovation.

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**093**
**Ovarian follicle extracellular vesicle-microRNAs mediated response of beef cows to environmental heat stress**

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**Application:** Identify EV-mediated molecular response in the ovarian follicles of beef cows under environmental heat stress.
**Introduction:** Elevated ambient temperature during the summer season is a major cause of stress in dairy and beef cows, leading to impaired reproductive function and fertility. Follicular fluid extracellular vesicles (FF-EVs) play an important role in intrafollicular cellular communication by mediating, in part, the deleterious effects of heat stress (HS). Here we aimed to investigate the changes in FF-EV miRNA cargoes in beef cows in response to HS during the summer season.
**Materials and Methods:** Dry beef cows ($n = 11$) were subjected to synchronization and stimulation for follicular development. Ovum pick-up (OPU) was conducted on animals in the summer (August 2021) and winter (February 2021) seasons. Rectal temperatures were collected at each OPU and environmental data were collected daily for three weeks before each OPU, which indicated that the temperature-humidity index values were 53.4 and 79.2 for winter and summer, respectively. FF samples were collected from each group (4 replicates/group; 8 mL FF/replicate) and subjected to differential and high-speed ultracentrifugation (120,000xg for 70 min x2) to isolate EVs. The EVs were characterized using a western blot, nanosight tracking analysis, and transmission electron microscopy. Total RNA was isolated from EVs and small-RNA library preparation and RNAseq (NovaSeq6000; Illumina) were performed.
**Results:** Small-RNAseq analysis of FF-EVs revealed that approximately 240 known miRNAs were detected in each season with 232 miRNAs being mutually expressed. Among these, miR-148a, miR-99a, and miR-10b were the most abundant in both seasons. Differential expression analysis identified 16 miRNAs including miR-10a, miR-10b, and miR-19a that were up-regulated while 8 including miR-26a, miR-181a, and miR-1246 that were down-regulated in the summer compared to winter group (FC $\geq$ 1.5; FDR < 0.1). Ontological classification analysis identified EGFR tyrosine kinase inhibitor resistance, ErbB signaling, and p53 signaling as the top pathways targeted by the elevated miRNAs in the summer group, while, cellular senescence, JAK-STAT signaling, and FoxO signaling were the top pathways targeted by the elevated miRNAs in the winter group. Sequence motif analysis revealed the appearance of two specific motifs in 13 out of the 16 upregulated miRNAs under HS conditions. Both motifs were found to be potentially bonded by specific RNA binding proteins including Y-box binding proteins (YBX1 and YBX2) and RBM42.
**Conclusions:** The FF-EV-miRNA profiles can be a good indicator of the cellular level responses to HS in beef cows with the potential interplay between miRNA motif and RNA binding proteins.

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## 094

## The impact of temporary or prolonged β-carotene deficiency during the transition period on the preovulatory granulosa cell transcriptomic profile at the time of breeding in dairy cows

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**Application:** The majority of dairy cows suffer from antioxidant (AO) deficiency, e.g., in β-carotene (bC), during the first few weeks of lactation, which creates oxidative and metabolic stress (Mary et al., Animal, 2021,15:100303). This deficiency can be minimised by good transition management and dietary supplements.
**Introduction:** Here, we hypothesised that maintaining cows in an optimal AO status throughout early lactation may positively influence the growing ovarian follicle compared with cows that experienced a transient period of bC deficiency around wk2 postpartum or experienced a prolonged period of bC deficiency from parturition until breeding (wk8).
**Materials and Methods:** RNA-sequencing was performed on granulosa cells of wk8 preovulatory follicles ($n$ = 23 cows) retrospectively in association with different blood bC patterns. These cows were kept in the same farm under a standard management system and same diet and were synchronized before ultrasound-guided transvaginal follicular aspiration at wk8. Only the pre-LH surge samples were included in this analysis ($n$ = 16). Differentially expressed genes (DEGs) were determined using DESeq2 (FDR < 0.05) between cows with: (1) high wk 2 bC (not deficient: >150 μg/dL) and high wk 8 bC (above optimal level: >350 μg/dL, Mary et al., 2021) (Always-High) ($n$ = 8); (2) low wk 2 and wk 8 bC (Always-Low) ($n$ = 4); and (3) low wk 2 and high wk 8 bC (Low-to-High) ($n$ = 4).
**Results:** We detected 164 DEGs in the *Always-High vs Always-Low* comparison, and 88 DEGs in the *Low-to-High vs Always-Low* comparison, only 23 of which were common. Only two DEGs were detected in *Always-High vs Low-to-High*. GO-term association (HyperGTest in R) identified that DEGs in *Always-High vs Always-Low* were associated with alterations in oxidoreductase activities, upregulated mitochondrial RNA processing and mitochondrial gene expression, estrogen metabolic processes, and cellular ketone, lipid and amino-acid metabolic processes, as well as downregulated autophagy, vesicle-mediated transport, and unsaturated fatty acid metabolic processes. In contrast, DEGs in *Low-to-High vs Always-Low* were associated with upregulated endosome-to-lysosome transport and vesicle-mediated transport between endosomal compartments suggesting increased autophagic activity, as well as downregulation of transcription, RNA stability and translation, and protein acetylation. The two upregulated DEGs in *Always-High vs Low-to-High* were NSUN6, an RNA methyltransferase, and JCAD, a Hippo signalling negative regulator, and therefore a promotor of follicular growth.
**Conclusions:** These results illustrate that optimal bC levels during the transition period may improve follicle quality. Restoring bC only by wk 8 can compensate, at least in part, for bC deficiency at wk2. However, these follicles may still exhibit active cellular repair mechanisms and disruption of cell proliferation after a period of bC deficiency early post-partum.

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## 095

## An investigation into the factors associated with nadir and peak milk pregnancy associated glycoproteins in seasonal-calving pasture-based dairy cows

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**Application:** Early detection of pregnancy is a useful management tool to improve herd fertility performance in seasonal-calving systems of milk production.
**Introduction:** The objective was to examine the effect of cow fertility index (FI; low <€85, medium €85–€119 & high ≥€120), parity (1, 2, 3 +), milk yield (MY: 25th, 50th, 75th & upper quartile), and sire on initial peak, nadir and post-nadir peak Pregnancy Associated Glycoprotein (PAG) S-N optical density values (test sample minus negative control).
**Materials and Methods:** Milk PAG S-N values were determined weekly from weeks 5–21 post insemination using the IDEXX Milk Pregnancy Test (IDEXX, USA). Only cows that conceived to first artificial insemination and maintained pregnancy were retained, resulting in observations on initial peak, nadir, and post nadir peak from 259 cows. Only sires with greater than 5 pregnancies were included in the analysis (range of 5 to 36) with mean (± SD) pregnancies per sire = 11.5 (± 6.8). PAG S-N values were normally distributed and were analysed using generalized linear mixed-models.

**Results:** Parity, FI, MY, and sire were all significantly associated with initial peak, nadir, and post-nadir peak PAG S-N values. Greater milk PAG S-N values were associated with greater FI for initial peak ($P < 0.0001$, low 1.43, [1.37, 1.50], medium 1.45, [1.39, 1.52] & high 1.69, [1.62, 1.75]), nadir ($P = 0.015$, low 0.45, [0.43, 0.48], medium 0.46, [0.44, 0.48] & high 0.48, [0.46, 0.50]), and post-nadir peak ($P < 0.0001$, low 1.78, [1.72, 1.85], medium 1.79, [1.73, 1.86] & high 1.90, [1.83, 1.97]). There was a trend for declining initial peak ($P < 0.0001$), nadir ($P < 0.0001$), and post-nadir peak ($P < 0.0001$) milk PAG S-N values with increasing parity number. Sire was significantly associated with initial peak ($P < 0.0001$; range 0.92–1.7), post nadir peak ($P < 0.0001$; range 1.33–2.20), and nadir ($P < 0.0001$; range 0.25–0.81) PAG S-N values. Cows in the lower quartiles (25th and 50th) for milk yield ($P < 0.001$) had greater PAG S-N initial ($P < 0.001$), nadir ($P < 0.001$) and post-nadir peak ($P < 0.001$) values compared with cows in the 75th and upper quartiles.
**Conclusions:** Milk PAG S-N values were influenced by a combination of phenotypic and genetic factors in pasture-based dairy cows.
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## 096
## Transcriptomics of corpus luteum and endometrium are altered in genetically divergent dairy cows in pasture-based dairy production

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**Application:** The research helps elucidate the molecular mechanisms within tissues of the reproductive tract that underpin phenotypic differences between breeds and phenotypic differences that occur in response to genetic selection in lactating dairy cows.
**Introduction:** The objective was to identify differentially expressed genes (DEG) in the corpus luteum (CL) and endometrium in genetically divergent groups of cows [Elite Holstein-Friesian (elite genetic merit for fertility & milk production), national average Holstein-Friesian (NA) and Jersey cows].
**Materials and Methods:** Following synchronised oestrus, biopsies of CL (d 7 and d 14 after estrus) and endometrium (d 14 only) were collected from Elite ($n = 20$), NA ($n = 14$) and Jersey ($n = 22$) cows, mRNA was extracted and sequenced, and DEG were identified using the limma package in R. Ingenuity Pathway Analysis (IPA) and Gene Ontology (GO) were used to identify biological functions and enriched pathways based on DEG.
**Results:** There were 145, 173, and 47 DEG for NA vs. Jersey, 196, 43, and 48 DEG for Elite vs. NA, and 268, 53,120 DEG for Elite vs. Jersey, in d 7 CL, d 14 CL and d 14 endometrium, respectively (false discovery rate $P \leq 0.05$). There was a greater number of DEG for d 7 CL compared with d 14 CL for the Elite vs. NA comparison. In d 7 CL, GO analysis for the comparison of Elite vs NA identified 80 DEG that were associated with "Immune System Process". The Panther Pathways analysis within GO categorized the bulk of the genes (>75%) as associated with inflammation mediated by chemokine and cytokine signalling, T cell activation, B cell activation, and integrin signalling pathway. The IPA analysis revealed involvement of CX3CL1, CX3CLR1, and CXCL10 in the neuroinflammation signalling canonical pathway, with greater expression in Elite cows. The IPA analysis of DEG in d 7 CL for Elite vs Jersey also identified immune system activation processes similar to the Elite vs NA comparison. There were fewer DEG identified in endometrium for Elite vs NA ($n = 48$). The biological function "quantity of leukocytes" was downregulated in Elite vs NA in the endometrium ($P \leq 0.05$). Greater expression of CAST was detected in the endometrium of Elite cows compared with Jersey and NA cows (LogFC 0.53 and 0.31, respectively).
**Conclusions:** In conclusion, CL and endometrium transcriptomes differed between Elite, NA, and Jersey cows, with an overall pattern indicating divergence between breed in immune system and inflammation pathways that may underpin differences in phenotypic fertility.

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## 097
## Pregnancy scanning for litter size is profitable across all regions, times of lambing and genotypes in southern Australia

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**Application:** Ultrasound pregnancy scanning enables sheep producers to improve the management of ewes of different litter sizes, including non-pregnant ewes, to increase reproductive performance and profit.
**Introduction:** Pregnancy scanning allows ewes to be separately managed by litter size class to: meet recommended condition score targets (to enhance survival and progeny productivity); allocate multiple bearing ewes to more sheltered lambing paddocks, and: select replacement ewes based on birth type. However, an Australian survey reported that 69% of respondents do not pregnancy-scan their flocks for

litter size (Howard and Beattie, 2018). This study aimed to establish the profitability of pregnancy scanning across flocks and regions in southern Australia. It was part of a project to build a stronger business case for pregnancy scanning, directly addressing the key perception by non-adopters that the business case is weak.
**Materials and Methods:** The profitability of pregnancy scanning for both pregnancy status and for litter size was modelled, across three different regions (long, medium and short growing seasons in the winter rainfall regions of southern Australia), three different genotypes (Merino, Merino x Terminal and a Maternal flock) and three different lambing times (autumn, winter and spring) using the Australian Farm Optimisation Model. The model and production assumptions are documented at https://australian-farm-optimising-model.readthedocs.io/en/latest/index.html. The study was extended to include the summer rainfall regions by using a simpler gross margin analysis.
**Results:** Implementing optimal nutritional management, paddock allocation and replacement strategies based on litter size was profitable in all agricultural regions of southern Australia for all genotypes and at all times of lambing. The average increase in profitability was $5.75 AUD/ewe scanned with a 400% return on investment. The profitability of scanning was sensitive to sheep meat prices, but not very sensitive to wool and supplement prices, nor overall flock reproductive rate.
**Conclusions:** Pregnancy scanning for litter size is a vital tool for improving reproductive rate, lamb (and ewe) survival rates and lamb growth rates. There is a strong business case via (i). selling of non-pregnant ewes ($1.75/ewe), (ii). better allocation of feed based on litter size ($2.00/ewe), (iii). allocating multiple bearing ewes to the best lambing paddocks and/or reducing mob size to enhance lamb survival ($1.00/ewe), (iv). accounting for birth type when selecting breeding replacements ($1.00/ewe), (v). ability to prepare the lambing feed budget in advance, (vi). earlier detection of reproductive failure, and (vii). ability to more rapidly re-mate non-pregnant ewes.
**Acknowledgements:** Funded by Meat and Livestock Australia and Australian Wool Innovation.

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## 098
## Ovarian stimulation with FSH increases *in vitro* embryo production in high AMH heifers in a dose-dependent manner

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**Application:** Cattle with greater circulating anti-Müllerian hormone (AMH) concentration present greater antral follicle population which in turn leads to greater embryo yield after *in vitro* embryo production. Circulating follicle stimulating hormone (FSH), however, is lower in high AMH cattle.
**Introduction:** The aim of this study, therefore, was to evaluate the effect of FSH dose on ovarian response and *in vitro* embryo production in high AMH heifers.
**Materials and Methods:** Pregnant Holstein heifers had a blood sample collected at 42 days of gestation and heifers with circulating AMH in the highest 25% of the population (> 378.3 pg/mL) were selected and enrolled in the study. Heifers ($n = 33$) were randomly assigned to receive either 0 (FSH0), 160 (FSH160) or 300 (FSH300) mg of porcine FSH in a crossover design. Follicle ablation (day 0) was performed to synchronise follicular wave emergence and FSH administration, consisting in four decreasing dose injections 12 h apart, was initiated 36 h later (day 1.5). Ovum-pick was performed 40 h after the last FSH administration (day 5), and cumulus oocyte complexes (COCs) were subjected to *in vitro* embryo production using standard procedures. Heifers were subjected to ovum pick-up at 49, 63 and 77 days of gestation with a "washout" interval of 14 days between sessions. Total follicle numbers were determined on day 5 using ultrasonography. Differences between treatment groups were evaluated using generalised linear mixed models and orthogonal polynomial contrasts.
**Results:** Total number of follicles at ovum pick-up, number of COCs and number of viable COCs increased linearly ($P < 0.0001$) with increasing FSH dose. There, however, was no evidence ($P > 0.3$) for an effect of FSH dose on COC recovery percentage nor percentage of COC's classified as viable. There was a linear increase in ($P < 0.0001$) the blastocyst percentage as FSH dose increased (FSH0: 23.95 ± 3.5; FSH160: 35.04 ± 3.9; FSH300: 42.80 ± 4.0). Consequently, there was a linear increase in ($P < 0.0001$) the number of blastocysts produced per heifer as the FSH dose increased (FSH0: 2.4 ± 0.3; FSH160: 4.3 ± 0.5; FSH300: 6.2 ± 0.6).
**Conclusions:** In conclusion, use of FSH before ovum pick-up increases ovarian response and *in vitro* embryo production in high AMH heifers. Furthermore, these effects are dose-dependent such that larger doses of FSH result in greater embryo production.

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**099**

## Effect of synchrony and expression of oestrus on fertility of lactating Jersey cows submitted to a Double-Ovsynch protocol for timed artificial insemination or artificial insemination after a synchronized oestrus

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**Application:** To improve fertility of high-producing Jersey cows at first service.
**Introduction:** Expression of oestrus at timed insemination is associated with increased fertility in lactating dairy cows (Pereira et al., 2016); however, the effect of expression of oestrus at timed AI for cows submitted to a Double-Ovsynch protocol has not been characterized. We hypothesized that lactating Jersey cows submitted to a Double-Ovsynch protocol would have more pregnancies than cows inseminated to a detected oestrus regardless of synchronization rate or expression of oestrus.
**Materials and Methods:** Lactating Jersey cows ($n$ = 1,138) were randomized within parity to 1 of 2 treatments for first insemination: (1) a Double-Ovsynch protocol [$n$ = 712 ; d 0 GnRH; d 7 $PGF_{2\alpha}$; d 10 GnRH; d 17 GnRH; d 24 and d 25 $PGF_{2\alpha}$; 32 h GnRH (d 26); 16 h TAI (d 27)] or (2) a protocol for synchronization of oestrus with twice-daily detection of oestrus based on AI to rubbed tail chalk from d 21 to 32 [$n$ = 426 ; d 3 GnRH; d 10 $PGF_{2\alpha}$ ; d 24 and d 25 $PGF_{2\alpha}$]. For Double-Ovsynch cows, expression of oestrus was recorded from 24 to 27 d and was categorized as occurring >24 h before or at insemination. Serial blood samples were collected during (d 24 and 27) and after the protocols (d 34) to assess synchronization rate which was defined as high progesterone (>0.5 ng/mL) at 24 and 34 d and low progesterone (<0.5 ng/mL) at 27 d. Binomial variables were analysed by logistic regression using the GLIMMIX procedure of SAS with the fixed effects of treatment, parity, and their interaction.
**Results:** Overall, synchronization rate was greater ($P$ < 0.01) for Double-Ovsynch cows than for Oestrus cows detected in oestrus and inseminated (91.9% vs 79.0%). For synchronized cows, pregnancies per AI were more ($P$ = 0.04) for Double-Ovsynch than for Oestrus cows (54.5% vs 48.7%). For Double-Ovsynch cows, expression of oestrus between 24 and 27 d was 28.2% with 4.2% and 25.1% of cows in oestrus >24 h before and at insemination, respectively. Overall, pregnancies did not differ ($P$ = 0.20) for Double-Ovsynch cows that expressed or did not express oestrus at insemination (59.5% vs 53.8%, respectively).
**Conclusions:** We accept our hypothesis that lactating Jersey cows submitted to a Double-Ovsynch protocol for TAI would yield more pregnancies than cows inseminated to a synchronized oestrus regardless of synchronization rate and expression of oestrus.
**Acknowledgements:** Supported by NIFA USDA CARE project 2021-68008-34105.

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**100**

## Reproductive performance of a new prostaglandin F2α-based timed artificial insemination protocol in sheep

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**Application:** This work facilitates the control of the reproductive cycle of ewes in a more practical, economical, and animal-enviromental welfare-friendly manner.
**Introduction:** Increasing the interval between two prostaglandin F2α (PG) doses up to 14–16 days apart (“long interval” protocols) leads to better reproductive outcomes in ewes after cervical timed artificial insemination (TAI; Fierro and Olivera-Muzante (2017)). These protocols can achieve conception rates comparable to progesterone-eCG based protocols (Fierro and Olivera-Muzante (2017)), but lower than obtained with pre-synchronized spontaneous estrus ewes (Olivera-Muzante et al., 2020), limiting its use. With the aims to concentrate oestrus and ovulations, and consequently improve the reproductive outcomes, this experiment evaluated the effect of pre-synchronized ewes with another PG dose seven days before (“3PG” protocol).
**Materials and Methods:** During the breeding season (CICOMA-SUL; 31° 03′ S-57° 13′ W; Uruguay), 253 Merino ewes grazing native basaltic pastures were selected. Based on their reproductive category (53 nuliparous; 200 multiparous), BCS (3.3 ± 4.0) and BW (40.0 ± 4.1) ewes were assigned to two groups; “2PG”: two 15 days-apart injections (Day -15 and 0; Delprostenate 160 μg/dose) and TAI at 56 ± 1.5 h after Day 0; Control, $n$ = 125) and, “3PG”: three PG injections (Day -22, -15 and 0) and TAI at 68 ± 1,5 h after Day 0; Treatment, $n$ = 128). Cervical TAI was performed using 150 million viable sperm/ewe from a fresh semen pool extended with skim milk from 12 Merino rams. Visual-physical state of cervical mucus at TAI (1-clear and scarce- beginning estrus to 6 -creamy or caseous- end of or post estrus); fertility (preg-

nant/total ewesx100), prolificacy (foetuses/pregnant ewes) and fecundity rates (foetuses/total ewesx100) on Day 60 were evaluated by trans-abdominal ultrasonography (3.5 MHz convex array transducer). Differences between groups were analyzed using procedure for categorical variables (CATMODE of SAS), considering ewe category, protocol, and their interactions.
**Results:** A category effect was observed ($P < 0.05$) in mucus grade, fertility and fecundity of 3PG protocol (nuliparous more advanced mucus grade at TAI, or lower fertility and fecundity than multiparous). There were observed no significant differences ($P > 0.05$) in overall fertility (66.4 and 71.1%), prolificacy (1.27 and 1.20) or fecundity (84.0 and 85.2%), between 2PG and 3PG protocols, respectively.
**Conclusions:** We concluded that 3PG protocol with TAI at 68 h did not significantly improve the reproductive outcomes of estrous synchronized ewes. The best time of TAI and interaction category-synchronization treatment should be evaluated in the future.
**Acknowledgements:** To Facultad de Veterinaria-Udelar, Uruguayan Wool Secretariat (SUL). Financed by CSIC I+D 3 project.

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## 101
## Influence of breed on the ovarian antral follicle population in sheep in Mexico

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**Application:** Select the best breeds to achieve high ovulatory responses in applied reproductive programmes.
**Introduction:** The success of embryo transfer programmes in sheep is affected by factors related to the donor. The response to a superovulatory treatment is determined by the number of 2 to 5 mm size antral follicles on the surface of the ovaries at the time the superovulatory treatment is started. The aim of the study was to evaluate the effect of the ewe breed on the number of the 2 to 5 mm size ovarian follicles.
**Materials and Methods:** The study was conducted in September 2022 in the sheep farm at Universidad Autónoma Chapingo, located in the central part of Mexico. In total 80 multiparous, healthy and in good body condition ewes including four breeds, Creole (C, $n = 20$), Katahdin (K, $n = 20$), Dorper (D, $n = 20$) and British Suffolk (BS, $n = 20$) were used. The number of 2 to 5 mm size follicles was determined by ultrasound (Aloka Prosound II, Japan) and a 7.5 MHz rectal probe, maintaining the animals in a standing position. For the evaluation the ewes were fasted from food and water for 18 h, fixed in a chute, the rectum was cleaned of feces, the probe lubricated with gel and introduced into the rectum until the bladder was identified. Then the ovaries were located and the number of follicles on both ovaries were counted. The results are presented as means and standard errors and were analyzed by analysis of variance with SAS. Statistical differences were considered at a $P < 0.05$.
**Results:** The mean number of follicles was 11.25 ± 0.5, 10.58 ± 0.6, 13.15 ± 0.8, and 11.83 ± 0.6 for C, K, D, and BS, respectively. The results were higher ($P < 0.05$) in D (13.15) compared to C (11.25) and K (10.58) ewes, but no other breed differences were recorded.
**Conclusions:** The results showed high follicle population in D ewes, meaning they could be stimulated with low amount of FSH in superovulatory treatments.

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## 102
## Follicular response to the intraovarian application of autologous platelet-rich plasma in ewes

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**Application:** The use of platelet-rich plasma (PRP) is an alternative to complement superovulation programmes in mammals, increasing the ovarian follicular population.

**Introduction:** In the ewe, the beginning of growth of small ovarian follicles as waves depends on the action of follicle stimulating hormone. The platelets contain a high concentration of growth factors, which have positive effects on the stimulation of endometrial and follicular growth. The objective of the study was to evaluate the ovarian follicular population after the intraovarian application of PRP.
**Materials and Methods:** The study was conducted on the Experimental farm at Universidad Autónoma Chapingo in Chapingo, México. Eleven multiparous, healthy and in good body condition Dorper ewes were used. Blood samples were collected from the jugular vein in 2.7 mL vacutainer tubes containing sodium citrate and were maintained at 4 °C during all the procedure. The samples were centrifuged at 1000 g for 30 min and the serum obtained was centrifuged for a second time at 1500 g for 10 min. Then the upper two-thirds of the tubes were discarded, keeping the lower third part with a higher platelet concentration ($12\ 500 \times 10^f$ platelets/mL). Through laparoscopy 50 μL of autologous PRP were administered in the right ovary of each ewe, while the left ovary received 50 μL of saline solution and was considered as control. The experimental design was completely random, and the treatments were: T1 (Control) = ovary with an application of saline solution and, T2 = ovary with an application of PRP. Next, the population of follicles $\geq$2 mm size on both ovaries were determined weekly, for six weeks, with an ultrasound (Aloka, Prosound 2, Japan) and a rectal transducer (7.5 MHz). Before each review session, the ewes were fasted from food and water for 18 h. The number of follicles was analyzed with GLM of SAS. The effect of treatment was considered significant with a $P < 0.05$.
**Results:** The follicular population was similar ($P > 0.05$) between PRP and control in weeks 0, 1, and 2 ($6.55 \pm 0.51$ vs $7.18 \pm 0.58$; $3.64 \pm 0.39$ vs $3.27 \pm 0.30$; $5.91 \pm 0.61$ vs $5.36 \pm 0.24$, respectively), however, there were significant differences ($P < 0.05$) for weeks 3 ($7.18 \pm 0.42$ vs $5.82 \pm 0.42$), 4 ($10.36 \pm 0.39$ vs $5.91 \pm 0.39$), 5 ($8.73 \pm 0.41$ vs $5.27 \pm 0.56$), and 6 ($8.91 \pm 0.65$ vs $5.91 \pm 0.37$).
**Conclusions:** In conclusion, the intraovarian application of autologous PRP increased the follicular population from week 3 to 6 post-treatment.

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## 103
## Does ewe genetic merit influence phenotypic performance for reproductive traits?

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**Application:** Reproductive efficiency can be defined as the ability of the ewe to become pregnant and produce two lambs per year and needs to be optimised in Irish sheep production systems. Therefore, number of lambs born per year is one of the key traits in the national sheep genetic indexes. This study looks at the validation of the genetic index for sheep in Ireland and examines the performance of animals ranked within the top and bottom 20% of animals being recorded.
**Introduction:** Differences in phenotypic performance are reflected in genetic evaluations with reproductive efficiency deemed one of the most important traits governing overall farm productivity. The objective of this study was to investigate the impact of the ewe's maternal genetic merit and country of origin (New Zealand or Ireland) on ewe reproductive traits.
**Materials and Methods:** The study was performed over a seven-year period (2016 to 2022) and consisted of three genetic groups: high maternal genetic merit New Zealand (NZ), high maternal genetic merit Irish (High Irish) and low maternal genetic merit Irish (Low Irish) ewes. Each group contained 30 Suffolk and 30 Texel ewes, selected based on either the New Zealand Maternal Worth (New Zealand group) or the Sheep Ireland €uro-star Replacement index (Irish groups). Ewes were mated via laparoscopic artificial insemination (AI) using fresh semen collected on the day of AI. Fourteen days post AI rams were introduced for a 20-day period allowing ewes an opportunity to be naturally mated for two repeat cycles. The impact of maternal genetic merit on reproductive traits such as conception rate to first service, barren rate, scanned litter size and number of lambs born were analysed using linear mixed models. For binary traits, the impact of maternal genetic merit on reproductive traits such as conception to first service were analysed using logistic regression.
**Results:** New Zealand ewes outperformed Low Irish ewes for reproductive traits including conception to first service and litter size ($P < 0.05$); High Irish ewes did not differ from either New Zealand or Low Irish ewes for conception to first service or litter size ($P > 0.05$).
**Conclusions:** Results demonstrate the suitability of New Zealand genetics for an Irish based production system but more importantly emphasise the role of a well-established genetic index in complimenting a breeding objective.

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## 104
## Administration of carbetocin prior to timed artificial insemination in ewes increases cervical penetration and prolificacy but not fertility following cervical insemination with frozen-thawed semen

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**Application:** Carbetocin could be a more ethical, cleaner, and cheaper alternative to equine Chorionic Gonadotropin in artificial insemination protocols.
**Introduction:** Fertility obtained by cervical artificial insemination with frozen-thawed ram semen is low (Evans, 1988) due to the characteristics of the ewe cervix (Moré, 1984), which prevents semen release into the uterine body reducing fertility (Salamon and Maxwell, 1995). Oxytocin improves cervical penetration, but requires frequent injections (King and Coetzer, 1996). Carbetocin is an analogue with longer half-life that might be used in a single dose. We evaluated cervical penetration, fertility, prolificacy of nulliparous, primiparous and multiparous Merino Dohne ewes with cervical or laparoscopic intrauterine timed artificial insemination after administration of carbetocin.
**Materials and Methods:** Three hundred and ninety ewes were synchronized with medroxy-acetate progesterone sponges (7 days), equine Chorionic Gonadotropin and prostaglandin F2a and were timed artificially inseminated (intracervical and intrauterine) with frozen-thawed semen. Half of the ewes received carbetocin (50 mg kg/PV intramuscular injection) 12 h before insemination. The average (±SD) weight of the ewes was: nulliparous ($n$ = 105) 43.5 ± 0.50 kg, primiparous ($n$ = 149) and multiparous ($n$ = 136) 50.4 ± 0.47 kg. The average (±SD) body condition score was 3.1 ± 0.25 (1–5, Jefferies 1961). Ewes were inseminated with frozen-thawed semen pooled from four rams (68.7 million progressive motile spermatozoa in 0.25 mL). Cervical penetration was measured with a scaled cannula during intracervical insemination. Pregnancy and multiple gestations were assessed by ultrasound scanning 50 days after insemination. Cervical penetration was considered as a function of the effects of carbetocin treatment, parity and their interaction. Fertility was analysed according to a binomial distribution with a GLIMMIX procedure, with the fixed effects of insemination method, carbetocin treatment and ewe parity. Multiple pregnancies were analysed with Fisher's exact test, with the data of primiparous and multiparous ewes combined.
**Results:** Cervical penetration was deeper (19.3 ± 1.80 mm) ($P$ < 0.05) in multiparous ewes than in nulliparous and primiparous ewes (13.7 ± 1.40 mm) and was affected by carbetocin in all parity groups ($P$ = 0.015). Fertility was higher in intrauterine (64.1%) compared to intracervical (19.9%) inseminated ewes ($P$ = 0.0001). Fertility was not affected by carbetocin or ewe parity. Multiple gestations were higher in intrauterine inseminated ewes (39.7%) compared to intracervical inseminated ewes (25.6%)($P$ = 0.0346). Carbetocin almost doubled (45.6%) the multiple gestations compared to the control ewes (25.6%)($P$ = 0.0090).
**Conclusions:** Carbetocin increased prolificacy in intrauterine insemination and cervical penetration depth but not fertility in intracervical insemination.
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## 105
## A new semi-automated method for cytological sampling of the endometrium of dairy cows- validation on *ex-vivo* organs

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**Application:** *Ex-vivo* validation of a semi-automated device on slaughterhouse-derived uteri for the collection of cytological samples from the endometrium for the diagnosis of subclinical endometritis (SE) in dairy cows by naive operators.
**Introduction:** Subclinical endometritis is one of the most frequent diseases affecting the reproductive tract of dairy cows. Evaluation of cytological smears is considered the gold standard to diagnose SE. In recent years, multiple techniques were developed to collect endometrial cytology samples such as low-volume lavage, cytobrush, and cytotape. Nonetheless, the search for a suitable method enabling the dissemination of SE diagnosis is still ongoing.

