AETE

Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

40ème COLLOQUE SCIENTIFIQUE

40th SCIENTIFIC MEETING

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Patrick Lonergan

Special Celebration

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Brescia, Italy, 5th and 6th September 2024

AETE

Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

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It is our great pleasure to welcome you to the Annual Scientific Meeting of AETE in the beautiful city of Brescia, Italy as we celebrate the 40th anniversary of our association. Brescia is not as famous as Roma, Venice, or Firenze but, as you could imagine, every Italian city has something special to offer. This year, Brescia, together with Bergamo, is the Italian capital of culture. This has unfortunately a sad reason: these two cities suffered severely during the Covid pandemic, but fortunately that's now just an old memory…

The city is connected to the most important European cities with 3 international airports: Linate, Bergamo, and Verona. A one-hour drive will take you to the beautiful Lake Garda and Lake Iseo. Brescia is also not far from Milan, Venice, and Verona and the city is very close to the wonderful Alpes. The "Centro Pastorale Paolo VI", located in the center of Brescia, will host us for the two-day meeting. The building dates back to the 17th century with a typical expression of Baroque art. Hoping for two days with sunny weather, the ancient garden is great to socialize and to share experiences with a cup of good Italian coffee.

The highlight of this year is the pre-conference workshop that will kick off our annual event. The workshop will be held at the agriculture high school ITAS G. Pastori, not far from the Paolo VI. Here all the facilities for theoretical and practical sessions are at our disposal, including a farm with 50 dairy cows.

A short walk from the Paolo VI will take us to the Piazza Arnaldo where we will have our Gala dinner enjoying music, good food and wine. Don't miss it! In the center of Brescia you will find some beautiful architecture such as the old cathedral (12th century), the new cathedral and several cozy squares. You can also visit the castle of Brescia built in the 13th century or have a walk in the beautiful piazza della Loggia where we will have the farewell party.

Dear colleagues, this is only a small part of what Brescia offers. Let's make this 40th anniversary meeting a memorable and inspiring celebration!

Pierluigi Guarneri
On behalf of the LOC

Marja Mikkola
On behalf of the Board of Governors

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All the original articles and abstracts are published in Animal Reproduction.
For citation purposes, Animal Reproduction should be the original source (**Animal Reproduction 2024, vol. 21, issue 3 …..**)

SUPEROVULATION / OPU-IVP / ET

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Dr. Patrick Lonergan

A.E.T.E. Medalist 2024

Commendation of Dr. Patrick Lonergan

for the AETE Pioneer Award 2024

The Association of Embryo Technology in Europe (AETE) has decided to give the AETE Pioneer Award 2024 to

Patrick Lonergan

Professor Pat Lonergan was born January 21st, 1967 in Dublin, Ireland, to Olive and Christy Lonergan. Pat is one of four children, he has two sisters, Helen and Tríona and one brother Gerard.
Pat's interest in agriculture began during childhood, working summers on his uncle Tom Lonergan's dairy farm in County Tipperary. Inspired by his uncle and supported by his parents, Pat enrolled at University College Dublin (UCD) in 1984, earning a bachelor's degree in Agricultural Science five years later. As part of his studies, he completed a practical year gaining hands-on experience in dairy, beef, sheep and pig production at UCD's Lyons Farm, near Dublin. Pat continued his academic journey at Lyons Farm, pursuing a Master's and PhD from 1989 to 1992 under the supervision of Professor Ian Gordon's, recipient of the 1995 AETE Pioneer Award. His postgraduate research focused on factors influencing *in vitro* embryo production (IVP), including morphology, follicle size, follicular fluid supplementation, and alternatives to serum, contributing significantly to the field's progress.

In March 1991, I received a COMNET EU-scholarship for a nine-month placement at UCD Lyons Farm, in Professor Gordon's laboratory, focusing on IVP. It was there that I first met Pat. My limited English was sufficient for daily needs but not for scientific discussions or understanding Prof. Gordon's detailed IVF manuals; Pat's enthusiastic and patient explanations of the IVP procedure greatly boosted my confidence. During this period, Pat's pioneering PhD research on priming ovaries with FSH before collection for IVF, culminated in his widely cited 1994 paper (Lonergan et al. 1994), and my first of many co-authored papers with Pat. During that time, Trudee Fair, who would later become Pat's wife and a distinguished scientist in follicle growth and oocyte competence, also joined Professor Gordon's lab at UCD's Lyons Farm, to undertake her Master's Degree. Together with Teresa Mogas and several other lab members, we shared memorable international Friday night dinner parties, often concluding with Irish coffees and indescribable dancing. It was a special chapter in all our lives.

After completing his PhD in 1992, Pat secured a one-year post-doctoral position at the Norwegian Veterinary School in Oslo. Following which, he moved to the Institut National de la Recherche Agronomique (INRA) in Nouzilly near Tours, France, where he spent the next four years collaborating with Pascal Mermillod on various aspects of oocyte and embryo development *in vitro*. During this period, he also participated in a European FP3 Consortium grant ('Ex Ovo Omnia'), coordinated by Franz Dessy at Louvain Le-Neuve, Belgium. Pat worked with and learned from many esteemed Scientists at INRA_Nouzilly, such as Pierre Mauléon, Marc-Antoine Driancourt, Danielle Monniaux, Yves Cognie, Gerard Baril, Eric Palmer, Yves Combarnous, Michel Terqui, Francoise Martinat-Botté, Francoise Berthelot, Jean-Louis Dacheux, Michel Courot, Jean-Pierre Signoret, Philippe Chemineau, Marie-Thérèse Hochereau-de Reviers, and many others, each contributing significantly to his professional growth and development.

In 1996, Trudee completed her PhD in Copenhagen and joined Pat in Nouzilly, they were married that year and their first child, Tadhg, was born in 1997 in Tours. The family returned to Ireland in Summer 1997, where Pat took up an EU-funded fellowship at UCD, with Maurice Boland (AETE Pioneer Awardee 2011). By September 2001, Pat was appointed as a College Lecturer in the Department of Animal Science and Production, and in 2009 was promoted to a Full Professor. During this period, I maintained close contact with Pat, and in September 1999, I returned to UCD Lyons Farm. It felt like time had stopped, everything unchanged since 1992. Pat, was now not just my friend, but also my PhD Mentor, this brought both joy and anxiety, in case I was not going to meet his expectations. Looking back, I can confidently say that I've done well. I hope Pat agrees with that sentiment. Together, we've co-authored 74 papers out of his >350 publications, a fact that fills me with immense pride. I look forward to increasing this number in the years ahead, knowing we have enough time until retirement.

Scientific collaborations have been, and continue to be, the cornerstone of Pat's distinguished career. He has forged extensive networks of colleagues both in Ireland and internationally, including Trudee Fair, Sean Fair, Stephen Butler, David Kenny from Ireland, Alfonso Gutiérrez-Adán, Spain, Fuller Bazer, Tom Spencer, Cindy Tian, USA. This is just a glimpse into the breadth of his collaborative efforts, which span numerous institutions and countries worldwide.

Pat dedicated much of his early career to optimising IVP, but his focus shifted in the early 2000s to maternal-embryo communication. He employed a combination of superovulation, artificial insemination, and *in vivo* culture in the sheep oviduct to delineate the impacts of oocyte maturation, fertilization, and embryo culture—both *in vivo* and *in vitro*—on embryo yield and quality. This work remains among the most cited papers, a source of great pride to me, as it was one of my PhD chapters (Rizos et al. 2002). The findings confirmed that oocyte quality predominantly determines blastocyst yield *in vitro*, whereas post-fertilization culture conditions primarily influence blastocyst quality. Building on these insights, Pat collaborated with Urban Besenfelder from the University of Vienna, utilizing Urban's sophisticated endoscopic transfer technique to conduct reciprocal transfers between *in vitro* and *in vivo* conditions.

Later, together with Jim Roche, Niamh Forde, and others, Pat investigated the impact of circulating progesterone on uterine biology and embryo development, leading to significant publications from 2009 to 2020 that established him and his team as leaders in this field. One pivotal study explored the global transcriptome of the endometrium from Day 5 to Day 16 in pregnant and cyclic cattle, examining normal and elevated progesterone conditions. This research illuminated how progesterone levels regulate endometrial gene expression, with low progesterone correlating to an altered endometrial transcriptome and delayed conceptus elongation. Furthermore, in another study he demonstrated that the embryo could benefit from elevated progesterone levels in the uterus even when not present during this period, highlighting progesterone-induced changes in the endometrial transcriptome as pivotal for conceptus growth.

Between 2011 and 2012, Pat conducted pioneering RNA Seq studies on bovine embryos, exploring their transcriptomic profile from blastocyst on Day 7 to elongated conceptus on Day 16. These findings were integrated with data from endometrial studies, creating a comprehensive profile of potentially secreted molecules in the conceptus that interact with endometrial receptors during the critical maternal recognition of pregnancy.

From 2010 to 2020, he also investigated how conceptus-induced changes alter the endometrial transcriptome, aiming to pinpoint when cows recognize pregnancy. By comparing endometrial samples from cyclic and pregnant heifers on Days 5, 7, 13, and 16, his research revealed that detectable differences first appeared on Day 16, largely attributed to conceptus-derived interferon-tau. In contrast, *in vitro* studies by Pat's group in 2018 and 2019 showed that exposing endometrial explants to oocytes, 2-cell embryos, or Day 5 morulae did not significantly alter their relative abundance. In addition, the group has shown that temporal changes in the endometrial transcriptome and uterine fluid composition are crucial for establishing uterine receptivity to implantation and ensuring successful pregnancy. These changes are orchestrated by conceptus-derived IFNT and maternally-derived progesterone from the corpus luteum, inducing gene expression in uterine luminal and superficial glandular epithelia. This supports transport and secretion into the uterine lumen, crucial for conceptus growth and development.

Pat's dedication to cattle reproduction remains unwavering. Recently, from 2021 to 2024 with preliminary results presented in abstracts and posters by his current PhD students, he conducted large-scale embryo transfer studies on commercial farms. These studies demonstrate that while beef-cross calves hold greater economic value compared to male dairy calves, there is potential for further economic gains through the transfer of purebred beef embryos. In addition, comparable pregnancy rates between fresh embryo transfers and AI in lactating dairy cows were achieved. A complimentary study on the incidence and timing of pregnancy loss highlighted greater losses from Day 32 to Day 62 following ET of IVP embryos, particularly with frozen embryos, while losses after Day 62 were minimal.

Pat's infectious curiosity, meticulous attention to detail and generous support have not only inspired me, but numerous other students to continue working in the field of Reproductive Biology. While his mentorship has fostered many lifelong friendships as well.

Pat's passion for science is boundless, but he also possesses another great passion, birdwatching. His dedication to observing and studying various bird species is truly inspiring, reflecting his keen eye and patience. Pat can identify numerous birds by their songs, behaviors, and appearances, often discerning subtle differences that others might overlook. His enthusiasm not only enriches his own life but also educates and inspires those around him, fostering a deeper appreciation for nature and its avian wonders. May his birdwatching adventures continue to bring him joy and fulfillment, opening new opportunities for discovery and connection with the natural world. His passion for birdwatching beautifully complements his professional life, showcasing the diverse interests and talents he brings to both work and personal pursuits.

To date, Pat has authored over 350 refereed international articles, supervised more than 45 PhD students and postdoctoral fellows, and secured over €15 million in funding from the EU, Science Foundation Ireland, and the Department of Agriculture in Ireland for research projects. This high output was recognized by the award of a Doctor of Science, Degree from the National University of Ireland in 2005. He has served as Associate Editor of Biology of Reproduction and Reproduction Fertility and Development and has been invited to give Keynote Lectures at >50 International conferences/institutes worldwide. He is ranked Number 1 in Ireland and Number 13 globally in Animal and Veterinary Sciences on Research.com, which ranks researchers based on a scientist's Discipline. While he is ranked in the top 2% of scientists in his field world-wide according to Stanford University listing, a publicly available database of top-cited scientists. His contributions to our field have been recognized with numerous honors and esteemed leadership positions. Notably, Pat was elected to the Elected Member of the Royal

Irish Academy in 2012, served as President of the International Embryo Technology Society in 2016, Elected Associate Member of the European College of Animal Reproduction in 2016, and held the position of Secretary for the AETE from 2004 to 2009, among others.

Pat Lonergan and Trudee Fair have built a beautiful family together, with four lovely children who bring immense joy and pride to their lives. Their three boys, Tadhg, Hugh, and Myles (who is also my beloved godchild), are energetic and full of life. Each one has their unique personality and interests, contributing to the dynamic and loving atmosphere of their home. Completing their family is their daughter, Iseult, who adds a special touch of sweetness and charm. The close-knit bond they share as a family is truly heartwarming, and it's always a delight to see them supporting and caring for each other in every aspect of life.

Pat's passion and dedication, both professionally and personally, are truly commendable. Pat, your commitment to excellence in your work and your enthusiasm for birdwatching exemplify the remarkable balance you maintain between your career and personal interests. You inspire those around you to strive for their best and to pursue their passions with the same sincerity. It's an honor for me to call you not only a colleague but also a good friend. Keep up the fantastic work and continue to let your passions shine.

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September 2024

Animal Reproduction

Thematic Section: 40th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryo transfer: past, present, future – a personal perspective

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How to cite: Lonergan P. Embryo transfer: past, present, future – a personal perspective. Anim Reprod. 2024;21(3):e20240068. https://doi.org/10.1590/1984-3143-AR2024-0068

Abstract

Embryo transfer is just one of a range of assisted reproductive technologies – often the last one in a sequence of others – that has revolutionised the cattle breeding industry. The number of in vitro-produced embryos transferred annually now surpasses the number derived by traditional superovulation by a factor of four. Although issues with cryotolerance of IVP embryos, embryo loss, and, in some cases, calf birth weight remain to be fully resolved, IVP embryos are likely here to stay as a tool for genetic improvement in dairy herds, offering increased flexibility in sire usage allowing multiple pregnancies from elite dam-bull combinations to be generated and the ability to produce more embryos per unit time than traditional superovulation. What follows is a short personal look back at the last 30 years; if you are looking for deep insights into the underlying biology regulating embryo development, this is not the place to look! Please refer to some of the excellent recent reviews and research papers cited herein.

Keywords: assisted reproductive technologies, bovine, IVF.

Introduction

When I received an email from the AETE President Marja Mikkola informing me that I had been selected to receive the 2024 Pioneer Award I have to admit that I was shocked, humbled and a little embarrassed, all at once – shocked, because it was completely unexpected; humbled, because I know the calibre of those who have previously received the award (see Table 1); and embarrassed, not because of false modesty, but because I genuinely consider there are many people more deserving than me. Furthermore, such awards are normally reserved for those near the end of their careers; I hope I still have a few productive years left! Nonetheless, it is a huge honour for me to accept the award and to be listed among those eminent previous recipients. It is a further delight to have my great friend and colleague, Dimitrios Rizos, himself a past President of AETE, present the commendation.

Having to write this short paper has allowed me to pause, 'take stock' and look back over my career to date. I remember very well, as a naïve PhD student, attending my first scientific conference (the annual meeting of the International Embryo Technology Society, IETS, in Bournemouth, UK, in January 1991). Not really knowing what to expect, on the first morning, myself and a fellow PhD student from Malaysia, Sharif Haron, walked from our hotel to the conference centre behind two 'cowboys' adorned with Stetsons and cowboy boots (pretty sure it was Charles Looney and Brad Stroud from Texas); we wondered what we were letting ourselves in for! I was starstruck seeing some of my 'heroes' from the literature for the first time, and realising that they were all (relatively!) normal people. This may be difficult for younger researchers to appreciate given the immediacy of information available online today, where everyone has a web presence in one form or another, but in the pre-internet world, at the risk of sounding old, things were very different.

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Received: May 20, 2024. Accepted: June 14, 2024.
Financial support: The author's work has been funded over the years from a variety of sources including Science Foundation Ireland, Department of Agriculture, Food and The Marine, Irish Research Council, and the European Commission.
Conflict of interest: The author has no potential conflicts of interest to disclose.

Table 1. Past locations of annual meetings of the AETE and past recipients of the AETE Pioneer Award.