**Materials and Methods:** We aimed to validate a novel, semi-automated method for collecting endometrial cytology samples, using slaughterhouse-derived uteri, and to compare it with the cytobrush as the gold standard. The newly proposed method achieved atraumatic collection of cytological material by the flexible construction of the swab's rod in combination with an automatised rotation system created by a 3D printer. We compared the time of sampling, measured as follows: time was counted when one of the sets was allocated in the portio vaginalis of the cervix until the endometrial cytology sample was collected, to illustrate the ease of use of the new device. *Ex-vivo* uteri were placed in a bovine AI training model. A total of 54 uteri were used for the experiment, from which 108 cytological smears and 216 cervical transit time measurements in seconds (s) were obtained by two naive operators. Furthermore, we compared the percentage of polymorphonuclear cells (PMNs) in smears obtained by both methods. Smears were stained by Diff-Quick and the PMN% was assessed after counting 300 nucleated cells by two blinded operators. We used the Wilcoxon signed-rank test to compare the times of sampling between the new device and the cytobrush. Lin's concordance correlation coefficient (CCC) was used to assess the inter-method/rater agreement on the PMN% in smears.
**Results:** Median (IQR) time of sampling using the new device— 30.2 s (30.9) was not different ($P = 0.57$) in comparison to cytobrush— 33.2 s (21.25). The CCC between smears taken by both methods to evaluate the reproducibility of PMN% was 0.87 (0.79–0.92). The CCC for each sampling method between raters 1 and 2, to evaluate the inter-rater reliability of the PMN% was for the new device 0.91 (0.87–0.94) and the cytobrush 0.88 (0.85–0.91).
**Conclusions:** The new method enables naive operators to take a sample as quickly as using the cytobrush, with similar inter-rater reliability of the PMN%.

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## 106
## Glucocorticoid and progestogen profiling in bovine skimmed milk, saliva and plasma using ultra-performance liquid chromatography high resolution mass spectrometry (UHPLC-HR-MS)

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**Application:** A sensitive analytically and biologically validated mass spectrometry-based method for progestogen and glucocorticoid profiling in different bovine matrices.
**Introduction:** The roles of progesterone (P4) and cortisol during the oestrous cycle and parturition have been well described. Both steroids have been repeatedly determined as single targets. With the emergence of increasingly sensitive MS based techniques, steroid profiling became of interest as it enables the analysis of several steroids in parallel. Both P4 and cortisol are metabolised within the body to biologically active metabolites. 5α–Dihydroprogesterone (5α-DHP) is an agonist of the P4 receptor. In few species, including the African elephant, the horse, and the hyrax, 5α-DHP has been identified as the main gestagen supporting pregnancy. Tetrahydrometabolites of P4 have been characterised as neurosteroids. Allopregnanolone (3α,5α-THP) has been shown to attenuate the response to stress (Patchev et al., 1996). To date, functional information about tetrahydrometabolites of glucocorticoids is scarce. However, they significantly reduced GABA-mediated chloride ion uptake in rat cortical microsacs possibly mediating stress response (Stromberg et al., 2005).
**Materials and Methods:** We have extended our previously published UHPLC-HR-MS method for progestogen profiling by several relevant glucocorticoids (Rehm et al. 2021). The method now comprises twenty-three steroids, twelve progestogens and eleven glucocorticoids. We analytically validated the method for three matrices (saliva, plasma, and skimmed milk). Subsequently, we applied the method for steroid profiling in plasma samples obtained daily from 5 cyclic cows and in skimmed milk samples collected from 5 cows post-partum for 7 consecutive days starting with birth.
**Results:** In plasma of cyclic cows, progestogen metabolites mirrored the profile of P4. P4 was most abundant followed by 3α,5β-THP and 3α,5α-THP (5.3 ng/ml, 1 ng/ml and 0.9 ng/ml respectively, one day prior to luteolysis). Glucocorticoid levels fluctuated across the oestrous cycle. Cortisol was most abundant followed by 3α,5β-tetrahydrocortisol (3α,5β-THF). The most abundant glucocorticoid in milk post-partum (pp) was 3α,5β-THF (6.38 ng/ml, day 0 pp) and the most abundant progestogen was pregnenolone/3β–dihydroprogesterone (0.7 ng/ml, day 0 pp) followed by 5α–DHP (0.4 ng/ml, day 0 pp).
**Conclusions:** In conclusion, steroid profiling which includes bioactive steroid metabolites gave a more complete picture. The planned extension of the method by further additional groups of steroids such as androgens will eventually allow the analysis of the complete steroidome. Assessing the steroidome during specific physiological conditions opens the way to study steroid feedback mechanisms to the brain and to elucidate the detailed function of biologically active steroid metabolites.

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## 107
## Impact of supplementing rumen-protected methionine during the periconceptional period in postnatal growth in beef cattle

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**Application:** Impact of supplementing rumen-protected methionine (RP-Met) during the periconceptional period in postnatal growth in beef cattle.
**Introduction:** Evidence suggests changes in maternal nutrition in the periconceptional period influence the postnatal growth period in cattle. The use of methyl donors in embryo culture during the first stages of embryogenesis has resulted in increased birth weight and 205-adjusted weaning weight in beef cattle (Estrada-Cortés et al., 2021). We hypothesized that feeding RP-Met during the periconceptional period will program bovine gestation to enhance postnatal growth.
Materials and Methods: A total of 114 multiparous cows were fed a roughage-based diet and randomized to receive corn gluten supplemented with 15 g of RP-Met (RP-Met; Smartamine M, Adisseo) or not (Con) from day −7 to +7 relative to timed artificial insemination. Estrus synchronization was conducted using the 7-Day CoSynch + CIDR protocol. After calving, 40 calves (Con = 19; RP-Met = 21) were considered for birth weight (BW) analysis. Female calves ($n$ = 34) were weighed at 2, 4, 6 months and weaning. Liver biopsies were collected for RNA-seq at 5 months of age. Fifteen random samples were used for Illumina RNA-seq. Enrichment analysis was conducted using IPA software. The statistical analysis for birth weight data included the treatment, sex, and the interaction of treatment and sex. Post-natal body weight data were analyzed as repeated measures where the model included the treatment, age, and the interaction of treatment and age.
Results: A treatment-by-sex interaction was observed ($P$ = 0.04) in BW. Male calves ($n$ = 6) from cows fed RP-Met showed greater BW than those from the Con group (Con = 31.9 ± 2.3 kg; RP-Met = 41.4 ± 2.3 kg), but no difference was observed in females. No difference in postnatal body weight was observed. When utilizing $P$ < 0.01 and a log fold change of < −1 and >1 as cutoffs, there were 129 genes downregulated in the RP-Met group and 24 genes upregulated. In the enrichment analysis, the top canonical pathways were related to the inhibition of immune system function in the RP-Met group.
**Conclusions:** Further research should be conducted to elucidate if the sexual dimorphism is a response to RP-Met supplementation in BW of male calves. Also, the long-term effects of the immune system's changes in the transcriptome of the liver in the RP-Met need to be further evaluated. This is an ongoing experiment where more data is being collected in female calves to observe the effect of RP-Met on muscle and adipose-tissue transcriptome.

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## 108
## Reproductive parameters in buffalo herds located in a tropical country

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**Application:** The first step to applying reproductive biotechnologies is to know how the natural reproduction of animals works; in the buffalo, this is essential because, despite being very close phylogenetically to cows, the buffalo has many differences in oestrus expression, follicle diameter size at ovulation, progesterone profiles (Bertoni et al., 2020).
**Introduction:** Tropical countries do not have seasons, but reproduction shows a seasonal pattern that has been mainly associated with the availability of forages. The production with buffaloes has become an alternative due to the quality of its milk and meat, the low production costs, and the novelty of the system that makes it advantageous to produce with buffaloes even in European and North American countries. The application of reproductive biotechnologies to increase production in the species is not widely used (Gimenes et al., 2015).
**Materials and Methods:** This study analyze the reproductive parameters during 2022 of two Colombian buffalo herds located in different regions: Puerto Boyaca and Uraba. Animals were maintained in low tropical rainforest with a temperature 23–32 °C, annual rainfall (2300 mm and 570 mm), altitude (2 – 190 mosl) for Uraba and Puerto Boyaca, kept in natural and improved pastures (Brachiaria humidi-

cola or decumbens), with water and minerals ad libitum. The pregnancies were obtained by natural mating, with a ratio of 1 male for every 40 females. A reproductive check-up was done by rectal palpation every three months. All the information was registered in specialized software for herd management. Descriptive statistics of parameters are presented, and comparisons were performed using the Mann-Whitney test.
**Results:** Data from 705 adult females were reported. The average age was 2.65 years (30 to 130 mo), and 8.58 years ( 38 – 170 mo), the birth rate was 57.36% and 59.35%, the inter calving period was 438 and 431 days, days calving- conception 128 and 116 days, first calving age 40,10 and 36,89 months for Uraba and Puerto Boyaca respectively. The intercalating period, calving to conception, shows no differences between farms (21 days), and it remains constant from the second through eighth calvings.
**Conclusions:** Despite knowing that reproductive parameters of buffaloes are better than reported for cows, it can be observed that first calving age they are low. A rapid onset of postpartum ovarian activity is observed. It is essential to observe that this parameter remains constant during the majority of the reproductive life of females. No differences are observed between the reproductive parameters of the two farms evaluated.
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## 109
## Estradiol downregulates myometrial progesterone receptor protein expression in periparturient ewes

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**Application:** Postnatal mortality of livestock due to premature birth ranges from 5–10% in the U.S. Therefore, the early delivery/fetal dysmaturation and mechanisms involved are economic problems for producers.
**Introduction:** Parturition requires activation of myometrial contractility, which may be driven by a withdrawal of progesterone and a rise in estrogen levels; however, estradiol can induce parturition without the withdrawal of progesterone. Thus, the mechanisms are unclear. The aim of this study was to evaluate if the progestogenic responsiveness of the uterus in periparturient Rambouillet ewes is affected by estradiol levels. We hypothesized that estrogen would affect progesterone receptor protein expression.
**Materials and Methods:** We administered estradiol (4 Silastic implants of 50 mg each; 200 mg/ewe) and Letrozole, a potent inhibitor of the aromatase enzyme (injections of 1 mg/kg in sesame oil [vehicle] 1:1 v/v; 50 mg/ml final concentration) in a $2 \times 2$ factorial arrangement. Ewes were randomly assigned to the following treatments: C (Empty implants + Letrozole vehicle, $n = 6$), E (Estradiol + Letrozole vehicle, $n = 6$), L (Empty implants + Letrozole, $n = 8$), and E+L (Estradiol + Letrozole, $n = 7$). All treatments began at d 139 to 142 of gestation, and ewes were euthanized 26 hr later for tissue collection. Maternal carotid blood samples were collected at slaughter to measure plasma estradiol levels by radioimmunoassay. Formalin-fixed cross-sections of the uterus were immunofluorescently stained for progesterone receptors and DAPI for nuclear counterstaining. One image of each entire stained cross-section was generated to evaluate receptor distribution, and confocal imaging of individual uterine compartments (uterine glands, myometrium, and endometrial epithelium) was generated for image analysis (ImagePro Plus). Statistical significance ($P < 0.05$) was assessed using the MIXED procedure of SAS with ewe as a random effect for: (1) estradiol concentration in systemic blood and (2) uterine progesterone receptor protein expression between treatments.
**Results:** Our results showed that: (1) the E group had a significantly greater estradiol concentration when compared with C and L (149.21 ± 55.93 vs 30.61 ± 11.73 and 56.90 ± 21.66 pg/mL, respectively, $P < 0.05$), and (2) that estradiol treatment downregulated myometrial progesterone receptor protein expression when comparing E vs C and E+L (27.05 ± 3.88 vs 42.00 ± 3.88, and 46.13 ± 3.88 intensity units, respectively, $P < 0.05$). No other difference was found for any other comparison.
**Conclusions:** Our results suggest that estradiol downregulates myometrial progesterone receptors, and thus progestogenic responsiveness, in late periparturient ewes, leading to activation of the myometrium and fetal delivery.
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## 110
## Metabolic memory affects energy and protein metabolism of the uterus in undernourished ewes during early gestation

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**Application:** Improve ewes body reserves to reduce the impact of nutritional restriction on reproduction.
**Introduction:** Our aim was to study the effect of undernutrition on uterus energy and protein metabolism in ewes with different body condition score at the beginning of the experiment.
**Materials and Methods:** During the breeding season in Spain, 36 multiparous Rasa Aragonesa ewes were divided into 2 groups with different BCS: BCS $\geq$ 2.75 (high, H, 2.9 ± 0.04) and BCS $\leq$ 2.25 (low, L, 2.1 ± 0.04). Body weight of H and L group was 61.9 ± 1.6 and 50.9 ± 1.7 kg, respectively. Both groups received a diet to cover energy and protein maintenance requirements for 20 days, after which they were randomly assigned to 2 nutritional treatments: 1.5 (control, C) or 0.5 (undernourishment, U) times the daily maintenance requirements. The first day of the experimental diet, ewes were estrous synchronized with intravaginal sponges for 12 days and mated with rams. Only pregnant ewes on day 5 were included in this study setting up 4 groups: high-iBCS control (HC, $n$ = 6), high-iBCS undernourished (HU, $n$ = 6), low-iBCS control (LC, $n$ = 9) and low-iBCS undernourished (LU, $n$ = 7). Ewes were slaughtered on day 5, and uterine content of glucose, non-esterified fatty acids (NEFA), b-hydroxybutyrate (BHB), albumin and globulins were determined. Variables were analyzed by ANOVA using a mixed procedure.
**Results:** Animals with high-iBCS presented higher number of recovered embryos than low-iBCS ($P$ < 0.05), and control ewes greater than undernourished ($P$ < 0.05). In low-iBCS group, control ewes presented higher uterine glucose concentration than undernourished ewes ($P$ < 0.05), but these differences were not observed in high-iBCS group. Only in low-iBCS group, undernourished ewes presented higher NEFA uterus concentration than control group ($P$ < 0.01), while only in high-iBCS group, control ewes presented higher BHB uterine concentration than undernourished ewes ($P$ < 0.05). Similarly, the response to undernourishment on uterine protein metabolism was dependent on the initial energy reserves of the animals. Ewes with a better metabolic status (high-iBCS and control animals) presented the highest albumin concentration in uterus ($P$ < 0.05), while the LU group presented the highest globulin uterus concentration ($P$ < 0.01), indicating a possible tisular alteration.
**Conclusions:** High-iBCS ewes are more efficient facing up to nutritional restriction using other energy sources than glucose (BHB; nutrient partitioning), reflecting an adaptive response to the nutritional restriction that is dependent on initial body condition. The low albumin concentration and the high globulin levels in ewes in a severe negative energy balance, indicate an alteration of the uterine tissue that may be associated with greater embryonic losses.

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## 111
## Replacement of eCG by FSH/LH during a timed artificial insemination protocol in crossbreed beef cows

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**Application:** The addition of specific hormones to estrus synchronization protocols can enhance the pregnancy rate per artificial insemination (P/AI).
**Introduction:** The utilization of eCG in synchronization protocols is currently questioned by an increasing animal rights movement against obtaining this hormone from pregnant mares. For that reason, there is a growing interest in exploring new alternatives. The objective of this experiment was to compare the P/AI using either an i.m. dose of eCG (300 IU; Vetegon®, Calier) or a subcutaneous dose of FSH/LH (30 IU; Pluset®, Calier) during the final stage of an eight-day progesterone-estradiol based timed artificial insemination (TAI) protocol.
Materials and Methods: For this experiment were used multiparous crossbreed beef cows, with Body Condition Scores of 4 and 5. All cows were examined by transrectal ultrasonography to determine their ovarian status, and treatments were randomly distributed among cows ($n$ = 275) with the presence of a corpus luteum or follicles >8 mm. On Day 0, cows received a progesterone intravaginal device (Plucelar 0,6®, Calier) and 2 mg of estradiol benzoate i.m. (EB®, Calier). On Day 8, the devices were removed, and 500 µg of cloprostenol i.m. (Veteglan®, Calier) and 1 mg of estradiol cipionate i.m. (ECP®, Calier) were administrated. Simultaneously estrous liquid ink tester (Pincelar®, Calier) was applied on the tail base, and differential treatments were utilized (eCG or FSH/LH). On Day 10, all cows were TAI using conventional frozen semen, and an additional dose of a GnRH analogous (Pluserelina®, Calier) was given only to cows that did not show estrus signs. On Day 32 after AI, a gestation diagnostic was conducted, and the data were analyzed using the Pearson Chi-Square Test procedure and Logistic Regression Analysis (Infostat®).

**Results:** The P/AI was not different between groups (eCG = 38.1% and FSH/LH = 45.45 %, $P = 0.23$). However, a correlation was observed between P/AI and estrous presentation ($P = 0,03$), resulting in a higher rate for those cows that showed estrus signs (eCG = 39.42 % and FSH/LH = 47.5 %).
**Conclusions:** In conclusion, 30 IU of FSH/LH can successfully replace the final dose of eCG that is usually employed at progesterone device removal.

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## 112
## Risk factors affecting walking activity and subsequent fertility in high yielding dairy cows submitted to fixed-time artificial insemination

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**Application:** The study of risk factors affecting estrous behavior measured by walking activity has an important economic, environmental and societal impact since it may enhance reproductive performance, raising therefore farm profits and reducing cow culling rate.
**Introduction:** Cows showing estrus during a fixed time artificial insemination (FTAI) have greater fertility than animals not showing estrus. Thus, this study determined (i) Associations between walking activity increase (WAI) and pregnancy per AI; (ii) Risk factors affecting WAI in dairy cows submitted to FTAI.
Materials and Methods: Lactating dairy cows ($n = 829$) received a new or previously used (5-or 10-days use) CIDR and were administered 100 μg gonadoreline (D-8). Five days later (D-3), CIDR was removed and two doses of prostaglandin-F2α was administered 24 h apart. Thirty-six h after CIDR withdrawal, 100 μg of GnRH was administered. Cows were served 22–24 h after GnRH application (D0). On D-8, ovarian structures were examined by ultrasonography to assess size of corpus luteum (CL) and the largest follicle. Following information was collected from all animals at D0: walking activity increase (from D-3 to D0), parity, number of previous AI, milk production and date of AI. Pregnancy (P/AI) was determined by ultrasonography 28 d after AI. Data were analyzed using GLIMMIX in SAS.
**Results:** Pregnant cows had a greater WAI than non-pregnant cows (226.04 ± 8.5 vs 181.9 ± 6.3 units; $P = 0.0003$). Walking activity AUC was associated with P/AI (0.581 ± 0.03; $P = 0.001$) with a sensitivity and specificity of 79.0% and 54.5%, respectively. The optimum WAI threshold predictive of P/AI was 206.8 unit. Each unit increase in milk production (kg) was associated with a decrease on WAI ($P < 0.01$) in $-3.50 \pm 0.7$ . Animals served during the cold season (October to April) and with a number of previous AI $\geq 3$ had a greater WAI than animals served during the warm season (215.6 ± 6.9 vs 172 ± 7.5 units; $P < 0.0001$) and with a number of AI <3 (220.1 vs 186.7 units; $P = 0.07$). Similarly, WAI was greater in cows that had a CL present at protocol initiation than animals with no CL (232.07 ± 9.1 vs 175.9 ± 7.0 units; $P = 0.001$). Conversely, neither parity nor previous P4 device use were associated ($P > 0.05$) with WAI.
**Conclusions:** In conclusion, WAI was associated with predicting P/AI with moderate sensitivity and poor specificity. Moreover, season and number of AI, days in milk, milk-production and ovarian status were associated with WAI in dairy cows submitted to a FTAI.

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## 113
## Precise regulation of ovarian function increased fertility of lactating dairy cows compared to detection of oestrus

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**Application:** These data provide a robust comparison of fertility outcomes in lactating dairy cows receiving first service following Double-Ovsynch or oestrus detection. Novel aspects of these data, related to identifying embryonic viability/presence prior to the first pregnancy diagnosis, will help to elucidate key pathways influencing fertility of lactating dairy cows.
**Introduction:** Fertility programs, such as Double-Ovsynch, increase pregnancy probability in comparison with AI after oestrus detection (Kim et al., 2020). It is unclear which mechanisms favour pregnancy establishment following Double-Ovsynch compared with natural oestrus. Preliminary evidence ($n = 110$ cows) suggested that a greater proportion of cows inseminated following oestrus had greater pregnancy loss from initial PSPB increase to 32–38 days post-AI in comparison with Double-Ovsynch (25.0 vs 3.0%; Minela et al., 2022). Cows with delayed onset of PSPB increase (>21 days post-ovulation) had greater pregnancy losses in comparison to earlier onset ($\leq$21 days post-ovulation; 25.0 vs 2.4%, respectively). Based on these outcomes a follow-up study was designed with sufficient power to further investigate potential differences in pregnancies/AI. The hypothesis was that Double-Ovsynch would result in greater pregnancies/AI in comparison with oestrus detection.
**Materials and Methods:** Lactating dairy cows receiving first service (between 69–94 DIM) were blocked by parity and randomly assigned to receive AI following oestrus detection ($n = 360$), or Double-Ovsynch ($n = 311$). Pregnancy diagnosis (between days 32–41 post-ovulation) and confirmation of ovulation were performed via ultrasonography. All cows were equipped with an ear tag that tracked activity and rumi-

nation in 2-h intervals (Allflex livestock intelligence). Oestrus characteristics and number of oestrus events prior to AI were collected on the Data Flow software.
**Results:** Cows inseminated following Double-Ovsynch had greater pregnancies/AI in comparison with oestrus detection (51.9 vs 36.3%; $P < 0.01$). Overall, primiparous cows had greater pregnancies/AI in comparison with multiparous cows (50.2 vs 37.9%; $P < 0.01$). Oestrus expression near timed-AI in Double-Ovsynch cows (85/300 cows) had no effect on pregnancies/AI (57.7 vs 50.5%, oestrus vs. no oestrus; $P = 0.52$). Pregnancies/AI did not differ between cows in the oestrus group ($n = 334$) with 0 vs $\geq$1 oestrus' detected in the pre-voluntary waiting period (33.3 vs 38.2%; $P = 0.91$).
**Conclusions:** These data help to support the utilisation of Double-Ovsynch as a strategy to enhance fertility of lactating dairy cows for first AI. Ongoing data analyses of within-cow PSPB concentration could help to elucidate whether premature pregnancy losses are the main contributor to the gap in fertility between oestrus detection and Double-Ovsynch.
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## 114
## Effects of prenatal testosterone excess on responsiveness of the neuroendocrine axis to estradiol positive feedback in first-generation ewes: a model for polycystic ovary syndrome

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**Application:** Prenatal testosterone exposure causes reproductive perturbations in sheep that closely mirror those seen in women with polycystic ovary syndrome (PCOS).
**Introduction:** Polycystic ovary syndrome is the most common infertility disorder affecting over 100 million women worldwide, and is characterised by ovulatory dysfunction, hyperandrogenism, and polycystic ovaries. Obesity plays a significant role in the development and severity of the PCOS phenotype. This study investigated the effects of prenatal testosterone excess from gestational days 60–90 and postnatal excessive weight gain on responsiveness to estradiol positive feedback of first-generation ($F_1$) ewes.
**Materials and Methods:** Suffolk ewes received testosterone propionate (T; 100 mg i.m.) or corn oil (C; vehicle i.m.) twice weekly from gestational days 60–90 (term = 147 d). At 5 mo of age, $F_1$ T lambs were assigned randomly to either a maintenance (100% of NRC requirements) or overfed diet (130% of NRC requirements) and control lambs were fed the maintenance diet. At 18 mo of age, $F_1$ ewes ($n = 8$/group; control, T-maintenance, and T-overfed) confirmed to be in seasonal anestrous received four silastic estradiol implants subcutaneously, designed to release physiological concentrations of estradiol (late-follicular phase). Blood samples were collected beginning 4 h prior to insertion of estradiol implants, every 2 h for 60 h for characterisation of the LH surge. Luteinising hormone concentration was assessed via a previously validated radioimmunoassay. Data were analysed as one-way ANOVA with Dunnet's post-hoc analysis.
Results: Onset of the LH surge was delayed ($P < 0.01$) in both T-maintenance (15.20 ± 0.58 h) and T-overfed (15.33 ± 0.53 h) groups compared to controls (11.33 ± 0.53 h). Duration of the LH surge was greater ($P < 0.05$) in T-maintenance (19.50 ± 1.76 h) and tended to be greater ($P = 0.06$) in T-overfed females (19.60 ± 1.58 h) compared to controls (14.29 ± 1.33 h). Area under the curve was not different ($P = 0.48$) between treatments (Control = 202.03 ± 33.99 AU; T-maintenance = 270.17 ± 42.99 AU; T-overfed = 228.48 ± 39.25 AU). Peak concentration was also not different ($P = 0.27$) between groups (Control = 29.82 ± 4.47 ng/ml; T-maintenance = 20.14 ± 5.29 ng/ml; T-overfed = 19.23 ± 5.91 ng/ml). Luteinising hormone surge amplitude was greater ($P < 0.01$) in controls (36.39 ± 2.89 ng/ml) compared to both T-maintenance (20.18 ± 3.23 ng/ml) and T-overfed females (16.65 ± 3.23 ng/ml).
**Conclusions:** Prenatal testosterone treatment disrupted several parameters of the estradiol-induced LH surge in sheep and postnatal overfeeding did not further exacerbate these surge defects. Delay in onset and reduced amplitude of the LH surge likely contribute to ovulatory dysfunction seen in this sheep model of PCOS phenotype.
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## 115
## Effect of manipulating follicular dynamics with GnRH during the oestrous cycle prior to first AI in lactating Holstein cows

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**Application:** This study furthers the understanding of strategies to enhance fertility of cows receiving AI following a detected oestrus.
**Introduction:** Lactating dairy cows in the US have the potential for pregnancy rates/AI near that of nulliparous heifers when the oestrous cycle prior to AI is manipulated with fertility programs to regulate antral age of the ovulatory follicle (OF) and corpora lutea number and regression (Minela et al., 2021). Roche et al. (1999) discovered that extended time of dominance >10 day resulted in reduced fertility of dairy cows. Time of dominance of the OF may fall into the >10 days category for a significant portion of cows that initiate a new follicular wave prior to day 12 of the oestrous cycle before AI. The current study tested the effect of exogenous GnRH on d 5 to 8 of the oestrous cycle prior to 1st AI to increase the percent of cows with three follicular waves. The hypothesis was that reduced dominance would result in greater pregnancy rates/AI following detected oestrus in 1st AI cows.
**Materials and Methods:** Lactating Holstein cows ($n$ = 1,109) were randomized by parity into two treatments: no treatment (controls) or 100 μg of cystorelin 5 to 8 days following a pre-voluntary waiting period oestrus. From 69 to 92 DIM, cows were inseminated 8 to 20 h after oestrus. Oestrus was detected using automated activity monitors. Pregnancy diagnosis was performed 31–40 days post-AI.
**Results:** Oestrus was detected in 35% of all cows during the pre-VWP (45–62 DIM). The detection rate was greater in primiparous compared with multiparous cows (49 vs 27%; $P < 0.001$). Treatment with GnRH increased the percent of cows that ovulated between 5–8 days after pre-VWP oestrus (82 vs 0%; $P = 0.001$) and the percent of cows with $^{3}2$ CL (86 vs 19; $P = 0.001$) during the oestrous cycle prior to AI. Treatment with GnRH did not affect average inter-oestrus interval ($P = 0.3$). There was no effect of treatment on pregnancy rate/AI (41 vs 40%).
**Conclusions:** Treatment with GnRH did not affect pregnancy rate/AI even though follicular dynamics was altered in most cows treated with GnRH in addition to increasing CL number in the cycle prior to oestrus. Analysis of within-cow daily measurements of the trophoblastic protein (PSPB), currently in progress, may provide a novel understanding of the effects of time of dominance on early pregnancy.
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## 116
## Expression of selected angiogenesis factors in growing homogenous and cavitary corpora lutea in cows

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**Application:** Explanation of the physiological mechanisms of the formation of different types of cows' corpora lutea.
**Introduction:** Corpora lutea in cattle have two morphological forms: homogenous, in which the entire gland is filled with luteal tissue, and cavitary, in which luteal tissue surrounds a centrally located cavity. The formation of a cavity may be associated with changes that occur in the first h and days after ovulation. Angiogenesis is one of the most important processes for the further development of the corpus luteum, and it is most intense in the first days of the development of the luteal tissue. The aim of the study was to determine the differences in the expression of factors involved in the angiogenesis process – Fibroblast Growth Factor 2 (FGF2) and Vascular Endothelial Growth Factor A (VEGFA) in both types of growing corpora lutea.
**Materials and Methods:** The study involved 10 Holstein-Friesian cows synchronized with a double injection of 500 μg cloprostenol 11 days apart. Only animals with clearly expressed oestrus symptoms were used. Corpora lutea (5 homogenous and 5 cavitary) were obtained by colpotomy within 6–8 days from the onset of oestrus. Additionally, blood was taken from each cow to determine serum progesterone concentration, measured by radioimmunoassay. Expression of FGF2 and VEGFA at the mRNA and protein levels were determined in the whole wall of luteal tissue by Real-Time PCR and Western blotting, respectively. Statistical analysis was carried out using the GraphPad PRISM program, the data were analyzed using the ANOVA test, then the Sidak multiple comparison tests.
**Results:** Mean serum progesterone values were similar (4.64 and 4.5 ng/mL for homogenous and cavitary corpora lutea, respectively). Higher FGF2 mRNA ($P < 0.01$) and protein ($P < 0.05$) expression in the homogenous corpus luteum than in cavitary ones was observed. Moreover, VEGFA mRNA expression did not show any differences between both structures ($P > 0.05$), while higher protein concentrations were found in the case of homogenous corpora lutea ($P < 0.05$).
**Conclusions:** The obtained results show higher expression of angiogenic factors in homogenous corpus luteum in comparison with its cavitary counterpart. Yet, the lack of differences in progesterone concentration may suggest that high angiogenic activity is shorter and more rapid in the presence of a cavity. As a consequence, progesterone concentration was similar in both types of corpora lutea. However, the full understanding of the observed differences requires further research.