Year	Meeting	Venue	Recipient
1984-1990	1-6	Lyon, France	-
1991	7	Cambridge, UK	-
1992	8	Lyon, France	-
1993	9	Lyon, France	Joachim Hahn, Germany
1994	10	Lyon, France	Charles Thibault, France
1995	11	Hanover, Germany	Ian Gordon, Ireland
1996	12	Lyon, France	Steen Willadsen, UK
1997	13	Lyon, France	Robert Moor, UK
1998	14	Venice, Italy	Pierre Mauléon, France
1999	15	Lyon, France	Alban Massip, Belgium
2000	16	Santander, Spain	Robert Cassou, France
2001	17	Lyon, France	Josef Fulka, Czech Republic
2002	18	Rolduc, Netherlands	AE 'Tony' Wrathall, UK
2003	19	Rostock, Germany	Ian Wilmut, UK
2004	20	Lyons, France	Torben Greve, Denmark
2005	21	Keszthely, Hungary	Jean-Paul Renard, France
2006	22	Zug, Switzerland,	Ray Newcomb, UK
2007	23	Alghero, Sardinia	Steph Dieleman, Netherlands
2008	24	Pau, France	Gottfried Brem, Austria
2009	25	Poznan, Poland	WR 'Twink' Allen, UK
2010	26	Kuopio, Finland	Yvan Heyman, France
2011	27	Chester, UK	Maurice Boland, Ireland
2012	28	St Malo, France	Danielle Monniaux, France
2013	29	Istanbul, Turkey	Tom McEvoy, UK
2014	30	Dresden, Germany	Klaus-Peter Brüssow, Germany
2015	31	Ghent, Belgium	Michel Thibier, France
2016	32	Barcelona, Spain	Henrik Callesen, Denmark
2017	33	Bath, UK	Cesare Galli, Italy
2018	34	Nantes, France	Patrice Humblot, France
2019	35	Murcia, Spain	Poul Hyttel, Denmark
2020	36	2020 Online	-
2021	37	2021 Online	-
2022	38	Utrecht, Netherlands	Hilary Dobson, UK
2023	39	Heraklion, Greece	Sabine Meinecke-Tillmann and Burkhard Meinecke, Germany
2024	40	Brescia, Italy	Patrick Lonergan, Ireland

At that conference, there were talks about alternative gonadotrophins for superovulation in cattle (Maurice Boland, Ireland), a new (!) technique called transvaginal ultrasound guided follicular aspiration of bovine oocytes (Martin Pieterse, The Netherlands), follicular dynamics in sheep and cattle (Marc-Antoine Driancourt, France), turnover of dominant follicles in cattle (Jim Roche, Ireland), oocyte maturation and sperm transport in superovulated cattle (Poul Hyttel, Denmark), cryopreservation of ova and embryos from livestock (Heiner Niemann, Germany), recipient management and embryo transfer (Peter Broadbent, UK), velogenetics for the reduction of generation interval (Michel Georges, Belgium), nuclear transplantation in cattle (Steen Willadsen, Denmark) and a look forward to the next 100 years of embryo transfer (George Seidel, USA). What a stellar line-up of speakers and topics! It was fantastic and I was hooked! As a result, I have only missed two IETS conferences since 1991, have served on the Board of Governors and was lucky enough to be elected President in 2009.

Later the same year, I attended my first AETE conference (the 7th meeting of the association) in Cambridge, UK, in September 1991. This was the first time the meeting was held outside of its

birthplace in Lyon, France (Ponsart et al., 2009; Thibier 2014). I drove from Ireland with a fellow PhD student, picking up Dr Ke-Huan Lu en route, a previous student of my PhD mentor, Prof. Ian Gordon, and one of the first to produce a calf from an IVF-derived embryo (Lu et al., 1987, 1988). It was a great meeting and my first exposure to those involved in embryo research in Europe. Back then, Michel Thibier, a central figure in the establishment of the AETE and President that year, would do a simultaneous translation into French of each presentation! There were invited talks on aspects of embryo production in vivo and in vitro (Heiner Niemann, Germany), rapid cryopreservation of bovine embryos (AM de Leeuw, The Netherlands), and extraspecies embryo transfer in equids (WR 'Twink' Allen, UK). On the centenary of the first paper to be published on embryo transfer in mammals (Heape, 1891), Chris Polge (UK), who is widely credited with discovering the cryoprotective properties of glycerol (Polge et al, 1949) – although apparently similar work by others predates it (Bernstein and Petropavlovski, 1937; Rostand, 1946) – and who had been instrumental in bringing the meeting to Cambridge, delivered the Walter Heape Memorial Lecture on novel reproductive biotechnologies. In that paper, he described the opportunities for large scale production of embryos in vitro, the potential of sex-sorted semen, embryo multiplication by nuclear transfer and genetic modification to create transgenics. In the three decades since he gave that presentation, many of these technologies have become well established in the tool box of assisted reproductive technologies available to farmers.

Figure 1. Prof. Ian Gordon (1928-2021) – a true pioneer in the field of livestock reproduction. Despite his international reputation and prolificacy in terms of publications, few published photos exist of him.

I attended most of the AETE meetings in the subsequent years and served on the Board from 2000 to 2007 (Secretary from 2002 to 2007). This was a very enjoyable time working with colleagues from across Europe; as Secretary and the only native English speaker on the Board at the time, I had the pleasure of editing the abstracts each year and through this activity I got to know all of the individuals involved in domestic animal embryo work in Europe. While I have not been able to attend as many meetings as I would have liked in recent years, I have dipped in and out at regular intervals and am happy to see that the AETE continues to be a very friendly group with great science and great social events.

Early beginnings - my introduction to the world of embryos

I owe a lot to my late uncle, Tom Lonergan, my father's brother, with whom I spent all of my childhood summers, working on his small dairy farm in County Tipperary in the south of Ireland. This is where my interest in agriculture was born. Mainly because of him, and with my parents' encouragement, I entered University College Dublin (UCD) as a student in autumn 1984, graduating five years later with a Bachelor's Degree in Agriculture. At the time, students were obliged to do a full 'practical year' (now called professional work experience) to gain experience in all of the main farm enterprises (dairy, beef, sheep, pigs and tillage). I spent much of that year at the University's Lyons Farm, located some 25 km from the main UCD campus in Dublin, which was later to become my main place of work.

I have always loved the challenge of identification. Outside of work, my passion is bird identification and I spend whatever spare time I have either immersed in books on bird identification or in the field watching birds. During the early part of my undergraduate degree in Agricultural Science at UCD, two modules stuck out – one was Agricultural Botany which involved the identification of common grasses and weeds and the other was Plant Pathology which involved, in part, identifying diseases on the leaves of a variety of crop plants. This was done using an identification key and I loved the challenge. I was convinced that I would follow this subject after graduation and become a plant pathologist. Then, in final year, we had Prof. Ian Gordon (Figure 1), himself a past recipient of the AETE Medal (1995) and the IETS Pioneer Award (1998), for the subject of Animal Reproduction. His lectures were an inspiration! One of his greatest characteristics as a teacher was his encyclopaedic knowledge of the literature. As undergraduate students we were riveted by his fascinating lectures, as he regaled us with entertaining anecdotes and stories from his own experience and from the literature (collecting urine from nuns in Italian convents to purify human menopausal gonadotropin was particularly memorable!). My 'passion' for plant pathology was quickly forgotten and I decided that further study in mammalian reproduction was for me.

I started my venture into the world of bovine embryology in 1989 when I began a Masters under the supervision of Prof Gordon, or 'Prof' as he was affectionately known by all of his graduate students. Gordon graduated from Nottingham University in 1951 before embarking on a prolific scientific career under the guidance of Sir John Hammond at Cambridge, widely regarded as the father of modern animal physiology. Gordon was a true pioneer in the field of livestock reproduction, particularly in the area of oestrous synchronisation, superovulation and non-surgical embryo transfer and, latterly, in the area of in vitro embryo production in cattle. He was a prolific author, publishing many scientific articles with some 280 to his credit, but in his later years at UCD, from where he retired in 1993, and in his retirement, he published 6 books. His first book, *Controlled Breeding in Farm Animals,* was published in 1983. This later evolved into a four-volume series entitled *Controlled Reproduction in Farm Animals* separately dealing with Cattle and Buffalos (1996), Sheep and Goats (1997), Horse, Deer and Camelids (1997) and Pigs (1999). The second edition of his book *Reproductive Technologies in Farm Animals*, first published in 2004, was published as recently as 2017. In addition, his masterpiece, *Laboratory Production of Cattle Embryos* was first published in 1994 with a second edition published in 2003. This tome was a true 'one-stop-shop' covering everything from the historical developments in IVF technology in cattle, through detailed chapters on oocyte recovery, oocyte maturation, sperm capacitation, IVF, embryo culture, cryopreservation, to embryo transfer. Although, a little dated given the rapid progress over the past 20 years, this book is still a 'must have' for anyone working in the area. And remember, all of this was written by painstakingly manually wading through hard copies of published journals and books of abstracts, without the aid of a 'Google Search' or 'PubMed' which we all rely on so much today. Ian Gordon died in 2021; indeed that was a bad year for domestic animal reproductive biologists – as well as Gordon, we lost George Seidel (Colorado State University), Twink Allen (University of Cambridge) and Keith Inskeep (West Virginia University), all pioneers in their own right.

During my Masters, I was based in what became known as the 'IVF Lab' at UCD's Lyons Research Farm. Little did I know then that I would, after a few years as a post-doc abroad, spend my entire career at Lyons. We were (and still are) lucky to be located very close to several major abattoirs from which, through their continued support, we have unlimited access to ovaries for research. During my Masters, I investigated various factors affecting the production of embryos in vitro including the effect of breed cross of donor, stage of the oestrous cycle and donor age on oocyte yield, the effect of follicle size on oocyte diameter and meiotic competence, the effect of temperature (10 v 30 degrees) and duration of storage (up to 24 h) of ovary collection on oocyte quality and the optimum heparin concentration for IVF.

After my Masters, I continued in the area of embryo production in vitro and completed a PhD, also under Prof Gordon's supervision, investigating various factors affecting in vitro embryo production including the effect of cumulus-oocyte-complex morphology, follicle size, follicular fluid supplementation and alternatives to serum during IVM on the outcome of IVP. In addition, we investigated the effect of priming the ovary with FSH prior to ovary collection on blastocyst yield after IVF. My first paper (and second most cited) was published in 1994 on the effect of follicle size on bovine oocyte quality and developmental competence following

maturation, fertilization, and culture in vitro (Lonergan et al., 1994). Looking back, it was a very simple study – very different from what is expected by journals nowadays.

It was at Lyons that I met and fell in love with Trudee – while I was finishing my PhD, she completed a Masters. We were very fortunate to be among Gordon's last cohort of graduate students. Amongst the meetings we attended together was the 12th International Congress on Animal Reproduction (ICAR) in The Hague in August 1992 – another feast of excellent science. Amongst the line-up of speakers, a young Poul Hyttel (AETE Pioneer in 2019) from the then Royal Veterinary and Agricultural University (KVL; now, the University of Copenhagen) gave a fantastic workshop on oocyte growth and development including beautiful electron microscopy images of oocytes during maturation in vivo and in vitro. Trudee decided almost then and there that she was going to do a PhD with Poul and in 1993 she was awarded one of the first EU Marie Curie International Fellowships to do just that (in collaboration with Torben Greve, AETE Pioneer in 2004). So began a lifelong friendship with Poul and Trudee's research career in oocyte biology.

In November 1992, after completing my PhD, I took up a one-year post-doctoral position at the Norwegian Veterinary School in Oslo working with Wenche Farstad. That was a fantastic year and gave me the opportunity to get to know all of Scandinavia; leaving Oslo at 4 am to drive to the nearest abattoir, in Hamar, was a bit of a shock to the system, as was getting stopped by the police for speeding at 6 am 'in the middle of nowhere' and receiving a very hefty fine (honestly officer, I wasn't going that fast)! I subsequently moved to the Institut National de la Recherche Agronomique (INRA) in Nouzilly near Tours in the Loire Valley, France, where I spent the next four years working with Pascal Mermillod on various aspects of oocyte and embryo development in vitro. INRA was a great place to work. Once I 'mastered' French, with the persistence and indulgence of people like Pascal, Nati Poulin ('Eh ben dis donc!') and others, I integrated completely. At the time, as is still the case today, INRA-PRMD (Physiologie de Reproduction des Mammifères Domestiques) was a melting pot of leading experts and their teams working on all aspects of mammalian reproduction including the recently retired Pierre Mauléon (first President of AETE), Marc-Antoine Driancourt (follicle development), Danielle Monniaux (follicle growth), Pascal Mermillod (oocyte maturation, bovine IVF), Yves Cognie and Gerard Baril (small ruminant reproduction), Eric Palmer (equine reproduction), Yves Combarnous (Molecular Endocrinology), Michel Terqui, Francoise Martinat-Botté and Francoise Berthelot (pig reproduction), Jean-Louis Dacheux (epididymis function), Michel Courot (andrology and male fertility), Jean-Pierre Signoret (animal behaviour), Philippe Chemineau (photoperiod), Marie-Thérèse Hochereau-de Reviers (spermatogonial stem cells) and many others. In addition, through involvement in a European FP3 Consortium grant ('Ex Ovo Omnia') during my time at INRA, coordinated by Franz Dessy at Louvain Le-Neuve in Belgium, I got to know many new colleagues from across Europe.

While in France, in 1994, KVL offered an oocyte/embryo-focused summer school in Copenhagen. This was a fully funded week-long series of lectures and practicals with many of the leading figures in oocyte and embryo biology at the time (including, from memory, Ian Wilmut, Jan Motlik, Taku Nagai, Barry Bavister, Heiner Niemann, Don Rieger, Frank Barnes, and many others). We had lectures by day and socialised at night in a very informal atmosphere that allowed students to rub shoulders with some of the 'greats' of the day; it was wonderful.

Trudee finished her PhD in Copenhagen in 1996 and came to France for the last year or so; during that time we got married and had our first child, Tadhg, who was born in Tours. We returned to Ireland in the summer of 1997 and that autumn I returned to UCD as a Post-doc working with Maurice Boland (also a recipient of the AETE Pioneer Award, in 2011) and was appointed to the faculty in the Department of Animal Science and Production in September 2001, where I have happily remained to this day.

Importance of collaboration

Collaboration has played a major role in my career. I have been lucky to establish a large network of excellent colleagues at home and internationally through national and international grants as well as through one-to-one contacts. This has resulted in the publication of many papers together and the co-supervision of a large number of graduate students. Apart from Trudee, with whom, at the time of writing, I have co-authored 72 papers, Dimitrios and I have published 74 papers together since our first in 1994 when he was a visiting Erasmus student and then from his PhD at UCD before he moved

to INIA-Madrid in 2004. Other notable collaborators include Sean Fair, University of Limerick (51 papers), Alfonso Gutiérrez-Adán, INIA-Madrid (44 papers), Stephen Butler, Teagasc (42 papers), David Kenny, Teagasc (33 papers), and Tom Spencer, University of Missouri (28 papers). There are many others – too many to mention – but of course, most of these publications arose from the hard work of a team of excellent graduate students (Table 2).

Table 2. List of graduate students (co-)supervised.

Student	Year	Masters/PhD	Thesis title
Martina O'Kearney Flynn	1998	Masters	Culture of bovine embryos in vitro
Garret Byrne	1999	Masters	Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and in vitro
Brian Enright	1999	Masters	Culture of in vitro produced bovine zygotes in vitro vs in vivo: implications for early embryo development and quality
Michael O'Leary	2001	Masters	Effect of Organic and Inorganic Selenium Supplementation on aspects of Reproduction and Tissue Concentration
Mark Kingston	2003	Masters	Factors Affecting Embryo Production in Cattle
Wendy Griffin	2004	Masters	The Effect of Dosing Propylene Glycol to Dairy Cows During the Early Postpartum Period, or to Heifers on Metabolic and Developmental Parameters Related to Fertility
Lisa Burke	2004	Masters	The Developmental Competence of Oocytes in Dairy Heifers and Cows
Catherine Foley	2005	Masters	In vitro production of bovine embryos from single oocytes
Adam Woods	2006	Masters	The Effect of Embryo Source and Recipient Progesterone Environment on Embryo Development in Cattle
Catherine Lawson	2008	Masters	The effect of omega-3 polyunsaturated fatty acids on early embryo development in cattle
Miriam de Feu	2008	Masters	The Effect of Genotype And Management Factors on Fertility And Nutrition in The Modern Holstein - Friesian Dairy Cow
Lydia O'Hara	2009	Masters	Effect of storage duration, storage temperature and diluent on the viability and fertility of fresh ram sperm
Michael McDonald	2014	Masters	The relationship between ear temperature and onset of oestrus and ovulation in beef heifers
Nicola Gillespie	2014	Masters	Taught Masters in Animal Reproduction
Christopher Johnston	2014	Masters	Taught Masters in Animal Reproduction
John Doyle	2014	Masters	Taught Masters in Animal Reproduction
Niamh Cantwell	2019	Masters	Studies affecting in vitro development of bovine oocytes and embryos
Evelyn Drake	2020	Masters	Evaluation of delayed timing of artificial insemination with sex-sorted spermatozoa on pregnancy/artificial insemination in seasonal calving, pasture-based, lactating dairy cows
Rachel White	2022	Masters	Incidence and treatment of endometritis and effect of dietary marine seaweed extracts on reproductive function in spring-calving, pasture-based lactating dairy cows
Jane Kennedy	2023	Masters	Factors affecting in vitro production of bovine embryos
Fabian Ward	2002	PhD	The Use of Ovum Pick-Up in Association with In Vitro Maturation, Fertilisation and Culture as an Aid to Improved Reproduction in Cattle
Dimitrios Rizos	2002	PhD	Studies on Development, Cryotolerance, Ultrastructural Morphology and Gene Expression in Bovine Embryos Produced In Vivo or In Vitro
Serafeim Papadopoulos	2003	PhD	Studies in the Production of Ruminant Embryos
Sean Fair	2005	PhD	Ewe breed difference in fertility after AI with frozen semen

Table 2. Continued...