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## 117
## Effect of delayed timing of artificial insemination with sex-sorted semen on pregnancy per artificial insemination in synchronized dairy heifers managed in a seasonal-calving pasture-based system

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**Application:** This study demonstrated that delaying the normal timing of AI of heifers with frozen-thawed sex-sorted semen by 8 h improved pregnancy per AI.
**Introduction:** The use of sex-sorted semen to generate dairy replacement heifers is growing rapidly as dairy producers seek to accelerate genetic gain, reduce dystocia in their herds and seek to produce fewer low-value male dairy calves. The objective of this study was to evaluate the timing of artificial insemination (AI) with frozen-thawed sex-sorted semen on pregnancy per artificial insemination (P/AI) in dairy heifers.
**Materials and Methods:** A six-day progesterone CO-Synch protocol was used for ovulation synchronization of dairy heifers, with timed AI (TAI) coincident with (TAI_0) or 8 h (TAI_8) after the second injection of gonadotrophin-releasing hormone (GnRH), corresponding to either 48 h or 56 h after removal of the PRID device. Pregnancy diagnosis was conducted by transrectal ultrasound scanning of the uterus 34 d after TAI ($n$ = 816 records available for analysis). Generalized linear mixed models were used to examine the effects of treatment on P/AI. Treatment ($n$ = 2), herd ($n$ = 11), treatment × herd were included as categorical fixed effects. Heifer body weight and Economic Breeding Index values for milk production, fertility, calving performance, beef carcass, cow maintenance, cow management, and health were included as continuous fixed effects. Heifer ID was included as a random effect.
**Results:** There were effects of treatment ($P$ = 0.048) and herd ($P$ = 0.002) on P/AI, but a treatment × herd interaction effect was not detected ($P$ = 0.65). Pregnancy per AI was 9.1 percentage points greater for heifers assigned to TAI_8 [59.1% (52.7 – 65.1%)] compared with heifers assigned to TAI_0 [50.0% (44.4 – 56.3%)]. Pregnancy per AI across herds was 54.5% and ranged from 38.2% to 75.2%.
**Conclusions:** This study demonstrated that delaying the normal timing of AI of heifers with frozen-thawed SS semen by 8 h improved P/AI. Studies to date, including the current study, evaluated the time of AI with sexed semen using frozen-thawed sexed semen, however similar studies, using fresh sexed semen are warranted.
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## 118
## Importance of estrual activity on pregnancy establishment and maintenance following timed embryo transfer in beef cattle

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**Application:** Determine the timepoint of pregnancy most influenced by estrual activity and how it relates to pregnancy success.
**Introduction:** Cows expressing estrus prior to fixed-time artificial insemination had a 27% increase in pregnancy success compared to non-estrual cows (Richardson et al., 2016). Preovulatory estradiol concentrations associated with estrus improved fertilization success, embryo quality, uterine environment, and pregnancy maintenance (Atkins et al., 2013; Larimore et al., 2015; Northrop et al., 2018). The objective of this experiment was to analyze pregnancy loss from day 7 to 90 by estrual status prior to embryo transfer.

**Materials and Methods:** Cows ($n$ = 604) were synchronized using the 7-day CO-Synch+CIDR® protocol and grouped by estrus expression prior to day 0 (Estrus: $n$ = 198), with non-estrual animals receiving GnRH and randomly assigned to estradiol (E2: $n$ = 202; 0.1mg estradiol 17-β) or no treatment (GnRH: $n$ = 204) on day 0. On day 7, all cows received an *in vivo* produced embryo of similar grade and stage, and estrual status was retrospectively determined using Estrotect® patch change (day 0 to 7) [E2-Estrual (E2E): $n$ = 177, E2-Non-estrual (E2N): $n$ = 25, GnRH-Estrual (GnRHE): $n$ = 110, GnRH-Non-estrual (GnRHN): $n$ = 93]. Pregnancy was determined on day 19, 24, 30, 55, and 90 by interferon-stimulated gene expression, pregnancy-associated glycoprotein abundance, progesterone concentration, and/or transrectal ultrasonography. Pregnancy was analyzed using PROC GLIMMIX with treatment, year, replicate, and the interaction of year and replicate as fixed effects. Pregnancy was analyzed as a repeated measure using PROC MIXED with treatment, day, and their interaction as fixed effects, and as probability of survival using PROC LIFETEST.
**Results:** There was no effect of treatment on percent pregnant on day 19 ($P$ = 0.37), day 55 ($P$ = 0.14), or day 90 ($P$ = 0.13). Percent pregnant tended to differ between treatments on day 24 ($^{xy}P$ = 0.07; Estrus = 47 ± 4%$^{x}$, E2E = 41 ± 4%$^{xy}$, E2N = 33 ± 10%$^{xy}$, GnRHE = 39 ± 5%$^{xy}$, GnRHN = 29 ± 5%$^{y}$), and differed on day 30 ($^{ab}P$ = 0.04; Estrus = 41 ± 4%$^{a}$, E2E = 38 ± 4%$^{a}$, E2N = 23 ± 9%$^{ab}$, GnRHE = 34 ± 5%$^{ab}$, GnRHN = 24 ± 4%$^{b}$). As a repeated measure, there was no effect of treatment by day ($P$ = 0.89), while there were significant effects of treatment ($^{ab}P$ = 0.04; Estrus = 49 ± 2%$^{a}$, E2E = 51 ± 2%$^{a}$, E2N = 40 ± 6%$^{ab}$, GnRHE = 48 ± 3%$^{a}$, GnRHN = 41 ± 3%$^{b}$) and day ($^{abcd}P$ < 0.0001; 19 = 47 ± 2%$^{a}$, 24 = 39 ± 2%$^{b}$, 30 = 33 ± 2%$^{c}$, 55 = 28 ± 2%$^{d}$, 90 = 27 ± 2%$^{d}$) on pregnancy. Pregnancy survival tended to differ ($P$ = 0.06) between treatments from day 7 to 90.
**Conclusions:** Estrual cows prior to embryo transfer (day 0 to 7) had greater pregnancy success during conceptus attachment and tended to have greater embryo/fetal survival to day 90 compared to non-estrual cows.
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## 119
## Necroptosis in bovine follicles and granulosa cells cultured *in vitro*

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**Application:** Understanding bovine follicle atresia for improvement of reproductive biotechnology.
**Introduction:** During follicular development, granulosa cells undergo functional and structural changes affecting their steroidogenic activity. Nearly all follicles regress and cell death throughout follicular development is an underlying mechanism of cell loss during follicular atresia. However, cell death in response to different factors can be loosely categorized as either apoptosis or a form of regulated necrosis. Necroptosis is a caspase-independent form of programmed cell death (PCD) executed by the receptor interacting protein kinase 1 (RIPK1)-RIPK3-mixed lineage kinase domain-like protein signaling cascade. Z-VAD-FMK is a well-known cell-permeant Pan Caspase Inhibitor of apoptosis. Cytokines, especially TNFα in combination with a pan-caspase inhibitor is sufficient to activate RIPK3-dependent necroptosis in some cell types. The aim of this work was to evaluate whether the inhibition of the caspase system will introduce necroptotic PCD in medium–size bovine follicles.
**Materials and Methods:** Small follicles (3 to 8 mm) from animals in luteal phase were used. Granulosa cells (from at least 30 individuals) were collected by aspiration and pre-cultured for 24 h on collagen coated plates. Whole undamaged follicles were dissected from ovaries and pre-cultured for 1 h. Cells and whole follicles were cultured *in vitro* with cytokines: Interferon gamma (IFNγ) (50 ng/ml), TNFα (50 ng/ml), TNFα + IFNγ alone and in combination with Z-VAD-FMK (100 μM) for 24 h. Effects of those treatments on the mRNA expression of RIPK1 and RIPK3 were analyzed with qRT-PCR. The treatment effect was considered significant at $P$ < 0.05 using $T$-test.
**Results:** In whole follicles relative expression of RIPK1 was increased by all factors and in granulosa cells by IFNγ and TNFα + IFNγ compared to control. Addition of Z-VAD-FMK to TNFα and TNFα + IFNγ increased RIPK1 expression in both types of cultures. TNFα+ IFNγ alone or with Z-VAD-FMK increased RIPK3 gene expression compared to the control in both culture types. IFNγ or TNFα only with co-stimulation with Z-VAD-FMK increased RIPK3 gene expression in cells and bovine medium-size follicles.
**Conclusions:** Our studies indicate that RIPK1 gene is expressed in the same pattern after incubation with cytokines alone or in combination with Z-VAD-FMK. In contrast, enhanced expression of RIPK3 gene was observed after treatment with Z-VAD-FMK. These results suggest that necroptosis in bovine mid-size follicles requires RIPK3 activation as alternative method of apoptosis during PCD. Our results clearly indicate that Z-VAD-FMK is able to prevent apoptosis by augmenting necroptosis.
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## 120
## Metabolic adaptation to lactation and resumption of ovarian cyclicity of dairy cows in contrasting facilities in half-time confinement plus grazing or total confinement

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**Application:** Feeding system, but not housing facilities, provided in combination with grazing alter milk production and resumption of ovarian cyclicity of dairy cows, although without evident differences in endocrine-metabolic profiles.
**Introduction:** Although confined systems promote greater milk production and have been associated with better energy balance compared to pasture-based systems, reproductive performance is not necessarily improved (Mee, 2012). There are no studies evaluating the effect of facilities provided when combining partial confinement with grazing. The aim was to compare the effect of nutritional strategies and housing facilities on milk production, metabolic indicators and ovarian cyclicity.
**Materials and Methods:** After calving, healthy multiparous autumn-calving Holstein dairy cows ($n$ = 33) were blocked and assigned to one of three treatments: compost barn-total mixed ration (CB-TMR), compost barn-grazing (CB-GRZ), and outdoor soil-bedded-grazing (OD-GRZ). Cows in CB-TMR were confined with TMR ad libitum, while CB-GRZ and OD-GRZ, graze pasture during half of the day and received a supplemental TMR in confinement. From calving until 90 days in milk (DIM), milk production and body condition score (BCS) were recorded daily and fortnightly, respectively. Serum non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), insulin, and insulin-like growth factor-1 (IGF-1) concentrations were determined. The probability of ovarian cyclicity was determined by ovarian ultrasonography at 21, 40, and 60 DIM. All variables were analyzed using the Glimmix procedure of SAS.
**Results:** Milk production was greater ($P < 0.0001$) in CB-TMR (44.0 ± 0.98 L/cow/day) than in CB-GRZ (34.4 ± 0.98 L/cow/day) and OD-GRZ (34.2 ± 0.98 L/cow/day), which did not differ. BCS tended to be higher in CB-TMR ($P = 0.05$) than OD-GRZ cows (2.76 ± 0.03 vs 2.66 ± 0.03, respectively), while CB-GRZ did not differ from either(2.69 ± 0.03). Serum concentrations of metabolites and hormones were not affected by treatment or the interaction between treatment and DIM. The probability of ovarian cyclicity was affected by the interaction between treatment and DIM ($P < 0.05$), as CB-TMR resulted in a lower proportion of cows with a corpus luteum at 21 and 40 DIM (11% at both times) than CB-GRZ or OD-GRZ cows (50 and 55%, respectively, at both times). Pasture-based treatments did not differ. At 60 DIM there were no differences between treatments (78, 80, and 67%, for CB-TMR, CB-GRZ, and OD-GRZ, respectively).
**Conclusions:** Housing facilities did not affect productive or reproductive performance of dairy cows. Although, metabolic indicators did not differ between treatments, pasture-based treatments produced less milk but exhibited an earlier resumption of ovarian cyclicity than confined cows.

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## 121
## Reproductive losses in beef cattle reared on an extensive system of production: effects of genotype and parity

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**Application:** Improvements in reproductive performance impact the profitability of the beef industry. Beef industry is currently facing several emerging societal/consumer issues, including animal welfare and food safety. Genetic selection is a permanent and cumulative approach to reducing the environmental impact of beef production.
**Introduction:** Reproductive losses are a major cause of reproductive failure in cattle with a concomitant financial loss to the beef industry (Shorten et al., 2015). Crossbreeding has been used to increase the weaning weight of calves per cow (Gregory and Cundiff, 1980); however, little information is available on the effect of heterosis on reproductive losses. This study compares the reproductive losses of purebred [Hereford (H) and Angus (A)] and crossbred (HA and AH) cows and the effect of the parity (Nulliparous (NP), Primiparous (PP) and Multiparous, (MP) cows.
**Materials and Methods:** This work is a retrospective study of the reproductive losses of breeding cows of two different purebreds and their crossbred used in a diallelic design. The dataset contained 2959 records of pregnancy diagnosis (PD), calving and weaning, registered over

13 years (1994–2006). MP and PP cows were mated with andrologically evaluated bulls during 80 days beginning on December 1st (summer, SH). The NP cows were mating during 45 days (late November to mid-January - spring – summer, SH). The PD was conducted 45 days after the mated period ended. The reproductive losses (number of weaned calves/total exposed cows * 100) were studied in three Periods: I) from the mating period to PD (number of cows diagnosed as non-pregnant/ total exposed cows * 100); II) from PD to calving (number of calving cows/ number of cows diagnosed as pregnant * 100); III) from calving to weaning (number of weaned calves/numbers of calving cows * 100). The means were compared with Tukey test ($P \leq 0.05$).
**Results:** The total reproductive losses were affected by the genotype ($P < 0.01$) and parity of the cows ($P < 0.01$). Only in period I, genotype and parity affected the reproductive losses ($P < 0.01$): on average, crossbred cows had less losses (0.15 ± 0.01) compared with purebred cows (0.23 ± 0.01). Primiparous cows had greater losses than the other parities (Primiparous: 0.35 ± 0.03 vs Nulliparous: 0.11 ± 0.02 vs. Multiparous: 0.15 ± 0.02 $P \leq 0.02$). No interaction between genotype and parity was detected.
**Conclusions:** It was concluded that crossbreeding is a tool to decrease the reproductive losses in beef meat breeding herds.
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## 122
## One-carbon metabolite supplementation with plane of nutrition during early gestation alters maternal serum metabolite concentrations in beef heifers

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**Application:** Increase nutrient availability to the fetus during maternal nutrient restriction.
**Introduction:** Maternal nutrient restriction during the first trimester alters developmental outcomes. One-carbon metabolites have the potential to change epigenetic events (Crouse et al., 2019), which are underlying mechanisms of developmental programming. Our objective was to evaluate the effect of one-carbon metabolite (folate, vitamin B12, choline, and methionine) supplementation with plane of nutrition during early gestation on maternal serum glucose, urea nitrogen, and non-esterified fatty acid concentrations, and glucose concentrations in allantoic and amniotic fluid at mid gestation.
**Materials and Methods:** Twenty-nine cross-bred Angus heifers were oestrous synchronized and artificially inseminated with female-sexed semen. At breeding (day 0), heifers were assigned to treatments in a 2 × 2 factorial design. Factors were targeted heifer daily gain: control (0.60 kg/day) vs restricted (−0.23 kg/day) and one-carbon metabolite supplementation (rumen protected choline [60 g/day] and methionine [10 g/day] in a ground corn carrier, and weekly injections of 320 mg folate and 20 mg vitamin B12) vs no supplementation (corn carrier and saline injections). Gain and supplement treatments were implemented from gestational day 0 to 63 and heifers were managed similarly from day 64 to day 161 ± 2 when they were necropsied. Blood samples were collected via jugular venipuncture on day 62 and 154. Allantoic and amniotic fluid were collected at necropsy. Data were analysed using the MIXED procedure of SAS with day as a repeated measure for serum but not for allantoic and amniotic fluid measurements, and significance declared at $P \leq 0.05$.
**Results:** Serum glucose was greater ($P = 0.02$) for heifers receiving the control diet compared with restricted (2.74 vs 2.53 ± 0.06 mM). A supplement × day interaction was present ($P = 0.04$) for urea nitrogen, with heifers receiving supplement having the lowest urea nitrogen concentration on day 62. A gain × day interaction was present ($P = 0.006$) for non-esterified fatty acid concentration, which were not different on day 154 and greater ($P = 0.005$) in restricted compared with control on day 62 (372 vs 181 ± 62 mM). Allantoic glucose concentration tended ($P = 0.09$) to be greater for heifers on the restricted plus supplemented diet compared with restricted no supplement and control with OCM supplement.
**Conclusions:** One-carbon metabolite supplementation with plane of nutrition alters the concentration of metabolites in the maternal serum and allantoic fluid potentially improving foetal development in compromised pregnancies.
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**123**

## Comparison of the transcriptomes of embryonic tissue and uterine tissue in heifers that established pregnancy following transfer of an IVP embryo generated using semen from two sires with differing field fertility

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**Application:** Pregnancy loss is considered one of the main causes of reproductive inefficiency in beef cattle industry. Results from this study could potentially lead us to identify sub-fertile bulls.
**Introduction:** The objective was to determine the differentially abundant genes between the conceptus and uterus of pregnancies produced by sires of known fertility. Our hypothesis was that the paternal genome would have a significant effect on transcript abundance between these tissues. The sires used have been previously reported to have significantly different levels of field fertility; however, they had passed all normal quality control standards for frozen thawed semen.
**Materials and Methods:** *Bos indicus* heifers ($n$ = 45) were subjected to estrus synchronization and embryo transfer with IVP embryos from either a high Fertility or a low Fertility sire, pregnancies were confirmed at slaughter. Samples were collected from the trophectoderm and uterus on days 25 and 36 of gestation for RNA sequencing. Total RNA was isolated from tissue samples using the RNeasy kit (QIAGEN; Hilden, Germany) per manufacturer's instructions. RNA sequencing was conducted using an Illumina platform. Sequences were aligned to the reference genome ARS-UCD1.2. Differentially expressed genes between sires and by tissue were determined using edge-R package from R. False discovery rate was 0.05.
**Results:** On day 25, 15,755 genes were identified in the trophectoderm between the sires, 11 genes were downregulated and 6 genes were upregulated in the Low fertility sire. In the uterus, only two genes were decreased. On day 36, in the trophectoderm the Low fertility Sire resulted in 23 downregulated genes and 4 upregulated genes. In the uterus, there were 8 downregulated genes for the Low Fertility Sire whereas 21 genes were upregulated. Gene ontology analysis reported differentially expressed genes in the Low Fertility Sire compared to the High Fertility Sire were associated with immunology and reproduction. Of particular interest, MHC-I gene was found to be downregulated in the trophectoderm on day 36, known for promoting maternal tolerance of the conceptus in mammals during the first trimester of gestation. Additionally, the *Spermatogenesis Associated Gene-22* responsible for gamete generation, homologous chromosome pairing at meiosis and meiotic DNA repair synthesis was also downregulated in the uterus on day 36.
**Conclusions:** These data suggest that field fertility records might be inaccurate for bull selection to improve reproductive performance. These genes could be responsible for differences in conception rates, paternal genome seems to play a bigger role than originally thought.
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## Factors associated with pre-weaning AMH concentrations in replacement dairy heifers born from nulliparous dams

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**Application:** The objective of this prospective, observational study was to investigate if certain factors are associated with greater pre-weaning AMH concentrations in female replacement offspring born from nulliparous heifers.
**Introduction:** Anti-Müllerian hormone (AMH) is secreted by granulosa cells of healthy, growing follicles and is positively correlated with ovarian reserve. Maternal and environmental factors such as nutrition, disease, lactation stage and endocrine disruptors are thought to have a profound impact on ovarian reserve development during early foetal life (Mossa et al., 2017). For genetic progress, it can be advantageous to breed dairy replacements from heifers to expedite the generation interval, however there is some evidence that nulliparous animals produce female offspring with smaller ovarian reserves compared with multiparous animals (Akbarinejad et al., 2018).
**Materials and Methods:** Seasonal, pasture-based dairy heifer calves from six Irish farms ($n$ = 156) born from nulliparous dams were blood sampled at an average of 60 days of age (range: 41–96) in spring 2022. Serum tubes were used for blood collection and the blood was centrifuged, serum separated and frozen on the same day as sampling. An enzyme-linked immunosorbent assay (ELISA) was used to test the samples (AMH (Bovine) ELISA, Ansh Labs) and all samples were tested in duplicate. Statistical analysis was performed with linear regression using the lme4 package (Bates et al., 2015) on the R statistical software program (R Core Team, 2015). 'R Core Team (2015)' is/are cited in the text but not provided in the reference list. Please provide it/them in the reference list or delete these citations from the text." /–>). Pre-weaning AMH concentration was the dependent variable and the independent variables tested included maternal variables such as breeding bodyweight indices, age at conception and estimated genetic values. Independent foetal variables that were tested included breed, age at sample collection and estimated genetic values. Initially, univariate analysis was performed with each independent variable

and only those with a *P*-value < 0.2 were included in multivariate analysis, where significance was only considered when *P*-value < 0.05. Farm was treated as a random effect in all models.
**Results:** The distribution of AMH concentrations was right skewed with a median concentration of 734.1 pg/ml and an interquartile range of 1129.42 pg/ml. For the linear regression, AMH concentration was transformed using the natural logarithm. Predicted transmitting ability for survival (a fertility trait) and the health subindex ($P < 0.05$) in the calf were significant in the final model.
**Conclusions:** Selection of replacement heifers based on genetic estimates for fertility and health may be beneficial in improving pre-weaning AMH concentration. Further work needs to be conducted to investigate if pre-weaning AMH concentration is a useful predictor of future fertility and longevity in seasonal pasture based dairy heifers.

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## 125
## Economic effect of hormone-based fertility programs in dairy farms

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**Application:** Economic consequences of different hormone-based fertility programs.
**Introduction:** Hormone-based fertility programs facilitate reproductive management. In most European countries, the use of hormones is relatively low, and thus extensive. Nevertheless, more intensive hormone-based fertility programs can be beneficial. This study aimed to compare the economic consequences of intensive and extensive hormone-based fertility programs under Dutch dairy production circumstances.
**Materials and Methods:** An existing individual cow-based, dynamic, and stochastic bio-economic simulation model (Edwardes et al., 2021) was extended. The dynamics (i.e., milk production, reproductive cycle and diseases) of a 200 cow-herd were simulated in daily time steps. Four scenarios of fertility programs were modelled. The baseline scenario reflected the current Dutch extensive hormone-based fertility program in which PRIDsynch was applied to an anoestrus cow, Ovsynch to a cystic cow, and prostaglandin to a sub-oestrus cow. The intensive hormone-based fertility programs are included in the Fixed-Time Artificial Insemination (FTAI) scenario, FTAI with Heat Detection (FTAI+HD) scenario, and the Heat Detection (HD) scenario. In FTAI, Double-Ovsynch was applied and ended with FTAI. In FTAI+HD, Double-Ovsynch was applied as in the FTAI scenario but with an additional oestrus detection probability after the insemination. In FTAI and FTAI+HD, Ovsynch was applied to non-pregnant cows with a corpus luteum (CL), and PRIDsynch was applied to non-pregnant cows without CL. In HD, PRIDsynch was applied to cows without oestrus and non-pregnant cows without CL and Ovsynch to non-pregnant cows with CL. For all intensive hormone-based fertility program scenarios, the annual mean net economic return (NER) was calculated, and compared with the baseline.
**Results:** The FTAI+HD scenario gave the highest annual revenues on milk production (€727,633) and the number of calves (€10,776). Slightly less annual revenues resulted from the FTAI and HD scenarios. The lowest annual revenues resulted from the baseline scenario with €698,878 for milk production and €8,125 for calf revenues. The FTAI scenario gave the highest costs (€234,276) followed by the FTAI+HD (€234,140), HD (€231,449) and baseline (€224,510) scenarios. Compared with the baseline, the highest NER was observed for the FTAI+HD scenario with €21,776 higher net revenues, followed by the FTAI and the HD scenarios with €18,885 and €9,543 higher net revenues, respectively.
**Conclusions:** Fertility programs with more intensive use of hormones gave economic advantages over the current Dutch fertility program.
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## 126
## Adenomyosis is associated with the changes in the expression of extracellular matrix and adhesion molecule genes in bovine uterus

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**Application:** Understanding bovine adenomyosis to develop therapeutics.
**Introduction:** Adenomyosis, associated with presence of endometrial glands (EGs) and stroma in myometrium, interferes with reproductive success in cattle but molecular mechanisms associated with pathology are not well understood. As less rigid extracellular matrix (ECM) is suggested to facilitate cell migration, expression of ECM components and adhesion molecules in endometrium and myometrium of cows with adenomyosis was evaluated.
**Materials and Methods:** Uterine tissue was collected from cows ($n$ = 107) slaughtered at a local abattoir and endometrium was separated from myometrium. Based on uterine tissue histology, animals were divided into 5 groups ($n$ = 4–6/group; 0-II), stage 0: healthy; stage IA: minimal endometrial invasion with single glandular ducts below endometrium-myometrium junction, stage IB: proliferation of EGs within perivascular connective tissue in myometrium and presence of foci within mucosal surface layer, stage II: transgression of EGs into myometrium, stage III: EGs within inner muscle layer of uterus, stage IV: EG nests within glandular serous uterine wall in myometrium up to the tunica serosa. We used real-time PCR and Taqman primers/probes to evaluate gene expression of ECM and adhesion molecules. Results were analysed using one-way ANOVA followed by a Tukey multiple-comparison test with the significance set at $P < 0.05$. Greater disease progression was observed in cows classified as III-IV, but insufficient samples were available for inclusion in the study.
**Results:** We observed adenomyosis stage dependent change in expression of *COL1A1*, *COL3A1* and *COL4A1* in endometrium and myometrium. In endometrium, expression of *COL1A1* was significantly higher in stage II as compared to all other categories ($P < 0.05$), *COL3A1* and *COL4A1* showed a significant decrease in stage IB and stage II of adenomyosis as compared to healthy endometrium. No change in *COL4A1* degrading enzyme, MMP2 was observed, but increasing trend in its expression could be noted. In myometrium, *COL1A1* increased significantly only in stage IA of adenomyosis, and expression of *COL3A1* and *COL4A1* was significantly higher in stages IB and II. Expression of *MMP2* was significantly decreased in stage IA. In case of adhesion molecules, expression of *ITGB3* was significantly decreased only in stage IB compared with healthy endometrium, and *SPP1* was decreased in stage IB and stage II. Among adhesion molecules investigated, only *SPP1* showed significant increase between healthy and stage IA myomterium.
**Conclusions:** Dysregulated uterine expression of ECM and integrins can facilitate cell migration in adenomyosis and affect reproductive function in cows.
**Acknowledgements:** Study supported by statutory funds from IAR&FR, Olsztyn and UE&LSci, Wrocław.

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## 127
## Transforming growth factor beta and its family members show alterations in expression in bovine uterus with adenomyosis

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**Application:** Understanding bovine adenomyosis to develop therapeutics.
**Introduction:** Adenomyosis is uterine dysfunction with presence of endometrial glands (EGs) and stroma in myometrium. It interferes with reproductive success in cattle. As it is associated with cell proliferation and differentiation which can be induced by transforming growth factor (TGF)ß, we aimed to evaluate expression of *TGF*ß and its family members in endometrium and myometrium of cows with adenomyosis.
**Materials and Methods:** Uterine tissue was collected from cows ($n$ = 107) slaughtered at a local abattoir and endometrium was separated from myometrium. Histology was used to divide animals into 5 groups ($n$ = 4–6/group), stage 0: no changes; stage IA: minimal endometrial invasion with single glandular ducts below endometrium-myometrium junction, stage IB: proliferation of EGs within perivascular connective tissue in myometrium and presence of foci within mucosal surface layer, stage II: transgression (greater degree of proliferation and penetration) of EGs to the myometrium, stage III: EGs within inner muscle layer of the uterus, stage IV: EG nests within the glandular serous uterine wall in myometrium up to tunica serosa. We used real-time PCR to evaluate uterine expression of *TGF*ß family genes and genes involved with cell differentiation. Results were analysed using one-way ANOVA followed by a Tukey multiple-comparison test with the significance set at $P < 0.05$. Insufficient samples from stage III-IV were available for inclusion in study.
**Results:** Alterations in uterine expression of *TGFß* and its family members in endometrium and myometrium were dependent on stage of adenomyosis. In endometrium, whereas *TGFß* and *ACVR2A* were significantly higher at stage IB compared with stage 0, *INHBA* was maximally expressed at stage II compared with all the other stages. Additionally, *ACVR2A* decreased in stage II compared with stage I. No dif-

ferences were observed in expression of *FST* and *ACVR2B*. The expression of *HOXA10*, regulator of endometrial function was significantly decreased at stages IB and II. In myometrium, whereas expression of *TGFß*, was higher at stage II compared with stage 0 and stage IA, *ACVR2A* and *FST* were significantly increased at stage II of adenomyosis compared with all the other stages. There was no change in expression of *HOXA10* expression across the stages, but expression of *CDKN3* was significantly decreased in stage IB.
**Conclusions:** Bovine adenomyosis is associated with alterations in uterine expression of *TGFß* and its family members which can interfere with endometrial remodeling during implantation period.
**Acknowledgements:** Study was funded by statutory grant from IAR&FR in Olsztyn and UE&LSci in Wroclaw.

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## 128
## The presence of a conceptus at day 14 modifies liver gene expression in ewes

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**Application:** Contribute to the knowledge about the signaling mechanisms between the mother and the embryo.
**Introduction:** The effects of the presence of conceptus on the liver transcriptome at Day 14 of the estrous cycle or pregnancy were investigated.
**Materials and Methods:** Adult Rasa Aragonesa ewes were mated to establish a pregnant ($n$ = 7) and cyclic ($n$ = 6) group. After slaughtered liver was collected and pregnancy was confirmed by the presence of an embryo. Liver sequencing was performed on a HiSeq4000 (Illumina Inc.). The quality control of the raw sequences was assessed with FastQC software. Statistical analysis was conducted using the edgeR pipeline (Robinson and Oshlack, 2010) and the Benjamini-Hochberg method was applied to control the false discovery rate (FDR).
**Results:** We found nine DEGs (differentially expressed genes) between pregnant and non-pregnant ewes in the liver. Pregnancy downregulates CYP2C9 (CytochromeP4502C9), involved in the metabolism of arachidonic to eicosanoid acids, by -380.8-fold-change. As arachidonic acid is an essential fatty acid and a precursor in the synthesis of prostaglandins (Bosetti, 2007), pregnancy downregulation in the liver could be a mechanism of saving arachidonic acid for prostaglandin synthesis to promote pregnancy. Also, pregnancy downregulated EFHB (EF-Hand Domain Family-Member-B) by -7.1-fold-change which regulates the amount of free calcium in the cytosol. Thus, pregnancy downregulation of this gene could be a strategy to maintain high levels of free calcium to fulfill the pregnancy requirements. On the other hand, pregnancy upregulates the expression of RNF213 (Ring Finger Protein 213) by 2.1-fold-change, which acts by inhibiting the lipolytic process. It has been shown that triglycerides are diverted from the uptake by the liver to the uterus during pregnancy (Ghio et al., 2011), thus, upregulation of this gene could be a mechanism to provide the necessary energy for the developing embryo and the uterus. ZNFX1 (Zinc Finger NFX1-Type Containing 1), an IFNt-induced gene involved in immune response (Wang et al., 2019), was also upregulated by 2.4-fold-change in pregnant animals. The liver is a key, frontline immune tissue, assuring the peripheral immune tolerance of the organism, relevant in the uterus during pregnancy.
**Conclusions:** In conclusion, the present study demonstrated that as early as day 14 maternal metabolic tissues such as the liver responded to the presence of the embryo by changing the transcriptome profile.