Student	Year	Masters/PhD	Thesis title
Ciara O'Meara	2005	PhD	Evaluation of semen for artificial insemination of sheep
Deirdre Corcoran	2006	PhD	Gene expression during development in bovine embryos
Niamh Forde	2007	PhD	Genomics of ovarian follicle development
Anna Zielak	2007	PhD	Identification of novel genes regulating ovarian follicle development
Fiona Carter	2009	PhD	Gene expression during bovine oocyte maturation and early embryo development
Naomi Smith	2009	PhD	Impact of maternal nutrition during gestation on offspring health and development
Lorraine Richardson	2011	PhD	Cervical function: explaining fertility differences among breeds of ewe
Sean Cummins	2012	PhD	The effects of predicted differences for fertility in dairy cows on productive efficiency and gene expression profiles in key tissues
Ian Hutchinson	2012	PhD	The effect of strategic supplementation with polyunsaturated fatty acids on the reproductive performance of lactating dairy cattle
Abdullah Al Naib	2013	PhD	In vitro embryo production in cattle: a tool to understand the regulation of sperm function and embryo development
Lydia O'Hara	2014	PhD	Progesterone regulation of conceptus development in cattle
Lilian Okumu	2010	PhD	Localisation of key genes in the bovine uterus during the oestrous cycle and early pregnancy
Satoko Matoba	2013	PhD	Studies of oocyte developmental competence in cattle
Beatriz Fernández-Fuertes	2016	PhD	Factors affecting sperm function in cattle
Shane Leane	2016	PhD	Nutritional effects on reproduction in pasture-based systems of dairy production
Francis Curran	2016	PhD	Nutritional effects on fertility in pasture-based systems
Federico Randi	2017	PhD	An integrated approach to improving the reproductive efficiency of season-calving cow herds in Ireland
Colin Byrne	2017	PhD	An examination of the effects of nutrition on age at puberty and subsequent fertility inn dairy-bred bulls
Edel Murphy	2018	PhD	Optimising semen processing procedures of liquid and frozen-thawed bull semen in a commercial artificial insemination centre
Claudia Passaro	2018	PhD	Embryo-endometrial interaction during early pregnancy in cattle
José María Sánchez Gómez	2018	PhD	Understanding conceptus-maternal interaction in cattle to improve embryo survival
Eber Rojas Cañadas	2018	PhD	Fertility phenotypes in season-calving pasture-based dairy cows
Clio Maicas	2019	PhD	Fertility of sex-sorted sperm in seasonal-calving pasture-based dairy herds
Beatriz Rodriguez Alonso	2019	PhD	Studies on embryo-maternal interaction in the oviduct of cattle
Benjamin Planells Codoner	2019	PhD	New molecular insights into sex determination and early differentiation in mice and cattle
Sandra Bagés Arnal	2020	PhD	Studies on maternal embryo communication in cattle
Stephen Coen	2022	PhD	Nutrition and genomic control of sexual maturation in the bull
Elena O'Callaghan	2022	PhD	Sire contribution to pregnancy establishment in cattle
Alan Crowe	2024	PhD	Use of assisted reproduction techniques to accelerate genetic gain and increase value of beef production in dairy herds
Laura Thompson	In progress	PhD	Effect of assisted reproduction techniques on foetal development and postnatal characteristics of calves
Eliza O'Shea Murphy	In progress	PhD	Accelerating genetic gain and improving beef output from dairy herds
Joanne Hanifin	In progress	PhD	Effect of heterospermic semen on fertility in cattle

Progesterone, the uterus and conceptus elongation

In vitro embryo production is a fascinating tool. It is possible to replicate in a petri dish the final stages of oocyte maturation in the follicle, fertilisation in the oviduct and the first week or so of embryo development in the oviduct and uterus. Thus, the early embryo is somewhat autonomous; it does not require contact with the female reproductive tract to reach the blastocyst stage and is capable of establishing a pregnancy after transfer to a uterus that itself has not been exposed to an embryo prior to the transfer. Having spent a lot of my early career trying to optimise the in vitro production of embryos, in the early 2000s I became more interested in maternal embryo communication; growing embryos in the lab was one thing but how they interact with the female reproductive tract to establish a pregnancy was much more interesting. We first used a combination of superovulation, artificial insemination and in vivo vulture in the sheep oviduct to tease out the respective impacts of oocyte maturation, fertilisation and embryo culture in vivo vs. in vitro on embryo yield and embryo quality. These data were published in one of my favourite, and our most cited, paper (Rizos et al., 2002) and were most recently kindly highlighted by Pete Hansen in his paper associated with the 11th International Ruminant Reproduction Symposium in Galway, Ireland in May 2023 (Hansen, 2023). The broad conclusion from these studies, was that the main factor determining blastocyst yield in vitro is the quality of the oocyte that goes into maturation while the main factor affecting blastocyst quality is the post-fertilisation culture environment. To a large degree, the developmental competence of the oocyte is 'set' once it is removed from the follicle and few, if any, protocols for in vitro maturation result in a consistent improvement in development above the typical 30-40% blastocyst rate (Lonergan and Fair 2016). These results were subsequently extended by us (Lonergan et al., 2003a,b) and others (Gad et al., 2012) by carrying out reciprocal transfers between in vitro and in vivo culture conditions.

We carried out a series of studies in collaboration with Urban Besenfelder and Viteslav Havlicek from the University of Vienna, using Urban's exquisite endoscopic transfer method, to place embryos (up to 50) into the oviducts of females in different metabolic states (nulliparous heifers, lactating and nonlactating postpartum cows) and were able to demonstrate that the ability of the oviduct to support early embryo development was compromised due to the metabolic stress associated with lactation (Rizos et al., 2010; Maillo et al., 2012, 2015). More recently, we showed that this is likely, at least partly, due to altered embryonic genome activation (Rabaglino et al., 2023). We also used the same model to understand asynchrony in the oviduct (Rodríguez-Alonso et al., 2020) and the effect of progesterone concentration on development to the blastocyst stage in vivo (Carter et al., 2010).

Together with Jim Roche, Niamh Forde and others we looked at the influence of circulating progesterone on various aspects of uterine biology and embryo development. The first of many papers from those studies described the endometrial transcriptome in heifers during the oestrous cycle and early pregnancy and how this is influenced by increasing progesterone concentrations after ovulation (Forde et al., 2009). This study led to many others through which we attempted to untangle the complex interplay between progesterone, uterine biology and pre-attachment conceptus development (Carter et al., 2010; Forde et al., 2010, 2011a,b, 2012 and others). For more details, see reviews by Spencer et al. (2016), Lonergan et al. (2016), and Lonergan and Sánchez (2020).

Progesterone priming of the uterus is essential for optimal pregnancy establishment. As the corpus luteum develops following ovulation, the uterus is exposed to increasing concentrations of progesterone which alter the transcriptome of the endometrium. By comparing the transcriptome of cyclic and pregnant bovine endometrium, it is clear that temporal changes in endometrial gene expression occur irrespective of whether the cow is pregnant or not and it is really only at the time of maternal recognition of pregnancy at around d 16 that major changes in gene expression are detectable between pregnancy and cyclic animals (Forde et al., 2011a). An adequate rise in progesterone after ovulation drives these normal temporal changes that occur in the endometrial transcriptome of cattle that are necessary for the establishment of uterine receptivity and the promotion of conceptus development. Forde et al. (2009) described the global transcriptome of the endometrium from Day 5 to Day 16 in pregnant and cyclic cattle under conditions of normal and elevated

progesterone and revealed how circulating concentrations of progesterone regulate endometrial genes. Those studies found that progesterone supplementation advances the normal temporal changes in endometrial gene expression, particularly for genes associated with energy sources or contributors to histotroph, which may contribute to advanced conceptus development on Day 13 and Day 16. In contrast, low progesterone was associated with an altered endometrial transcriptome and retarded conceptus elongation (Forde et al., 2011b, 2012). Interestingly, the embryo does not have to be present in the uterus during the period of progesterone elevation in order to benefit from it (Clemente et al., 2009), supporting the concept that the positive effect on conceptus growth is mediated via progesterone-induced changes in the endometrial transcriptome.

Around the same time, we carried out one of the first RNA Seq studies on bovine embryos to describe the transcriptomic landscape of the developing embryo from the blastocyst stage on Day 7 to the elongated conceptus on Day 16 (Mamo et al., 2011) and combined these data with similar data from the endometrium to provide a comprehensive list of potentially secreted molecules in the conceptus that interact with receptors on the endometrium and vice versa during the critical window of maternal recognition of pregnancy (Mamo et al., 2012).

We investigated conceptus-induced changes in the endometrial transcriptome to address the question of how soon the cow knows she is pregnant (Forde et al., 2011a). By comparing endometria from cyclic or pregnant heifers on Days 5, 7, 13 and 16, the earliest we could detect differences between cyclic and pregnant animals was on Day 16, by which time most of the changes are due to conceptus-derived interferon-tau. Interestingly, interferon-tau (IFNT) mRNA is detectable in the bovine embryo from day 6 (late morula/early blastocyst stage) onwards (Wrenzycki et al., 1999). Furthermore, bovine blastocysts secrete IFNT into culture medium in vitro (Larson et al., 2001; Kubisch et al., 2004). Culture of bovine endometrial explants in vitro with Day 8 blastocysts leads to an increase in the transcript abundance of several interferon-stimulated genes (ISGs; e.g., *ISG15, MX2* etc), demonstrating that the endometrium can respond to blastocyst-derived IFNT. In contrast, exposure of explants to oocytes, 2-cell embryos or Day 5 morulae did not alter their relative abundance (Passaro et al., 2018, 2019). While some authors, including us, failed to detect a response of the endometrium to pregnancy before approximately Day 15/16 (e.g., Forde et al., 2009), others have reported altered expression of several genes including ISGs by Day 7 (Sponchiado et al., 2017). Thus, there is compelling evidence that the blastocyst as early as Day 7 produces IFNT and that this induces a response in the endometrium. However, whether this interaction has any significant role in pregnancy establishment is probably questionable given that it is possible to transfer embryos into a uterus up to about Day 16 and establish a pregnancy (Betteridge et al., 1980). Indeed, we have shown that the effect of IFNT on the endometrium is very acute, with exposure for as little as 3 h in vitro increasing mRNA expression of a range of ISGs (Talukder et al., 2023).

Conceptus elongation

The relationship between circulating progesterone and uterine receptivity has been well described (reviewed by Spencer et al., 2016; Lonergan and Sanchez, 2020). Elevated progesterone concentrations in the first week after conception have been associated with accelerated post-hatching conceptus elongation, mediated through advancement in the regular temporal changes in the uterine endometrial transcriptome (Forde et al., 2009) and alterations in the uterine lumen fluid (ULF) composition (Simintiras et al., 2019a).

As stated above, the success of in vitro fertilization (IVF), where embryos are made in the laboratory, demonstrates that contact with the female reproductive tract is not necessary in order for the embryo to reach the hatched blastocyst stage. However, the characteristic elongation of the ruminant conceptus prior to implantation is dependent on secretions from the uterus as evidenced by the fact that it does not occur in vitro and does not occur in vivo in the absence of uterine glands (Gray et al., 2002). This highlights the key role played by the uterine endometrium in driving the elongation process via endometrial secretions which compose the uterine lumen fluid. Temporal changes of the endometrial transcriptome and uterine fluid composition are necessary to establish uterine receptivity to implantation and, in

turn, are pivotal to the success of pregnancy establishment. These modifications are regulated by conceptus-derived IFNT together with maternally-derived progesterone from the corpus luteum, to induce expression of genes in uterine luminal and superficial glandular epithelia for transport and/or secretion into the uterine lumen to support growth and development of the conceptus (Simintiras et al., 2019a,b,c). Interestingly, elongation also appears to be associated with oocyte quality as we have observed over numerous studies that IVP blastocysts transferred in groups to the same uterus elongate at different rates. This is important because short (retarded) conceptuses have a different gene expression pattern to their longer age-matched counterparts (Barnwell et al., 2016, Ribeiro et al., 2016) and such short conceptuses produce less IFNT and fail to elicit an appropriate response from the endometrium around the time of pregnancy recognition (Sánchez et al., 2019).

Beef on dairy

Despite working with embryos and IVF for all of my career, it is only relatively recently that we have had the opportunity to carry out some large-scale embryo transfer studies at a commercial scale (Crowe et al., 2024b). These studies have been carried out in an era when the use of both sex-sorted dairy semen (to generate replacement females) and conventional beef semen (to generate all remaining pregnancies) is increasing in the dairy herd, facilitating genetic gain in replacement females while enhancing the beef value of surplus calves (reviewed by Crowe et al., 2021). Although beef-cross calves have greater economic value than male dairy calves, further gains are potentially feasible through the transfer of purebred beef embryos.

Since 2017, the number of in vitro-produced (IVP) embryos transferred has surpassed the number derived by traditional superovulation, now accounting for approximately 80% of all bovine embryos produced and transferred (Viana, 2023). According to the latest data available from the International Embryo Technology Society, almost 1.2 million IVP embryos were transferred worldwide in 2022 compared to 370,000 in vivo derived embryos (Viana, 2023). Although issues with cryotolerance (i.e., freezability) of IVP embryos, embryo loss, and, in some cases, calf birth weight remain to be fully resolved, IVP embryos are likely here to stay as a tool for genetic improvement in dairy herds, offering increased flexibility in sire usage allowing multiple pregnancies from elite dam-bull combinations to be generated and the ability to produce more embryos per unit time than traditional superovulation.

To test the feasibility of using IVF in our seasonal pasture-based system of production in Ireland, we recently carried out a large-scale field trial to examine fertility in lactating dairy cows following timed AI or timed ET with fresh or frozen, beef or dairy, IVP embryos (Crowe et al., 2024b). Pregnancy rates for embryos transferred fresh were comparable with those achieved after AI. However, consistent with other studies, embryonic loss was increased with IVP embryos compared to AI. A subsequent study (Crowe et al., Forthcoming) examining the timing and incidence of pregnancy loss in the same cohort of cows from service event to parturition revealed that the largest proportion of pregnancy loss occurred before Day 18 (AI and ET). Pregnancy loss from Day 32 to Day 62 was greater following ET compared with AI, particularly with frozen embryos while losses after Day 62 were small (≤ 3.5%) in all groups. The percentage of cows that calved following fresh ET was similar to AI (both greater than frozen ET). Further work is clearly required to improve the likelihood of pregnancy establishment and reduce embryonic and fetal mortality following transfer of a cryopreserved IVP embryo. It is likely, based in recent studies, that some of this loss is due to delayed attachment in IVP embryos (Crowe et al., 2024a)

Pregnancy loss in dairy cows is a major contributor to reproductive inefficiency at herd level (Wiltbank et al., 2016; Berg et al., 2022). Greater embryo mortality presents an obstacle to more widespread use of IVP embryos, particularly in seasonal systems of production with a compact breeding season. This is particularly true in seasonal, pasture-based systems of production with a short, well-defined, breeding season such as that operated in Ireland. In his paper, written as entertainment during the Covid-19 pandemic, Pete Hansen (Hansen, 2020) addressed the incompletely fulfilled promise of embryo transfer in cattle, asking why pregnancy rates are not greater than they are given that embryo transfer bypasses any

potential issues relating to oocyte quality, fertilisation and oviduct function. Nonetheless, pregnancy success is generally similar for ET and AI. This would suggest that issues around embryo quality and/or technical improvements in the methodology of ET and recipient management still remain.

Conclusion

The world of animal reproductive biology is a small one; most of us know, or know of, each other (and most of us review each other's papers!). I consider myself very lucky to have the job I have; I look forward to going to work every day. Through science, we have been able to visit virtually every corner of the globe and spend time with colleagues, many of whom have become close over the years. There are not a lot of jobs that offer such perks. Our knowledge of reproductive biology and the regulation of early embryo development in domestic animals has come on in leaps and bounds over the past three decades. However, there is still much to discover. My brother, Gerard, often teases me by asking why we have not yet solved the 'problem' of cow fertility. We're too clever I tell him; sure, that would be like turkeys voting for Christmas!

Acknowledgements

Thanks to Marja Mikkola, Cesare Galli, Claire Ponsart, Pascal Salvetti, and Michel Thibier for help sourcing some old AETE proceedings. Thanks to the many students and post-docs with whom I have worked over the years and the few special ones that have gone on to become collaborators. I am proud of all the students with whom I have had the pleasure of working and am delighted to see some of them have remained in the field and become successful in their own right. Thanks also to excellent technical assistance at Lyons over the years including Mary Wade, Pat Duffy, and especially Michael McDonald who is central to all of our animal-based research. Thanks to all of my collaborators over the years; long may the collaboration continue! Thanks to my parents, Christy and Olive, for their support throughout my career and, of course, to Trudee and the 'kids', Tadhg, Hugh, Myles and Iseult for their love. Finally, I would like to thank again the board of AETE for giving me this honour.

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Author contributions

PL: Conceptualization, Writing – original draft, Writing – review & editing.

President´s letter

President´s letter

Dear friends and colleagues

It is my great pleasure to welcome you to the Annual Scientific Meeting of AETE in the beautiful city of Brescia, Italy as we celebrate the 40th anniversary of our association. The first meeting of AETE was held back in 1984, with Pierre Mauléon as the very first President of our society; Jeff Mahon, Vice President and Treasurer; and Jean-Claude Plat, the first Secretary. Since these early times AETE has grown, flourished and evolved to what it is today – a vibrant and friendly society that fosters connections between its members to interact and exchange knowledge across Europe and worldwide with our partner societies IETS and SBTE, as well as numerous national societies.

This milestone anniversary is a perfect occasion for reflection and celebration. We will revisit the decades via the path that has led our society and the ART industry to our current achievements and where we are heading. Our outstanding speakers from Europe, Brazil and the US will share their diverse perspectives, guiding us through this voyage. In addition to these invited talks, the program will include two practical workshops, the student competition and oral presentations of selected abstracts. With four poster sessions, there will be ample opportunity to discuss with the presenters about their latest research. We are also thrilled to honor Patrick Lonergan with the Pioneer Award of 2024 for his distinguished career.

Kicking off the event, the Local Organizing Committee, chaired by Pierluigi Guarneri, has prepared a Preconference Workshop. The workshop, focused on bovine species including the buffalo, will delve into the origins of fertility, starting from the *in utero* period to the management of young animals, collection of oocytes, and finally grading of oocytes and IVP embryos. This event will take place at the Agricultural Technical Institute barn facilities a short distance from our conference venue.

The scientific sessions will be hosted at The Centro Pastorale Paolo VI. The LOC has prepared a delightful social program starting with a Welcome Reception taking place in the beautiful garden of Paolo VI. Our Gala Dinner will be an evening under the arches of the Vita restaurant accompanied by jazz music and followed by dancing. Since we are in the "home" of pizza, what better way to bid farewell than with a party at a local pizzeria, enjoying a buffet of delicious pizzas and Italian specialties. After the main event, the conference will conclude with an exciting post-conference tour to Avantea in Cremona.

I extend my heartfelt thanks to everyone involved in organizing this special anniversary meeting. The dedication of the LOC and the board of AETE have been instrumental in making this event a success. I am deeply grateful to our loyal sponsors and new supporters for their generous contributions. A special thank you to our invited speakers as well as all researchers sharing their latest work and all members and friends of our society for making this event possible.

Let's make this 40th anniversary meeting a memorable and inspiring celebration!

Marja Mikkola
President of AETE

Commercial Embryo Transfer Activity in Europe 2023

Collated by Helene Quinton

National data collectors

Country	Collector	Comment
Austria	Eva DACHSBERGER and Friedrich FUEHRER	
Belgium	Isabelle DONNAY	
Czech Republic	Jiri SICHTAR	
Denmark	Henrik CALLESEN	
Estonia	Ants KAVAK	
Finland	Anna OKSA-PULLIAINEN	
France	Serge LACAZE	
Germany	Hubert CRAMER	
Hungary	Ferenc FLINK	
Italy	Giovanna LAZZARI	
Latvia	Ilga SEMATOVICA	
Lithuania	Rasa NAINIENE	
Netherlands	Anna BEKER VAN WOUNDENBERG and Erik MULLAART	
Norway	Tjerand LUNDE	
Poland	Jedrzej JASKOWSKI	
Portugal	Joao Nestor CHAGAS E SILVA	
Romania	Stefan CIORNEI	
Russian Federation	Victor MADISON	
Slovakia	Dalibor POLAK	No Activity
Slovenia	Janko MRKUN and Aleksandar PLAVSIC	
Spain	Pablo BERMEJO	
Sweden	Renee BÅGE	
Switzerland	Andreas FLEISCH	
Turkey	Hakan SAGIRKAYA	
United Kingdom	Jake OLIVIER	

Declared bovine *In vivo* embryo production

	Dairy				Beef				All		
Country	Collec-tions	% Coll. with sexed semen	Embryos & ova	Transferable embryos	Collec-tions	% Coll. with sexed semen	Embryos & ova	Transferable embryos	Collec-tions total	Transferable embryos total	Embryos/ collection
Austria	978	2%	8 264	5 558	20	10%	249	98	998	5 656	5,7
Belgium	0		313	189	0		3 058	1 891		2 080	
Czech Republic	334	0%	2 852	1 593	0		0	0	334	1 593	4,8
Denmark	964	0%	8 599	6 302	51	0%	489	413	1 015	6 715	6,6
Estonia	0		0	0	2	0%	0	0	2	0	0,0
Finland	371	2%	3 306	2 371	3	0%	50	37	374	2 408	6,4
France	5 624	23%	47 577	29 520	1 487	4%	14 964	8 301	7 111	37 821	5,3
Germany	4 058	0%	34 734	23 459	247	0%	2 740	1 546	4 305	25 005	5,8
Hungary	449	100%	2 480	1 125	26	4%	239	186	475	1 311	2,8
Italy	2 218	56%	21 685	14 860	105	0%	882	682	2 323	15 542	6,7
Latvia	0		0	0	0		0	0			
Lithuania	3	0%	18	18	0		0	0	3	18	6,0
Netherlands	3 116	0%	21 998	14 468	0		0	0	3 116	14 468	4,6
Norway	330	3%	1 870	1 683	20	0%	275	247	350	1 930	5,5
Poland	270	47%	1 404	1 061	6	0%	122	44	276	1 105	4,0
Portugal	104	95%	922	443	25	0%	179	109	129	552	4,3
Romania	11	0%	127	73	88	0%	570	386	99	459	4,6
Russian Federation	357	68%	2 324	1 258	95	0%	1 224	672	452	1 930	4,3
Slovenia	28	61%	156	127	2	0%	15	8	30	135	4,5
Spain	262	84%	2 471	1 248	114	14%	1 325	674	376	1 922	5,1
Sweden	245	2%	2 005	1 387	0		0	0	245	1 387	5,7
Switzerland	336	70%	4 105	2 721	15	20%	208	161	351	2 882	8,2
Turkey	240	66%	1 999	1 480	0		0	0	240	1 480	6,2
United Kingdom	478	0%	3 898	1 923	1 246	0%	9 478	4 263	1 724	6 186	3,6
Total	**20 776**	**16%**	**173 107**	**112 867**	**3 552**	**3%**	**36 067**	**19 718**	**24 328**	**132 585**	**5,4**

Declared bovine *In vitro* embryo production (OPU-IVP)

	Dairy				Beef				All		
Country	OPU	% OPU with sexed semen	Oocytes	Embryos	OPU	% OPU with sexed semen	Oocytes	Embryos	OPU	Embryos	Embryos /OPU
Finland	554	0%	5 356	1 796	0		0	0	554	1 796	3,2
France	1 741	3%	22 865	5 959	83	7%	1 115	552	1 824	6 511	3,6
Germany	1 709	0%	22 667	6 572	204	0%	3 088	556	1 913	7 128	3,7
Hungary	10	70%	41	8	2	0%	9	4	12	12	1,0
Italy	364	0%	4 062	1 106	0		0	0	364	1 106	3,0
Netherlands	6 402	0%	72 192	16 503	0		0	0	6 402	16 503	2,6
Norway	184	0%	1 587	489	0		0	0	184	489	2,7
Poland	132	89%	1 585	380	0		0	0	132	380	2,9
Russian Federation	187	100%	880	133	0		0	0	187	133	0,7
Spain	1 055	93%	8 968	1 836	172	10%	1 389	260	1 227	2 096	1,7
Switzerland	321	55%	4 003	1 238	6	33%	44	14	327	1 252	3,8
Turkey	3	0%	21	1	0		0	0	3	1	0,3
United Kingdom	579	0%	4 541	1 663	952	0%	11 011	4 157	1 531	5 820	3,8
Total	**13 241**	**12%**	**148 768**	**37 684**	**1 419**	**2%**	**16 656**	**5 543**	**14 660**	**43 227**	**2,9**

Declared bovine *In vitro* embryo production – abattoir

	Dairy				Beef			
Country	Donors	% with sexed semen	Oocytes	Embryos	Donors	% with sexed semen	Oocytes	Embryos
Finland	5	0%	91	3				
Hungary	60	67%	580	193				
Italy					836	0%		
Netherlands	179	0%	23 130	5 481				
Slovenia	0		0	0	199	0%	562	76
Spain	270	0%	5 390	1 040	2	0%	11 240	2 716
Turkey	386	6%	3 234	1 081				
Total	**900**	**0%**	**32 425**	**7 798**	**1 037**	**0%**	**11 802**	**2 792**

Declared bovine embryo technologies – embryo genotyping

	Sexed embryos		Genotyped embryos	
Country	In Vivo	In Vitro	In Vivo	In Vitro
France	477	0	701	46
Germany	0	0	242	346
Netherlands	0	0	0	7 046
Total	**477**	**0**	**943**	**7 438**

Declared bovine embryo transfers and exports– *In vivo*

Country	Dairy				Beef				Total embryos transferred
	Fresh embryos transferred	Frozen embryos			Fresh embryos transferred	Frozen embryos			
		Domestic transferred	Foreign transferred	Exported		Domestic transferred	Foreign transferred	Exported	
Austria	2 079	2 731	47	298	37	54	6	0	4 954
Belgium	35	195	0	0	386	1 935	0	0	2 551
Czech Republic	1 033	0	0	0	0	0	0	0	1 033
Denmark	4 403	1 886	0	10	132	151	0	0	6 572
Estonia	0	0	0	0	0	0	51	0	51
Finland	678	1 154	42	0	6	23	17	0	1 920
France	14 072	12 530	1 016	131	2 893	3 809	175	503	34 495
Germany	9 951	12 367	0	864	344	1 021	0	0	23 683
Hungary	652	490	7	0	31	69	19	6	1 268
Italy	6 580	1 860	0	0	85	0	0	0	8 525
Latvia	0	0	1	0	0	0	37	0	38
Lithuania	0	0	0	0	0	0	0	0	0
Netherlands	2 550	10 412	0	0	0	0	0	0	12 962
Norway	28	1 889	30	0	50	30	200	0	2 227
Poland	646	531	589		36	0	0		1 802
Portugal	131	220	33		39	47	43		513
Romania	68	21	10	0	386	7	0	0	492
Russian Federation	284	511	0	0	0	1 170	300	0	2 265
Slovenia	0	0	0	0	0	0	0	0	0
Spain	606	430	184	0	131	397	103	84	1 851
Sweden	856	1 052	0	0	0	0	0	0	1 908
Switzerland	504	1 480	275	657	17	7	53	22	2 336
Turkey	929	227	0	0	0	0	0	0	1 156
United Kingdom	875	477	837	19	1 576	1 613	184	229	5 562
Total	**46 960**	**50 463**	**3 071**	**1 979**	**6 149**	**10 333**	**1 188**	**844**	**118 164**

Declared bovine embryo transfers and exports – *In vitro*

	OPU				Abattoir		
Country	Fresh embryos transferred	Domestic frozen embryos transferred	Foreign frozen embryos transferred	Embryos exported	Fresh embryos transferred	Domestic frozen embryos transferred	Total embryos transferred
Finland	0	729	0	0	0	0	729
France	2 563	2 497	352	0	0	0	5 412
Germany	1 885	1 808	0	0	0	0	3 693
Hungary	10	0	0	0	0	0	10
Italy	415	0	0	0	67	0	482
Netherlands	5 217	4 666	0	0	0	0	9 883
Italy	415	0	0	0	67	0	482
Norway	0	347	30	0	0	0	377
Poland	123	89					212
Portugal		24	32				56
Russian Federation	5	40	0	0	6	0	51
Spain	987	618	54	0	680	1 310	3 649
Switzerland	157	494	104	0	0	1	756
Turkey	0	0	0	0	12	35	47
United Kingdom	433	1 087	0	369	0	0	1 520
Total	**11 795**	**12 399**	**572**	**369**	**765**	**1 346**	**26 877**

Declared embryo production, transfer and export in other species – *In vivo*

		Embryo collection		Embryo transfer			
Species	Country	Collections	Viable embryos	Fresh embryos	Frozen domestic	Frozen foreign	Exported embryos
Sheep	Hungary	2	8	0	0	0	0
	Portugal	13	25	23	9		
	Romania	10	50	50	0	0	0
	Russian Federation	140	1 745	1 065	131	0	0
	Spain	13	55	55	121	129	0
	Sweden	0	0	0	0	230	0
	United Kingdom	4 104	31 449	353	76	0	0
	Total	**4 282**	**33 332**	**1 546**	**337**	**359**	**0**
Goat	United Kingdom	20	200	0	0	0	0
Horse	Estonia	5	6	5	0	0	0
	Finland	17	7	7	0	0	0
	France	1 590	731	875	0	0	0
	Hungary	3	3	3	0	0	0
	Portugal	74	52	52	52		
	Romania	53	32	32	0	0	0
	Russian Federation	26	16	14	1	0	0
	Spain	0	25	0	0	0	0
	Sweden	50	32	32	0	4	0
	Total	**1 818**	**904**	**1 020**	**53**	**4**	**0**
Rabbit	Romania	23	99	99	0	0	0

Declared embryo production, transfer and export in other species – *In vitro*

		Oocyte collection			IVP embryo transfer			Exported embryos
Species	Country	OPU conv	Oocytes	Embryos	Fresh embryos	Frozen domestic	Frozen foreign	
Sheep	Serbia	2	22	6	0	0	0	0
Horse	Estonia	0	0	0	0	0	4	0
	Finland	2	1	0	0	0	0	0
	France	0	0	0	0	0	75	0
	Hungary	46	793	100	0	7	76	0
	Italy	3 357	43 541	6 667	11	840	0	3 813
	Portugal	26	283	63	0	0	0	0
	Russian Federation	32	42	0	0	0	0	0
	Spain	181	1 519	276	0	0	0	0
	Total	**3 644**	**46 179**	**7 106**	**11**	**847**	**155**	**3 813**

INVITED LECTURES

Animal Reproduction

Thematic Section: 40th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

40 years of AETE: the contribution of scientists and practitioners to the progress of reproductive biotechnologies in Europe

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How to cite: Galli C, Lazzari G. 40 years of AETE: the contribution of scientists and practitioners to the progress of reproductive biotechnologies in Europe. Anim Reprod. 2024;21(3):e20240061. https://doi.org/10.1590/1984-3143-AR2024-0061

Abstract

This conference celebrates the 40th anniversary of AETE. Over the past 40 years, AETE has served as a forum for scientists, practitioners, and students working in assisted animal reproduction in livestock species. AETE conferences have reflected developments in the field, from basic to applied science, as well as regulatory changes in assisted animal reproduction practices. Europe has led the way in these developments for many years, progressing from artificial insemination, embryo transfer, and cryopreservation to semen sexing, in vitro production of embryos, cloning by nuclear transfer, genomic selection, and the rescue of highly endangered species. These significant contributions were made possible by the support of funding agencies, both at the national and European levels, promoting cooperation between scientists and practitioners. Assisted reproduction, and animal breeding more generally, face opposition from various groups, including animal rights activists, vegetarians, proponents of organic farming, environmentalists, certain political parties, and increasing regulatory burdens. These challenges seriously affect funding for scientific research, the work of practitioners, and the breeding industry as a whole. It is crucial to invest time and resources in communication to remind the public, politicians, and regulators of the achievements in this field and the contributions made to the food supply chain and the care of the rural and natural environment.

Keywords: assisted reproduction techniques, biotechnologies, AETE, Europe.

Introduction

Europe has always been at the fore front and a leader in the development of assisted reproduction biotechnologies, more specifically termed ART (Assisted Reproduction Technologies). Lazzaro Spallanzani (Italy, 1729-1799) was the first to perform the first successful artificial insemination in a bitch (Lonergan, 2018) and Walter Heape (United Kingdom,1855-1929) was the first to perform embryo transfer (Betteridge, 2003). Artificial insemination and embryo transfer were, and still are, the cornerstones of reproductive biotechnologies in mammalian reproduction. Following our forebears, many pioneers, both in academia and in practice, stepped in to bring reproductive biotechnologies to what we know and practice today, as well as opening new windows for younger generations to look into the future. This paper will not pretend to provide a complete and referenced review of 40 years of reproductive biotechnologies in Europe; rather, it offers a personal perspective. Having worked for 40 years both in academia and in industry, I will discuss on how science and practice have fed each other, primarily through scientific societies like AETE (Association of Embryo Technology in Europe), whose 40th anniversary we are celebrating this year.

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Received: May 7, 2024. Accepted: June 20, 2024.
Financial support: None.
Conflicts of interest: The authors have no conflict of interest to declare.

Scientific societies like AETE in Europe, or IETS (International Embryo Technology Society) internationally, as well as national societies, have played an important role in advancing the field by bringing together scientists and practitioners around the table to discuss findings in research laboratories, the needs of the industry and practitioners and, most importantly, creating a forum for students to present their work and network with the community during 2 to 3 days meetings, that always included social events to facilitate this. The never-ending struggle of the Board of Governors of these societies and the program chairs has always been, and still is, to find the right balance to attract both scientists and practitioners. Moreover, an important part of such meetings are the exhibitors, not only because they provided the sponsorship to pay part of the expenses, but primarily to showcase newly developed tools, consumables, disposable and reagents required as the procedures developed and the regulatory requirements dictated.

Forty years ago, much of the work presented and discussed was related with cattle, but over the years, reproductive biotechnologies have widened their application to include other livestock species like small ruminants, pigs, buffaloes, horses, etc. but also endangered and exotic species, genomics selection, stem cells and genome editing.

In the paper celebrating the 30th year of AETE (Thibier, 2014) there is a detailed narration of the birth and evolution of AETE. In this paper I will attempt to outline what European scientists and practitioners have contributed to the advancement of reproductive biotechnologies, not only in Europe but globally, and how they have found in the AETE their home.

The preamble to Assisted Reproduction Technologies in Livestock

Many of the ARTs in use today were developed in Europe and disseminated around the world through exchange visit of scientists or veterinarians or during conferences. One very well know was the meeting organized by Tim Rowson at the animal research station in Cambridge, UK, in 1972, on the collection and surgical transfer of cattle embryos (Betteridge, 2003). Many of the attendees at this meeting were then the founders of IETS in 1974 in Colorado, where George Seidel also started a strong program at Colorado State University for the collection and transfer of cattle embryos. For about a decade, the focus was on the refinement of superovulatory protocols, which are substantially the same as those used today (Lonergan and Sánchez, 2022), the optimization of the flushing and recovery protocol, and the replacement of the surgical embryo transfer with the non-surgical transcervical method (Wright, 1981). The first successful cattle embryo cryopreservation was also achieved in Cambridge by Ian Wilmut (Wilmut and Rowson, 1973) resulting in the birth of a calf named Frosty II.

Although the practitioners in the decade 1984-1994 were working to improve MOET (Multiple Ovulation and Embryo Transfer) or, more generally, in vivo derived embryos, many research laboratories concentrated on the in vitro production of embryos. Only a few years earlier, in1978, Louise Brown was born following in vitro fertilization performed by Robert Edwards (Steptoe and Edwards, 1978), who conducted his experimental work at the Animal Research Station in Cambridge, again working with bovine oocytes. All these successful events in Europe served as a strong starting point for scientist and practitioners to continue their promising work.