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## 129
## Ageing is associated with molecular and functional modifications of endometrial physiology in cattle

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**Application:** Reproductive ageing is associated with decline in fertility in mammals. Considering the irreplaceable contribution of the uterus to pregnancy success and outcomes, determining the impact of ageing on this organ is essential to identify strategies that preserve its biological functions in older females.
**Introduction:** Ageing is associated with an inflammatory process that affects the functions of most organs. Although age-related dysfunction of cell cycle and activation of inflammation-related pathways has been reported in bovine uterus, effect of ageing on endometrial physiology remains a matter of debate in cattle and more generally in mammals. Using an experimental model of cloned bovine females, the objective of our study was to evaluate the effect of ageing on the endometrium using transcriptome profiles patterns and the responses of cultured endometrial cells to interferon tau, the major factor of pregnancy recognition in ruminants.
**Materials and Methods:** The study was conducted using 6 young primiparous bovine clones aged 4–5 years and 7 old nulliparous clones aged 13–14 years generated from the same somatic cell line and reared under similar conditions. Plasma progesterone concentration was determined at days 2, 8, 14, and 22 post-estrus cycle synchronization. Using endometrial biopsies and a custom bovine array (23,926 unique transcripts), gene expression profiles were determined in four individuals per group at 15 days post-estrus. Differentially expressed genes were identified using the fold change rank ordering method. Pathway enrichment analyses were performed using Ingenuity Pathway Analysis software. Primary cultures of endometrial cells were derived from biopsies collected at 15 days post-estrus ($n$ = 3/ group) then incubated with recombinant interferon-tau (IFNT, 100 ng/mL) for 1 h and 24 h. Transcript levels of interferon- dependent genes were quantified by RT-qPCR.
**Results:** Progesterone profiles were similar between the two groups of females. Between young and old bovine endometrium, transcriptome analyses unveiled 1286 differentially expressed genes, and activation of pathways related to inflammation, immunity, metabolism, and cellular processes. In primary glandular epithelial cells treated with rIFNT for 1 h, *MX1* and *RSAD2* transcript levels were significantly lower in old versus young bovine females ($P$-value $\leq$ 0,01 and $P$-value $\leq$ 0,05 respectively). In stromal cells, *RSAD2* transcript level was significantly lower in old females at 1 h ($P$-value $\leq$ 0,001).
**Conclusions:** By affecting the ability of the endometrium to respond to the embryo, we suggest that age-related modifications of endometrial physiology may account for reproductive failures of uterine origin. Additional experiments are required to support our hypothesis.

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## Association between nonesterified fatty acids concentration and subclinical hypocalcemia at calving with early lactation clinical diseases and fertility in grazing dairy cows

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**Application:** Research in peripartum diseases and fertility of dairy cows in grazing conditions contributes to specific preventive measures.
**Introduction:** This study describes the association between subclinical hypocalcemia (SCH; defined as blood calcium concentrations <2.10 mM) and high non-esterified fatty acids (NEFA; defined as blood NEFA concentrations >0.6 mM) at calving with early lactation disease and reproductive performance during a full lactation.
**Materials and Methods:** Dairy cows ($n$ = 646), were selected from 13 commercial grazing dairy herds in Uruguay. Retained placenta (RP), metritis and clinical mastitis (CM) cases were recorded during the first 30 days in milk (DIM) for a year. Blood samples were taken from each cow within 0–4 days DIM for NEFA, calcium, magnesium and phosphorus determinations and body condition score (BCS) was recorded. To evaluate the association between the health events and risk factors (parity, BCS and metabolites), multivariable logistic regressions models were developed. To evaluate the association between the risk factors mentioned above and early lactation disease with reproductive performance (i.e. first insemination and pregnancy rates), LSM were analysed by Glimmix. For all regression models, herd was considered as random effect. Variables or their interaction with parity, with a $P \leq 0.10$ remained in the models. Statistical significance was set at $P \leq 0.05$ and statistical tendency at $P \leq 0.10$.
**Results:** Overall, 16.9% of the cows recorded a first case of CM and 4.2% recorded retained placenta-metritis (considered as a single outcome). Logistic regression models showed that CM was associated with parity [OR = 0.42, $P < 0.01$], and SCH [OR = 1.75, $P = 0.04$] and tended to be associated with suboptimal BCS, [OR = 0.66, $P = 0.07$]. Moreover, RP-metritis tended to be associated with high NEFA as a risk factor [OR = 2.2, $P = 0.06$] and negatively affected pregnancy rates, (healthy cows = 75% ± 0.04 vs cows with RP-metritis = 49% ± 0.1, $P = 0.03$). In addition, pregnancy rates tended to be affected by parity (PP = 70% ± 0.08 vs MP = 55% ± 0.08, $P = 0.07$) and by high NEFA concentrations (low NEFA = 77% ± 0.04 vs high NEFA = 68% ± 0.05, $P = 0.09$). Finally, AI rate tended to be affected by the interaction between parity and NEFA (MP low NEFA = 82% ± 0.05 vs MP high NEFA = 70% ± 0.05, $P = 0.07$).
**Conclusions:** Data show the association between BCS, NEFA and Ca at calving with health status and reproductive performance during early lactation of PP and MP dairy cows on grazing systems.

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## 131
## Follicular characteristics and pregnancy rates to AI in Holstein heifers treated with two protocols with prolonged proestrus and inseminated with sexed semen

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**Application:** High-fertility timed-AI (TAI) protocols facilitates the application of sexed semen in Holstein heifers.
**Introduction:** The widespread use of sexed semen requires the development of TAI programs with high fertility (Bo et al., 2018). Two experiments were designed to evaluate of the length of insertion of the progesterone (P4) device on follicular characteristics and P/AI in Holstein Heifers treated with the oestradiol/P4 based protocol, named J-Synch, and inseminated with sexed semen.
**Materials and Methods:** Holstein heifers ($n$ = 14 in Experiment 1 and 336 in Experiment 2) were used. On Day 0, all heifers received 2 mg oestradiol benzoate (Over, Argentina) and a device containing 0.7 g P4 (Sincrover, Over). The P4 device was removed on Day 6 in the 6-d J-Synch group and on Day 7 in the 7-d J-Synch group. All heifers received 150 μg D(+) cloprostenol (Prostal, Over) at device removal and were tail painted for oestrus detection. In Experiment 1, heifers were scanned twice daily from device removal to ovulation. In Experiment 2, heifers with >30% of the tail-paint rubbed off by 72 h after device removal were inseminated at that time, whereas those without the tail-paint rubbed-off received 10 μg buserelin (Gestar, Over) and were inseminated 12 h later. All heifers in Experiment 2 were inseminated with sexed semen from 6 bulls (Sexcel, ABS, USA) that were equally distributed among groups and were examined for pregnancy 30 days after AI. Data were analysed using ANOVA in Experiment 1 and GLM mixed procedure for binary data with a logit link in Experiment 2.
**Results:** In Experiment 1, the interval from device removal to ovulation tended ($P$ = 0.08) to be longer in the 6-d J-Synch group (96.0 ± 5.8 h) than in the 7-d J-Synch group (82.5 ± 5.0 h). The diameter of the largest follicle at the time of device removal and before ovulation did not differ among groups (8.1 ± 1.5 and 13.7 ± 0.7 mm vs 10.1 ± 1.5 and 1.9 ± 0.6 mm, for the 6 and 7-d J-Synch groups, respectively). In Experiment 2, although oestrus expression did not differ (86.4%, 146/1169 vs 87.4%, 146/167 for the 6 and 7-d J-Synch, respectively), P/AI was greater ($P$ < 0.05) in those in the 7-d J-Synch (49.1%, 82/167) than those in the 6-d J-Synch group (37.9%, 64/169).
**Conclusions:** Delaying the removal of the P4 device by one day in the J-Synch protocol resulted in higher P/AI in Holstein heifers inseminated with sexed semen.
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## 132
## Nutrient restriction during early gestation in dairy cattle impairs ovarian development in the offspring

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**Application:** Bovine fertility may be enhanced by avoiding nutritional deficiencies in pregnant dams.
**Introduction:** Female beef calves born to mothers exposed to a nutritionally restricted diet in early gestation have been shown to have a reduced total number of ovarian follicles (ovarian reserve; Mossa et al., 2013). The aim of this work was to investigate the impact of maternal nutrient restriction from shortly before conception to early gestation on the development of the reproductive tract in female progeny in dairy cattle.
**Materials and Methods:** Holstein-Friesian heifers ($n$ = 42) homogenous for age (14–17 mo) and weight (366.2 ± 41.1 kg) were randomly assigned to three experimental groups and, starting 10 days before artificial insemination (AI), were individually fed at: (i) 0.6 of their maintenance energy requirements (M) up to day 80 (Nutrient Restricted, NR80;$n$ = 16) or (ii) 120 DG (days of gestation; NR120,$n$ = 16), and (iii) 1.8 M until 120 DG (Control, C; $n$ = 10). Estrus cycles were synchronized, and heifers were inseminated with sex-sorted semen from a single sire. Pregnancy was diagnosed and confirmed via ultrasound (MyLab Omega, Esaote, with 4–10 MHz sectorial probe) 28 and 55 days after AI, respectively. After the end of the differential diet, all heifers were group fed ad libitum until calving. Twenty-two single

female calves were born (NR80 = 8; NR120 = 9; C = 5); body weight (BW) and height at withers (H) were measured regularly until slaughter at 4.5 mo. Ovaries were measured, weighed, all visible antral follicles were counted, and cumulus oocyte complexes (COCs) were collected. Data were analyzed with R software with One-way ANOVA and mean contrast separated with Tukey post-hoc test. Results are expressed as mean ± SEM.
Results: BW at birth was lower in NR80 than C calves ($P < 0.05$) and similar between NR80 and NR120 (C = 41.4 ± 1.1; NR80 = 36.7 ± 0.6; NR120 = 38.3 ± 1.2 kg), while BW at slaughter and H were similar among groups. Ovarian volume was similar among groups. Ovarian weight was lower ($P < 0.05$) in NR120 compared to C and similar between NR120 and NR80 (C = 10.4 ± 1.3; NR80 = 7.4 ± 0.9; NR120 = 6.7 ± 0.5 g) and not correlated to BW at slaughter (R = 0.05). NR120 heifers had less ($P < 0.05$) visible antral follicles than C whereas no difference was detected between NR80 and C (C = 197.2 ± 36.5; NR80 = 150.1 ± 20.9; NR120 = 104.2 ± 10.7). Fewer COCs were retrieved ($P < 0.05$) from NR120 and NR80 compared to C ovaries (C = 75.8 ± 12.57; NR80 = 48 ± 3.5; NR120 = 48.2 ± 6.68).
Conclusions: Maternal exposure to undernutrition from preconception to day 120 of gestation resulted in a reduction of ovarian weight, visible antral follicles and retrieved COCs in their female offspring indicating a potential impairment of the size of the ovarian reserve.
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## 133
## Heifers in large, high-producing dairy herds have lower age at first calving

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**Application:** Optimal age at first calving is an important key performance indicator for lifetime production and dairy farm profit, as well as for prospective health, fertility and longevity. Many herds have potential to decrease the age at first calving, thereby increasing profit and reducing greenhouse gas emission from the rearing of recruitment heifers.
**Introduction:** Despite long-term advisory efforts aiming for 24–25 months of age at first calving, improvements have been slow. The national, Swedish mean has been above 27 months for long, but in year 2022, it decreased below 27 months for the first time for heifers of both major dairy breeds, Holstein and Swedish Red. The objective was to improve reproductive herd health management based on identified risk factors.
**Materials and Methods:** Data from 2,024 dairy herds in the Swedish national dairy herd recording scheme were used to describe the current state and investigate associations between explanatory variables (herd size, housing/milking system, breed, organic or conventional production, milk yield and region) and the dependent variable age at first calving, using a multivariable linear regression model.
**Results:** The results showed that age at first calving was higher in herds with crosses of Swedish Red and Holstein (27.7 months, $P = 0.005$,) than in herds without a dominant breed (27.1 months). Herds with ≥200 cows had lower age at first calving (26.7 months, $P \leq 0.002$) than herds with <100 cows (27.8 months). Organic herds had lower age at first calving (27.1 months, $P = 0.02$) compared to conventional herds (27.6 months). Herds with free-stall systems, irrespective of milking system, had lower age at first calving (27.2 months, $P = 0.02$) than herds with tie-stall system (27.8 months). Age at first calving decreased with increasing herd milk yield ($P < 0.001$), 26.0 months in the highest-yielding herds (≥11,619 kg ECM) and 29.4 in the lowest-yielding herds (<9,791 kg ECM). There was substantial variation ($P < 0.001$) in age at first calving in herds in different Swedish counties, with a range from 26.0 months in the northernmost counties to 28.6 months further south.
**Conclusions:** In conclusion, although the national mean age at first calving recently has improved to below 27 months, there is still potential for improvement for most of Swedish dairy herds. Notably, in the largest and most high-yielding herds the age at first calving was lowest, implying that intensive production systems have successful management strategies to learn from in the reproductive herd health advisory service.

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## Effect of circulating estradiol and progesterone concentrations on FSH release profile of beef heifers submitted to an estradiol/progesterone-based synchronization protocol

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**Application:** To better understand the effect of steroidal hormones on modulation of FSH release during follicular emergence in *Bos indicus* heifers.
**Introduction:** It is known that circulating estradiol (E2) and progesterone (P4) modulate gonadotropin release in cattle. However, there are few reports about FSH profile during an E2/P4-based protocol. This study evaluated the effect of circulating P4, from intravaginal devices with higher vs lower P4, and the effect of E2 concentrations, from a pre- vs post-deviation follicle, on FSH release and follicular development in Nelore heifers submitted to an E2/P4-based synchronization protocol.
**Materials and Methods:** Nelore heifers ($n$ = 23; 24.0 ± 2.7 mo old) were previously synchronized to have a post-deviation 7-d old follicle (HighE2) or a pre-deviation 2-d old follicle (LowE2), without CL on d0. On d0, heifers received 1.5 mg E2 benzoate and the P4 treatments: a new 2g intravaginal device (HighP4) or a 1g device previously used for 14d (LowP4). Blood samples were collected every 12 h until d5 and ultrasound evaluations were performed daily until d7. Thereafter, devices were removed and heifers were reassigned to a new replicate (cross-over). P4 concentrations were evaluated by chemiluminescence-immunoassay, FSH and E2, by radioimmunoassay. Data were analyzed by GLIMMIX (SAS 9.4) as 2 × 2 factorial arrangement. Treatments and interaction were considered as fixed effects and replicate as random effect. FSH concentrations over time were analyzed as repeated measures, separately from d0 to follicular emergence (60 h), and from emergence to deviation (120 h). Differences were declared when $P \leq 0.05$.
**Results:** Only data from heifers that had follicular emergence were analyzed (HighE2/LowP4 = 9; HighE2/HighP4 = 9; LowE2/LowP4 = 8; LowE2/HighP4 = 8). No interaction was detected. Groups HighE2 had higher circulating E2 (5.4 ± 0.9 vs 2.7 ± 0.5 pg/mL) and a greater follicle on d0 (10.7 ± 0.4 vs 6.7 ± 0.3 mm). Mean circulating P4 over time was higher in HighP4 groups (3.6 ± 0.1 vs 2.4 ± 0.1 ng/mL). Before emergence, FSH concentrations were not affected by P4 but were higher in HighE2 groups. After emergence, there was no effect of E2, whereas HighP4 groups had higher FSH concentrations. Day of emergence was not affected by treatments (2.9 ± 0.1). However, in HighP4 groups, deviation occurred later (5.8 ± 0.2 vs 5.2 ± 0.2 d) and the number of subordinate follicles at deviation was lower (7.5 ± 0.6 vs 9.6 ± 0.7).
**Conclusions:** In *Bos indicus* heifers, circulating E2 at the beginning of an E2/P4-based protocol affected FSH concentrations before follicular emergence, whereas P4 concentrations affected FSH concentrations between emergence and deviation, resulting in a slight delay in deviation time.
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## Effects of subluteal circulating progesterone concentrations on ovarian dynamics in prepubertal beef heifers

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**Application:** To understand the effects of subluteal circulating progesterone (P4) concentrations on ovarian dynamics in prepubertal Nelore heifers.
**Introduction:** Much is known about the effects of P4 on follicular growth (FG) in pubertal heifers and cows. However, its effects on ovarian dynamics of prepubertal *Bos indicus* beef heifers are still unclear. This study aimed to evaluate the effects of subluteal P4 concentrations on ovarian dynamics in prepubertal heifers.
**Materials and Methods:** Prepubertal Nelore heifers ($n$ = 21; 320.3 ± 4.7 kg of body weight; 22.8 ± 0.5 mo old) underwent follicular ablation on d-1. On d0, heifers were randomly assigned to two treatments: Control (CON; $n$ = 11): heifers received a sham intravaginal device (IVD) without P4; or Subluteal P4 (SP4; $n$ = 10): heifers received a 1 g P4 IVD previously used for 14d. The IVD were kept until d22. Ultrasound evaluations were performed daily from d0 to d26 to evaluate follicular wave (FW) dynamics. Blood samples were collected daily from d0 to d24 for circulating P4 (determined by chemiluminescence immunoassay). Statistical analysis was done by GLIMMIX (SAS 9.4), considering treatments and body weight as fixed effects, and age as a random effect. Differences were declared when $P \leq 0.05$.
**Results:** Five heifers from SP4 ovulated during the study (1st FW: $n$ = 2; 2nd FW: $n$ = 2; 3rd FW: $n$ = 1) and were removed from the subsequent analyses. Mean circulating P4 was higher in SP4 than in CON, from d1 to d22. Heifers in SP4 group had higher mean circulating P4 over time (1.1 ± 0.1 vs 0.3 ± 0.1 ng/mL), greater maximum diameter of dominant follicle (DF; 14.4 ± 0.6 vs 12.2 ± 0.5 mm), and greater FW interval (8.7 ± 0.5 vs 6.6 ± 0.5 d). There was no effect of treatment on the number of FW (CON: 4.1 ± 0.3 vs SP4: 3.6 ± 0.2), nor on FG rate (1.1 ± 0.1 mm/d). During the first FW, SP4 heifers had greater maximum diameter of DF (13.6 ± 0.6 vs 11.6 ± 0.1 mm), lower FG rate

(0.9 ± 0.1 vs 1.1 ± 0.1 mm/d), and greater interval to the second FW (9.5 ± 0.6 vs 7.2 ± 0.8 d). Considering the second FW, SP4 heifers had greater maximum diameter of DF (13.4 ± 0.7 vs 11.5 ± 0.5 mm), and greater interval to the third FW (7.5 ± 0.9 vs 6.0 ± 0.5 d), although no difference was detected on FG rate.
**Conclusions:** Exposure of prepubertal heifers to subluteal P4 increased the maximum diameter of the DF and the interval between FW. These effects were detected during the first and second FW, when the higher concentration of circulating P4 in SP4 was more pronounced than in CON.
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**136**

## Association of transient, delayed or persistent elevated non-esterified fatty acids concentration around parturition with pregnancy rate in grazing dairy cows

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**Application:** Management to limit the duration of elevated non-esterified fatty acids (NEFA) concentration around parturition could reduce the negative effect of circulating NEFA on subsequent reproductive performance.
**Introduction:** Elevated concentrations of blood metabolites related to energy balance around parturition, such as NEFA, can reduce reproductive performance. Elevated NEFA concentrations measured once within 14 d prepartum or within 14 d postpartum are negatively associated with pregnancy rate (Overton et al., 2017). Studies that evaluate the association of transient, delayed or persistently elevated NEFA concentration around parturition with pregnancy rate are lacking.
**Materials and Methods:** Holstein primiparous ($n$ = 346) and multiparous ($n$ = 626) cows from four grazing dairy farms were blood sampled for NEFA determination prepartum (10 to 7 d before calving) and postpartum (5 to 8 d after calving). Time to pregnancy was defined as the interval in days from calving to the last insemination before the pregnancy diagnosis. Pregnancy diagnoses were performed by transrectal palpation or ultrasonography by the farm veterinarian. Censoring time was 150 days in milk. Cox proportional hazards regression models were used to analyse pregnancy rate. Non-esterified fatty acids dynamics (Low: prepartum $\leq$ 0.30 mM and postpartum $\leq$ 0.60 mM, Transient: prepartum > 0.30 mM and postpartum $\leq$ 0.60 mM, Delayed: prepartum $\leq$ 0.30 mM and postpartum > 0.60 mM and Persistent: prepartum > 0.30 mM and postpartum > 0.60 mM), parity (1, 2, and 2+) and calving month were included as class variables. Farm was included as a random effect. Variables were retained when $P \leq 0.10$. Statistical significance and tendency were set at $P \leq 0.05$ and $P \leq 0.10$ respectively.
**Results:** Non-esterified fatty acids dynamics were associated with pregnancy rate. Compared with the Low group (reference), pregnancy rate in Transient cows did not differ (HR = 0.84, $P$ = 0.19) while Delayed cows tended to have a decreased pregnancy rate (HR = 0.77, $P$ = 0.08) and Persistent cows had a decreased pregnancy rate (HR = 0.73, $P$ = 0.02). Compared with parity 2, parity 1 cows had an increased pregnancy rate (HR = 1.31, $P$ = 0.02), while parity 2+ were not different (HR = 0.90, $P$ = 0.36).
**Conclusions:** Data indicate that cows that overcome elevated NEFA concentration before 5 to 8 d after calving maintain reproductive performance, while cows with persistently elevated NEFA concentration had an impaired reproductive performance.
**Acknowledgements:** Funding was provided by Elanco Animal Health (Greenfield, IN) and the University of the Republic, Uruguay.

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**137**

## Supplementing the diet of twin-bearing Merino ewes with rumen protected arginine during mid-to-late gestation increases circulating arginine concentration

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**Application:** Improve survival rates of twin Merino lambs through maternal supplementation of rumen-protected (RP) arginine during mid-to-late gestation.
**Introduction:** High incidences of twin lamb mortality in Merino sheep have both a significant financial impact and raise welfare concerns within the industry. Mortality rates are greater in twin born lambs with approximately 12 million twin lambs dying in Australia each year. Arginine is critical during fetal growth due to its haemodynamic role in utero-placental development. The aim of this study was to determine the effect of maternal RP-arginine supplementation on circulating levels of arginine in the ewe and lamb attributes associated with viability.
**Materials and Methods:** From day 100 of gestation ewes were fed a pellet, pea and barley mix ration supplemented with either 0, 4, 8, 12 or 16 g RP-arginine per day ($n$ = 12 ewes per treatment) through to parturition. Lamb parameters of meconium staining, immunoglobulin G measured by a radial-immunodiffusion assay, rectal temperature, blood glucose measured by glucometer (Abbott Freestyle Optium Neo®, Victoria, Australia) were recorded at birth.
**Results:** Supplementation with 16 g RP-arginine increased circulating arginine and total amino acid concentrations in the ewes 4 h post feed compared with control ($P < 0.05$); however, no differences were observed between the other treatments. Birthweight (4 g: 4.00 ± 0.2 g; 8 g: 4.02 ± 0.2 g; 12 g: 3.96 ± 0.3 g; 16 g: 4.03 ± 0.2 g; CTL: 3.82 ± 0.2 g) did not differ between the treatments. Lambs born to ewes in the 8 g RP-arginine treatment group had greater blood glucose at birth compared with control (3.3 ± 0.6 mmol/L and 2.0 ± 0.3 mmol/L respectively; $P < 0.05$). Rectal temperature of lambs from the 16 g treatment were higher at 4 h post birth compared with control lambs ($P = 0.05$); however, no differences in rectal temperature were observed at 24 h post birth between treatments. Parturition difficulty of ewes, meconium staining of lambs and lamb IgG at birth did not differ across treatments.
**Conclusions:** Supplementation with RP-arginine effectively increased circulating arginine levels in the pregnant ewe, and rectal temperature and blood glucose of their lambs, but the responses were not consistent across the doses investigated. Although birthweight was unaffected, improved thermoregulation and glucose have the potential to improve survival of twin lambs, with further investigation required in a larger cohort.
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## 138
## Effect of administration of mycobacterium cell wall fraction during the peri-ovulatory period on embryo production following superovulation in virgin dairy heifers

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**Application:** Assisted reproductive technologies are effective tools for increasing the rate of genetic gain through multiplication of genetics from elite animals. Improvements in the effectiveness of such technologies can result in greater production efficiency of livestock production systems.
**Introduction:** Pro-inflammatory cytokines play an important role in regulating several reproductive events in the early post-oestrus period, including ovulation, corpus luteum formation, and early embryo development. The objective of this study was to determine whether induction of a pro-inflammatory response during the peri-ovulatory period could improve embryo production following superovulation in virgin dairy heifers.
**Materials and Methods:** The study was conducted on a commercial dairy using virgin, Holstein heifers ($n$ = 13; age 12–18 months). Prior to enrolment, animals were randomly assigned in a $2 \times 2$ crossover design to receive saline or Amplimune (NovaVive), a commercial immunostimulant, consisting of a fraction of the cell wall from *Mycobacterium phlei*. Animals were subjected to superovulation as described previously (Chebel et al., 2008), except that dominant follicle removal was performed and animals received an intravaginal progesterone insert 36 h prior to administration of exogenous follicle stimulating hormone (240 mg, Folltropin, Vetoquinol). The intravaginal insert was removed in the evening of Day -1 and animals received their assigned treatments, 5 mL via intramuscular administration, in the morning of Day 0 (day of presumptive oestrus). Embryo recovery was performed on Day 7 and recovered structures were evaluated microscopically. Data were analysed by analysis of variance using a general linear model.
**Results:** Animals treated with Amplimune had a lesser ($P < 0.04$) number of structures recovered (9.5 ± 1.1 vs 13.3 ± 1.12), but there was no difference between saline and Amplimune for the number of viable embryos produced (6.8 ± 1.1 vs 5.7 ± 1.1). There was also no difference between saline and Amplimune for the number of degenerate embryos (3.0 ± 1.0 vs 1.8 ± 1.0), number of unfertilized oocytes (2.8 ± 0.6 vs 2.1 ± 0.6), number of grade 1 embryos (3.9 ± 0.8 vs 3.1 ± 0.8), and number of grade 2 embryos (1.7 ± 0.5 vs 2.0 ± 0.5) recovered.
**Conclusions:** Administration of the commercial immunostimulant Amplimune during the peri-ovulatory period reduced the total number of structures recovered following superovulation but did not affect the number of viable embryos produced nor the quality of embryos

recovered. Results of the study indicate that stimulation of a pro-inflammatory response during the peri-ovulatory period does not enhance embryo production following superovulation in virgin dairy heifers.

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## 139
## Post-natal anti-Müllerian hormone profiles for Australian Merino and Suffolk ewe lambs

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**Application:** Understanding anti-Müllerian hormone (AMH) changes and differences between sheep breeds within the Australian sheep industry enables determination of ranges or values that may identify animals of superior reproductive potential. Commercially, producers can prioritise animals for joining, minimising losses and improving productivity.
**Introduction:** In sheep, AMH predicts ovarian reserve (Torres-Rovira et al., 2014) and first mating fertility (Lahoz et al., 2012). Therefore, AMH may be a suitable phenotypic marker of fertility in sheep; however, AMH profiles have not been established for breeds utilised in Australia. The objective of this study was to identify pre-pubertal profiles of circulating AMH levels in Suffolk and Merino ewe lambs.
**Materials and Methods:** Fortnightly blood samples were collected from two to 18 weeks of age from 42 ewe lambs ($n$ = 20 Suffolk and $n$ = 22 Merino). Plasma AMH was determined using an ovine specific AMH ELISA kit (Ansh laboratories, Texas, USA). Differences between breeds were determined using an ANOVA, unbalanced design (Genstat 18th Edition, VSC international). Data presented as Mean ± SEM.
**Results:** Compared with Suffolk lambs, AMH levels were higher ($P$ < 0.01) in Merino lambs at 2 (13.1 ± 2.57 vs 2.8 ± 0.93 ng/mL), 6 (26.1 ± 2.46 vs 11.1 ± 1.55 ng/mL), 10 (10.8 ± 1.37 vs 5.6 ± 0.92 ng/mL) and 18 weeks (1.55 ± 0.21 vs 0.81 ± 0.08 ng/mL). For each breed, two AMH profiles were evident. In 13 Suffolk lambs (PEAK), AMH peaked between 6 and 8 weeks of age (15.1 ± 1.37 ng/mL) and decreased thereafter, with no distinct peak observed in the remaining animals (FLAT). Average AMH differed between these cohorts of Suffolk lambs (FLAT; 1.5 ± 0.22 vs PEAK; 6.0 ± 0.39 ng/mL, $P$ < 0.05). In contrast, an AMH peak was observed in all Merino lambs at 6 weeks of age. However, in 14 lambs this peak was preceded by a trough at 4 weeks (AMH = 3.8 ± 2.76 ng/mL), no drop observed in remaining animals (AMH = 19.4 ± 3.76 ng/mL) ($P$ < 0.05).
**Conclusions:** There appears to be two distinct AMH profiles for both Suffolk and Merino ewe lambs. Similar to Sarda ewe lambs, Suffolk ewe lambs show a single AMH peak at approximately 6 weeks of age, or a gradual increase in AMH. In contrast, and not previously described, AMH peaked in all Merino ewe lambs; however, a proportion of animals displayed a distinct drop immediately prior. Current data demonstrate post-natal AMH profiles differ between breeds; whether this is reflected in differences in ovarian development requires investigation.

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## 140
## Effect of forage allowance on growth, endocrine and reproductive variables in the progeny of beef cows grazing campos grasslands

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**Application:** Improve beef cattle productivity in pastoral systems adjusting forage allowance during the gestation-lactation cycle.
**Introduction:** Nutrition in utero and early stages of life program the development and functioning of different organs, affecting animal productivity. The objective of this study was to evaluate the effect of different forage allowances during gestation and lactation on parameters that describe growth and development, metabolic status and reproductive performance on the progeny.
**Materials and Methods:** Angus, Hereford and Angus x Hereford multiparous pregnant cows were used in a complete randomized block design, assigned to different forage allowances (FA) during the gestation-lactation cycle. The levels for high (HFA) and low (LFA) were 8 and 4 kg DM/kg live weight (LW) in fall, 4 and 4 kg DM/kg LV in winter, 12 and 8 kg DM/kg LV in spring, and 8 and 4 kg DM/kg LV in sum-

mer (Sollenberger et al., 2005). At weaning, females calves ($n$ = 17) were reared at the same forage allowance (>3.31 kg DM/kg LW). LW was measured and blood samples collected monthly to measure serum insulin-like growth factor-I (IGF-I) concentrations. Puberty was estimated by weekly ovarian ultrasonography starting at 250 kg of LW. At 18 months of age, antral follicle count (AFC) during three ovum pick up sessions 21 days apart was measured. The cumulus-oocyte complexes were conditioned and transferred to the *in vitro* production laboratory to evaluate embryonic development up to day 8. Data was analysed using general mixed models and frequencies in SAS.
**Results:** Growth and development from birth to weaning were similar between HFA and LFA calves. During rearing, IGF I concentrations did not differ in HFA (276 ± 25.2) and LFA (272 ± 23.8 ng/mL; $P$ = 0.9). Puberty occurred at similar age in HFA and LFA (459 ± 11 days). LFA heifers tended to be heavier (272 ± 8.9; $P$ = 0.05) and had a greater AFC (19.7 ± 2.3; $P$ = 0.02) than HFA heifers (247 ± 9.4 kg and 13.5 ± 1.4 follicles). Number of total viable oocytes (HFA = 96 vs LFA = 109; $P$ = 0.97), cleavage rate (day 3) (HFA = 68.4 ± 4.9 vs LFA = 68.1 ± 5.4%; $P$ = 0.96) and blastocyst rate (day 8) (HFA = 14.6 ± 3.6 vs LFA = 16.8 ± 3.1%; $P$ = 0.63) were similar among groups.
**Conclusions:** The dynamics of forage allowance imposed on the cows during the gestation-lactation cycle, had little benefit on AFC of LFA but not HFA heifers.
**Acknowledgements:** To CSIC-UdelaR for partially financing this study.

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## 141
## Impact of environmental conditions on pregnancy rate of beef cows inseminated at fixed time

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**Application:** Interannual changes in environmental conditions affect pregnancy rate at fixed time artificial insemination (FTAI) in beef cattle.
**Introduction:** Heat stress decreases feed intake, milk production and impairs reproduction. The hypothesis of this study was that environmental conditions around the time of FTAI affect the pregnancy rate of beef cows.
**Materials and Methods:** Data from 224 FTAI programs of 42 commercial farms, including Angus x Hereford females ($n$ = 35 341 heifers and $n$ = 25 805 lactating cows) from eight years of records (2010–2017) were utilized. The breeding season lasted from October to February in the Southern hemisphere. Only heifers that had an Andersen score $\geq$3 enter the program, using the synchronisation protocol described by Cunha et al. (2020). Ten days before FTAI lactating cows with $\geq$40 days post-partum, cycling or in shallow anestrus (follicle $\geq$8 mm) were selected for the program. Lactating cows received 2 mg of oestradiol benzoate intramuscularly on day -10 and a dose of 400 IU of eCG by the time of intravaginal device removal on day -3. Farms were divided into three regions and weather data were compiled from stations closer to the clusters of farms. Data included minimum, maximum, and mean temperature, humidity, wind speed, rainfall, radiation and temperature humidity index (THI), 21 days before and after FTAI and for the average of the 42 days. Correlations between pregnancy rate and parity, year, region, and farm were determined using Proc Corr of SAS. Subsequent analyses were performed using pregnancy at FTAI and environmental variables 21 days before and after each date of FTAI. For the complete database, a linear mixed model was used to evaluate the associations between pregnancy at FTAI with all the independent categorical effects using PROC MIXED.
**Results:** Year and parity were the effects associated with pregnancy rate ($P$ < 0.05), with no significant interactions. The pregnancy rate was greatest in 2011 (5 119/7 756, 0.66) and lowest in 2013 (5 556/9 921, 0.56), and 2013 was significantly different from all other years with the exception of year 2010 (2 708/4 476, 0.605). The pregnancy rate in heifers (22 618/35 341, 0.64) was greater than in cows (15 483/25 805, 0.60). Minimum (18.4 ± 2.2 °C), maximum (31.0 ± 3.6 °C), and mean (24.0 ± 2.6 °C) temperatures in the 21 days post FTAI were greater in 2013 than in other years. THI in the 21 days pre (70.7 ± 3.6) and post (72.0 ± 3.0) FTAI were also greatest in 2013.
**Conclusions:** High THI, minimum and maximum temperatures 21 days before and after FTAI in year 2013 reduced pregnancy rate in grazing beef cows.

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## 142
## Estimating the stage of the oestrous cycle in Japanese black beef cattle based on the number of large follicles

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**Application:** Monitoring the number of large follicles (LFs) by ultrasonography could be used to estimate the stage of the oestrous cycle for hormonal treatment initiation, thereby improving the efficiency of timed artificial insemination (timed-AI).
**Introduction:** The stage of the oestrous cycle at the initiation of hormonal treatment affects the efficiency of timed-AI for cattle reproduction (Dirandeh, 2014). In the first follicular wave, one large dominant follicle (DF) is typically observed, whereas in the second, an atretic DF and a new DF are observed as two LFs ($\geq$10 mm in diameter) that have passed the emergence phase. This study investigated whether the stage of the oestrous cycle could be estimated by monitoring the LF number.
**Materials and Methods:** The size and number of follicles and corpus luteum (CL) were recorded weekly by ultrasonography in Japanese black beef heifers ($n$ = 37) and cows ($n$ = 212) from a single farm in Japan. If the LF disappeared from the first examination to the second, it was presumed that ovulation had occurred. If there was no LF and CL, it was assumed as day 1 or 2 of the cycle (day 0 = day of oestrus). The stage of the oestrous cycle at the second examination was estimated as follows; (1) any day from day 3 to 7, if there was an LF at the first examination and that had disappeared and a new CL was identified at the second examination; and (2) day 8 or 9, if there was no LF at the first examination, and a new CL was identified at the second examination. For case (2), the cycle stage prior to the first examination was estimated as day -8 or -7. A third examination was also performed in some heifers and cows. The relationship between the LF number and the stage of the oestrous cycle was analysed by the Chi-square test.
**Results:** The number of LFs was associated with the stage of the oestrous cycle ($P < 0.01$). In cows with one LF, 80.9% (140/173) were in the first half (by day 10) of the cycle. In cows with two LFs, 77.8% (35/45) were in the second half (after day 11). There was no difference between heifers and cows.
**Conclusions:** The number of LFs was related to the stage of the oestrous cycle. Estimating the stage of the oestrous cycle based on the number of LFs could be useful for determining the initiation of hormonal treatment.

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## 143
## Effect of a new intravaginal progesterone device on the progesterone concentrations and estrous rates of dairy cattle

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**Application:** The new intravaginal progesterone ($P_4$) releasing device (referred to as ProB) can help improving the fertility of dairy cattle.
**Introduction:** The manufacturing capability of the sustained P4 releasing device is important for improving the fertility of dairy cattle through the synchronization programs. ProB consists of a T-shaped spine coated with silicone rubber, loaded with 1.3 g $P_4$. The objective of the present study was to examine P4 concentrations after insertion of ProB into the vaginal tract of ovariectomized cows. In addition, the effect of Ovsynch plus ProB protocol on estrous rates was evaluated in postpartum anestrous dairy cattle.
**Materials and Methods:** Experiment 1. Ovariectomized cows (03 Holstein cows and 01 Red Sindhi cow) with live weight of 457.5 ± 85.0 kg, were randomly inserted ProB ($n$ = 5; 1.3 g of progesterone) and CIDR ($n$ = 2; 1.38 g of progesterone, Zoetis). After 7 days of insertion, all intravaginal devices were removed from cows (Day 0 = insertion). Blood samples were collected daily via the tail venipuncture during 7 days of insertion and 1 day after removal. The concentrations of plasma $P_4$ was analyzed by ELISA. Experiment 2. One hundred days postpartum, anestrous Holstein cows were randomly assigned into one of two treatments: cows which received ProB-based protocol (ProB; $n$ = 100) and cows which received CIDR-based protocol (CIDR; $n$ = 102). In the protocol, cows received the first GnRH treatment with $P_4$ insertion, followed by a treatment with $PGF_{2\alpha}$ 7 days later and a second GnRH treatment 24 h later. Estrus was detected by observations twice daily. Progesterone concentrations and estrus rates were compared between two groups using the t-test. $P$ values less than 0.05 were considered to be statistically significant in all analyses. Data are presented as the mean ± SD.
**Results:** Experiment 1. In ProB group, $P_4$ levels increased dramatically and reached a peak of 9.10 ± 1.78 ng/mL at day 1. Then, the levels dropped gradually to 2.93 ± 0.55 ng/mL at day 7. In cows inserted CIDR, $P_4$ concentration showed similar fluctuations as the ProB group. Progesterone concentrations did not differ between both groups of treatments (ProB = 4.86 ± 2.46 ng/mL vs CIDR = 5.76 ± 1.81 ng/mL; $P > 0.05$). Progesterone concentrations reduced to less than 1 ng/mL after the removal in both groups. Experiment 2. There was no difference in the rate of estrus between ProB group and CIDR group (79.0% vs 69.6%; $P > 0.05$).

**Conclusions:** Our findings suggested that ProB can be used in the estrous synchronization protocol in dairy cattle.

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## 144
## The effect of exogenous GnRH at the time of artificial insemination on luteinizing hormone in lactating Holstein cows

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**Application:** Investigate the effect of GnRH and estrous expression at the time of breeding on LH profile.
**Introduction:** Administration of gonadotropin releasing hormone (GnRH) at the time of artificial insemination (AI) was shown to improve pregnancy rates for dairy cows displaying low intensity of estrous expression (Burnett et al., 2022). However, the physiological mechanism underlying this association remains unclear. Therefore, this study aimed to evaluate the effect of GnRH at supposed time of AI on the profile of luteinizing hormone (LH) in spontaneous estrous from lactating Holstein cows.
**Materials and Methods:** A total of 42 lactating Holstein cows were enrolled. Animals received a synchronization protocol and had their estrous cycle followed through plasma progesterone and ovarian ultrasonography until detection of the subsequent spontaneous estrous event. On the following estrus detected by an automated activity monitor, cows were randomly assigned into two experimental groups: GnRH ($n$ = 21), cows received an injection of 100 μg of GnRH (Fertiline, Vetoquinol), and Control ($n$ = 21), cows received an injection of 2 mL of saline solution at supposed time of AI (considered Time 0). Blood samples were collected prior to treatment and hourly for the following 6 h to determine LH concentrations. Mixed effect models were conducted using RStudio version 2022.12.0 + 353.
**Results:** A total of 42 animals were used in the analysis. Ovulation was observed in 40/42 of the cows following an estrous event. The average LH before treatment was 2.0 ng/mL (0.11–7.58 ng/mL). The LH tended to decrease and was below 1 ng/mL 3-h post-treatment in most animals 28/42. Control cows had lower circulating LH 1-h post-treatment (Control = 1.15 ± 1.6; GnRH = 3.16 ± 2.36; $P$ < 0.001) compared to GnRH cows. LH concentration did not differ between groups 3-h post-treatment ($P$ = 0.55). There was no association between intensity of estrous expression and LH concentrations ($P$ = 0.52).
**Conclusions:** In conclusion, this study demonstrated that intensity of estrous was not associated with LH levels in spontaneous estrous of lactating Holstein cows. The administration of GnRH at AI was shown to increase LH 1-h post-treatment. Cows presumably past their LH surge were less affected by exogenous GnRH, potentially because of a depletion in the LH reserve in the pituitary gland. Therefore, improvement in LH promoted by GnRH could elicit benefits on dairy cow's fertility. Future research is needed to elucidate the role of GnRH during AI on spontaneous estrous of lactating Holstein cows.
**Acknowledgements:** Resilient Dairy Genome Project (LSARP-2020) and the Natural Sciences and Engineering Research Council (RGPIN-2020-05433).

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## 145
## Evaluation of different reproductive management protocols in ewe-lambs aimed to advance the age of pregnancy onset

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**Application:** Advancing pregnancy onset using GnRH or glycerol treatment in combination with the ram-effect restricts the unproductive period.
**Introduction:** Ram-effect, hormones, and nutritional flushing (e.g. glycerol) are routinely used to induce ewes oestrus (Valasi et al, 2012; Porcu et al, 2017, 2020). This study aimed to compare the efficacy of the ram-effect alone or combined with GnRH or glycerol treatment when used to advance the onset of oestrus and pregnancy in ewe lambs.
**Materials and Methods:** Prepubertal ewe-lambs ($n$ = 112) with an average body weight (BW, Kg ± s.e.m) of 33.8 Kg ± 0.2, were divided into four groups homogeneous for body weight: ram-effect only (CON), $n$ = 31; ram-effect and hormone treatment (GnRH), $n$ = 33; ram-effect and glycerol treatment (GLY), $n$ = 22; ram-effect, hormone, and glycerol treatment (GLY/GnRH), $n$ = 26. On Day 1, GnRH and GLY/GnRH groups received one injection of a GnRH analogue (gonadorelin 40 μg/head), GLY and GLY/GnRH groups received the glucogenic treatment

(120 mL/head) in drinking water till Day 8. On Day 8, CON and GLY groups were joined with fertile rams (ratio 1/10) fitted with crayon markers and managed as a single flock. GnRH groups were subjected to ultrasound scanning. Animals with detected corpora lutea, received an injection of PGF2α analogue (100 μg/head), and then were joined with rams. The remaining ewe-lambs received a second dose of gonadorelin. On Day 15, they were checked again and the ones showing corpora lutea were injected with a PGF2α analogue, while the others received a third injection of gonadorelin and all the animals were joined with rams. Pregnancies were confirmed within 30 days by ultrasound scanning.
**Results:** The efficacy of the protocol among different groups was determined by assessing differences in the number of days required to achieve pregnancy rates of 25%, 50% and 75%, costs and incomes over the entire life cycle of the replacement lambs, compared to control. The GnRH-treated groups achieved 25% pregnancy rates earlier than the GLY and CON groups ($P<0.01$ - GLM, Fisher Pairwise Comparisons). Thereafter, all the treated groups showed a lower number of days to reach the 50% pregnancy rate ($P<0.01$), when compared to CON group. At the 75% threshold, the groups maintained similar differences. GLY/GnRH and GnRH treatments had similar responses, showing no synergistic effect between GLY and GnRH.
**Conclusions:** Both hormone and glycerol treatment, combined with the ram-effect, can advance pregnancy onset and increase the days in milk of ewe-lambs thus improving the dairy farm income.

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**146**

## Impact of pre- and postnatal nutrition on number of kisspeptin- and neurokinin 3 receptor-immunopositive neurones in the arcuate nucleus of sexually mature heifers

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**Application:** Developmental changes occurring in response to nutritional extremes prenatally and during the early postnatal period are believed to be imprinted in the genome and in some cases, manifested later in life. Therefore, understanding the long-term effects of pre- and postnatal nutrition on reproductive neuroendocrine function is essential.
**Introduction:** Neurokinin B, which acts via the neurokinin 3 receptor (NK3R), plays a crucial role in the control of the episodic release of gonadotropins. Our previous studies have shown that sexually mature heifers exposed to pre- and postnatal nutritional extremes (restricted feed intake or obese) had increased secretion of luteinising hormone in response to exogenous administration of senktide, an NK3R agonist. Therefore, we hypothesized that heifers exposed to nutritional extremes during the pre- and postnatal period have an increased number of NK3R-immunopositive neurones in the arcuate nucleus (ARC) of the hypothalamus.
**Materials and Methods:** Heifers were programmed nutritionally using a $3 \times 2$ factorial arrangement of pre- and postnatal nutritional regimens. Beginning at 90 days of pregnancy, Bos indicus-influenced cows ($n = 95$) were fed to achieve body condition scores (BCS; 1-9 scale) of 3-3.5 (L; thin), 5.5-6 (M; moderate), or 7.5-8 (H; obese) by onset of the third trimester and maintained thereafter. Heifer offspring were weaned at 3-3.5 months of age and assigned to either a low- (L; 0.5 kg/day) or a high-gain (H; 1 kg/day) diet until 8 months of age, then fed a common diet until puberty. For the current experiment, heifers ($n = 18$; 6/group) representing LL, MH, and HH combinations were ovariectomised postpubertally (17 months of age) and received oestradiol replacement to maintain basal oestradiol concentrations at 2-6 pg/mL. Brain tissues were harvested at ~53 months of age and hypothalamic tissues were dissected and sectioned using a microtome. Sections containing the ARC were processed for double-label immunofluorescence to determine kisspeptin and NK3R immunoreactivity.
**Results:** Total number of kisspeptin- and NK3R-expressing neurones in the ARC did not differ among nutritional treatments. Additionally, no significant differences were observed for the number of kisspeptin- and NK3R-expressing neurones for each subregion of the ARC (rostral, middle, and caudal).
**Conclusions:** Nutritional extremes during the pre- and postnatal period did not alter the number of kisspeptin- and NK3R-immunopositive neurones in the ARC of sexually mature heifers. We are currently performing confocal microscopy to investigate if the percentage of kisspeptin neurones that colocalise NK3R is impacted by perinatal nutritional extremes.
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## 147
## The difference in the amount of circulating miRNA in precocious and non-precocious beef heifers at weaning age

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**Application:** Selecting beef heifers for sexual precocity may decrease the time animals are kept on the farms reducing the beef production cycle duration. Understanding the mechanism evolved may allow to development of tools to anticipate selection around weaning age.
**Introduction:** Late puberty is a challenging trait in Bos indicus beef heifers, some present spontaneous anticipation of puberty, and identifying the factors that differentiate these heifers can allow the selection at an early age (i.e., weaning).
**Materials and Methods:** Blood samples were collected at the weaning procedure (∼9 months) from more than 300 heifers (Bos indicus) kept under the same management and accompanied by the SysGen system (which uses phenotypic data for animal selection). At the end of the first breeding season (heifers were around 18 months), 25 heifers were selected from the pregnant animals and 25 from the non-pregnant in the first breeding season. RNA was extracted from plasma samples by the Trizol method, transcription of RNA into DNA was done with the MiRCURRY LNA RT kit, and the expression of the candidate miRNAs was evaluated for RT-PCR using the miRCURY kit. The amount of circulating miRNAs were calculated by the 2-ΔΔCt procedure, test t was used to analyze the data.
**Results:** The heifers were born with similar weight (31.5 + 0.5 kg; 31.8 + 0.4 kg), but the pregnant ones gained more weight from birth until weaning ($P = 0.03$) 0.8 + 0.1 kg/day and non-pregnant 0.7 + 0.1 kg/day; the pregnant had a higher weaning weight ($P = 0.004$; 228 + 2.8 kg) compared to the non-pregnant 214 + 4.2 kg. The higher daily weight gain was maintained from weaning to the yearling ($P = 0.001$; 0.5 + 0.01 kg/day and 0.4 + 0.01 kg/day, respectively), which resulted in a higher weight at the yearling age ($P = 0.0001$) in pregnant 312 + 4 kg compared to the non-pregnant 283 + 6 kg heifers. From birth to yearling age, pregnant heifers gained 280 + 4 kg and the non-pregnant 251 + 6 kg ($P = 0.0001$). No difference was observed in the relative amount of miRNA 122, 127, 363, 154, 27b, 126, 205, and 125 between pregnant and non-pregnant at weaning age. miRNA 192, which is associated with diabetes, increased by 57% ($P = 0.007$) in the pregnant (2.15) when compared to non-pregnant (1.22) heifers. The miR-29 was more abundant ($P = 0.023$) in the pregnant (2.50) than in the non-pregnant heifers (1.21); the miR-29 is related to the time of life and reproduction.
**Conclusions:** Precocious pregnancy in heifers kept under the same management seems to be more related to metabolism difference than greater dry matter intake. Some miRNAs related to liver metabolism (122) were not increased in pregnant heifers. This speculation is being tested in an ongoing experiment; food consumption will be evaluated individually in Nellore heifers maintained under the same management.
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## 148
## Effects of prenatal and postnatal nutrition on α-melanocyte-stimulating hormone neuronal projections to kisspeptin neurons in the arcuate nucleus of beef heifers

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**Application:** Nutritional manipulation during the prenatal and early juvenile period may alter the developmental trajectory of hypothalamic neurocircuitries controlling gonadotropin-releasing hormone (GnRH) secretion, thus programming puberty and subsequent fertility in the female bovine.
**Introduction:** The proopiomelanocortin (POMC)-derived peptide, α-melanocyte-stimulating hormone (αMSH), is an integral component of the neuronal networks that mediate the effects of nutrition in the control of reproductive functions for its excitatory actions on GnRH and kisspeptin neurons. Leptin not only plays an activational role regulating POMC/αMSH expression, but also plays an organisational role modulating the development of hypothalamic neurocircuitries during perinatal development. Therefore, we hypothesised that either maternal undernutrition or obesity during late gestation, in combination with a low or high rate of body weight gain in heifer offspring during the juvenile period, would alter the magnitude of αMSH neuronal projections towards kisspeptin neurons in the arcuate nucleus (ARC).
**Materials and Methods:** Brahman×Hereford cows ($n = 36$) bearing female pregnancies were fed to achieve a body condition score (BCS) of 3-3.5 (thin, L), 5.5-6 (moderate, M), or 7.5-8 (obese, H) by ∼6 mo of gestation and maintained at the target BCS until calving ($n = 12$/group). Heifer offspring from each group were weaned at ∼3.5 mo of age and allocated randomly to be fed to attain a low (L; 0.5 kg/d) or high rate of BW gain (H; 1 kg/d) until 8 mo of age and then placed on a common growth diet. This 3 × 2 factorial design generated six combinations

of maternal-postnatal treatments (LL, LH, ML, MH, HL, and HH). Heifers were euthanized at ~14 mo of age. Hypothalamic sections containing the ARC were processed for double-label immunofluorescence to determine αMSH (excitatory) projections towards kisspeptin neurons.
**Results:** Pre- and postnatal dietary treatment did not alter the number of kisspeptin-expressing neurons in the ARC. Approximately 41.2% of kisspeptin-immunopositive neurons were in close apposition to αMSH projections across all groups. No treatment effects were observed for the percentage of kisspeptin neurons in close apposition to αMSH-immunoreactive projections or the average number of αMSH innervations per kisspeptin neuron.
**Conclusions:** Maternal undernutrition or obesity during late gestation, in combination with a low or high rate of body weight gain in heifer offspring, did not alter the magnitude of αMSH neuronal projections towards kisspeptin neurons in the ARC. These findings are in agreement with our previous studies demonstrating that prenatal nutritional extremes did not impact age at puberty in heifers.
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## 149
## Hormone concentrations in different sub-species of beef cattle while subjected to a nutritional challenge

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**Application:** Sub-species differences in beef cattle and their resilience to pregnancy loss.
**Introduction:** Seventy percent of the world's beef cattle population are located in tropical or sub-tropical environments. These cattle (Bos *indicus*-influenced) are more resilient to pregnancy loss while receiving a lesser amount of nutrients when compared to Bos taurus cattle. However, the mechanism behind this added resilience is still unknown.
**Materials and Methods:** Angus (*Bos taurus*), Brangus (*Bos indicus*-influenced), and Brahman (*Bos indicus*) mature, multiparous-cows were randomly assigned (day -30) to either a 100% maintenance diet (control; $n = 22$), or a 70% maintenance diet (restricted; $n = 23$) for 45 days. Blood was collected via venipuncture on days -30, -7, -2, 0, 1, 2, 3, 4, 5, 6, 7, 9, 12, and 15 to measure circulating progesterone, insulin, and IGF-1. All cows were submitted to a 5-day CO-Synch + CIDR synchronization protocol on day -7. Data were analyzed using mixed models with SAS (version 9.4; SAS for Windows).
**Results:** Weight change was different between control and restricted cows after day -30 ($P = 0.0001$). Breed of cow influenced plasma insulin with Brangus having increased insulin on days -30, -7, -2, 0, 7, 9, 12, 15 compared with Angus ($P < 0.01$). Furthermore, Brangus had greater plasma insulin than Brahman ($P < 0.05$) on all days previously listed except day 12 ($P > 0.10$). Differences were also observed on day -2 with Brahman plasma progesterone being greater than Brangus ($P = 0.0116$). Moreover, Brangus cows had greater circulating progesterone than Brahman on day 9 ($P = 0.05$). Circulating IGF-1 steadily decreased from day -30 to day 15, resulting in an IGF-1 day effect ($P < 0.0001$). There was a breed x day interaction ($P < 0.001$) where Brahman had greater plasma IGF-1 on day 9 than Brangus ($P = 0.04$) and tended to have greater plasma IGF-1 than Brangus on days 7 and 15 ($P = 0.05$, $P = 0.09$; respectively). On day 15, restricted cows had greater plasma insulin than control ($P = 0.03$).
**Conclusions:** Multiple day x breed effects were found in this experiment. This work showed that different breeds of beef cattle have differing circulating hormone levels. Since few treatment effects were found, it is still unclear what allows Bos *indicus*-influenced cattle to possess a greater resilience to pregnancy loss while nutritionally challenged.
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## 150
## Indomethacin infusion through multiple routes and the resulting Prostaglandin F2α metabolite production during late embryonic development in beef cattle

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**Application:** To reduce the occurrence of late embryonic mortality in beef cattle.
**Introduction:** In beef cattle, the underlying physiological mechanisms and endocrine profiles associated with late embryonic/early fetal mortality, during the period of active placentation (gestational day 28-60), remain relatively unknown. The objective of this pilot study was to inhibit uterine derived prostaglandin production through indomethacin infusions, a substance shown to inhibit cyclooxygenase 1 and 2 pathways, to further characterize the physiological role of prostaglandins during embryonic development in cattle.

**Materials and Methods:** Pregnant and open cycling beef cows were randomly divided into four treatment groups. These consisted of pregnant cows receiving indomethacin systemically through jugular infusion ($n$ = 1, treatment 1), pregnant cows receiving indomethacin directly into the uterine artery ($n$ = 2, treatment 2), cyclic cows receiving indomethacin through trans-cervical infusion ($n$ = 4, treatment 3), and cyclic cows receiving indomethacin through systemic infusion ($n$ = 4, treatment 4). A standard 5-day CO Synch + CIDR synchronization protocol was used herein. Insemination occurred on day 0 ($n$ = 14) and viable pregnancies were confirmed by the presence of fetal heartbeat via trans-rectal ultrasound on day 27 and checked daily to confirm viability of the pregnancy until day 37. On day 28, a subset of cows ($n$ = 3) were fitted with a coccygeal vein polyethylene catheter that was inserted 75 cm into the caudal vena cava for indirect sampling of the uterine ovarian drainage. Indomethacin was administered every 6 h to treatment 1 and 2 from day 28 to day 37 at either 270 mg ($n$ = 1) or 320 mg ($n$ = 2) dosages, along with 6-h blood collections via catheter. Treatment 3 and 4 received indomethacin every 12 h at either 80 mg ($n$ = 4), 160 mg ($n$ = 4) dosages, and blood was collected via venipuncture every 12 h. Serum concentrations of prostaglandin F2$\alpha$ metabolite were measured with a validated commercial ELISA (Cayman Chemical).
**Results:** Throughout all treatment groups, indomethacin did not reduce prostaglandin F2$\alpha$ metabolite ($P$ > 0.05). However, treatment 3 caused a treatment x dosage x time interaction ($P$ = 0.019). All pregnant cows maintained pregnancy throughout the trial. Data were analyzed using mixed models with SAS (version 9.4; SAS).
**Conclusions:** It is still unclear if the route of administration, or the dosage, of indomethacin is the culprit for failing to inhibit uterine derived prostaglandin production. Future research will include placing an osmotic pump within the oviduct to deliver indomethacin directly into the uterine horn.
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## Reproductive transcriptome of bovine pregnancies with high, low concentrations of pregnancy-associated glycoproteins following Artificial Insemination

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**Application:** Investigate the molecular events that contribute to pregnancy losses in cattle.
**Introduction:** Cows that undergo Late Embryonic mortality (LEM) have lower concentrations of Pregnancy Associated Glycoproteins (PAG). Studies from our group have demonstrated that low PAG pregnancies derived from *in vitro* fertilization (IVF) are characterized by impaired placenta development. This study aimed to compare the transcriptome of caruncle and chorioallantois between beef cows with high and low concentrations of PAG following Artificial Insemination.
**Materials and Methods:** Bos taurus cows ($n$ = 30) were artificially inseminated on day 0. Serum progesterone and PAG concentrations were determined via validated Radioimmunoassay and ELISA, respectively, on days 36 and 40, and pregnant cows were classified as high PAG ($n$ = 8) or low PAG ($n$ = 7). Pregnant cows were harvested on days 36 ($n$ = 3 High PAG and $n$ = 4 Low PAG) and 40 ($n$ = 5 High PAG and $n$ = 3 Low PAG), and caruncle and chorioallantois samples were collected. Total RNA was isolated using the RNeasy kit (QIAGEN; Hilden, Germany). The RNA sequencing was conducted using an Illumina platform. Differentially expressed genes (DEGs) were determined using edge-R package from R. Functional enrichment analyses were performed using the Toppgene suite.
**Results:** Progesterone concentrations were not different ($P$ > 0.05) between the High and Low PAG groups on days 36 (1.7 ± 1 ng/mL vs. 4.2 ± 0.8 ng/mL, respectively) and 40 (5.3 ± 0.7 ng/mL vs. 5.7 ± 0.9 ng/mL, respectively). The PAG concentrations, however, were significantly greater ($P$ < 0.05) in the High vs. Low PAG groups on days 36 (8.5 ± 0.7 vs. 1.4 ± 0.58 ng/mL, respectively) and 40 (12.9 ± 1 ng/mL vs. 2.3 ± 1.5 ng/mL, respectively). On D36, one gene was differentially expressed in chorioallantois, and 66 were differentially expressed in caruncle samples. On D40, five genes were differentially expressed in chorioallantois and 26 were differentially expressed in caruncle samples. Functional enrichment analyses revealed upregulated processes in Low PAG caruncles associated with responses to type-I interferons on D36, and regulation of cell secretion and proliferation on D40. Although peripheral PAG concentrations differed significantly between groups, PAG transcripts were not differently expressed in chorioallantois from days 36 and 40.
**Conclusions:** High vs. Low PAG pregnancies derived from AI have distinct transcriptomic profiles, but the low PAG group had no apparent effects on placenta development as observed in low PAG IVF pregnancies from previous studies.
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## 152
## The effect of high or low concentration of progesterone during diestrus and its association with intensity of oestrus in lactating Holstein cattle

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**Application:** Greater intensity of oestrous has been shown to improve fertility and this study demonstrated this could be due to progesterone (P4) concentration during diestrus.
**Introduction:** The objective of this study was to determine the effect of different concentrations of progesterone (P4) during the oestrus cycle on the intensity of oestrous expression detected by an automated activity monitor (AAM).
**Materials and Methods:** Lactating Holstein cows ($n$ = 125) were randomly assigned to 1 of 2 treatments. All cows were enrolled onto a presynchronization protocol, starting on day -27 relative to the final estrus, composed of the administration of GnRH and a P4 insert, 7 d later an injection of PGF2α and insert removal, and a second injection of GnRH 48h later. Cows were then submitted to the same hormonal protocol as the presynchronization program starting on d 7 of the estrous cycle and received an injection of estradiol cypionate (E.C.P) on -2 d of the study. Cows in the high P4 (HP; $n$ = 61) treatment received no additional treatment. Cows in the low P4 (LP; $n$ = 64) treatment received extra PGF2α injections on day – 15, - 14.5, and 14 and again on day – 10, - 9, -8.5, and – 3 of the protocol. Blood samples were harvested to quantify the concentration of P4 throughout the study. Individual activity was monitored continuously by a leg mounted AAM. Using the AAM, an oestrus alert was determined when the relative increase (RI) in activity of the cow exceeded 100% of the baseline activity. Data was analyzed using mixed linear regression models in SAS.
**Results:** Concentration of P4 was greater for HP cows on day – 8 and – 3 of the study, as expected. At the time of the estrus alert, cows on the HP treatment had lower P4 concentration compared with cows on the LP (0.78 ± 0.14 ng/mL vs. 1.36 ± 0.11 ng/mL, respectively). The proportion of cows that did not show oestrus was greater for HP than for LP (18.2 % vs. 5.1%), however, cows in the HP treatment had greater RI compared with cows on the LP treatment (398.5 ± 21.1 RI vs. 312.4 ± 19.8 RI, respectively). There was no difference in the duration of oestrus.
**Conclusions:** In conclusion, cows enrolled the HP treatment had fewer cows expressing oestrus, however they had greater concentration of P4 during diestrus and had greater relative increase at oestrus compared with cows that were enrolled in the LP treatment.