Multiple ovulation and embryo transfer

The basis for the successful production of embryos in vivo is superovulation, and the understanding of the dynamic of follicular development is necessary to exploit the ovarian reserve of oocytes (Monniaux et al., 1983, 2014; Monniaux, 2012). Much of the work was done at INRA and often presented at AETE conferences. The protocol in the early days relied on the use of PMSG (now called eCG) but it had undesired side effects due to its long half -life (Monniaux et al., 1983; Vos et al., 1994). A better understanding of follicular dynamics and follicular wave synchronization has allowed the optimization and development of more user-friendly protocols, but major advances in number of viable embryos produced have not been achieved (Bo and Mapletoft, 2014). The development and use of recombinant b-FSH did not

improve the results over the products extracted from pituitary glands (Wilson et al., 1993). Therefore, practitioners still rely today on pituitary extract of porcine origin (Folltropin, Pluset, Stimufol, 2 of these produced in Europe) or sheep origin (Ovagen) with the limitation that being a purified extract, there is inevitable batch to batch variation affecting their efficacy.

In vitro embryo production

The birth of the first baby by IVF sparked an interest in animal IVF, especially in livestock species, particularly in cattle. Although the first calf obtained by IVF was born in US using in vivo matured oocytes (Brackett et al., 1982), the practical application required the use of immature oocytes harvested from ovaries and matured in vitro to metaphase II. Several European scientists contributed significantly to the in vitro maturation of livestock oocytes (Fulka et al., 1982) demonstrating oocyte developmental competence (Staigmiller and Moor, 1984) and finally, the culture of viable embryos in vitro (Gandolfi and Moor, 1987).

The potential value of in vitro technology quickly caught the interest of investors and the industry. Operations like Ovamass, associated with University College Dublin in Ireland, and Animal Biotechnology Cambridge on the Huntington Road premises in Cambridge, were established with the aim of producing large number of embryos from beef donors to be implanted into dairy cows. Similar operations were established in the Netherlands, France and Italy. Europe quickly became the leader in the production of embryos from slaughtered animals (Galli and Lazzari, 1996), as witnessed by the data published annually by the IETS Data Retrieval Committee.

The use of ovaries from slaughtered animals was very useful for research and for beef animals. However, from a genetic selection perspective, especially for dairy, it had to be done on live animals. In fact, in the years following the steps performed in the human field to obtain the first IVF baby also veterinarians started to practice ovum pick up on cows. The first attempts to use ultrasound guided follicular aspiration for embryo production in vitro were reported by Callesen et al. (1987) and further developed by Pieterse et al. (1988, 1991). Using a human endovaginal probe adapted for the use in cattle, Pieterse reported a recovery rate of 55%, the repeatability of the procedure and the absence of side effects on the donor cows.

Although the procedures for embryo production in those days still required major laboratory refinements that came later on (Galli and Lazzari, 1996), the basics of OPU described by Pieterse et al. are still the same as those used today by many practitioners. Recovery rates have improved to over 70% due to the use of better ultrasound equipment with 6 or 7 MHz convex array probes that provide a better resolution on smaller follicles or the use of gonadotrophin priming that increases the size of smaller follicles. The OPU technique was initially applied on problem cows that did not respond to superovulation (Kruip et al., 1994; Looney et al., 1994), but it was later applied on a wider scale, including on pregnant cows, heifers and prepuberal heifers. (Galli et al., 2001). It is difficult and often not relevant to make comparisons between different data set since there are so many variables involved, most of which are not even manageable. Beef breeds perform better than dairy, dry cows do better than lactating ones and cows perform better than heifers. In vitro produced embryos cultured in presence of serum and/or co-culture had a reduced cryotolerance. For several years, until the culture media were improved, the surrogate sheep oviduct was used to produce freezable embryos (Rizos et al., 2002; Lazzari et al., 2010).

Associated with suboptimal in vitro culture systems the embryo developing in vitro were responsible for the so-called LOS (large offspring Syndrome), especially when embryos were originating from nuclear transfer and other invasive micromanipulations (Farin et al., 2010). The underlying mechanisms were initially described by Young working in Edinburgh (Young et al., 1998; Lazzari et al., 2002b). Due to the deregulation of imprinted genes, LOS resulted in offspring that was well above average birth weight, including placenta hypertrophy and hydroallantoids causing dystocia at parturition and increased stillbirth rate. Although the incidence of the phenomenon has decreased due to the better culture media devoid of fetal calf serum, it has not completely disappeared.

Although Europe led in the development of the technology and its practical application, in several AI organization and amongst practitioners, it did not follow the global trend whereby

in vitro produced embryos are rapidly replacing in vivo derived ones today according to IETS data retrieval committee. In Europe, two-thirds of the bovine embryos produced still come from MOET, and in vitro produced embryos are mainly used by bull testing organizations.

Another species where in vitro embryo production is impacting breeding programs and practitioner activities is the horse. The implementation of ICSI (IntraCytoplasmic Sperm Injection), another technique developed in the human field (Palermo et al., 1992) to bypass male infertility, found application in the horse to bypass zona hardening of the oocytes matured in vitro collected from the slaughterhouse or by ovum pick up (Lazzari et al., 2002a; Galli et al., 2007). ICSI is revolutionizing the horse breeding industry because of difficulties in capacitating the stallion spermatozoa, the low quality of many frozen semen samples or the limited availability of semen of dead stallions in addition to the sub-fertile or old mares unusable by conventional in vivo flushing (Lazzari et al., 2020; Claes and Stout, 2022), where once again Europe is leading the way. According to the AETE Data Retrieval Committee, the number of in vitro produced horse embryos is greater than the one produced by in vivo flushing. This is also supported by the competitive advantage that ICSI embryos can be cryopreserved very successfully, both by slow freezing or vitrification, making it possible for a seasonal breeder like the horse to produce embryos also outside the breeding season for transfer during the breeding season or for marketing. The pregnancies and the foals obtained from ICSI embryos are normal and do not exhibit phenotypical abnormalities like the LOS observed in ruminants. This technique is also rapidly developing both in South and North America, as it has for bovines.

Embryo/semen sexing and Genomic selection of livestock

Having the offspring of the desired sex has always been the desire of all breeders. When PCR came on the market in 1988, sexing of cattle embryos became a reality for many cattle breeding organization, especially in Europe for dairy breeds (Bredbacka et al., 1995; Thibier and Nibart, 1995), and also for individual practitioners when portable kits and simplified protocols were developed for field use. The procedure required to take a biopsy from the embryos (5 to 10 cells) had to be done carefully w/o damaging the embryo too much, and the embryos and fresh transfer was the preferred protocol. Embryo sexing is also used in horses where there is no sexed semen available at the commercial level (Lazzari et al., 2020; Coster et al., 2023). While embryo sexing allows to know the sex of the embryo, the use of sexed semen predetermines the sex of the embryo allowing the production of the desired sex only. The refinements and the commercialization of the semen sexing technology was later done in US but the initial groundbreaking experiments on separating X and Y sperm were performed by Jane Morrell (current Board Member of AETE) at the National Institute for Medical Research in London (Morrell et al., 1988). The technique of embryo biopsy, superseded by sexed semen for sex selection, has remained relevant with the introduction of genomic selection (Hayes et al., 2009). Several cattle breeding organization in Europe were quick to implement genomic selection of embryos before transfer or freezing (Ponsart et al., 2014) to accelerate selection primarily on the male line, to select the bull of the next generation and avoid the birth of unwanted bull calves. Similar work has been undertaken also on equine embryos (Coster et al., 2024).

Cloning by nuclear transfer

Another dream of the animal breeders was to achieve the quality and uniformity typical of plant breeders where cloning is widely used. Cloning mammals is more complicated and the first experiments were actually performed in amphibians (Gurdon, 1962). Despite attempts by many laboratories to clone mice and a controversial publication by an Austrian investigators (Illmensee and Hoppe, 1981) claiming success, the mouse turned out to be more difficult to clone than livestock.

Steen Willadsen a Danish veterinarian working in Cambridge (an AETE pioneer awardee), was a key player in cloning sheep and cattle both by blastomere separation and by nuclear transfer (Willadsen, 1986). Embryo cloning, as developed by Willadsen, had clear limitations on the number of nuclei available in each morula (20 to 30 cells) used, and the process of serial

cloning had limitation after the first round. Despite this, embryo cloning was taken up by newly established cloning companies interested in cattle breeding in North America. However, it became clear that, together with the technical difficulties, the phenotype of the embryo was unpredictable, and the interest waned.

It was again thanks to European scientists, with the cloning of Dolly the sheep (Wilmut et al., 1997) that cloning, or better defined as Somatic Cell Nuclear Transfer, regained attention. The possibility to clone an adult animal of known phenotype clearly makes the difference and re-ignited the interest of the industry as well as scientists. After Dolly several other mammals were cloned from somatic cells in Europe including the bovine (Galli et al., 1999), the horse (Galli et al., 2003), the rat (Zhou et al., 2003), the mouflon through interspecies nuclear transfer (Loi et al., 2001) to mention a few.

Studies were also undertaken, especially by Yvan Heyman (Heyman et al., 2007) to demonstrate that the products originating from cloned animals did not differ from non-cloned controls. Despite all these efforts and the pioneering role of many European scientists, cloned animals and their products are not allowed to enter the food chain in Europe. Cloning by somatic cell nuclear transfer is still not efficient, especially in ruminants but works better in pigs and horses.

The reprogramming of the genome of a differentiated cells provided an unprecedented opportunity for scientists interested in understanding the epigenetic events underlying cell differentiation (Yang et al., 2007; Matoba and Zhang, 2018). The unravelling of the mechanisms involved in genome differentiation and reprogramming will be important to increase the efficiency of SCNT. However, these advancements will probably not come from European scientists or industry since the funding of the EU that supported most of the past European successes described above is no more available and directed to other "politically correct" priorities.

Stem cells and genetic engineering

In 1981 Martin Evans, working at Cambridge, UK, published a seminal paper to describe the derivation of embryonic stem (ES) cells from the mouse embryo (Evans and Kaufman, 1981). This work earned him the Nobel prize in 2007 shared with Mario Capecchi and Oliver Smithies for the development of "gene targeting", concept largely used today for genome editing. The use of embryonic stem cells became fundamental to generate the knock out mouse models to understand the function of any given gene in the genome (Robertson et al., 1986).

Given the potential of embryonic stem cells several laboratories attempted to derive ES cells from livestock species (Notarianni et al., 1991) as it would provide an unlimited source of cells for cloning. However, it turned out to be a daunting task (Galli et al., 1994). Interest in stem cells was also driven by the possibility of genetic engineering as it was done in the mouse, but the molecular pathways were only partially understood (Lazzari et al., 2006). The undifferentiated state could only be kept for a limited time in culture and it was not until the conditions for human ES cells were worked out (Thomson et al., 1998) that the derivation of stable bovine ES cells was reported (Bogliotti et al., 2018). Currently the interests in livestock ES cells is mainly academic since cloning can be done with somatic cells and it appears that there is no advantage to using less differentiated cells for nuclear transfer compared to fully differentiated ones (Sung et al., 2006).

The interest in generating livestock carrying genetic modification was present also in Europe after the pioneering work in USA of Brinster (Hammer et al., 1985) by microinjecting the pronucleus of the zygote as it was done in the mouse. Several animals carrying transgenes of pharmaceutical interest were generated (Clark et al., 1989; Niemann et al., 1996) but the efficiency of the system was low making the projects very expensive and, in the long term, unsustainable.

The breakthrough to generate genome edited animals came with the discovery of programmable nucleases in the last ten to fifteen years. First, the Zinc fingers nucleases (Urnov et al., 2010), then the TALENs (Joung and Sander, 2013) and the Crispr/Cas9 (Jinek et al., 2012) opened a new era in the genetic modification of animals and plants. Unfortunately, most of the work behind these developments and applications took place in North America as in Europe the "phobia" against GMOs has cut the funding to scientists, driven away companies

and investors. Interestingly, the basic discovery behind the CRISPR/Cas9 technology was done by F.J. Mojica a Spanish scientist working in Alicante (Mojica et al., 1993; Lander, 2016).

On the 25th anniversary of the birth of Dolly (Galli and Lazzari, 2021) we are witnessing a revision of the European policy on these techniques, defined as New Genomic Techniques (NGT) (https://ec.europa.eu/food/plant/gmo/modern_biotech/new-genomic-techniques_en). Someone would say better late than never. This revision is primarily considered for plants but still not for animals despite the vast number of livestock genome edited already generated (Bishop and Van Eenennaam, 2020) for agricultural purposes in many parts of the world except Europe.

Currently in Europe we are using genome editing techniques in the field of xenotransplantation to create pigs whose organs, tissues or cells could be transplanted to humans (Fischer and Schnieke, 2022; Galli, 2023) or to generate animal models of human genetic diseases (Aigner et al., 2010; Porta-Sanchez et al., 2023). All this work is at the R&D phase, as the regulatory pathway for approval through the regulatory agencies has yet to be tested.

Assisted Reproduction Technologies for conservation biology

An area where ARTs are put at work at its best and Europe is leading the way is for the Biorescue project (https://www.biorescue.org/), a race against time to save the Northern White Rhino, an iconic species where only two female are living on earth and are based in Kenya. This project besides being unique in its scope it will also serve as a template for other endangered species. Several European institutions are involved in this project covering the clinical area on live animals for oocyte recovery and embryo transfer (Leibniz Institute for Zoo and Wildlife Research, Berlin), the politics and logistic (Safari Park Dvůr Králové, Czech Republic), embryo production (Avantea, Cremona), stem cell biology (Max Delbrück Center for Molecular Medicine) and ethic (Department of Comparative Biomedicine and Food Science, Padova). To date the project has been very successful with the initial trials with Southern White Rhino females to develop and validate the technique that produced embryos and two lines of ES cells (Hildebrandt et al., 2018). In 2019 we initiated the OPU and embryo production on the two NWR female left (Najin and Fatu, mother, and daughter). Soon we realized that only the daughter was producing embryos therefore we stopped collecting the mother. To date we have produced and cryopreserved 30 NWR embryos and many more from SWR (Hildebrandt et al., 2023). In the meantime, we have been working to develop embryo transfer. The challenges are many including the preparation of a vasectomized teaser bull to detect exactly when the surrogate recipients are in estrus. To date one pregnancy has been established with a SWR embryo (unpublished). To widen the genetic base to be able to have a self-sustaining population we are also using stem cell technologies to generate oocytes and spermatozoa in vitro through iPSc (induced pluripotent stem cells) (Hayashi et al., 2022; Zywitza et al., 2022). The application of ART in conservation biology is viewed with suspicion by the stakeholders who, for a long time, opposed their introduction. Therefore, it is imperative that an ethical assessment is in place before, during, and after the procedures are performed, both on the animals and in the laboratory (Mori et al., 2021; Biasetti et al., 2022).

Final considerations

ARTs and related techniques have made huge progress in the past 40 years, both in livestock and wildlife species. This progress has been fostered by several circumstances. First, by the public funding made available at the national and, above all, the European level. This has facilitated the propensity of laboratories to exchange scientists, collaborations between research groups, and presentations of original work at conferences. In the last decade or more, such funding for livestock research is no longer available, hastening competition rather than collaboration, as the driving forces to attract funding are now the number of publications or the number of patents at the expense of innovative, reproducible, and sharable work. Second, by the number of public institutions and practitioners, with companies or cooperatives that created a critical mass of knowledge and work with direct practical implication that required

solutions. Third, the scientific societies like AETE with annual meetings fostered the exchange of ideas, discussions with regulators and interactions in presence between members that, with the digital era and the recent pandemic, had suffered a lot.

Looking ahead the prospects are not very optimistic. Alongside the reduction of funding, there is also a growing opposition to animal breeding. This opposition arises not only for ethical reasons but also due to concerns about environmental impact and other trendy topics in today's political discussions. Unfortunately, these discussions often overlook the role of assisted reproduction in a broader context, including its significance in human fields, which are strongly interconnected. As for the future, I believe it will largely be in the hands of the younger generations. While they are being trained with modern techniques and tools, it's important for them not to forget the lessons of the past. By learning from history, they can better plan for the future using the new techniques and instruments available today.

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Author contributions

CG and GL: Funding acquisition, Writing – original draft, Writing – review & editing.

Animal Reproduction

Thematic Section: 40th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Evolution over the last 40 years of the assisted reproduction technologies in cattle - the Brazilian perspective I - timed artificial insemination

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How to cite: Sales JNS, Pugliesi G, Carvalho LR, Simões LMS, Lemos LA, Vicente MP, Silva RRR, Baruselli PS. Evolution over the last 40 years of the assisted reproduction technologies in cattle - the Brazilian perspective I - timed artificial insemination. Anim Reprod. 2024;21(3):e20240034. https://doi.org/10.1590/1984-3143-AR2024-0034

Abstract

The reproductive efficiency of the herd is correlated with higher productivity in livestock. Reproduction biotechniques, such as ovulation synchronization protocols, are important to optimize production and accelerate genetic profit in beef and dairy herds. The objective of this review is to describe the evolution over the last 40 years of the artificial insemination (AI) and the timed-AI (TAI) protocols in cattle from a Brazilian perspective. TAI protocols are based on synchronizing emergence of the wave of follicular growth, controlling circulating progesterone (P4) concentrations, stimulating the final growth of the follicle and inducing a synchronized ovulation. Hormonal alternatives that optimize the response at the end of the protocol and strategies to induce final follicle growth and ovulation in categories of females with low expression of estrus are described. Furthermore, the potential positive effect of previous exposure to injectable P4 on fertility of *Bos indicus* and *Bos taurus* cows is also discussed.

Keywords: ovulation, estrus, synchronization, P4.