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## The effect of high or low concentration of progesterone during diestrus and its association with the LH surge and PGF2α metabolite in lactating Holstein cattle

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**Application:** Progesterone (P4) concentrations during diestrus is associated with fertility, and this study demonstrated this could be due to more ideal LH and PGF2α metabolite (PGFM) responses.
**Introduction:** The objective of this study was to determine the effect of progesterone concentration during the oestrus cycle on circulating LH and PGFM.
**Materials and Methods:** Lactating cows ($n$ = 17) were randomly assigned to 1 of 2 treatments. Cows were enrolled into a presynchronization protocol, starting on day -27, composed of the administration of GnRH and a P4 insert, 7 d later an injection of PGF2α and insert removal, and a second injection of GnRH 48 h later. All cows were then resubmitted to the identical presynchronization program again, starting on d 7 of the oestrous cycle and received an injection of estradiol cypionate (E.C.P) on -2 d. Cows in the high P4 (HP; $n$ = 8) treatment received no additional treatment. Cows in the low P4 (LP; $n$ = 9) treatment received extra PGF2α injections on days -15, -14.5, -14, -10, -9, -8.5, and -3. Blood samples were collected, to quantify the peak LH concentration after E.C.P. administration, every 2 h until ovulation. Ovulation was confirmed by transrectal ultrasonography. An estradiol/oxytocin challenge for PGFM was performed on day 16 of the treatment. E.C.P (0.5 mL) was administrated 4 h before the intravenous treatment of oxytocin (5 mL). Blood samples were collected at -15, 0, 15, 30, 45, 60, 90, 120, and 180 min relative to the oxytocin injection. Data was analyzed using mixed linear regression models in SAS.
**Results:** Concentration of LH tended to be lower for HP than LP cows (0.49 vs 0.58 ng/mL). Duration from E.C.P. administration to peak LH was longer for cows in the HP treatment compared with the LP treatment (37.3 ± 6.3 h vs 28.3 ± 4.8 h). The duration of the LH peak was greater in the LP treatment compared with the HP treatment (8.7 ± 1.0 h vs 6.3 ± 1.6 h). The duration from peak LH to ovulation was shorter

in the LP treatment compared with the HP treatment (26.4 ± 2.3 h vs 35.1 ± 5.7 h). Concentrations of PGFM were greater for the LP treatment than the HP treatment (107.8 pg/mL vs 92.5 pg/mL).
**Conclusions:** In conclusion, cows that were exposed to lower concentrations of P4 during diestrus tended to have greater LH concentrations and greater circulating concentrations of PGFM following an oxytocin challenge in the subsequent oestrous cycle.

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**154**
**Effect of management with low stress handling on indicators of behaviour and reproductive efficiency at TAI in Nellore heifers**

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**Application:** Evaluate high stress handling compared to low stress handling during TAI in Nellore heifers.
**Introduction:** Reproduction is one of the main biological process and stressors has been commonly reported as negative influencing factors for reproduction. The present study evaluated the effect of different stress levels during herd management (high vs low stress handling) on the behaviour and the reproductive efficiency upon fixed-time artificial insemination (TAI). The frequency of kicks, vocalization and escape velocity of Nellore heifers was evaluated during management to perform TAI as well as pregnancy/AI.
**Materials and Methods:** A total of 801 Nellore heifers with 14 months of age from farms located in Mato Grosso, Brazil were used. The heifers were divided blocked based according to body weight (334.52 ± 1.17; Kg) at the beginning of the cyclicity induction protocol (D-24). The control treatment (CON; $n = 402$) was managed in a high stress handling (conducted aggressively and reactively) and the experimental treatment (RAT; $n = 399$) was managed in a low stress handling (conducted calmly and quietly). The heifers were synchronized to received a TAI (D0). Both treatments used the same protocol for estrus synchronization (P4+E2+eCG+PGF). At TAI (D10), the [BJPO3] escape velocity at the exit of the crush (walking, trotting, running), the frequency of kicks and vocalization were evaluated individually. The heifers of each treatment remained in separate pastures throughout the experiment, until the day of pregnancy diagnosis (D40). The pregnancy/AI (P/AI) was evaluated 30 days after the TAI (D40). Statistical analyses were performed using GLIMMIX of SAS 9.4.
**Results:** The frequency of heifers running out of the crush after AI (D10) was greater in CON management compared with RAT [CON = 51.5% (207/402) vs RAT = 27.5% (110/399); $P < 0.001$]. The rate of kicking during handling on the crush was greater on the CON [CON = 20.9% (84/402) vs RAT = 16.8% (67/399); $P = 0.06$]. There was an effect of treatment on the animal's vocalization during restraint at the time of AI [CON = 5.2% (21/402) vs RAT = 3.0% (12/399); $P = 0.05$]. There was a tendency for an effect of treatment on P/AI [CON = 43.0% (173/402) vs RAT = 47.6% (190/399); $P = 0.09$]. An interaction of treatment*escape velocity from the crush was found. Across all heifers that ran out of the crush, the heifers from the low stress handling had greater P/AI [RAT = 47.6% (58/110) vs CON = 39.6% (82/207); $P = 0.02$].
**Conclusions:** The low stress handling improves the behaviour and the reproductive efficiency of Nellore heifers.
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**155**
**Effect of lactation number on pregnancy losses in dairy cattle**

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**Application:** To help implement strategies for management to reduce pregnancy losses in the most prone population (first, second, or third and more lactation cows) on dairy farms.
**Introduction:** Pregnancy losses are one of the most critical problems on dairy farms. Pregnancy losses between 50 and 60% have been reported. This means that for every two cows diagnosed as pregnant in the first 30 days after artificial insemination (AI), just one will calve (Fricke and Wiltbank, 2022). Fertility is higher in younger than older cows, but there are no reports about the differences in pregnancy losses in both populations (Middleton et al., 2018). However, this study aimed to evaluate the differences in pregnancy losses between dairy cows in different lactation numbers.
**Materials and Methods:** Pregnant cows of first (L1; $n = 468$), second (L2; $n = 169$), and third or more lactations (L3+; $n = 144$) were evaluated. The first, second, third and fourth pregnancy diagnoses were evaluated at 35 ± 7, 50 ± 7, 110 ± 7 and 210 ± 7 days (respectively) after

AI by ultrasound. Statistical analysis was performed by JASP software with the Chi-square test, including lactation number and pregnancy diagnosis period. Values of $P < 0.05$ were considered significant.
**Results:** At the second pregnancy diagnosis, the pregnancy losses of L1 (5.33%) were lower ($P < 0.05$) compared to L2 (11.21%) and L3+ (13.94%). At the third pregnancy diagnosis, there were no differences in pregnancy losses ($P > 0.05$) between L2 and L3+ (17.93% and 17.20%, respectively), in this same period, there were no pregnancy losses for L1. At the fourth pregnancy diagnosis, the pregnancy losses in L3+ (3.51%) were higher ($P < 0.05$) than L1 (0%) but lower ($P < 0.05$) than L2 (8.87%). The total pregnancy losses in L1 (5.33%) were lower ($P < 0.05$) that L2 and L3+, with no difference ($P > 0.05$) between these last groups (33.81% and 31.33%, respectively).
**Conclusions:** Pregnancy losses were different between the cows of different lactation number. First lactation cows had very few pregnancy losses during the different periods of pregnancy diagnosis. Most of the pregnancy losses occur in second lactation cows. The main efforts to avoid pregnancy losses must be focused on second-lactation cows.

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## 156
## Pharmacokinetics of long-acting injectable progesterone in ewes

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**Application:** Understand the pharmacokinetics of long-acting injectable progesterone to be able to replace progesterone devices in hormonal protocols for oestrus synchronisation in ewes.
**Introduction:** In hormonal protocols, the luteal phase is normally mimicked by the intravaginal insertion of progesterone-based devices. However, these implants can cause vaginitis, its use is expensive, and the hormone content that remains in the device after its use causes environmental contamination. Thus, this study aimed to compare the pharmacokinetics of two doses of injectable long-acting progesterone in ewes.
**Materials and Methods:** Santa Inês adult multiparous, clinically healthy ewes ($n = 30$) weighing 49.2 ± 2.1 kg (mean ± SD) were used in the study. Before the administration of injectable progesterone - P4i (Progesterone BioRelease Technologies LLC of Lexington, USA), all animals received two doses of a prostaglandin analogue (120 μg, i.m., cloprostenol; Agener União, São Paulo, Brazil) separated 7 days. The animals were allocated to three experimental groups, receiving 150 mg of P4i (group G150), 75 mg of P4i (group G75), and a control group (saline solution, group CON), administered intramuscularly to all animals 24 h after the second dose of cloprostenol (Day 0). Blood samples were collected immediately before the application of the treatments and daily (morning and night) until ovulation or up to 5 days after the application of P4i. Serum progesterone concentrations were measured using solid-phase radioimmunoassay. Data were compared using a mixed model, with time as the repeated effect, treatment, time, and their interactions as the main effects. Significant differences were considered when $P < 0.05$.
**Results:** Both P4i doses maintained progesterone concentrations at luteal levels throughout all the studied period ($\geq$1 ng/mL). Progesterone concentrations were greater in both treated groups than in CON ewes until Day 4. Twelve h after the application, and on Days 3 and 4, concentrations were greater in G150 than G75 ewes. From Day to Day 5, the concentration decreased in both treated groups, without differences between groups on Day 5.
**Conclusions:** The progesterone formulation used was efficient to maintain progesterone luteal concentrations for at least 5 days with slight differences between the doses used.
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## 157
## Follicular dynamic and fertility performance of synchronized Aragoneza ewes treated with Kisspeptin-analogue C6

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**Application:** Alternative solutions to the current synchronization programs for timed artificial insemination (TAI) of ewes are of crucial importance for the ovine industry.
**Introduction:** Neuropeptide kisspeptin and its receptor, KiSS1R, play a major role, in governing the timeline of puberty onset and promoting ovulation by stimulating gonadotrophin-releasing hormone (GnRH) secretion. The suboptimal lifespan of naturally secreted kisspeptin gives a very poor pharmacodynamic characteristic. To overcome this issue, a synthetic KISS1R agonist, mainly based on the kisspeptin backbone, was created and named KisspeptinC6 (Decourt et al., 2016). The evaluation of KisspeptinC6 on follicular dynamic and fertility was tested in this controlled field study.
**Materials and Methods:** Eighty-two mature Rasa Aragonesa ewes (liveweight LW: 56.9 ± 3 kg; BCS: 3.1 ± 0.6) were synchronized using a 14-day progestogen-based program. A blood sample was collected from each animal one week before sponge introduction and on the day of sponge insertion: ewes were categorized as "anoestrous" when both samples $\leq$0.5 ng/ml of progesterone quantified by chemiluminescence. The ewes were randomized according to LW, BCS, and cyclicity, and assigned to one of the three groups: Negative CTR, no treatment applied at sponge removal ($n$ = 20); eCG ($n$ = 20), 480 IU equine chorionic gonadotropin (eCG) i.m. administered at sponge removal, and KisspeptinC6 ($n$ = 42), ewes received 15 nmol KisspeptinC6 by i.m. injection 24 h after sponge removal. Ewes were transrectally ultrasound scanned from the time of sponge removal Day 0 every 24 h until day 4, to determine the timing of ovulation. Ewes were cervically inseminated 55 h after sponge removal with fresh semen from five different rams. Data were analyzed using a non-parametric Fisher exact test for two proportions.
**Results:** Ovulation rate between the 3 different groups was (30%$^{a}$, 95%$^{b}$, and 52.4%$^{a,b}$, for Negative CTR, eCG, and KisspeptinC6 respectively, $P < 0.01$). The average timing between AI and ovulation, for ovulated ewes, was 44.0 ± 2.5$^{b}$, 27.8 ± 2.1$^{a}$, and 29.5 ± 1.7$^{a,b}$ h for Negative CTR, eCG, and KisspeptinC6 respectively ($P = 0.04$). In the absence of ovulation, animals presenting a persistent follicle, above 7mm in diameter 4 days after sponges removal were classified as "Cystic", the incidence of Cystic condition in the treatments was 10%$^{a}$, 5%$^{a}$ and 24%$^{a,b}$, for Negative CTR, eCG, and KisspeptinC6 ($P = 0.04$). Pregnancy per AI was evaluated by transabdominal ultrasound examination at 30 and 50 days after AI; the results were 5%$^{a}$, 40%$^{b}$, and 2.4%$^{a}$, for Negative CTR, eCG, and KisspeptinC6 respectively ($P = 0.001$).
**Conclusions:** From the results of the current study, Kisspeptin analog C6, at the dose of 15 nmol, does not achieve fertility performances comparable with those of eCG on synchronized ewes.

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## 158
## Influence of calving interval on the carbon footprint of lactating dairy cows under the life cycle assessment metric

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**Application:** The current demand for sustainable development has motivated the implementation of practices and technologies for agricultural production aimed at reducing greenhouse gas (GHG) emissions. The Life Cycle Assessment (LCA) has been used to map environmental impacts and support the development of technologies and the use of mitigation solutions.
**Introduction:** LCA studies of milk production in the tropics have shown that herd diet, genetics and fertility are factors that influence estimates of $CH_4$ emissions. The fertility of the herd, evaluated by the calving interval index (CI), influences the composition of the herd. The objective of this study is to show the influence of herd fertility on the results of $CH_4$ emissions, based on the sensitivity analysis of the LCA of milk production in a production system in different hypothetical scenarios.
**Materials and Methods:** The carbon footprint of milk production was estimated based on the LCA. The study followed ISO (2006a) 14040 and ISO (2006b) 14044 requirements. OpenLCA 3.11.1 software was used for data modeling and estimation of $CO_2$ equivalent emissions ($CO_2$ eq.) from milk production. The frontier considered was cradle-to-farm-gate comprising the stages of animal management, use of natural resources, energy, inputs and waste management, direct and indirect emissions (IPCC, 2019). Data were collected on a farm located in the state of Minas Gerais, with a compost barn system and an average production of 32 liters per day in Holstein cows. A comparison was made between production and $CO_2$ eq./L of milk (corrected for fat and protein content) of cows with an CI of 13 or 15 months. It was estimated a lactation period of 11 and 13 months for cows with an CI of 13 or 15 months, respectively. A 93% persistence of lactation was estimated for Holstein cows with a 15-month CI (Cole and Null, 2009; Biassus et al., 2010).
**Results:** The total GHG emissions for 1 kg of milk ($CO_2$ eq./L of milk) was 0.657 when the CI index was 13 months. However, for the 15-month CI, the amount kg $CO_2$ eq./L of milk produced was 0.703 (7% increase). This value does not consider the reduction in feed efficiency due to the increase of days in milk (DIM) in the emission of $CO_2$ eq.

**Conclusions:** It is concluded that reproductive efficiency is important to reduce the amount of $CO_2$ eq. per liter of milk produced.
**Acknowledgements:** The work was funded by Cargill® Brazil.

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## HEALTH AND REPRODUCTION

### 159
### App-led survey of dairy and suckler cattle congenital defects by veterinary practitioners in a co-operative veterinary practice

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**Application:** This veterinary practitioner-led data-recording model is latent with possibilities for metastasis across other clinically relevant conditions.
**Introduction:** Our knowledge on the epidemiology and typology of bovine congenital defects is predominantly based on veterinary diagnostic laboratory or university hospital data. These aspects may differ in field-generated data. Hence, the objective of this project was to field-test a mobile phone app designed for use by veterinary practitioners to collect clinical case data on routine farm visits. Ultimately, the aim was to assess the potential for practitioner-gathered clinical information, allied with analytics, to generate a repository consisting of an image gallery and associated clinical notes.
**Materials and Methods:** During the autumn 2020/spring 2021 calving season, 35 vets in 21 veterinary practices distributed nationally across 15 counties in Ireland participated in the project. Information and images were collected during routine dairy and suckler farm visits (mainly calvings) onto a mobile phone using Typeform. The questionnaire consisted of 15 questions; three photos could be collected/case.
**Results:** In total, 90 congenital defect cases were reported; 59 in dairy (n=57 Holstein/Friesian) and 28 in beef cows' (10 Limousin, 8 Charolais) calves. The dairy cows were most commonly bred by Holstein/Friesian (19) or Aberdeen Angus (16) sires and the beef cows by Charolais (9) or Limousin (8) sires. Both the dairy (17) and the beef (8) dams were most commonly bred by stock bulls, though breeding method was poorly recorded (53 missing). The majority of both dairy (46) and beef cows (18) were multiparous. The majority of calves were singletons (83), born at fullterm (86) mainly at assisted calvings (48). The three most common body systems affected by the defects were the musculoskeletal (45), digestive (30) and multiple systems (5). The three most common defects recorded were intestinal atresia (17)/hydrops (10), schistosomus reflexus (19) and palatoschisis (7). The most common reasons why the farmer called the vet to attend these cases were, in descending order, to assist at a dystocia (hydrops and schistosomus), to euthanise a calf (atresia) or to address a calf health problem (cleft palate). On the majority of farms, no (49 farms) or only one case (10) of the recorded deformity had been seen previously.
**Conclusions:** The key value points arising from this pilot project were 1) app convenience of use by busy practitioners, 2) recording of analysable information, 3) generation of a unique photo-archive and 4) ease of model upgrade based on practitioner feedback.

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### 160
### Cross-neutralisation between Bovine Viral Diarrhea Virus (BVDV) type 1 and 2 after vaccination with a BVDV-1a modified-live-vaccine

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**Application:** Two species of BVD virus exist: BVDV-1 and BVDV-2. This study provides evidence that serum from animals immunized with a modified-live-vaccine containing a BVDV-1 strain can neutralise BVDV-2 strains *in vitro*.

**Introduction:** BVDV-1 and BVDV-2 are responsible for major reproductive problems in cattle, resulting in large economic losses. Since there are many genotypes within BVDV-1 and BVDV-2 species, it is important to know whether the use of a monovalent vaccine containing a BVDV-1a strain (Mucosiffa®) could protect cattle against other genotypes, including those belonging to the BVDV-2.
**Materials and Methods:** A study to obtain the "12 months fetal protection" indication for Mucosiffa® was conducted in 2015-2017. Fetal protection was demonstrated in an experimental challenge with the BVD-1f-Hanover strain inoculated 363 days after vaccination (Achard et al., 2018). Twenty seronegative heifers were vaccinated (D0) and blood samples were taken at D28, D203 and D363 before challenge. For the present study, we used sera from 9 heifers to test their ability to neutralise several strains of BVDV: 1a-NADL, 1f-Hanover, 1e, 1b and two BVD-2a strains. All these strains are non-cytopathic (NCP) except the BVDV-1a-NADL which is cytopathic (CP). We used a microplate neutralisation protocol with a constant amount of virus as described by Meyer et al. (2021). Each strain-serum pair was tested 4 times. The reading was taken after immunoperoxydase staining. Virus neutralizing antibody titers (VNT) were expressed as the effective dose of 50% (ED50) calculated by the Spearman-Kärber method. A two-way ANOVA with repeated measures was used for the kinetic analyses by group.
**Results:** We identified three different patterns of VNT, depending on the strains.

- BVDV-1a and BVDV-1b: rapid increase until D28 and then stability in time.
- BVDV-1e and BVDV-1f-Hanover: rapid increase until D28, stability until D203 and then slightly decrease.
- BVDV-2a (two strains): increase until D203 and then stability. At D28, VNT were lower than for the BVDV-1 strains.

Statistical analysis showed no significant difference ($p > 0.05$) in VNT between BVDV-1f-Hanover, BVDV-1e and the two BVDV-2 strains at D203 and D363.
**Conclusions:** Our study showed that sera from cattle vaccinated with Mucosiffa® were able to neutralise strains of BVDV-2a. Interestingly, neutralizing antibody titers against these BVDV-2a strains and the BVDV-1f-Hanover strain were similar from D203. The vaccine has been shown to be effective against an experimental challenge using this latest strain. Although confirmation by experimental challenge with a BVDV-2a strain is necessary, we can hypothesize that the vaccine is clinically effective against infection with a BVDV-2a strain.

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## 161
## Prediction of subclinical endometritis in postpartum dairy cows from circulating cell-free miRNA profiles

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**Application:** Current diagnosis of subclinical endometritis (SCE), relying today on invasive uterine cytology and/or biopsy sampling, would benefit from the use of blood-based diagnostic biomarkers.
**Introduction:** Subclinical endometritis is an unresolved inflammation of the endometrium of postpartum dairy cows, affecting fertility. Due to the role of miRNAs in disease development and their use as markers in other species, this study evaluated the cell-free circulating miRNA profiles of SCE cows and their value as blood-based diagnostic biomarkers.
**Materials and Methods:** Healthy (H, $n = 6$) and SCE ($n = 11$) cows characterized by endometrial cytology and biopsy were blood sampled at 21 and 44 days postpartum. These cows are a subset of the animals enrolled in a larger study regarding SCE. Following extraction of cell-free plasma miRNAs and RNA-seq analysis, differential abundance analysis of miRNAs was performed with the DESeq2 R package (adjusted *P*-value of 0.05), and discriminant analysis was used to combine the differently abundant miRNAs in a single variable and create a predictive model.
**Results:** 34 miRNAs with differential abundance between H and SCE cows were identified. From these, 22 were more abundant and 12 were less abundant in SCE than in H cows. No main effect of the postpartum stage was observed. Overall, when used as a stand-alone SCE marker, no miRNA provided a good prediction of the diseased animals. Stepwise discriminant analysis revealed that among these 34 differently abundant miRNAs, miR425-3p, miR151-3p, and miR30b-5p contributed most to the identification of H and SCE animals. The combination of these three miRNAs in a single variable from subsequent canonical analysis allowed the prediction of the health status in 15/17 (88%) animals at 21 days postpartum. This canonical variable presented a sensitivity and specificity of 90.9% and 83.3%, respectively.

**Conclusions:** Although apparently confined to the endometrium, SCE is associated with distinct circulating miRNA profiles. Despite the fact that no individual miRNA presented a reasonable prediction of the disease as a stand-alone marker, the present results show that it is possible to combine multiple miRNAs to develop a blood-based diagnostic biomarker of SCE with acceptable accuracy.
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## 162
## Specificity of circulating markers for uterine inflammation in pasture-fed, seasonal-calving dairy cows

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**Application:** Identify dairy cows at risk of chronic postpartum uterine inflammation by measuring circulating markers.
**Introduction:** Delayed recovery of the reproductive tract from natural inflammatory processes associated with postpartum involution compromises subsequent reproductive function (de Boer et al., 2015). We hypothesised that cows identified with both purulent vaginal discharge (PVD) and cytological endometritis (uterine inflammation; UI) would have greater plasma concentrations of serum amyloid A (SAA) and α1-acid glycoprotein (α1-AGP) than cows with no uterine inflammation (NUI).
**Materials and Methods:** A subset of mixed-age Holstein-Friesian or Holstein-Friesian x Jersey cows from a 'parent' experiment (Hendriks et al., 2022) were retrospectively classified into UI and NUI groups ($n$ = 14 and 19, respectively), based on uterine polymorphonuclear neutrophils (PMN) ≥5% or ≤1% and PVD score ≥3 or ≤1, respectively (0 to 5 scale), at day 35 postpartum. Plasma samples were analysed for α1-AGP (Life Diagnostics) and SAA (TriDelta Development Ltd.) at day -8, 0, 4, 7, 14, 19, 28, and 35, relative to calving. A repeated measures ANOVA (PROC MIXED; SAS) for effect of day, inflammatory status, and their interaction, on log10 transformed concentrations of SAA and α1-AGP was undertaken. Fixed effects of parity, and random effects of parent experiment farm and treatment were included. Means presented for SAA and α1-AGP are back-transformed. Significance was determined as $P < 0.05$.
**Results:** Mean PMN (±SD) for NUI and UI groups were 0.2 (±0.42%) and 53.4 (±29.33%) and mean PVD scores were 1.0 (±0.00) and 3.4 (±0.51), respectively. There was a uterine health status by day interaction for α1-AGP ($P < 0.01$), and an effect of day for SAA and α1-AGP (both $P < 0.001$). Mean SAA for UI and NUI, respectively, were 32.2 and 24.1 μg/mL ($P = 0.26$), and α1-AGP were 1.40 and 1.26 mg/mL ($P = 0.56$). Between day -8 and 0, SAA increased from 20.8 to 86.9 μg/mL, and α1-AGP from 0.67 to 0.83 mg/mL, decreasing thereafter to 13.3 μg/mL at day 21 for SAA and 1.38 mg/mL at day 35 for α1-AGP.
**Conclusions:** SAA and α1-AGP are not specific circulating markers for unresolved postpartum uterine inflammation. Further work to identify markers of uterine inflammation could provide potential targets for treatment to improve reproductive outcomes.
**Acknowledgements:** Funding from New Zealand MBIE and dairy farmers to DairyNZ Inc. (DRCX1302) and from AgResearch SSIF.

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## 163
## Characterisation of reproductive tract microbiome profile of healthy, Bovine Genital Campylobacteriosis infected cattle

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**Application:** This study could be useful for the development of microbiome assay for Bovine Genital Campylobacteriosis in cattle.
**Introduction:** Bovine reproductive tract harbours multiple species of organisms that potentially play the key role in reproductive tract health, preventing pathogenic microorganisms' growth as well as maintaining fertility and pregnancy. *Campylobacter fetus* subspecies *venerealis* (CFV) is a pathogenic bacterium transmitting to cows during breeding by asymptomatic carrier bulls causing Bovine Genital Campy-

lobacteriosis (BGC). The objective of the present study is to characterise the bovine reproductive tract microbiome and potentially identify biomarker(s) for BGC immunity.
**Materials and Methods:** A total of 12 cycling heifers were selected, 6 were vaccinated once using a commercially available vaccine against BGC and six were left unvaccinated (control). Following oestrus synchronization (15 days post-vaccination) using two doses of PGF (Prostaglandin F2 alpha) 9 days apart, heifers were intravaginally challenged with a freshly prepared inoculum of pure live CFV bacteria ($27x10^8$ colony-forming units/heifer). Vaginal mucus samples were collected in phosphate buffer solution (PBS) at different timepoints, 12 times post-vaccination at 2-days intervals, and 8 times weekly post-challenge. The sample dates were aligned with the expected oestrus cycle phases and confirmed by ultrasonography. DNA was extracted from mucus sample and tested using qPCR and submitted for 16S rDNA amplicon Illumina sequencing (V5-V8 region). QIIME2 (*v*2020.11.1) bioinformatics pipeline was used for processing, analysing, and visualizing microbiome data.
**Results:** The mean abundance of vaginal microbiome population was associated with vaccination, oestrus, and challenge. Heifers were CFV qPCR negative before challenge and the percentage of heifers that were qPCR positive at days 2, 7, 14 and 28 after the challenge was 66.7%, 50%, 50% and 16.7% for nonvaccinated heifers and 0%, 33.33%, 66.67% and 16.67% for vaccinated heifers respectively. Vaccinated heifers eliminated the infection sooner than the nonvaccinated heifers (4 vs. 7 weeks). In both treatments, *Firmicutes* were more abundant than *Bacteroides*, *Proteobacteria* and *Actinobacteria*. *Streptococcus spp*., was higher in abundance and increased during oestrus than dioestrus in both treatment groups. In nonvaccinated heifers, *Campylobacter spp.* appeared 2 days following challenge compared to 14 days in the vaccinated heifers. In addition, *Arthrobacter sp.* (unassigned) were more abundant in oestrus compared to dioestrus in both treatments. Alternatively, *Proteobacteria seems* to increase after challenge, particularly *Acinetobacter*.
**Conclusions:** The microbiome profile of *Proteobacteria* and *Actinobacteria* changed depending on oestrus and dioestrus before and after challenge with CFV in vaccinated and nonvaccinated heifers.
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## 164
## Intrauterine infusion of honeybee product in healthy postpartum dairy cows is safe

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**Application:** Reduce antibiotic use by using honey products for uterine infection in dairy cow.
**Introduction:** The advocated treatment of postpartum endometritis in dairy cows is intrauterine infusion of antibiotics. In the context of rising antibiotic resistance worldwide, legislative restrictions for antibiotics use, and increasing number of organic dairy farms, there is need for alternative therapeutic approaches. Honeybee product (Propohoney) could constitute such alternative due to its antibacterial qualities. This study aimed to test the hypothesis that propohoney has no short- or long-term deleterious effects when infused into the uterus of healthy postpartum dairy cows.
**Materials and Methods:** 41 healthy Holstein cows with intact uterus and normal estrous cycle were divided into 8 different groups recieved an Intrauterine Infusion of: G1 ($n = 6$) 30 mL of propohoney 5% and G2 ($n = 5$) 30 mL of propohoney 2% (Organic Buckwheat Honey with 5% and 2% propolis respectively); G3 ($n = 5$) 30 mL of Honey (Organic Buckwheat Honey); G4 ($n = 5$) 19 g Cephapirin Benzathine (Metricure, Merck); and G5 ($n = 5$) 30 mL of Alcohol 5%, within 30-50 days in milk (DIM). In G6 ($n = 3$) cows were not infused. In G7 ($n = 8$) and G8 ($n = 4$) propohoney 5% and Honey were infused in cows with more than 50 DIM respectively. Trans-rectal ultrasonography, vaginal examination, cytological, and bacteriological evaluations of the uterus via cytobrush sampling at 0 h (before infusion), 48 h, 96 h and 192 h were performed to assess the acute response of the uterus. Uterine biopsies were performed for histologic evaluation on day 0 (before infusion) and 14 (after infusion). With continuous variables, a mixed linear model was made with the group, time, and the interaction. For non-continuous variables, a Proportional Odds Models with a post-hoc test for significant variables was performed. The treatment effect was considered significant at $P < 0.05$.
**Results:** The cytological evaluation showed that the percentage of polymorphonuclear cells in the uterus reached the utmost level ($53.8 \pm 30\%$) rapidly at 48 h before returning to the initial level of 96 h for cows treated with Propohoney 5% ($P < 0.001$) compared with the other groups. The results of the uterine bacterial culture were negative. No changes were measured for all the other parameters.
**Conclusions:** Within the limitation of the study, intrauterine infusion of the Propohoney in healthy postpartum dairy cows stimulates a local transient immune response that could be potentially advantageous in cases of endometritis.

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## 165
## Intrauterine infusion of TNF as possible treatment of metritis in cows

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**Application:** Possibility of usage of a non-antibiotic substance – tumor necrosis factor (TNFα) in treatment of metritis in dairy cows.
**Introduction:** Metritis is inflammation of all layers of the uterus which has significant influence on bovine fertility (Baranski et al., 2013). It involves Gram - negative bacteria, which produce toxins, such as lipopolysaccharides (LPS). LPS stimulates mechanisms of immune response, among which is secretion of proinflammatory mediators, such as cytokines, including interleukins (ILs), TNFα, prostaglandins (PGs) and leukotrienes (LTs) (Sheldon et al., 2006).
**Materials and Methods:** 16 healthy Holstein-Friesian cows with no previous metritis history were used in this study. Metritis was induced by administration of 10 mL Escherichia coli (strain O25:K23/α/:H1) suspension containing 109 colony-forming units (cfu)/ml. 12 h after bacteria inoculation cows were randomly assigned to one of four treatments consisting of intrauterine infusions and intramuscular injections:

- group 1 – control ($n = 4$): 10 mL of 0.9% saline both intrauterine and intramuscular,
- group 2 ($n = 4$): 10 mL of 0.9% saline intrauterine and 500 μg of PGF2α intramuscular,
- group 3 ($n = 4$): 1 μg/uterine horn of TNFα intrauterine and 10 mL of 0.9% saline intramuscular,
- group 4 ($n = 4$): 1 μg/uterine horn of TNFα intrauterine and 500 μg of PGF2α intramuscular.

Blood samples were collected at: -12 h, -6 h, 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h of the experiment, where '0 h' was the time of drug administration. Rectal temperature was measured and transrectal ultrasound exams were performed at each of these times. Concentration of PGFM, PGE2, LTB4 and LTC4, were determined in plasma samples by ELISA. Collected data were analysed by using the repeated measure ANOVA test followed by Bonferroni's Multiple Comparison Test (GraphPad PRISM).
**Results:** Intrauterine infusion of bacteria suspension caused inflammation, which was observed by the temperature increase and the presence of inflammatory fluid in uteri. 12 h after bacteria inoculation, thickening of the uterine wall was also observed.
Six h after inoculation, a rapid increase in plasma LTB4 was observed ($P < 0.05$). Administration of TNFα and aPGF2α caused a decrease of LTB4 secretion ($P < 0.05$). Moreover, an increase of PGFM concentration was observed after the TNFα administration ($P < 0.05$), compared with the control group ($P > 0.05$). During the experiment, no changes in LTC4 and PGE2 secretion were observed.
**Conclusions:** TNFα, similarly to aPGF2α, can inhibit the development of inflammatory processes in the uterus by the modulation of pro-inflammatory mediators. Intrauterine administration of TNFα, during experimentally induced metritis, reduced LTB4 concentration, additionally augmenting PGF2α secretion. There is a need for further development of the described treatment.

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## 166
## The use prepartum additives (plant extracts) in dairy cows influences behavior and mother-calf bond at calving

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**Application:** To reduce the use of antibiotics in animal production, natural alternatives such plant extracts, have been used to substitute the use of Monensin.
**Introduction:** Different types of plant extracts have been used and their effects on the production, health and behavior of cows and calves have been evaluated. The use of additives at the end of gestation and their influence on behavior and mother-calf bond at birth has not been evaluated. The objective of this work was to determine the influence of additives use during the last month of gestation on the behavior and mother-calf bond at birth.
**Materials and Methods:** The experiment was carried out at the Facultad de Agronomía, Paysandú-Uruguay. Three treatments were evaluated [Control, Plant Extracts, and Monensin] from 30 days before calving. The Plant Extracts mix including: Trans cinnamaldehydes (>1000 ppm), Polyphenols (>450 ppm), Total curcuminoides (>280 ppm) and Piperine (>15 ppm). Sixty Holstein cows were used (20/treatment) with visual diurnal behavior, therefore nocturnal calvings were not included. During calving, were calculated (minutes): foetal expulsion (begin abdominal contraction-total expulsion of the calf), parturition-sternal position calf, parturition-standing calf and standing-suckling calf. The data was analyzed including in the statistical model the treatment, parity (primiparous/multiparous) and their interactions as fixed effects and type of delivery (eutocic/dystocic) as covariate. In foetal expulsion time, assisted calvings were excluded.
**Results:** Time of foetal expulsion time was greater ($P = 0.04$) in Control (45.5 ± 7.5 min, $n = 7$) than in Monensin (24.3 ± 7.5 min, $n = 4$), but Plant Extracts (42.7 ± 7.5 min, $n = 7$) was not different from the other treatment. Time of standing-suckling was lower ($P = 0.02$) in Plant Extracts (29.3 ± 10.9 min, $n = 13$) than in Control (75.1 ± 10.9 min, $n = 9$), but Monensin treatment was not different from the other groups. In primiparous cows, time parturition-sternal position calf was lower ($P = 0.02$) in Plant Extracts (3.3 ± 2.7 min) than in Control (10.7 ± 2.7 min), but Plant Extracts was not different from the other treatment. Parturition-sternal position calf, parturition-standing calf and calf weight did not differ between treatments.
**Conclusions:** Calves born from cows fed with Plant Extracts had less time from standing to sucking than controls, and they ranked intermediate between the Control and Monensin treatment in time to fetal expulsion. Calves born from primiparous Plant Extracts cows had less time from calving to the sternal position than the Control primiparous cows. Data suggest that Plant Extracts had positive effects on the behavior and mother-calf bond at calving, and therefore having an impact on better conditions of animal welfare.

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## Shotgun metagenomic sequencing to study the uterine microbiota in dairy cows diagnosed with clinical or subclinical endometritis

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**Application:** To identify the microbial composition of samples collected from postpartum uterus of dairy cows diagnosed with clinical (CE) or subclinical endometritis (SCE).
**Introduction:** Clinical endometritis is characterized by uterine bacterial dysbiosis and inflammatory changes in endometrium. 16S rRNA sequencing is used for culture-independent study of bacteria, however, shotgun metagenomic sequencing offers a more detailed characterization of the bacteriome at species level. We aimed to explore the metagenomic composition of uterine samples from postpartum dairy cows diagnosed with CE or SCE.
**Materials and Methods:** Uterine cytobrush samples were collected at 21 days post-partum from 23 Holstein cows from two farms and processed for metagenomic sequencing. At day 35 post-partum, cows were assessed for uterine health status and divided into groups: CE ($n = 7$) was defined as the presence of mucopurulent/purulent vaginal discharge with $\geq$5% endometrial neutrophils; SCE ($n = 7$) as no abnormal vaginal discharge and $\geq$5% endometrial neutrophils; and nine cows were diagnosed as healthy due to no abnormal vaginal discharge and <5% endometrial neutrophils. After DNA extraction, sequencing and taxonomic assignment were done on Illumina platforms. Taxonomy, counts, and sample metadata were assembled in *metagenomeSeq* package in RStudio. Less abundant species (cumulative count across all samples <100) were filtered out before further analyses. Log-fold change (LFC) was used under zero-inflated log-normal mixture modeling to compare the relative abundance of species among healthy, CE, and SCE cows. Results with $P < 0.05$ were considered significant.
**Results:** *Peptoniphilus indolicus* (LFC = 2.46) and *Fusobacterium necrophorum* (LFC = 2.45) were more abundant in CE than healthy cows. *Alistipes finegoldii* (LFC = 5.04) and *Fusobacterium necrophorum* (LFC = 2.41) had greater abundance in CE than SCE cows, while *Nocardia otitidiscaviarum* (LFC = 1.55) was more abundant in SCE than CE cows. Our data indicated that the microbial profile of SCE cows was not significantly different in comparison to healthy cows.
**Conclusions:** The microbiota composition in CE versus SCE or healthy cows, is characterized by a higher relative abundance of 'usual suspects' of uterine infection such as *Fusobacterium necrophorum* and *Peptoniphilus indolicus*. The microbiota of SCE and healthy cows are similar. Our results based on shotgun metagenomics sequencing are in agreement with our current knowledge about microbial infection and CE and confirm the lack of association of SCE with potential uterine pathogens.
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## 168
## Characterisation of immune cell populations induced from two types of pre-breeding vaccination in Brahman cows

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**Application:** Characterise changes in peripheral leukocyte populations following two types of pre-breeding vaccinations.
**Introduction:** A relationship exists between immune and ovarian function; identification of leukocytes involved in vaccine response would narrow research focus to which cells may be negatively affecting the corpus luteum. Our objective was to identify changes in select leukocyte populations after live or inactivated pre-breeding vaccination.
**Materials and Methods:** Brahman and Brahman-cross females were given prostaglandinF2α on day -3 to induce luteolysis. On day 0, cows were randomly assigned to treatment; modified-live virus vaccination (MLV, $n = 8$; BoviShield Gold FP5VL5), inactivated virus vaccination (IV, $n = 8$; ViraShield 6VL5HB), or saline (CON, $n = 6$). Blood samples were collected into 50mL conical tubes containing K3-EDTA to isolate peripheral blood mononuclear cells (PBMC) on days 0, 4, 6, 8, 10, 14 from all animals, and from different subsets ($n = 10$) on 28, 46, and 65. Density gradient centrifugation with Ficoll Paque-PLUS allowed for collection of PBMC, which were incubated with propidium iodide (cell viability) and antibodies for surface cell markers CD4, CD8, CD25, CD14, CD86, and CD335. Antibody stained PBMC were evaluated with an Amnis FlowSight flow cytometer and data processed with IDEAS 6.2 software. Approximately 10,000 single cells were acquired per sample. Leukocyte percentages from days 0-14 were analyzed in SAS as a repeated measure using the MIXED procedure, including treatment, day, and their interaction in the model, while days 28, 46, and 65 were each evaluated by an ANOVA using the MIXED procedure.
**Results:** Treatment had no effect on day 0-14 leukocyte populations ($P \geq 0.25$), but CD14+, CD335+, CD86+CD335-, CD4+, CD8+, CD25+, and CD25+CD4+ populations differed over time ($P \leq 0.03$). The interaction of treatment and time affected CD14+ (monocyte), CD25+ (activated T-cell) and CD25+CD4+ (T-regulatory) cells ($P \leq 0.05$). On day 2, MLV females had an elevated monocyte population compared with CON. Activated T-cells decreased from day 0 to 2 in all treatments and gradually returned to levels similar to day 0. T-regulatory cells were variable over time but were similar within each sample day among treatments. Cytotoxic T-cells (CD8+) on day 28 and natural killer cells (CD335+) on day 46 were increased in vaccine treatments ($P \leq 0.03$) compared with CON. T-helper cells (CD4+) and cytotoxic T-cells tended to be decreased on days 65 and 46, respectively, in vaccinated animals ($P = 0.08$).
**Conclusions:** Vaccination and time impacted leukocyte populations. The association between reproduction and immune function indicates further research is needed to understand how leukocyte changes affect this relationship.
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## Macrophage and cytokine dynamics in cervical ripening in cows

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**Application:** Dystocia in cows accounts for majority of the parturition-related abnormal conditions and causes significant economic losses. Inadequate cervical ripening is considered one of the causes.
**Introduction:** In rodents, macrophages are associated with cervical ripening (Yellon et al., 2019). In cows, neutrophils infiltrate the cervix in late pregnancy (Yamanokuchi et al., 2022). However, the detailed process in cervical ripening is unknown. This study aimed to observe macrophages and mRNA expressions of interleukin (IL)-1α, 1β, 6, 8, and 10 and TNFα in the cervical tissue and cytokine dynamics in the cervical mucus from late pregnancy to calving to clarify the normal cervical ripening mechanism in cattle.
**Materials and Methods:** Cervical mucus was collected from 41 Japanese Black cows at 200, 230, 260, and 274 days of pregnancy and at 7-day intervals thereafter. Cervical biopsy samples were collected at 200, 260, and 274 days of pregnancy and at 7-day intervals thereafter. Sectioned specimens were subjected to multiplex fluorescent immunostaining using anti-Iba-1, anti-iNOS, and anti-CD163 antibodies, and the macrophage infiltration rate was calculated. Cytokine concentrations in the cervical mucus were presented as concentration per protein weight. Total RNA was extracted from the cervical tissue and mucus, and mRNA expression of the six cytokines were analysed using real-time PCR. Repeated measures two-way analysis of variance was performed on cytokine concentrations and macrophage infiltration rate, and Tukey-Kramer test was applied when significant differences were observed.
**Results:** Macrophage infiltration was observed 5–6 weeks before the calving week. There was a strong correlation ($r = 0.80–0.97$) between the expression sites of Iba-1 and iNOS. Additionally, IL-6 mRNA expression increased 3 weeks before calving ($P < 0.05$). The IL-1α, IL-1β, IL-8, and TNFα concentrations in the cervical mucus increased ($P < 0.05$) 0–3 weeks before calving as compared to those at 12–14 weeks before calving; IL-8 showed the largest increase among the six cytokines. The mRNA expression of IL-1α increased 3 weeks before and during the calving week, while that of IL-8 mRNA increased 2–3 weeks before and during the calving week ($P < 0.05$).

**Conclusions:** These results indicate that cervical ripening begins 5–6 weeks before parturition in cows, when M1 macrophages infiltrating the cervical tissue produce large amounts of IL-6. Moreover, the inflammatory cells infiltrate the cervical mucus, and IL-1α, IL-1β, IL-8, and TNFα levels increase toward parturition. Particularly, IL-8 is strongly involved in cervical ripening.
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## 170
## Associations between postpartum uterine luminal fluid volume and long-term reproductive performance in lactating Holstein dairy cows

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**Application:** Cows with intrauterine fluid more than 11.0 mm diameter evaluated by trans-rectal ultrasonography around ten days postpartum had lower reproductive performance throughout the lactation period.
**Introduction:** Poor conception rate at first artificial insemination (AI) and the existence of repeat breeder (RB) cows that did not become pregnant within three services adversely affect herd reproductive performance. Therefore, it would be valuable to be able to identify cows that are predicted to have lower reproductive performance in the early postpartum period for efficient reproductive management. Our objective was to evaluate the uterine condition in the early postpartum on reproductive performance in lactating dairy cows.
**Materials and Methods:** Lactating Holstein dairy cows ($n$ = 52, parity 3.7 ± 1.9, milk yield 39.8 ± 7.5 kg/day; mean ± SD) were evaluated. Around ten days postpartum (mean 10.2 day, median 10.0 day; calving day = 1 day), we evaluated intrauterine fluid diameter and uterine diameter by trans-rectal ultrasonography. In addition, we conducted vaginal examinations for detection of purulent vaginal discharge (PVD) with a vaginal speculum. Also, we measured body condition score (BCS) and back fat thickness (BFT) before calving (-7 to -1 day) and same day of uterine evaluation; changes of BCS and BFT from before to after calving were calculated as ΔBCS and Δ BFT, respectively. Reproductive performance indexes including first AI conception rate, days open and RB rate were evaluated. First AI conception rate and RB rate were analyzed using multiple binary logistic regression, and days open was analyzed using multiple linear regression analysis. The independent variables were intrauterine fluid diameter (≤11.0 mm, >11.0 mm), uterine diameter (≤50.0 mm, >50.0 mm), parity (1, ≥ 2), PVD (-, +), ΔBCS, and ΔBFT.
**Results:** No variables were associated with first AI conception rate. Intrauterine fluid diameter was associated with days open and RB rate. Days open was longer in cows with >11.0 mm (170.0 ± 88 days) than those with ≤11.0 mm (127.0 ± 62 days) ($P$ = 0.04). RB rate was higher in cows with >11.0 mm (47.8%) than those with ≤11.0 mm (15.8%) ($P$ = 0.01).
**Conclusions:** Existence of intrauterine fluid more than 11.0 mm diameter around ten days postpartum increased days open and RB rate in lactating Holstein dairy cows. This index could aid in the detection the lactating dairy cows with low reproductive performance throughout the lactation period.
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## Associations between the postpartum uterine and vaginal microbiota and the subsequent development of purulent vaginal discharge varies with dairy cow breed and parity

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**Application:** Up to 30% of cows are affected by purulent vaginal disease, which reduces their fertility. The findings of this study suggest that therapeutic interventions used to treat purulent vaginal discharge may need to differ depending on the parity and breed of dairy cow affected.
**Introduction:** The objective of this study was to characterize the species composition and functional potential of the vaginal and uterine microbiota at one week postpartum in dairy cows diagnosed with or without purulent vaginal discharge (PVD) at three weeks postpartum.

**Materials and Methods:** Cytobrush samples of the vagina and uterus were collected at one week postpartum from 36 Holstein-Friesian (7 primiparous and 29 multiparous) and 29 Jersey (10 primiparous and 19 multiparous) cows. Microbial DNA was isolated from each sample and processed for shotgun metagenomic sequencing. Differential abundance analysis was performed using the LIMMA package in R. Breed, parity, PVD status, and the interaction of parity × PVD status and the interaction of breed × PVD status were included as fixed effects.
**Results:** The odds of multiparous cows being diagnosed as PVD+ was less compared with primiparous cows (OR = 0.21). Thirty-one percent of PVD- cows and 48% of PVD+ cows had purulent discharge at week 1. In the vagina of primiparous cows, differences between PVD- and PVD+ cows were minor, but multiparous PVD+ cows had greater abundance of *Fusobacterium necrophorum*, *Trueperella pyogenes*, *Porphyromonas levii*, and greater functional potential for amino acid synthesis and energy metabolism compared with PVD- cows. The uterus of primiparous PVD+ cows had lesser abundance of *Bacteroides heparinolyticus* compared with PVD- cows. In the uterus, differences included greater functional potential for cellulose biosynthesis and fucose catabolism in multiparous PVD+ cows compared with PVD- cows. In the uterus of primiparous PVD+ cows, functional potential for gram-negative cell wall synthesis and for negative regulation of tumor necrosis factor signaling was lesser compared with multiparous PVD+ cows. In the vagina of Holstein-Friesian PVD+ cows, the abundance of Cavibacter abscessus was greater whereas in the vagina of Jersey PVD+ cows the abundance of *Catenibacterium mitsuokai*, *Finegoldia magna*, *Klebsiella variicola*, and *Streptococcus anginosus* was greater compared with PVD- cows. In the uterine microbiota of Holstein-Friesian cows, the functional potential for spermidine biosynthesis was reduced compared with PVD- cows.
**Conclusions:** Differences in the species composition and functional potential of the vaginal and uterine microbiota between PVD- and PVD + cows were dependent on parity and breed.
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## 172
## Plasticity of bovine endometrial stem cells decreases in cows with puerperal endometritis

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**Application:** Potential new tools to treat endometritis.
**Introduction:** Endometritis is of relevance in cattle husbandry, current treatments rely on antibiotics, thus new therapeutic implementations are welcome. Adult stem cells are a potential promising therapeutic tool, of particular relevance are endometrial stem cells. This study aimed to evaluate the plasticity of endometrial stem cells.
**Materials and Methods:** Endometrial biopsies were obtained from puerperal Holstein cows (3-6 years and and 20-60 d postpartum). Transrectal palpation, vaginoscopy, endometrial cytology and bacteriology were used to diagnose endometritis and cows were grouped in: healthy (HC), with subclinical (SCE) and clinical endometritis (CE), according to Leblanc et al. (2002). Endometrial tissue was digested with collagenase-I (3.5 mg/mL) in DMEM medium for 2 h, the lysate was filtered through 40-micron mesh, centrifuged (800 g x 10min) and seeded in DMEM +10% serum and antibiotics. Cell cultures were expanded and flow cytometry for mesenchymal stem cell markers CD44, CD90 and MHCI-II was performed. Cells were subjected to trilineage mesodermal differentiation using STEMdiff™ Trilineage Differentiation Kit (Stemcell Technologies, Cat No. 05230; Vancouver, Canada) for 7 and 14 days. In order to confirm the actual differentiation, RT-PCR was performed using primers for AGGRECAN and SOX9 (chondrogenic), SPARC and RUNX2 (osteogenic) and PAPF1 for adipogenic differentiation. Western blots (Aggrecan, Sparc and Pparγ) were performed. Nonparametric test (Kruskal-Wallis) was used to compare groups, value of $\alpha = 0\ 05$.
**Results:** Six cell lines were studied (2 per group). Surface markers CD44 (68.5%; 76.2% and 56.6%,) and CD90 (98%; 89% and 88%) respectively for HC, SCE and CE were identified in cells. MHCII expression in all groups was negligible (below 3%). Judged by specific staining, intense osteogenic and chondrogenic differentiation was found in all samples at days 7 and 14. Adipogenic differentiation was not achieved for CE group (confirmed by RT-PCR; amplification of PPAF in HC/SCE, but not in CE group). Aggrecan, but not Sparc protein was detected in differentiated cells from HC, both proteins were detected in samples from SCE and none in samples from clinical endometritis. Noteworthy, Pparγ protein was not detected in any sample, but in the positive control.
**Conclusions:** Endometrial biopsies from healthy puerperal cattle and of those with subclinical endometritis yielded populations of adult cells with multipotency similar to mesenchymal stem cells. Cells from cows with clinical endometritis, had however a limited potential of differentiation and did not differentiate into adipogenic lineage, suggesting a deleterious effect of inflammation on the biogenesis of endometrial stem cells.
**Acknowledgements:** Funding: FONDECYT-REGULAR 1210349, ANID-CHILE.

### Reference

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## 173
## Prevalence of bacterial and fungal abortifacient pathogens in bovine foetuses and placentae – A national study

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**Application:** This study establishes the diagnostic value of submitting a bovine foetus to determine the primary bacterial and fungal abortifacient pathogens likely to cause abortion, in a statistically robust sample size.
**Introduction:** Diagnosis of the causes of bovine abortion is an international challenge. However, each country has its own profile of abortifacients necessitating national-level diagnostic surveillance. Hence, the objective of this study (part of a larger project on bovine foetal mortality diagnostics) was to establish the national prevalence of primary abortifacient pathogens in bovine foetal material submitted to the Irish Veterinary Laboratories Service (VLS).
**Materials and Methods:** Sampling of bovine (dairy and suckler) foetal material (abortions and stillbirths with uninflated lungs, +/- placentae) was carried out in 2020/2021 at the six Regional Veterinary Laboratories of the VLS. The foetus and placenta, if available, were examined for gross abnormalities. Straight crown-rump length (sCRL) was measured. A sample of foetal stomach content and a swab of placenta were collected and plated on blood, Brucella (incubated in 8% $CO_2$ at 37°C) and XLD agar (incubated in aerobically at 37 °C). The sample was also plated on Sabouraud's agar if gross lesions suggestive of fungal infection were observed. Plates were examined daily for seven days.
**Results:** The foetal carcasses originated from 855 individual herds (1 to 8 submissions/herd). Herd size ranged from 1 to 750 (median 110). In total, 1181 entire foetal carcasses were examined with (305) or without (876) placenta. The median, minimum and maximum sCRL of the foetuses was 75, 22 and 130 cm, respectively, implying median, minimum and maximum gestational ages of approximately 223 days, 108 days and fullterm, respectively. Of the 1,061 stomach content samples cultured, primary pathogens were detected in 281 (26.4%); Trueperella pyogenes (124), Salmonella Dublin (69), other Salmonella species (6), Bacillus licheniformis (31), Listeria monocytogenes (42) and Aspergillus species (9). All Brucella abortus cultures were negative. Of the 186 placentae cultured, primary pathogens were detected in 55 (29.6%); Salmonella Dublin (22), Bacillus licheniformis (21), Trueperella pyogenes (12), Listeria monocytogenes (4) and Aspergillus species (2). Two primary pathogens were cultured from the same placental sample in six cases.
**Conclusions:** These results indicate that approximately 30% of bovine foetal mortality (between ~4 and 9 mo.) in this national cattle population could be attributed to primary (mainly bacterial) abortifacients detectable using routine culture methods. This raises the question as to the causes of the remaining 70% of cases.

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## 174
## Predictive value of uterine cytology macrophages for the persistence of subclinical endometritis in postpartum dairy cows

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**Application:** Use uterine cytology polymorphonuclear neutrophils (PMN), macrophages (Mφ) and their ratio (PMN:Mφ) for prediction of persistence of subclinical endometritis (SCE).
**Introduction:** Postpartum SCE frequently evolves to a persistent status that causes subfertility. Diagnosis of SCE has been based in PMNs proportion in uterine cytology. Although Mφ are also involved in the pro-inflammatory and resolution processes, their diagnostic significance for SCE is unclear. The aim of this study was to relate uterine cytology PMN and Mφ content, as early as 21 days postpartum (DPP), with subsequent recovery or persistence of inflammation in postpartum dairy cows.
**Materials and Methods:** Cows without postpartum clinical disease or treatments ($n$ = 134) were sampled at 21 DPP and allocated to groups Healthy ($n$ = 71; PMN < 18%) or SCE ($n$ = 63; PMN $\geq$ 18%). Cows were then reassessed at 42 DPP to confirm the Healthy status and evaluate the Recovered ($n$ = 40, PMN < 5%) or Persistent ($n$ = 23, PMN $\geq$ 5%) SCE status. Cytology parameters were compared between groups by one-way ANOVA. A discriminant analysis evaluated PMN, Mφ and PMN:Mφ at 21DPP in SCE cows, and a canonical discriminant analysis combined Mφ and PMN:Mφ at 21DPP in a single variable to create a predictive model for SCE persistence.
**Results:** At 21DPP, PMN proportion was higher in Persistent than in Recovered SCE cows (45.1 ± 2.7 vs. 34.3 ± 2.0, $P$ < 0.01), in both groups being higher ($P$ < 0.001) than in Healthy cows (3.5 ± 1.5). The Mφ proportion was higher ($P$ < 0.0001) in Recovered SCE cows (5.8 ± 0.5) than in Healthy (2.5 ± 0.4) and Persistent SCE (1.3 ± 0.6) cows. The ratio PMN:Mφ was higher ($P$ < 0.0001) in Persistent SCE cows (75.8 ± 7.9) than

in Healthy (4.8 ± 4.6) and Recovered SCE (9.4 ± 6.0) cows. The canonical formula – 0.2446xMφ-0.0084xPMN:Mφ – showed a sensitivity of 0.91, specificity of 0.85, positive predictive value of 0.78 and negative predictive value of 0.94 for SCE persistence.
**Conclusions:** The joint evaluation of PMN and Mφ proportions and the PMN:Mφ ratio in uterine cytology, as soon as 21DPP, assist in predicting the persistence or recovery from SCE in postpartum dairy cows, allowing identification of cows requiring additional therapy and/or follow-up. The low Mφ proportion in persistent SCE cows may represent an impairment of Mφ recruitment to the uterus due to failure in inflammatory resolution pathways, whereas in Healthy cows may represent either a low inflammatory stimulus or a faster recruitment response occurring earlier than 21 DPP.
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## Bovine Genital Campylobacteriosis: *Campylobacter fetus* subsp. *venerealis* infection disrupts transcription of uterine receptivity and pregnancy maintenance related genes in bovine endometrial epithelial cells

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**Application:** Molecular signatures of bovine endometrial epithelial cells (bEECs) infected by *Campylobacter fetus* subsp *venerealis* (*Cfv*) may reveal diagnostic biomarkers and therapeutic targets for Bovine Genital Campylobacteriosis (BGC).
**Introduction:** BGC is a venereal disease of cattle caused by *Cfv*. Infection of females causes infertility, embryo mortality, and early abortion. Despite the worldwide distribution and economic impact, BGC pathogenesis remains elusive. As bEECs are involved in embryo implantation and pregnancy maintenance, this study evaluated their response to *Cfv* infection.
**Materials and Methods:** To simulate natural infection during luteal phase, *in vitro* cultures of bEECs were assembled from 4 healthy luteal phase uteri, exposed to 5 ng/mL of progesterone for 48 h before co-culture with *Cfv*. The bEECs with 90% confluence and >93% purity were challenged (6 h) with *Cfv* or non-stimulated (control). Total RNA was extracted and submitted to RNA sequencing. For the identification of differentially expressed genes (DEGs), a generalized linear model fitting was performed using Bioconductor package EdgeR, considering paired samples and treatment (control vs infected) as model additive factors. The DEGs were filtered using a $1 \leq$ log2-fold change $\leq -1$ and an adjusted *P*-value $\leq 0.05$.
**Results:** 280 DEGs were identified in *Cfv*-infected cultures, including 224 upregulated and 56 downregulated. These sets of DEGs revealed significant enrichment in 22 GO terms for biological processes ($p < 0.001$), mainly related to inflammatory immune response. The top 10 most significantly represented pathways were related to inflammation and associated immune response, including the IL-17 ($n = 15$ genes), cytokine-cytokine receptor interaction ($n = 31$), TNF ($n = 19$) and NFKB ($n = 15$). Several DEGs were previously implicated in embryo implantation ($n = 46$), pregnancy ($n = 163$), and uterine receptivity ($n = 7$). Regarding uterine receptivity, *Cfv* infection upregulated the transcription of genes *LIF* (2.4-fold), *IL6* (6.4-fold) and *IL23A* (2.3-fold), previously implicated in the physiological maternal-fetal immune tolerance; in contrast, *Cfv* infection downregulated the transcription of the solute carrier genes *SLC52A3* and *SLC2A12*, involved in embryo nutrition and pregnancy maintenance.
**Conclusions:** The results provide evidence that *Cfv* dysregulates the transcriptome of bEECs at an early stage of infection, ultimately compromising uterine homeostasis. Several genes involved in uterine receptivity, embryo implantation and pregnancy maintenance were disturbed by *Cfv* infection, which may contribute to infertility, early embryonic mortality and abortion associated with BGC.
**Acknowledgements:** This study was supported by Fundação para a Ciência e a Tecnologia (FCT) and Fundo Europeu de Desenvolvimento Regional (Project PTDC/CVT-CVT/30145/2017, Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA; Project UIDB/00276/2020, FCT) and Associate Laboratory for Animal and Veterinary Science (LA/P/0059/2020 - AL4AnimalS).