Introduction

In the last 40 years, assisted reproductive technologies (ARTs) have transformed the dairy and beef production industries leading to significant improvements in the quality and quantity of animal products. Among them, artificial insemination (AI) and embryo transfer (ET) are the most widely used biotechnologies (Baruselli et al., 2019). In recent years, a significant increase in the use of AI has been observed due to the development of timed-AI (TAI) protocols which allowed AI of cyclic and anestrus cows and heifers to be performed at a pre-determined time, without the necessity of estrus detection (Baruselli et al., 2017).

The first TAI protocol developed was based on the association of prostaglandin F2α (PGF2α) and GnRH (Pursley et al., 1995). Denominated *Ovsynch*, this protocol consisted of the administration of GnRH on a random day of the estrous cycle, with the aim of inducing ovulation and the consequent emergence of a new follicular wave. Furthermore, 7 days later, a dose of PGF2α was administered to regress possible corpus luteum (CL). Finally, 48 hours later, a second dose of GnRH was administered to synchronize ovulation. *Ovsynch* is widely

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Received: March 18, 2024. Accepted: June 14, 2024.
Financial support: LRC, MPV and RRRS receive grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Financial Code 001). LAL receive grant from the National Council for Scientific Development (CNPq) and JNSS receive productivity grant from the National Council for Scientific Development (CNPq).
Conflicts of interest: The authors have no conflict of interest to declare.

used in dairy cows, however, to obtain satisfactory results a high ovulation rate at the first GnRH is necessary. In cows (Vasconcelos et al., 1999) a greater response to the synchronization of ovulation and conception was observed when the protocol was started between days 5 and 12 of the estrous cycle. Thus, pre-synchronization protocols were developed to optimize the response to the first GnRH of *Ovsynch*.

After a few years, some hormonal modifications were carried out in order to improve the final response to the ovulation synchronization protocol. In the early 2000s, a protocol based on P4 and estradiol (E2) was reported (Bo et al., 2003; Baruselli et al., 2004), and is currently the most used in Brazil. The insertion of a P4 intravaginal device simultaneously with administration of E2, at a random moment of the estrous cycle, induces synchronization of the emergence of a new follicular wave (Bo et al., 2003). The period of permanence of the P4 intravaginal device controls follicular growth and ovulation (Prata et al., 2020). After removing the source of P4 (PGF2α and P4 device withdrawal), another hormonal treatment is used to synchronize ovulation (sources of E2 and/or GnRH; Sales et al., 2012). TAI is performed 48 hours after P4 removal. In addition, the use of equine chorionic gonadotropin (eCG) at the time of P4 device removal increased final follicular growth and pregnancy per AI (P/AI) in different categories (Sales et al., 2011a). However, in primiparous cows the results were more pronounced (Sales et al., 2016).

The TAI using E2 and P4 is well-established in suckled *Bos indicus* cows and substantially increases the reproductive efficiency of beef herds, leading to numerous economic benefits related to beef production (Baruselli et al., 2019). The TAI programs reduce the calving interval, increase conception at the beginning of the reproductive period, and increase the number of pregnant cows during the breeding season and the number of calves with greater genetic merit from AI (Baruselli et al., 2018). Due to these characteristics, interest has increased in the use of TAI in beef cows using resynchronization programs (Pugliesi et al., 2019). Thus, the objective of this review was to show the evolution over the last 40 years of the AI and TAI protocols in cattle from a Brazilian perspective.

Artificial insemination

The majority of bovine herds in tropical and subtropical areas (such as Brazil) are composed of *Bos indicus* because of their greater adaptability to high temperature and humidity, as well as, to the yearly variability in the feed supply (Baruselli et al., 2018). However, a long period of postpartum anestrous is observed in these animals, characterized by follicular emergence sustained by the release of FSH, but compromised final growth of the dominant follicle, and, consequently, absence of ovulation (Baruselli et al., 2018). These changes in the final follicular growth are due to the reduction of LH pulsatility after follicular deviation due to the calf presence and the reduced availability of forage (Jolly et al., 1995; Yavas and Walton, 2000). In cows in which the nutritional requirement is not met because of low feed availability, deficient GnRH secretion and consequently, reduced LH release are observed (Jolly et al., 1995; Montiel and Ahuja, 2005). The reduction of GnRH secretion occurs due to the negative feedback in the hypothalamus promoted by the increase in the concentrations of neuropeptide Y, NEFA, and beta-hydroxybutyrate produced by the mobilization of body fat (Hess et al., 2005). In addition to the nutritional effects, the calf's presence blocks the secretion of GnRH by the hypothalamus through the action of released endogenous opioids (Williams et al., 1996). As a result, AI based on detection of estrus was rarely used in beef and dairy cows managed on pasture.

In 2002, the Brazilian market for AI traded approximately 7.0 million doses of semen, and only 5.8% of dairy and beef females were artificially inseminated. Twenty years later (2023), 24.7 million doses of semen were commercialized and 20 to 23% of the females of the total number of the national herd were inseminated, a huge increase due to TAI (Baruselli, 2024). The increase in the AI market in Brazil occurred simultaneously with the development of the TAI technology. In 2023, 91.2% of females were inseminated by TAI in Brazil. The TAI annual growth rate has been 31.8% in the last 20 years, resulting in great advances and benefits to the meat and milk industries.

Timed-artificial insemination protocols

Currently, the TAI protocols in beef and dairy cattle are well established, in which P/AI between 30 and 65% are observed (Sales et al., 2015, 2016; Wiltbank et al., 2015; Baruselli et al., 2017). As mentioned before, in *Bos indicus* lactating beef cows, a long period of postpartum anestrus is observed (Baruselli et al., 2004). Due to this physiological condition, the main TAI protocol used in postpartum *Bos indicus* beef cows is based on the association of E2 and P4. Similar protocols are also used in dairy cows in Brazil, contrasting with the protocols based on GnRH and PGF2α that are predominant in the other countries. The standard protocol used based on GnRH and PGF2α is Ovsynch (Pursley et al., 1995). The response (ovulation of the preovulatory follicle at the end) to the TAI protocol based on E2 and P4 is approximately 80% in *Bos indicus* lactating beef cows (Sales et al., 2012) and approximately 85% in dairy cows in GnRH-based protocols and PGF2α when pre-synchronization protocols are used (Souza et al., 2008; Silva et al., 2018). In dairy cows, the Double-Ovsynch protocol has presented a better synchronization result, with ovulation in response to the first GnRH of around 82% and P/AI of 49.7% (Souza et al., 2008). However, some limitations (long protocol of 28 days and too many handlings) may limit the more extensive use of this protocol.

TAI protocols are based on synchronizing the emergence of a new follicular wave either by inducing follicular atresia by the simultaneous administration of P4 and E2 (Baruselli et al., 2017) or ovulating a dominant follicle with a GnRH treatment with at the onset of the TAI protocols (Wiltbank et al., 2015). At the end of a protocol, it is necessary to reduce circulating P4 concentrations by removing the P4 device (exogenous source) and administering PGF2α analog to induce luteolysis (endogenous source), so that, ovulation may occur. Lastly, it is necessary to stimulate the final growth of the preovulatory follicle and to induce a synchronized ovulation (using either GnRH or E2 esters), which allows insemination to be performed at a predetermined moment (Baruselli et al., 2017).

Synchronization of follicular wave emergence

Two ways to synchronize the emergence of a follicular wave in the TAI protocols are to promote ovulation of a dominant follicle by administration of GnRH or to induce follicular atresia by the association of P4 and E2.

The Ovsynch protocol (GnRH/PGF2a-based) is by far the most popular among dairy producers (Norman et al., 2009) and uses GnRH to promote ovulation and, consequently, synchronization of wave emergence (Pursley et al., 1995). Although Ovsynch fulfills the three premises for ovulation synchronization, it is not highly efficient (64%) when given at a random day of the estrous cycle (Vasconcelos et al., 1999). Aiming at improving the response to the first GnRH in the Ovsynch protocol, presynchronization protocols have been adopted to increase the proportion of cows within the ideal interval to be started on the protocol, with the largest follicles responsive to the first GnRH (Moreira et al., 2001; Souza et al., 2008). Amongst the presynchronization protocols, Double-Ovsynch has shown the best synchronizing results when compared to Presynch-Ovsynch, with mean ovulation to the first GnRH and P/AI of 82.0% and 49.7%, respectively (Souza et al., 2008). However, there are some limitations with Double-Ovsynch, such as long duration (28 days) and too many cow-handling that may restrict its use. Thus, there is still the need to develop more practical presynchronization protocols. Recently, our research group developed a new presynchronization method by induction of the largest follicle with an intravaginal P4 device 10 days before Ovsynch in lactating crossbred dairy cows, named P4synch (Silva et al., 2018; Sales et al. 2019). In the first study (Silva et al., 2018), P4synch had similar follicular diameter at the time of the 1st GnRH (Double-Ovsynch 17.2±0.7mm and P4synch 18.6±0.9mm; P= 0.28), ovulation rate to the 1st GnRH [Double-Ovsynch 86.3% (44/ 51) and P4synch 81.2% (39/48); P=0.50] and P/AI [Double- Ovsynch 39.0% (89/228) and P4synch 40.1% (85/212); P=0,85]. In the second study (Sales et al., 2019), the pre-synchronization rate (presence the follicle with >12 mm on D0) for P4synch group was 97.8% (45/46). There was difference between groups for presence of CL on D0 (P4E2: 80.4% [37/46] and P4synch: 37.0% [17/46]; P = 0.001), follicular diameter on D0 (P4E2: 15.0 ± 0.8mm and P4synch: 21.0 ± 0.8mm; P = 0.001), at the time ovulation induction (P4E2: 13.9 ± 0.9mm and P4synch: 17.6 ± 0.6mm; P = 0.001) and TAI (P4E2: 15.2 ± 0.7mm and P4synch: 17.2 ± 0.8mm; P = 0.05). Furthermore, there was no difference between groups for synchronization rate (presence de follicle with > 12

mm on TAI; P4E2: 76.1% [35/46] and P4synch: 80.4% [37/46]; P = 0.61), follicular persistence after ovulation induction (P4E2: 8.7% [4/46] and P4synch: 15.2% [7/46]; P = 0.34] and P/AI (P4E2: 37.4% [67/179] and P4synch: 42.4% [72/170]; P = 0.35]. The use of P4-intravaginal devices for periods longer than 10 days results in the development of larger and longer-lasting follicles compared to the natural patterns (Díaz et al., 2015). Cows with P4 devices develop large follicles due to the absence of a pre-ovulatory luteinizing hormone (LH) peak and maintenance of sub-luteal P4 concentrations (Short et al., 1979). Largest follicles are capable of ovulating after long periods (15 days) of P4 blockage (Lucy et al., 1990). Thus, the largest follicle (more than 10 days of growth) may be used in a presynchronization protocol as a more practical tool to precede Ovsynch.

Estradiol esters (E2 benzoate [EB], E2 valerate [EV], and E2 cypionate [EC]) have different pharmacokinetics after administration to animals. Some studies have shown that EB has a shorter half-life and higher peak concentration than EV or CE (Colazo et al., 2003). These pharmacological differences between E2 esters alter reproductive physiology responses during ovulation synchronization protocols (Sales et al., 2012). Some E2 esters are used to induce both synchronization of follicular wave emergence (Bo et al., 2003) and ovulation (Baruselli et al., 2004; Sales et al., 2012). In ovulation synchronization protocols, the most used E2 ester to synchronize follicular wave emergence is EB, and the E2 ester used to synchronize ovulation is EC. The EB can also be used to synchronize ovulation, but additional management is needed during the ovulation synchronization protocol (Sales et al., 2012). Moreover, EV can be used for both synchronization functions and has the advantage of not increasing the number of handlings necessary for the ovulation synchronization protocol (Bo et al., 1993). These differences in the characteristics of E2 esters are related to the size of the ester chain. The longer the ester chain, the lower the solubility in water and the longer the period required for absorption (Mapletoft et al., 2002). Recently, our research group evaluated the effect of EV on follicular dynamics and fertility of lactating *Bos indicus* cows subjected to the ovulation synchronization protocol. In Experiment 1, the occurrence of estrus and P/AI were similar between EB and EV administrated on D0 (P = 0.12 and P = 0.82, respectively). In Experiment 2, P/AI tended to be lower (P = 0.07) in cows inseminated 48 hours after removal of the P4 device when EV was administered at the beginning of the ovulation synchronization protocol compared to TAI at 54 hours. In Experiment 3, the occurrence of estrus (P = 0.12) and P/AI (P = 0.56) were similar between EB and EV administered on D0 and associated with EC on D9 and TAI at 48 hours after P4 device removal (Table 1). Thus, protocols using EV without exogenous ovulation induction require adjustments in the timing of AI from 48 to 54 hours after P4 device removal. However, combining EV at the beginning of the protocol and EC on D9 to induce ovulation allowed TAI to be performed 48 hours after P4 device removal in *Bos indicus* cows. Thus, the use of EV and P4 at the beginning of the ovulation synchronization protocol represents a less expensive alternative (no need to administer PGF2α), with less management (one fewer treatment; less handlings) and with similar reproductive efficiency to of the one with EB in *Bos indicus* cows subjected to TAI protocols. However, EV must be associated with EC so that TAI can be performed 48 hours after the removal of the P4 device (Sales et al., 2024b).

Table 1. Effect of estradiol ester used during progesterone-based timed-AI (TAI) protocols in suckled *Bos indicus* cows on the occurrence of estrus and pregnancy per AI (P/AI).

Variable	Occurrence of estrus, % (n/n)	P/AI, % (n/n)	P	
			Estrus	P/AI
Experiment 1				
EB/EC	78.3 (148/189)	52.1 (215/413)	0.17	0.82
EV	72.5 (129/178)	51.8 (207/400)		
Experiment 2				
TAI 48h	73.7 (160/217)	36.4 (79/217)	0.34	0.07
TAI 54h	77.9 (169/217)	45.2 (98/217)		
Experiment 3				
EB/EC	70.9 (156/220)	43.2 (95/220)	0.12	0.56
EV/EC	76.6 (160/209)	45.5 (95/209)		

EB/EC group - 2 mg of estradiol benzoate (EB) on D0 and 265 µg of cloprostenol sodium, 300 IU of equine chorionic gonadotropin (eCG) and 1 mg of estradiol cypionate (EC) on D9. EV group - 5 mg of estradiol valerate (EV) on D0 and 300 IU of eCG on D9. P4 - intravaginal progesterone (P4) device. EV/EC group - 5 mg of EV on D0 and 300 IU of eCG and 1 mg of EC on D9.

Circulating P4 concentrations and eCG

During the TAI protocol, it is necessary to control P4 for a certain period to prevent premature ovulation, control the final growth of the dominant follicle, and allow ovulation. To achieve this, the intravaginal P4 device must remain in the females for 7, 8, or 9 days to allow controlled follicular growth and prevent ovulation before AI. After this, it is necessary to drastically reduce circulating P4 to allow synchronized ovulation after administration of the ovulation inducer (Prata et al., 2020). The time of exposure to an intravaginal P4 device depends on the animal category. In primiparous cows, it is necessary to keep the P4 device for a longer period to allow for greater follicular growth, greater ovulation, and improved P/AI. In a recent study (Carvalho et al., 2023), a greater period of permanence (9 days) of P4 device in an E2/P4-based TAI protocol increased [7P4 = 25.0% (47/188) vs 9P4 = 41.1% (79/192); P = 0.01] P/AI in suckled *Bos indicus* primiparous cows. However, in multiparous cows, despite differences in expression of estrus (7D protocol induced lower expression of estrus compared to 8 or 9 D protocols), P/AI was similar among treatment due to the additional effect of GnRH treatment at AI on fertility of females not displaying estrus (Prata et al., 2020).

The use of eCG has shown positive effects on P/AI in herds with anestrous cows, early postpartum cows (less than 2 months postpartum), cattle with inadequate body condition score (<2.75 on a scale from 1 to 5), and in cows with compromised growth of the dominant follicle due to too high circulating P4 concentrations toward the end of the ovulation synchronization treatment (Sales et al., 2011a). It was observed that eCG given on the day of P4 vaginal device removal increased final growth of the preovulatory follicle, neither interfering with the number of ovulatory follicles nor with the time of ovulation. Due to the greater follicle growth, eCG-treated cows had higher ovulation incidence and P/AI. The effects of eCG on follicular dynamics and fertility were observed in multiparous and primiparous cows but were more pronounced in primiparous cows (Sales et al., 2016). In general, eCG increased final growth of the dominant follicle without altering the synchronization of ovulation. Such characteristics allowed for insemination of females at a predetermined time. Similar follicular dynamic results were described previously, in which the final growth of the dominant follicle was significantly greater in eCG-treated cows (1.45 mm/day) compared with control (0.90 mm/day; Sales et al., 2011a). The growth of the dominant follicle is stimulated by eCG due to its affinity for LH and FSH receptors present in follicular granulosa cells (Murphy and Martinuk, 1991). It is known that LH binds to granulosa cell receptors, triggering a cascade of reactions that synthesize catalytic enzymes responsible for producing steroids and consequently stimulate the final growth of the dominant follicle (Carroll et al., 1992). Similarly, eCG would increase E2 production stimulated by the follicular synthesis of cytochrome P450 17A mRNA (Soumano et al., 1998). Thus, eCG would act as a gonadotrophic support, similarly to LH, stimulating the growth of the dominant follicle, especially in low BCS anestrous and primiparous females. In this regard, an interesting strategy to use eCG has been proposed for primiparous *Bos indicus* cows in TAI programs (Pugliesi et al., 2022). In primiparous Nelore cows, splitting the commonly used eCG dose (300IU) over two time points (2 days before and at the time of removal of the P4 device) resulted in a 6.8% increase in P/AI compared to the administration of a single dose at P4 device removal (Pugliesi et al., 2022). Also, in a subsequent study (Sales et al., 2024a), it was observed that splitting the dose or increasing the eCG dose to 400IU positively impacted the P/AI of primiparous cows with BCS ≤ 2.75, but no effects were detected on multiparous cows.

For primiparous cows, an eCG splitting effect was observed on the size of the dominant follicle, as cows receiving eCG in two moments (150 or 200 IU, two times) of the synchronization protocol had a larger follicle and greater P/AI than cows administered eCG only at the time of P4 device removal. In addition, primiparous cows receiving 400 IU eCG, regardless of BCS, had greater P/AI than cows from other treatments. Administering 400 IU to cows with low BCS also resulted in greater P/AI than all other treatments assigned to this category.

Synchronization of ovulation

Estradiol esters, such as EB and EC, have been used for inducing synchronized ovulation in beef cows. Administration of EC at the time of P4 device removal on Day 8 or EB on Day 9 resulted in

ovulation at 68.5 and 70.2 hours after P4 device removal, respectively (Sales et al., 2012). In addition, the P/AI was similar between cows treated with EB on Day 9 (57.5%; 277/482) and EC on Day 8 (61.8%; 291/471; Sales et al., 2012). The traditional protocol in which EB is administered on Day 9, however, requires one extra handling (Day 0, EB + P4 device insertion; Day 8, PGF2α + P4 device removal; Day 9, EB; and Day 10, TAI), implying a disadvantage for reproductive management. To decrease the number of times, cows have to be handled, EB was administered at the time of P4 device removal. This administration of EB at the time of P4 device removal on Day 8 resulted in anticipated ovulation (59.4 hours after P4 device removal) and the resulting P/AI was satisfactory when TAI was performed 48 hours after P4 device removal (Ayres et al., 2008). The TAI 54 hours after P4 device removal resulted in a decreased P/AI, probably because TAI was performed near the time of the synchronized ovulation. Another report (Cavalieri et al., 2002) also described lower P/AI when EB was administered at the time of P4 device removal (Day 8) compared to EB administered 24 hours after device removal (Day 9). In another study, our group (Crepaldi et al., 2019), aimed to minimize the number of handlings during protocols for TAI in beef cows treated for induction of ovulation with EB at the time of P4 device removal. In this study, EB administration and P4 device removal were performed 10 hours later (Day 8.5; EB8.5 group) than at the conventional time (Day 8). Thus, cows of the EB8.5 group were submitted to TAI 38 to 42 hours or 44 to 48 hours after P4 device removal (D10). The ovarian response and P/AI of *Bos indicus* cows were similar among treatment group [EB8.5 = 60.1 (200/333), EB on D9 = 66.7 (232/348) and EC on D8 = 66.0 (233/353)]. These outcomes were observed due to the distinct response for induction of a preovulatory LH surge release when E2 esters are used in the treatment protocol. In a previous study, cows submitted to TAI that were treated with EC for induction of ovulation had a preovulatory release of LH surge 31 hours later than cows treated with EB (Sales et al., 2012). This difference in timing of the pre-ovulatory release of LH surge resulted in an expected delay of 10 hours at the time of ovulation. The delay by 10 hours in EB administration and P4 device removal, as compared with the timing when there are typically administrations of EB in the EB8.5 group, resulted in a day and timing of ovulation (Day 11 AM) similar to the EC administered on Day 8 (AM) and EB administered on Day 9 (AM). This adjustment in time of EB treatment and P4 device removal allowed the TAI to occur in the morning and afternoon of day 10 of the treatment protocol without hampering reproductive efficiency.

Time of artificial insemination (TAI)

Several groups have studied the appropriate timing of AI relative to the onset of estrus or ovulation in cows (Pursley et al., 1998; Dransfield et al., 1998; Roelofs et al., 2006). The general consensus is that later AI (>12 hours after the onset of estrus) usually results in greater fertilization rates but lower embryo quality when compared to insemination closer to the onset of estrus (Dalton et al., 2001; Saacke, 2008). For example, a large field study that included 17 herds and 2,661 breedings demonstrated that inseminating >24 hours after the onset of estrus resulted in a dramatic reduction in the frequency of pregnancy compared to inseminations performed between 4 and 12 hours after the onset of estrus (Dransfield et al., 1998). For TAI protocols, the time of insemination depends on the ovulation inducer used. Usually, TAI is performed 48 to 54 hours after EC administration on D8, 24 to 30 hours after EB administration on D9 (Sales et al., 2012), and 16 hours after GnRH administration (Wiltbank et al., 2015).

Unfortunately, the optimal interval for TAI with non-sorted sperm may not be compatible with the use of sex-sorted sperm for several reasons, including the potentially reduced lifespan of sex-sorted sperm in the female reproductive tract (Maxwell et al., 2004), fewer numbers of sorted sperm/straw (DeJarnette et al., 2008) and possible pre-capacitation induced by the sorting procedure (Lu and Seidel, 2004). In a small field trial, it has been reported an increased P/AI in heifers receiving AI 18–24 hours after the observed onset of estrus, as compared to those inseminated at 0–12 hours (Schenk et al., 2009). It is therefore reasonable to expect that decreasing the insemination-ovulation interval may be critical for achieving greater P/AI with sex-sorted sperm following TAI. A study conducted by our research group verified that increasing the interval between P4 device removal and TAI, such that most cattle were bred 0 to 12 hours before the synchronized ovulation, improved P/AI in TAI programs using sex-sorted sperm (Sales et al., 2011b).

Some strategies are used at the time of AI to optimize the response to the TAI protocol in *Bos indicus* cows. The use of GnRH at the time of TAI increased P/AI in cows that did not show estrus (52.7 [n = 393] vs. 38.1% [n = 420]; P = 0.001), in cows with BCS < 3.0 (57.1 [n = 723] vs. 48.6% [n = 698]; P = 0.001), and in primiparous cows (50.1 [n = 465] vs. 41.9% [n = 497]; P=0.001 Alves et al., 2021). Another alternative is the use of hCG, a glycoprotein hormone that has similar activity to LH. However, it has a longer half-life. In bovine females, its use can induce ovulation by binding to LH receptors in the granulosa and theca cells in ovarian follicles (De Rensis et al., 2008). In a recent study by our research group (Teixeira et al., 2022), the use of hCG increased P/AI in cows that did not show estrus (Control = 42.9% vs. hCG = 53.3%; P=0.04). Furthermore, the pregnancy at natural breeding tended to be greater in cows that showed estrus and received hCG (P=0.09).

Previous exposure to injectable P4 in TAI protocol

In *Bos indicus* lactating beef cows, the post-partum anestrous period is long (Ruiz-Cortés and Olivera-Angel, 1999), negatively affecting the herd's productive and reproductive indexes (Montiel and Ahuja, 2005). Despite the benefits of the TAI protocols, part of the cows do not respond to the synchronization of ovulation protocols due to a drastic reduction in LH pulse-frequancy observed mainly in primiparous cows (Sales et al., 2016) and in undernourished cows with low BCS (Grimard et al., 1995; Diskin et al., 2003).

In postpartum *Bos indicus* cows, it is necessary to stimulate the hypothalamus to release GnRH and to increase LH pulse-frequency which would allow for the final growth of the dominant follicle and ovulation. The positive effects of ovulation synchronization protocols in anestrous cows are mainly due to the stimulation of exogenous P4 on the pulsatility of GnRH and LH (Rhodes et al., 2002), allowing for ovulation of a pre-ovulatory follicle early in postpartum (Baruselli et al., 2017). During the early postpartum period, circulating P4 reduces the expression of E2 receptors in the hypothalamus by interfering with the hormone receptor-negative feedback in GnRH secretion (Day, 2004). However, in underfed cows with low BCS or primiparous, the final growth of the dominant follicle is hampered, resulting in small follicles at the time of TAI and 21% of cows do not respond to TAI protocols (Sales et al., 2016). Thus, in females that do not respond to the TAI protocol, the period of exposure to P4 during the ovulation synchronization protocol may not be sufficient to increase the LH pulsatility needed for ovulation to occur. Thus, treatment with P4 in anestrous cows increased follicular fluid E2 concentration due to increased LH pulse-frequency and its LH receptors on granulosa and theca cells in pre-ovulatory follicles (Rhodes et al., 2002). Some studies have shown that the use of P4 stimulates return of cyclicity in lactating dairy cows (Lucy et al., 2001). Recently, our research group conducted studies to evaluate the effect of injectable P4 (P4i) on the reproductive efficiency of postpartum *Bos indicus* and *Bos taurus* cows submitted to TAI. In the first study (Simões et al., 2018) the effect of previous exposure to P4i in TAI protocols on follicular growth and P/AI of postpartum *Bos indicus* cows was evaluated. In this study, the cows received 150 mg of P4i 10 days before the TAI protocol (D-10; Figure 1). The P4i treatment increased the follicular diameter at the beginning of the TAI protocol and on the day of removal of the P4 device. In addition, cows receiving P4i were 1.68 times more likely to become pregnant after TAI than the control group. Similar outcomes were observed in *Bos taurus* beef cows (Simões et al., 2024), in which the P4i treatment previous to TAI protocol increased P/AI (Control 45.6% [118/259)] and P4i 54.8% [142/259]; P=0.03). In another study (Santos et al., 2018) using 988 postpartum Nelore cows in adequate BCS (~3.0), a P4i treatment preceding the ovulation synchronization protocol did not influence P/AI (Control 64.7% [322/498] and P4i 62.9% [308/490]; P = 0.55) and cyclicity 30 days after TAI (Control 39.8% [70/176] and P4i 39.6% [72/182] P = 0.78). Thus, probably in cows with adequate BCS, postpartum LH pulsatility should allow growth and ovulation of a preovulatory follicle. This difference in fertility after P4 treatment is probably due to the BCS of the animals in the different studies. In the study by Simões et al. (2018), the cows were nutritionally impaired which resulted in low BCS. Nutritionally deficient cows have lower postpartum LH pulsatility associated to the formation of metabolites (Nonesterified fatty acids, Beta-hydroxybutyrate and acetate), endorphins and

peptides (mainly neuropeptide Y) known to produce negative feedback, blocking hypothalamic GnRH release (Hess et al., 2005). Thus, treatment with P4i before ovulation synchronization protocols may have increased LH secretion (Day, 2004), which resulted in greater P/AI. Similar outcomes were observed in *Bos taurus* dairy cows (Simões et al., 2023), in which the P4i treatment previous to TAI protocols increased P/AI (30 days: Control - 52.1% [122/234] and P4i – 56.2% [144/256]; and 60 days: Control - 49.6% [115/232] and P4i - 53.5% [136/254]). In addition, in the subgroup of cows without CL on D-7, the P/AI at 30 and 60 days after TAI was greater in cows of the P4i group (30 days: Control-NoCL - 32.7% [17/52] and P4i-NoCL - 50.7% [35/69]; P = 0.04; and 60 days: Control-NoCL 31.2% [15/48] and P4i-NoCL 50.0% [33/66]; P = 0.05). In this study, the cows received 300 mg of P4i 7 days before of the TAI protocol (D-7).

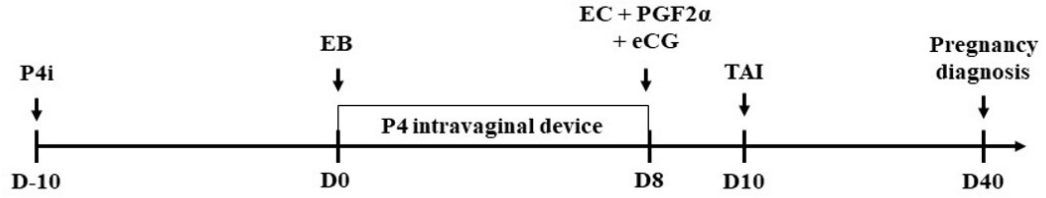

Figure 1. Progesterone-based timed-AI (TAI) protocol used in Brazil in suckled *Bos indicus* cows. P4i: 150 mg of injectable P4; Intravaginal device containing 1 g of progesterone; EB: 2 mg of estradiol benzoate; EC: 1 mg of estradiol cypionate; PGF2α: 500 µg of cloprostenol sodium; eCG: 300 IU of equine chorionic gonadotropin; TAI: Timed artificial insemination.

Conclusion

In the last 40 years, TAI was the main ART used that has enabled the expansion of AI in dairy and beef farms in Brazil, increasing service rates and genetics. The TAI protocols in beef and dairy cows are well established and hormonal manipulation of follicular and luteal dynamics in ovulation synchronization programs for TAI consist of synchronizing the emergence of a new wave of follicular growth, controlling the length of the P4 phase by progestogens e prostaglandins and steroids (PGF2α and estrogens) and inducing the synchronized ovulation of the dominant follicle. Recently, several fine-tuning adjustments in the protocols, such as the P4i strategy (previous exposure to injectable P4) brought a significant increase in fertility in *Bos indicus* and *Bos taurus* females.

Acknowledgements

The authors are grateful to the CNPq, CAPES and FAPEMIG for the financial support and Farmers of Brazil for allowing the use of their animals and facilities for several studies.

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Author contributions

JNSS: Conceptualization, Resources, Supervision, Visualization, Writing – review & editing; GP: Conceptualization, Visualization, Writing – review & editing; LRC: Conceptualization, Visualization, Investigation, Writing – review & editing; LMSS: Conceptualization, Visualization, Investigation; LAL: Investigation; MPV: Investigation; RRRS: Investigation; PSB: Conceptualization, Supervision, Visualization, Writing – review & editing.

Animal Reproduction

Thematic Section: 40th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

40 'wild' years: the current reality and future potential of assisted reproductive technologies in wildlife species

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How to cite: Mastromonaco G. 40 'wild' years: the current reality and future potential of assisted reproductive technologies in wildlife species. Anim Reprod. 2024;21(3):e20240049. https://doi.org/10.1590/1984-3143-AR2024-0049

Abstract

Over the past 40 years, assisted reproductive technologies (ARTs) have grown significantly in scale and innovation, from the bovine embryo industry's shift from in vivo derived to in vitro produced embryos and the development of somatic cell-based approaches for embryo production. Domestic animal models have been instrumental in the development of ARTs for wildlife species in support of the One Plan Approach to species conservation that integrates in situ and ex situ population management strategies. While ARTs are not the sole solution to the biodiversity crisis, they can offer opportunities to maintain, and even improve, the genetic composition of the captive and wild gene pools over time. This review focuses on the application of sperm and embryo technologies (artificial insemination and multiple ovulation/in vitro produced embryo transfer, respectively) in wildlife species, highlighting impactful cases in which significant progress or innovation has transpired. One of the key messages following decades of efforts in this field is the importance of collaboration between researchers and practitioners from zoological, academic, governmental, and private sectors.

Keywords: assisted reproduction, embryo technologies, biodiversity conservation, wildlife species.

Introduction

Decreasing wildlife population trends are coupled with concerns of ecosystem disruption that have far-reaching impact on animals and humans alike. The International Union for Conservation of Nature Species Survival Commission's (IUCN SSC's) One Plan Approach to conservation promotes in situ and ex situ connectivity in population management to enhance species sustainability goals (Byers et al., 2013). Assisted reproductive technologies (ARTs) can have a significant impact on achieving species recovery targets through long-term storage of genetic material as assurance against on-going or future depletion of diversity. While ARTs are not the miracle solution for fighting extinction, they are a powerful supporting tool for genetic management across time. Interest in the application of ARTs in wildlife species began in the mid-1970s with the transfers of flushed 'exotic' embryos into recipients of related livestock species (Kydd et al., 1985; Dresser, 1986). They gained momentum in the mid-1980s with the landmark paper by Jonathan Ballou, a researcher focused on the genetic and demographic challenges of small population management (Ballou, 1984). The wide range of reproductive strategies across vertebrate taxa (see Figure 1 for sperm and oocyte diversity) significantly influenced the development of ART programs, and the importance of domestic animal models was recognized early on (Wildt et al., 1986). This review will touch briefly on the progress of artificial insemination (AI) as a conservation breeding tool and focus on the potential for embryo technologies, specifically multiple ovulation embryo transfer (MOET) and in vitro produced embryo transfer (IVPET), to meet the growing threats to species sustainability.

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Received: April 18, 2024. Accepted: June 25, 2024.
Conflicts of interest: The authors have no conflict of interest to declare.

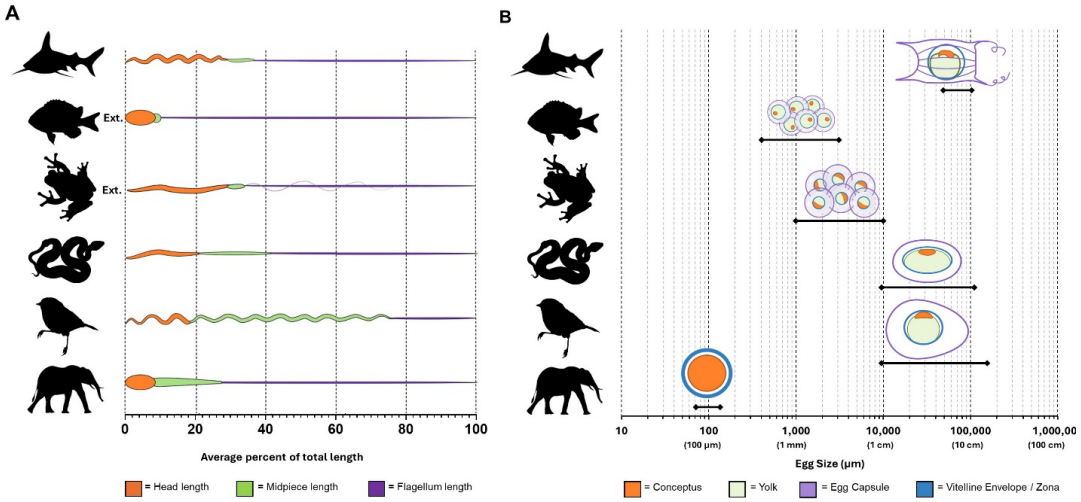

Figure 1. Diversity in gamete morphology across vertebrate taxa. Stylized sperm and egg drawings depict broad similarities and differences among Chondrichthyes, Osteichthyes, Amphibia, Reptilia, Aves, and Mammalia. There are further species-specific differences that are not captured in this schematic. (A) proportion of sperm head, midpiece and tail lengths among vertebrate classes. Total sperm lengths range from 20 – 350 µm depending on the species. Ext. = external; only sperm morphology from external (and not internal) fertilizers are shown. Sperm figure modified from Kahrl et al. (2022) by removing sperm images from internal fertilizers for Osteichthyes and Amphibia; licensed under a Creative Commons Attribution 4.0 International License (CC BY 4.0; http://creativecommons.org/.licenses/by/4.0/); (B) cellular and extracellular layers encompassing the eggs (or oocytes) of different vertebrate classes (Menkhorst and Selwood, 2008). Egg diameters range from 100 – 100,000 µm depending on the species. Egg images were created with BioRender.com (agreement number TG26OWIEYQ). Silhouette illustrations were contributed by various authors under public domain license (CC0 1.0 license) from PhyloPic (http://phylopic.org).