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## The transcriptome profile of bovine uterine endometrial epithelial cells isolated from cows diagnosed with metritis early postpartum provides evidence for inflammatory memory within the uterus

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**Application:** Determine if bovine endometrial epithelial cells (BEEC) carry cellular memory of an early postpartum uterine disease.

**Introduction:** Uterine disease in postpartum dairy cows creates an inflammatory condition compromising its function later postpartum, even after the infection resolution. A possible mechanism leading to abnormal uterine function and infertility in diseased cows involves "inflammatory memory," where epithelial cells remember past infections through an epigenetic mechanism (Naik and Fuchs, 2022). We hypothesized that uterine disease early postpartum would reprogram BEEC and their subsequent response to lipopolysaccharide (LPS) *in vitro*, thus providing evidence for inflammatory memory.
**Materials and Methods:** Primary BEEC were isolated from the uteri of primiparous Holstein cows diagnosed (7.6 ± 1.7 d postpartum) as healthy ($n$ = 3) or with metritis ($n$ = 5) and slaughtered at 29.1 ± 1.7 d postpartum. Based on uterine luminal contents postmortem, cows were classified as containing pus ($n$ = 3) or clean ($n$ = 5). Cells were seeded separately in 12-well plates with growth medium and incubated (38.5 °C, 5% $CO_2$) until 90% confluency (approximately 4 days in culture). Cells were then treated with medium only (control) or LPS (1 μg/mL) and harvested after 24 h for RNA isolation. RNA sequencing libraries were constructed from individual cell lines ($n$ = 16; 3 healthy control, 3 healthy LPS, 5 metritis control, and 5 metritis LPS) and sequenced on a single NovaSeq S2 PE100 flow cell. Statistical analyses were performed in R using glmLRT from edgeR. A transcript was considered differentially expressed (DEG) if the false discovery rate P<0.05. Enriched gene ontology terms were identified using DAVID and ShinyGO.
**Results:** There were 634 DEGs (202 upregulated, 432 downregulated) for cells derived from metritis compared with healthy uteri in untreated control cultures. The enriched biological processes for upregulated genes included extracellular-matrix organization and cell-cell adhesion. In LPS treatment, there were 533 DEGs (210 upregulated, 323 downregulated) in metritis compared with healthy and enriched biological processes of extracellular structure organization and blood-vessel development. Cells from healthy uteri and challenged with LPS had a greater number of DEG (1789; 1126 upregulated, 663 downregulated) relative to the LPS response in metritic uteri (871 DEG; 637 upregulated, 234 downregulated). Similar results were found in the pus vs. clean classification.
**Conclusions:** Early postpartum metritis changed the transcriptome of cultured BEEC and their response to LPS at one month postpartum. These data provide evidence for a cellular memory of the postpartum disease that may explain differences in fertility for healthy and metritic cows later postpartum.
**Acknowledgements:** Supported by the NICHD award R01HD092254.

**Reference**

Naik, S., Fuchs, E., 2022. Nature 607 (7918), 249–255.

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## Postpartum uterine disease reduces Forkhead box A2, a critical regulator of uterine gland function, within the uterus of lactating dairy cows

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**Application:** Reduce fertility losses caused by early postpartum uterine disease by understanding the mechanisms through which disease damages uterine glands that are essential for fertility.
**Introduction:** Postpartum uterine involution involves the restoration of functional uterine glands that are essential for fertility. The impact of uterine disease on this process is unknown. Forkhead box A2 (FOXA2) is a transcription factor expressed within uterine glandular epithelium (GE) and is essential for their function (Kelleher et al., 2019). We assessed the impact of uterine disease on FOXA2 protein and mRNA in cows with or without uterine disease.
**Materials and Methods:** Primiparous Holstein cows were diagnosed with metritis (M; $n$ = 16) or were Healthy (H; $n$ = 14) and were either antibiotic-treated (H, $n$ = 8 and M, $n$ = 7) or not-treated (H, $n$ = 6 and M, $n$ = 9) at 7-10 days postpartum and slaughtered at 29.1 ± 1.7 days postpartum. The uterine lumen was flushed with saline and classified as either clear (C; $n$ = 17) or containing purulent (pus, P; $n$ = 13) material. A cross section of the uterus was stained for FOXA2 protein (immunohistochemistry). The pixel intensity of FOXA2 staining in the GE was measured in the deep (D), middle (M), and superficial (S) endometrium using ImageJ (5 measurements per layer in single GE cells) and subtracted from background (surrounding stromal cell stain). Caruncular and intercaruncular endometrium were collected for RNA isolation and sequencing. Stranded RNA-seq libraries were constructed and sequenced on a single NovaSeq S2 PE100 flow cell with an average of 50 million paired reads per sample. The number of FOXA2 reads was measured. Data were analyzed using PROC MIXED of SAS.
**Results:** FOXA2 protein expression decreased ($P$ < 0.001) from the deep to superficial endometrium [57.7, 35.9, and 21.7 (SEM = 3.4) for D, M and S, respectively]. There was an effect of uterine disease ($P$ = 0.043) on FOXA2 protein because, compared with C cows, the P cows had lesser pixel intensity (33.2 ± 4.1 vs 43.7 ± 3.3) across all layers of the endometrium (P vs C). The reduced protein expression of FOXA2 in the GE was associated with a decreased number of RNA sequence reads for FOXA2 in caruncular and intercaruncular endometrium in the P cows [303.7 ± 50.0 vs 457.9 ± 39.9; P vs C; $P$ < 0.016). There was no effect of antibiotic treatment on FOXA2 expression.
**Conclusions:** Uterine disease early postpartum may delay fertility in postpartum dairy cows by decreasing glandular function through a mechanism involving decreased expression of FOXA2.
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**Reference**

Kelleher, A.M., DeMayo, F.J., Spencer, T.E., 2019. Endocrine Reviews 40, 1424–1445.

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## Vaginal and uterine microbiome in metritic versus healthy dairy cows at disease diagnosis (day 7 to 14 postpartum), after clinical cure (day 28 to 35 postpartum) and at mid-lactation (day 80 to 165 postpartum)

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**Application:** Determine if the uterine microbiome that differs for metritic and healthy cows at disease diagnosis also differs following clinical cure (early postpartum) and then later postpartum during the breeding period.
**Introduction:** Metritis is associated with dysbiosis within the uterine microbiome (Galvao et al., 2019). One of its negative impacts is decreased fertility. One possible explanation for decreased fertility is that the microbiome in early postpartum metritic cows is maintained following clinical cure and remains during the breeding period. We therefore assessed the changes in the vaginal and uterine microbiome at disease diagnosis, following clinical cure, and at mid-lactation (breeding period).
**Materials and Methods:** First-parity Holstein cows diagnosed with metritis ($n = 9$) and a healthy control ($n = 10$) were enrolled at 7 days postpartum (dpp) and followed weekly until 35 dpp for collection of vaginal swabs and then slaughtered at 80 dpp (5 metritis and 5 control; 79.0±7.5 dpp) or 165 dpp (4 metritis and 5 control; 165.0±4.9 dpp) for collection of uterine body tissue. The DNA was extracted from vaginal swabs and uterine body tissue and the V4 hypervariable region of the 16S rRNA gene was amplified and sequenced. The most prevalent ASV genera in metritis and control were analysed using mixed models in SAS. The β-diversity was analyzed using PAST4.03. Data were for 3 groups: 7 and 14 dpp (diagnosis), 28 and 35 dpp (early-lactation following clinical cure) and 80 and 165 dpp (mid-lactation).
**Results:** The five most prevalent genera at diagnosis were Fusobacterium, Ureaplasma, Bacteroides, Porphyromonas and Helcococcus whereas in mid-lactation uterine body the most prevalent genera were Alistipes, Anaerococcus, Escherichia, Lactobacilus and Muribaculaceae. Compared with control, the metritic cows from diagnosis to clinical cure had greater ASV counts for Bacteroides ($P = 0.011$), Fusobacterium ($P < 0.001$), Porphyromonas ($P < 0.001$) and Helcococcus ($P < 0.001$). We did not detect an effect of metritis versus control on the uterine body microbiome at mid-lactation. Based on microbial β-diversity, the microbiome of the uterine body at mid-lactation differed from the vaginal microbiome in metritis cows. This difference was less in control cows.
**Conclusions:** We concluded that metritis was defined by pathogenic bacteria at diagnosis that were no longer present at mid-lactation. Furthermore, the microbiome in mid-lactation was similar for cows diagnosed as metritis or healthy early postpartum. It is unlikely that the microbiome of early lactation persists into the breeding period and explains lesser fertility in cows with early postpartum uterine disease.
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**Reference**

Galvao, K.N., Bicalho, R.C., Jeon, S.J., 2019. Journal of Dairy Science 102 (12), 11786–11797.

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## Investigation of the vaginal microbiota of dairy cows through genetic sequencing of short (Illumina) and long (PacBio) reads and associations with gestational status

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**Application:** A species-level analysis can help to establish an association between vaginal microbiota and fertility, enabling the identification of biomarkers.
**Introduction:** The vaginal microbiota has been shown to be important in local immune regulation and may play a role in reproduction and fertility. Next-generation sequencing technologies are used to characterize the bovine microbiota, mainly using short-read sequencing (Illumina). However, the limitation of this technique is its inability to classify bacteria at the species level. Therefore, we aimed to distin-

guish the bovine vaginal microbiota at the species level using long-read sequencing (PacBio). In addition, the vaginal microbiota of pregnant cows after artificial insemination (AI) was compared with that of infertile animals.
**Materials and Methods:** Thirteen Holstein cows had vaginal swabs collected prior to AI. DNA was extracted and subjected to Illumina and PacBio sequencing to characterize the V4 region and entire 16S rRNA gene, respectively. Pregnancy was diagnosed 30 d after insemination using ultrasonography. The vaginal microbiota of cows that became pregnant (PREG) was compared to that non-pregnant (NP). Statistical analyzes (Minitab 18) included Analysis of variance with Tukey's test ($p \leq 0.05$) to compare relative abundances of phyla (Illumina and PacBio). The correlation between the alpha diversity indices (number of genera; Chao, Simpson and Shannon) obtained by the two methodologies used the Spearman test ($p \leq 0.05$). And Linear discriminant analysis and effect size (LEfSe) methods to associate specific bacterial species with each methodology.
**Results:** Through the PacBio platform, 366 509 readings were obtained, and 631 586 readings were obtained using Illumina. It was possible to identify 27 vs 28 phyla and 651 vs 662 genera using PacBio and Illumina sequencing, respectively. PacBio analysis identified 476 species; however, there was a significant variation between technologies in richness (number of genera and Chao index, $p \leq 0.05$) but not in diversity (Simpson and Shannon indices). The vaginal microbiota of seven pregnant cows was compared to six non-pregnant. The most abundant phyla in all the samples evaluated were Firmicutes (58%) and Bacteroidetes (32%). Linear discriminant analysis and LEfSe analysis detected 19 bacterial genera that showed statistical significance between PREG (unclassified Clostridiales, unclassified Firmicutes, *Helicobacter*, and *Intestinimonas*) and NP (*Pseudomonas* and *Jeotgalicoccus*).
**Conclusions:** There was statistical significance in the linear discriminant analysis and LEfSe between PREG and NP groups in 19 bacterial genera. Illumina sequencing was able to detect as many genera as PacBio, but it underestimated the richness. PacBio sequencing failed to classify most reads to the species level, indicating the need for further investigation.

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## Seasonal heat stress effects on body temperature, cortisol concentrations and uterine inflammation in postpartum dairy cows

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**Application:** Improve dairy cow fertility by reducing heat stress impact during hot seasons and, thereby, increase production of dairy farms.
**Introduction:** Summer heat stress is a main cause of decreased production and fertility in dairy cows. Current climate change has amplified heat stress as a main threat to health and productivity of dairy cows in hot climates. This study evaluated the impact of season-associated heat stress in body temperature, hair cortisol concentrations and postpartum uterine inflammation in Holstein-Friesian cows from two dairy farms in south Portugal.
**Materials and Methods:** Seasons were classified through the THI of the 6 hottest hours of the day, in summer (THI $\geq$ 72) and winter (THI < 72). In each cow ($n$ = 66), vaginal and rectal temperature were measured, eye thermography (emissivity 0.98 at 1 m, FLIR®EX8) evaluated, hair collected from neck for cortisol measurement (DRG®, salivary ELISA KIT), uterine cytology performed at 36 ± 3 days postpartum, in summer ($n$ = 35) and winter ($n$ = 31). Uterine cytology was performed just anterior to the cervix with a cytobrush adapted to the universal insemination gun. Samples were air dried and colored with diff-quick protocol when they arrived at the laboratory. Statistical analyses were conducted using the SAS for windows, data normality tested, regression analyzes perfomed using a General Linear Model (GLM) and differences between seasons were determined by Mann-Whitney-Wilcoxon Test. Statistical significance was set at $P < 0.05$.
**Results:** Vaginal, rectal and ocular temperatures increased ($P < 0.05$) with increasing THI during the previous 60 days (THI60). Hair cortisol also increased with increasing THI60 ($P < 0.01$), with hair concentrations, reflecting accumulated levels during the previous 60 days, with greater concentrations ($P < 0.01$) in summer than in winter (20.2 ± 7 ng/mg vs 14.3 ± 5.2 ng/mg respectively). The postpartum uterine cytology proportion of polymorphonuclear neutrophils (PMN) was lower ($p < 0.01$) in summer than in winter (2.5 ± 5 vs 27 ± 11.2). However, the summer increase in cortisol concentrations was not related ($p = 0.33$) with the observed decrease in uterine cytology proportion of PMN.
**Conclusions:** Summer associated heat stress significantly increased body temperature and cortisol concentrations and decreased the uterine inflammatory response in postpartum dairy cows. However, cortisol was not a main player in the apparent heat stress induced uterine immunosuppression, and other heat stress-induced modulators may be compromising uterine immunity.
**Acknowledgements:** The work was funded by FCT UID/CVT/0027/2020, LA/P/0059/2020-AL4Animals, PDR2020-101- 03112 and SFRH/BD/148804/2019 grant (L Capela).

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## 181
## Clinical mastitis and pregnancy rate in primiparous and multiparous grazing dairy cows

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**Application:** Research on parity-related differences in the impact of clinical mastitis (CM) on pregnancy rate under grazing conditions contributes to specific preventive measures.
**Introduction:** Although Cruz et al. (2021) reported greater odds for CM in multiparous (M) than primiparous (P) grazing dairy cows in the first 90 days in milk (DIM), the incidence of CM cases in the first 14 DIM was greater in P cows. In that study, pregnancy rates were lower in M cows and, as expected, in cows with CM. As far as we know it is not known if CM affects the pregnancy rate in a parity-dependent manner.
**Materials and Methods:** Holstein primiparous ($n$ = 375) and multiparous ($n$ = 663) cows from four grazing dairy farms were selected at 10 days before calving. Clinical mastitis was diagnosed by trained personnel according to Pinzón-Sánchez and Ruegg (2011) and registered during the first 30 DIM. Pregnancy diagnoses were performed by transrectal palpation or ultrasonography by the farm veterinarian. Time to pregnancy was defined as the interval in days from calving to the insemination that led to conception. Censoring time was 305 DIM. Within parity, chi-square tests were used to evaluate the proportions of pregnant cows according to health status. Cox proportional hazards regression models were used to analyze pregnancy rates. The farm was included as a random effect.
**Results:** In the first 30 DIM, 13.9% of P cows and 17.4% of M cows had CM. For primiparous cows, the proportion of pregnant at 305 DIM in healthy cows was 71.5% and in CM cows 73.1% ($P$ = 0.82); for multiparous cows, the proportions were 66.4% and 43.0%, respectively ($P$ < 0.0001). Primiparous cows had a higher pregnancy rate (HR = 1.22, $P$ = 0.02) than multiparous cows. Overall, a first case of CM within 30 DIM was associated with a decreased pregnancy rate (HR = 0.59, $P$ = 0.005). However, CM interacted with parity: in cows that suffered a first case of CM within 30 DIM, primiparous cows had an increased pregnancy rate (HR = 1.65, $P$ = 0.03) compared to multiparous cows.
**Conclusions:** Data confirms a higher pregnancy rate in primiparous than multiparous cows. A category-dependent impact of CM during the first 30 DIM on pregnancy rate was found only in multiparous cows, while no effect was found in primiparous cows; CM diminished pregnancy rate in M cows.
**Acknowledgements:** Funding was provided by Elanco Animal Health (Greenfield, IN) and the University of the Republic, Uruguay.

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## 182
## Why does gestational dietary protein restriction in heifers, affect the subsequent health and development of the male neonatal calf?

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**Application:** We examine the effects of protein restriction, as experienced by Northern breeder herds during gestation, on the developing calf during periconception and first trimester. We show that the environment experienced in utero shapes the neonate and its ability to survive and develop to adulthood.
**Introduction:** The placenta and fetus respond to maternal dietary perturbations in a sexually dimorphic manner (Copping et al., 2014; Micke et al., 2015). This has significant consequences in the male as survival, during gestation and at birth is reduced, compared to the

female (Copping et al., 2021). Neonate survival is dependent upon the ability to thermoregulate, stand, suckle, and ingest colostrum immediately after birth. Thermoregulation is influenced by thyroid hormones and BAT (brown adipose tissue), both influenced by gestational diet. In two-year old heifers restricted first trimester protein restriction increased thyroid hormones in the male neonate, enabling upregulation of UCP1 expression, thereby increasing thermogenesis, a means by which low birthweight calves can increase heat production (Micke et al., 2011; Copping et al., 2014; Fainberg et al., 2018).
**Objective:** Does periconception or first trimester protein restriction alter thyroid hormone and associated neonatal survival characteristics in yearling heifers.
**Materials and Methods:** 60 d prior to AI, 360 nulliparous heifers (12 mo) were assigned to equal periconception (PERI; -60 to 23 dpc) treatment groups, high and low protein (HPeri and LPeri). Each was trained to feed individually in stalls High (71 MJ ME, 1.18 kg CP/heifer/day) or Low (63 MJ ME, 0.62 kg CP/heifer/day) diet. Heifers were FTAI with semen from one bull on day 0. At 23 dpc half of each group was swapped to the alternative post-conception treatment (POST; 24 to 98 dpc), high (HPost: 102 MJ ME,1.49 kg CP/heifer/day) or low (LPost: 98 MJ ME, 0.88 kg CP/heifer/day), yielding four groups: [HPeri-HPost (HH), HPeri-LPost (HL), LPeri-HPost (LH), LPeri-LPost (LL)] in a two-by-two factorial design. At calving, heifers were monitored 24 h/d. Calf measurements and data collection was completed within 15 minutes of birth (prior to sucking) and monthly until 23 mo of age.
**Results:** Periconception diet affected FT4 levels at birth in male calves ($P < 0.05$). FT3 was significantly correlated with milk intake in the male and with weight gain to weaning.
**Conclusions:** Dietary protein restriction alters thyroid hormone production consequent to effects upon ADG, milk intake and fetal growth rate in male progeny of yearling heifers. We note the earlier intervention period (periconception) affected FT4 levels at birth rather than FT3 previously observed. The enhanced appetite observed may be prenatally programmed as gene expression in appetite regulating hypothalamic neuropeptides was altered in these progeny.
**Acknowledgements:** ARC.

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## 183
## Dietary betaine to ameliorate heat stress in gestating Merino ewes

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**Application:** In ruminant species, heat stress results in decreased reproduction and productivity and betaine is proposed as a dietary mitigation method.
**Introduction:** Heat stress ultimately compromises the maintenance of physiological parameters (Hopkins et al., 1978), reproductive functions (Kleemann & Walker, 2005) and overall performance (Sevi et al., 2001). The use of dietary betaine in livestock feeds is increasing, showing potential to protect from the effects of heat stress in ruminants (Digiacomo et al., 2016). The aim of this study was to examine the effects of dietary betaine supplementation on the physiological responses of gestating Merino ewes exposed to naturally high ambient temperatures.
**Materials and Methods:** One-hundred and sixty Merino ewes, aged between five to eight years old (83.0 ± 6.9kg), were selected for this study conducted at the Turretfield Research Centre, Rosedale, South Australia. Ewes were randomly assigned into two replicates ($n = 40$/treatment/replicate; 4 weeks apart), receiving either a control diet or 2 g betaine/ewe/day from mating to parturition. Respiration rates (RR) and rectal temperatures (RT) were collected on days exhibiting high ambient temperatures (>30 °C). RT was collected using a handheld digital thermometer and RR was measured by counting flank movements through blinded video recordings, and expressed as mean breaths/minute (bpm). Ambient conditions were recorded via an onsite weather station, and used to calculate the temperature humidity index (THI). Heat stress severity was then categorised based on the THI range as follows, $\leq$67 = no stress (thermoneutral), 68–74 = mild, 75–78 = moderate, 79–83 = severe and $\geq$84 = extreme) adapted from (Livestock Conservation Inc 1970). All data were analysed using SPSS software with significance represented by $P < 0.05$.

**Results:** Ewes receiving betaine had decreased RT on experimental days of severe (−0.2 °C, $P < 0.04$) and extreme heat stress (−0.3 °C, $P < 0.01$), as well as a reduction in mean RR (118 vs 122 breaths/min, $P = 0.007$) when compared with control ewes. In addition, a negative correlation was observed between mean ewe RT and lamb live weight 4 h post-partum ($r = -0.19$; $P = 0.033$).
**Conclusions:** These data indicate that dietary betaine supplementation at 2 g/ewe per day provides some improvements in the physiological responses initiated during heat stress. Therefore, betaine may be a beneficial supplement for the management of gestating ewes during periods of high heat load.
**Acknowledgements:** We gratefully acknowledge The Davies Livestock Research Centre and The South Australian Research and Development Institute for ongoing research support.

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## 184
## Risk factors for reproductive tract disease in clinically healthy dairy cows: A case-control study

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**Application:** Better understand the determinants of reproductive tract disease in dairy cows.
**Introduction:** Up to half of dairy cows may have reproductive tract disease in the second month of lactation, which is a risk factor for reduced fertility. However, the pathogenesis of these infections and inflammation is not fully understood. The objective was to investigate associations of early postpartum inflammatory and metabolic markers with reproductive tract disease in Holstein cows.
**Materials and Methods:** Holstein cows ($n = 1{,}517$) from 2 herds in Ontario, Canada were examined to assess clinical and subclinical health conditions. Total Ca, haptoglobin, and non-esterified fatty acids were measured in serum at 2 and 6 ± 2 d postpartum, and β-hydroxybutyrate (BHB) at 4, 8, 11, and 15 ± 2 d. Cows with difficult calving, twin birth, retained placenta, metritis, displaced abomasum, mastitis, or lameness were excluded ($n = 606$; 40% of the total), and the remainder were examined at 35 d for purulent vaginal discharge (PVD) and for endometritis (≥6% polymorphonuclear cells in endometrial cytology sampled with cytobrush). Cows were classified as healthy (no PVD or endometritis; $n = 628$), subclinical endometritis (endometritis, no PVD; $n = 146$), PVD (no endometritis; $n = 66$), or clinical endometritis (both endometritis and PVD; $n = 71$). Metabolites were categorized based on ROC curve cutpoints associated with pregnancy at first AI, and data were analyzed with multivariable logistic regression models.
**Results:** Among cows without other clinical disease, 16% had subclinical endometritis, 8% had clinical endometritis, and 7% had PVD only. Compared with clinically healthy cows, the adjusted odds ratio (AOR; 95% confidence intervals) of having subclinical endometritis were greater in cows with elevated haptoglobin at d 6 (≥0.47 g/L; AOR: 2.11; 1.39 to 3.19; $P < 0.001$) or elevated BHB at d 15 (≥1.2 mM; AOR: 2.23; 1.30 to 3.83; $P < 0.01$). Risk factors for clinical endometritis were hypocalcemia (≤2.22 mM; AOR: 2.16; 1.25 to 3.71; $P < 0.01$) and elevated haptoglobin at d 6 (AOR: 4.27; 2.54 to 7.19; $P < 0.001$), and for PVD were primiparity (vs. multiparous; AOR: 3.40; 1.83 to 6.32; $P < 0.001$) and elevated BHB at d 15 (AOR: 2.81; 1.23 to 6.41; $P = 0.01$).
**Conclusions:** The pathology of chronic reproductive tract disorders likely involves the failure of metabolic adaptation to lactation and dysregulation of inflammation after parturition.
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## 185
## A reverse vaccinology approach to design a multi-epitope vaccine against *Campylobacter fetus* subsp. *venerealis*

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**Application:** The design and *in silico* evaluation of a vaccine against *Campylobacter fetus* subsp. *venerealis* may assist in the development of an effective vaccine for the control of Bovine Genital Campylobacteriosis.
**Introduction:** Bovine Genital Campylobacteriosis is worldwide distributed and responsible for reproductive losses with a significant economic impact. However, without effective vaccines available globally, the control of Bovine Genital Campylobacteriosis has been challenging. This study aimed to develop a multi-epitope vaccine against *Campylobacter fetus* subsp. *venerealis* using a reverse vaccinology approach.
**Materials and Methods:** The proteome of *C. fetus* subsp. *venerealis* strain NCTC 10354 was screened for the identification of potential vaccine targets. A total of 1849 proteins were evaluated for subcellular location, virulence potential, allergenic potential, antigenicity, physicochemical properties, similarity to the host proteome, and transmembrane helices. From these, two proteins were selected for prediction of B-cell and T-cell epitopes. The conservancy of the antigenic, non-allergenic, non-toxic and water-soluble epitopes was assessed in 31 *C. fetus* subsp. *venerealis* strains. The most suitable epitopes were then used to design a multi-epitope vaccine, which was analysed for antigenicity, allergenicity, toxicity, solubility and physicochemical properties. The vaccine tertiary structure was predicted and subjected to model refinement, and stability was improved through disulfide engineering. Codon optimisation was performed for an efficient expression in *Escherichia coli* K12 and cloning was simulated *in silico* using a pET-30a(+) vector. *In silico* immune simulations were carried out using C-ImmSim software.
**Results:** The proteins FliK and OmpA were selected as the most promising targets for vaccine development. From these proteins, 15 conserved epitopes were selected for vaccine design. The vaccine included a multi-epitope fragment of 241 amino acids comprising 2 epitopes from OmpA and 13 from FliK linked by GPGPG linkers, connected to the cholera toxin subunit B by an EAAAK linker. The vaccine construct was predicted to be antigenic, non-allergenic, non-toxic, and soluble upon overexpression. The protein sequence was successfully cloned *in silico* into a PET-30a(+) vector. Additionally, the immune simulation evidenced an efficient stimulation of the immune response after exposure to the vaccine.
**Conclusions:** Overall, this study developed a novel vaccine candidate suitable for further *in vitro* and *in vivo* experimental validation, which may become a useful tool for the control of Bovine Genital Campylobacteriosis.
**Acknowledgements:** This study was supported by Fundação para a Ciência e a Tecnologia (FCT), Project PTDC/CVT-CVT/30145/2017; Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Project UIDB/00276/2020 and Associate Laboratory for Animal and Veterinary Science (LA/P/0059/2020 - AL4AnimalS).

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## 186
## Postpartum uterine disease and granulosa cell inflammation impact bovine ovarian function and cellular steroidogenesis

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**Application:** The research conducted here investigates how uterine diseases in dairy cattle contribute to subfertility.
**Introduction:** Postpartum uterine disease (UTD) occurs in approximately 40% of dairy cows (Sheldon et al., 2009). UTD is caused by pathogenic bacteria and associated with follicular fluid lipopolysaccharide (LPS) accumulation. We hypothesised that UTD and granulosa cell inflammation compromise follicular and corpus luteum function.
**Materials and Methods:** Uterine health status was assessed in 17 primiparous, lactating Holstein cows by observing vaginal discharge. Animals were categorised as having UTD before (metritis) or after (endometritis) postpartum d 14. Blood collection and transrectal ultrasonography were performed daily from 7 to 35 d postpartum to evaluate oestradiol, progesterone, size of the largest follicle and corpus luteum after ovulation. Analysis was performed with the mixed procedure of SAS with fixed effects of treatment, day, and appropriate interactions. To determine if granulosa cell inflammation impacts luteal cell steroidogenesis, abattoir derived granulosa cells were initially cultured with LPS (0, 0.1, 1, and 10 μg/mL; $n = 6$). After 24 h, granulosa cells were cultured for 8 d in the absence of treatment in luteinisation medium supplemented with 10 μM forskolin and 2 μg/mL insulin. Cell cultures were designed as a randomised complete block and analysed with the mixed procedure of SAS with fixed effects of treatment, day, and the interaction.
**Results:** Disease before or after d 14 did not affect the time to ovulation or the diameter of the preovulatory follicle. Metritis tended ($P = 0.08$) to reduce follicular growth from d 7 until d 17 postpartum compared with cows without metritis. There was no effect of metritis on oestradiol concentration during the period from d 7 until 17 d postpartum compared with cows without metritis. Luteal growth was not

affected by disease in either period. There was a day post-ovulation by endometritis interaction for progesterone whereby cows with endometritis had lower progesterone initially, but greater progesterone by 10 d. ($P < 0.001$). After 24 h, LPS increased cellular IL6 and *IL8* expression, and reduced oestradiol accumulation by 93.3% compared with medium alone ($P < 0.05$). After 8 d, progesterone accumulation was reduced if granulosa cells had been previously exposed to 1 or 10 μg/mL LPS ($P < 0.05$). The expression of genes associated with progesterone synthesis (*STAR* and *HSD3B1*) and cholesterol abundance were not affected in luteal cells after LPS exposure ($P > 0.05$).
**Conclusions:** These results indicate that UTD and granulosa cell inflammation influence ovarian function and may contribute to subfertility.
**Acknowledgements:** USDA-NIFA 2020-67015-21015.

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