Sperm Technologies: Impact of Artificial Insemination

The past century demonstrated the potential of sperm-focused technologies. In wildlife species, insemination of females under natural or stimulated cycles continues to be the most effective ART for offspring production. To date, artificial insemination (AI) has been attempted successfully in ~100 species across diverse taxa (Swanson and Penfold, 2018) with reptiles just beginning to benefit from an increased investment in the development of ARTs (Perry, 2021). Protocols for external fertilizers, such as fishes and amphibians, have also advanced, and will be discussed in the section below on in vitro embryo production. The power of AI stems from its widespread application due to the lower investment of resources (funds, specialized equipment, technical expertise), but also because the embryo is developing in its natural environment, precluding the need for in-depth knowledge of pre-implantation embryo development.

Sperm from wild mammals is collected by electroejaculation or post-mortem epididymal dissection, and more recently by urethral catheterization (Lueders et al., 2012; Prieto et al., 2014). The latter approach will be beneficial as animal welfare guidelines continue to change, making minimally invasive alternatives a necessity. In non-mammalian species, sperm samples are obtained by massage, artificial vagina, or catheterization (reviewed by Prieto et al., 2014). For many mammals, birds and fishes, sperm handling solutions are commercially available from related domestic animals and humans. In species where a commercial sperm extender is unavailable or not providing the desired outcomes, modification of a commercial product (e.g., by altering glycerol concentration) or optimization of a 'home-made' recipe is required.

Fresh/chilled sperm has produced viable offspring across all taxa (reviewed by Blanco et al. (2009) (birds); Kouba et al. (2009) (amphibians); Beirão et al. (2019) (fishes); Mastromonaco and Songsasen (2020) (mammals/birds); Perry (2021) (reptiles)); however, implementation of ARTs for long-term genetic management requires access to sperm over extended periods of time for the infusion of novel or minimally represented genes. As a result, sperm cryobiology has been an active field of study resulting in hundreds of published papers detailing the effects on pre- and post-thaw sperm characteristics. Sperm cryopreservation and use in ARTs have been successful

in many of the species attempted, even if just a one-time success. Species exhibiting chilling sensitivity traits can be challenging despite extensive efforts to optimize species-specific techniques. In these cases, enhancement of standard protocols may provide additional protection to sperm membranes to improve post-thaw motility and integrity, such as the use of specialized density gradients or oviductal extracellular vesicles in cheetahs (*Acinonyx jubatus*) (Crosier et al., 2009; Ferraz et al., 2020). More recently, several researchers shifted their focus to freeze-drying sperm, and preliminary trials showed promising outcomes (Kaneko et al., 2014; Anzalone et al., 2018). The possibility of eliminating the dependence on liquid nitrogen makes this an attractive method for field collections and resource-restricted conservation biobanks.

Insemination of naturally cycling or hormonally primed females using either fresh, chilled or frozen-thawed sperm has been accomplished through a variety of methods: non-surgically using transcervical (mammals) / transcloacal (birds, reptiles) sperm deposition, or surgically using laparotomic or laparoscopic sperm deposition into the uterine cavity or oviduct (Swanson and Penfold, 2018). Notably, for AI to be successful, the female must be at the right stage of the ovarian cycle to receive the sperm, except perhaps in species with long-term sperm storage capabilities within the female (e.g., bats: 6 months; lizards: up to 1.5 years; sharks: 1-2 years; snakes: up to 7 years; Holt and Fazeli (2016). The important topic of ovarian stimulation will be discussed further in the embryo technologies section below.

One of the greatest challenges for wildlife ARTs is advancing the technique from research to application. With limited access to research animals and materials, protocol development and implementation can take multiple decades. Thus, many of the AI births to date have been proof-of-concept attempts that generated media attention without further progress. The slow advancement and relatively low success rates present a significant challenge when advocating for the inclusion of ARTs in conservation breeding programs. Despite these drawbacks, AI has been used not only to achieve ex situ targets (sustainability of captive populations), but also to support in situ needs (recovery of the species in their native ranges).

Spotlight: Common Bottlenose Dolphin (*Tursiops truncatus*)

Throughout the 2000s, Todd Robeck and Justine O'Brien spear-headed a comprehensive sperm-based approach that included fresh-chilled, frozen-thawed and sex-sorted sperm inseminations in bottlenose dolphins. AI offered an opportunity to overcome the challenges of managing the genetics and demographics of a closed captive population in a species that is difficult to translocate between institutions. Ovarian synchronization, manual semen collection and sperm cryopreservation protocols were developed, and intra-uterine inseminations resulted in 60-70% pregnancy rates for fresh-chilled, frozen-thawed un-sorted and frozen-thawed sex-sorted sperm (O'Brien and Robeck, 2006; Robeck et al., 2013). To date, 30 bottlenose dolphin calves have been born from AI using sex-sorted frozen-thawed sperm with a sex predetermination rate of 93% (United Parks & Resorts, 2024), making it one of the greatest achievements in wildlife AI. Building on this work, successful inseminations have resulted in pacific white-sided dolphin (*Lagenorhynchus obliquidens*; Robeck et al., 2009), orca (*Orcinus orca*; Robeck et al., 2004), and beluga whale calves (*Delphinapterus leucas*; O'Brien et al., 2008). Although ART development has primarily benefitted marine mammals in managed care, the groundwork has been laid for possible application in future wild cetacean recovery efforts.

Embryo Technologies: Enhanced Potential for Genetic Rescue

With the on-going pressure to preserve and restore biodiversity, there is an urgency to include female genetics in the wildlife biobanks. This requires investment in embryo technologies, specifically the acquisition of a) oocytes for fertilization or cryopreservation, b) embryos for transfer or cryopreservation, and c) somatic cells for production of gametes and embryos. These techniques are dependent on species-specific knowledge of the female's reproductive biology and the embryo's early development. Despite the additional challenges, the need to ensure female genetic contribution to conservation populations has stimulated concentrated efforts in various threatened species.

The 1970s and 80s sparked an interest in interspecific transfers to investigate the possibility of using domestic animal surrogates to gestate endangered species embryos. These early attempts included the transfers of in vivo derived (IVD) embryos from Grant's zebra (*Equus quagga boehmi*) and Przewalski's horse (*Equua ferus przewalskii*) into domestic mare, bongo antelope (*Tragelaphus eurycerus*) into common eland antelope (*Taurotragus oryx*), and more (Dresser, 1988; Allen and Wilsher, 2020). The subsequent decades brought a re-focusing of attention onto basic reproductive biology to expand the knowledge of reproductive anatomy, physiology, and gamete biology (Pukazhenthi and Wildt, 2004; Herrick, 2019). Thus, while live births from embryo technologies have been documented, the outcomes have not been as consistent and widespread as AI, apart from fishes and amphibians. Currently, embryo technologies are not being implemented as a population management strategy in mammals, birds, or reptiles.

a) Ovarian Cycle Monitoring and Control

The female component of ARTs requires monitoring or control of ovarian dynamics in the oocyte donors and embryo recipients. For offspring-endpoint AIs, both natural and 'artificially' synchronized (i.e., application of exogenous hormones or alteration of environmental conditions such as changes in temperature or humidity) cycles are used. When using natural cycles, as in the giant panda, non-invasive hormone monitoring (urine and feces) is a necessity since blood sampling and ultrasonography are not possible in species that cannot be routinely handled (Kersey et al., 2010). In contrast, many elephants are trained for blood collection allowing serum LH levels to be used to time inseminations during natural cycles, a feat that is possible only due to the double LH peaks in African and Asian elephants (*Loxodonta africana* and *Elaphas maximus*, respectively; Thitaram and Brown, 2018). Aside from the species that can be trained for blood sampling, the role of non-invasive hormone monitoring in conservation breeding programs cannot be underestimated. Fecal hormone profiles have helped shed some light on the complexities of embryonic diapause, follicular stasis, and more. Similarly, the use of transrectal ultrasound in animals that can be chute trained has been instrumental in elucidating ovarian dynamics and establishing the timing for inseminations and embryo transfers (Pennington et al., 2019).

Ovarian synchronization with exogenous hormones has been successfully applied in many mammalian species using protocols generally adopted from farm and laboratory animals with certain species-specific modifications to optimize outcomes. In wild felids, gonadotropin dosages based on domestic cat studies proved difficult to extrapolate to diverse wild cat species on size alone due to species-specific differences in sensitivities to eCG and hCG: ocelots (*Leopardus pardalis*; 9 kg) required double the dose than cheetahs (35 kg), whereas clouded leopards (*Neofelis* nebulosa; 15 kg) required the same dose as domestic cats (2 kg) (Thongphakdee et al., 2018). Studies in domestic cats and cheetahs demonstrated that progestin priming prior to gonadotropin administration normalized the endocrine response thereby improving ovulation, corpus luteum function and oocyte developmental potential (Stewart et al., 2012; Crosier et al., 2017).

Acquisition of multiple oocytes from donor females requires ovarian super-stimulation in monovulatory species, as well as certain polyovulatory species. Application of exogenous hormones (e.g., FSH) to enhance follicular recruitment must maintain a balance between producing a maximum number of follicles and ensuring the retrieval of competent oocytes. Domestic cattle hormone regimens have formed the basis of many wildlife super-stimulation trials, ranging from wild bovids (e.g., banteng (*Bos javanicus*)) to antelopes (e.g., addax (*Addax nasomaculatus*)) (Sontakke, 2018). Notably, studies in domestic cattle demonstrated that extrapolation between even the more closely related species and subspecies, namely holstein (*Bos taurus*), gir (*Bos indicus*), and murrah cattle (*Bubalus bubalis*), is problematic due to the inherent differences in follicular waves, antral follicle populations, and follicle and corpus luteum diameters (Baldrighi et al., 2022).

For non-mammalian species, similar approaches employing exogenous hormones have been documented. In amphibians, both spermiation and ovulation can be induced with injections of hCG or GnRH; however, in certain species such as the Puerto Rican crested toad, hormones alone were not effective in stimulating ovulation, and altered environmental

conditions (e.g., hibernation at low temperatures) were required prior to hormone administration to enhance outcomes (Kouba and Vance, 2009). In both fishes and amphibians, addition of dopamine antagonists to GnRH protocols improved ovulation and fertilization (Van Eenennaam et al., 2008; Silla et al., 2021). In birds, on the other hand, inseminations have been timed to natural laying cycles (Swengel and Tuite, 1997), with alterations in photoperiod being used to induce egg laying outside of the breeding season (Zhu et al., 2017).

b) Multiple Ovulation Embryo Transfer (MOET)

MOET has not been widely attempted in wild mammals. Retrieval of multiple embryos from naturally bred or artificially inseminated donor females are either transferred fresh to recipient females or cryopreserved for future use; an effective method for increasing a female's lifetime reproductive output. Despite the abundance of data from domestic cattle studies highlighting the increased competence of IVD embryos compared to in vitro produced (IVP) embryos (Ferré et al., 2020), wildlife researchers interested in obtaining female genetics have focused on IVPET. Thus, aside from the early attempts on interspecies ET and efforts in species of commercial importance (e.g., dromedary camels (*Camelus dromedarius*), red deer (*Cervus elaphus*)), MOET has been applied in only a handful of species of socio-cultural, economic, and nutritional relevance to indigenous communities (wood bison (*Bison bison athabascae*), yak (*Bos grunniens*), llama (*Lama glama*), alpaca (*Vicugna pacos*); Mastromonaco, 2024). The lack of traction with MOET in conservation breeding programs has been due to difficulties in accessing and handling invaluable donor females. However, even in domestic cattle, there has been a shift away from MOET towards IVPET, with the number of transferable IVP embryos now surpassing the number of IVD embryos produced annually; a change that corresponds with the increasing efficiency of IVP systems (Ferré et al., 2020).

Spotlight: Wood Bison (*Bison bison athabascae*)

Since 2006, Gregg Adams has led a systematic plan to establish a biobank of disease-free sperm and embryos from free-ranging wood bison to mitigate the effects of tuberculosis and brucellosis in wild herds (Bison Integrated Genomics (BIG) Project, 2022). Domestic cattle synchronization and super-stimulation protocols have been somewhat effective in wood bison, particularly with modifications (i.e., single slow-release FSH dose) to reduce handling frequency and stress (Toosi et al., 2013). Interestingly, exogenous hormones could overcome seasonal constraints to produce competent oocytes with successful development in vitro but not in vivo, suggesting that differences in the oviductal environment negatively affected IVD embryo development in the anovulatory season (Palomino et al., 2020). Subsequent attempts to produce embryos across ovulatory and anovulatory seasons focused on retrieving oocytes for IVP (Zwiefelhofer et al., 2022a). To date, >20 calves have been born from AI, MOET and IVPET using fresh-chilled, frozen-thawed and sex-sorted sperm (reviewed by Acevedo and Barfield, 2023). With progress now being made on the collection of sperm and oocytes from free-ranging wood bison (Zwiefelhofer et al., 2022b), the wood bison project is one of the applications of the One Plan Approach to conservation that will include biobanking and ARTs; a potential model for the wisent, another bison species at risk (Duszewska et al., 2022).

c) In Vitro Produced Embryo Transfer (IVPET)

IVPET is the goal of many wildlife ART programs interested in preserving female genetics. Both sperm and oocytes are taken outside their 'natural habitats' and moved ex vivo into the culture dish requiring in-depth knowledge of gamete maturation, fertilization, and embryo development to ensure post-implantation success. Studies have highlighted the differences between IVP and IVD embryos, with the latter typically resulting in greater success post-thaw, -implantation and -birth (Ferré et al., 2020). While domestic and laboratory animal models have been instrumental in generating basic protocols, species-specific requirements have made advancement challenging in many wildlife species. Aquatic external fertilizers (fishes and amphibians), which release large numbers of oocytes and do not require the final embryo transfer step to produce live young, have experienced the greatest successes with IVP embryos. Aside from these cases, embryo technologies are not currently being implemented in species restoration programs. Wildlife IVP embryos have been created by both conventional (IVF) and advanced (somatic cell nuclear transfer; SCNT) methods to begin populating the conservation biobanks with female genetics (Mastromonaco, 2024). Optimistically, in vitro gametogenesis (IVG) may be contributing to the growing list of banked gametes

and embryos in the coming years. To date, animals born from IVPET have been scarce such that announcements of confirmed pregnancies or births continue to generate media attention.

i) In Vitro Fertilization (IVF)

Challenges with in vitro maturation, fertilization, and culture (IVM-IVF-IVC), typically reported as low polar body extrusion, cleavage and blastocyst rates in domestic species, are also experienced in many wildlife species (Mastromonaco, 2024). For instance, ovulation of immature (MI) oocytes and difficulties with in vitro maturation (<20% MII) in domestic dogs results in the need for in vivo matured oocytes in gray wolf (*Canis lupus*) IVPET trials (Nagashima and Songsasen, 2021). Similarly, a lack of optimal IVM systems for domestic cats (~60% MII) drives the outcomes in wild felid studies (Thongphakdee et al., 2020), and poor in vitro capacitation in domestic horse sperm leads to the use of intracytoplasmic sperm injection (ICSI) in zebra, Przewalski's horse (Gambini et al., 2020), and white rhinoceros (Hildebrandt et al., 2023). Inadequate IVC conditions responsible for the developmental 'block' at the time of embryonic genome activation in novel species has required transfer of embryos in the early cleavage stages in springbok and blesbok (*Antidorcas marsupialis* and *Damaliscus pygargus*, respectively; Chatiza et al., 2013), Eld's deer (*Rucervus eldii*; Thongphakdee et al., 2017), and reindeer (*Rangifer tarandus*; Lindeberg et al., 2021). In non-mammalian species, yolk-laden oocytes have made cryopreservation significantly more challenging than the smaller mammalian oocytes (Diwan et al., 2020). Despite these setbacks, researchers have not only generated IVF embryos but produced offspring of genetic value to the captive and wild populations.

Spotlight: Brown Brocket Deer (*Subulo gouazoubira*)

Over the past decade, the work of José Duarte on brown brocket deer highlights the challenges in developing IVF programs for novel species. Using a multi-disciplinary approach to neotropical deer genetic preservation involving cytogenetics, molecular genetics, and reproductive biology, Duarte and colleagues have been investigating the full spectrum of ARTs (Rola et al., 2021a). Common brown brocket deer pregnancies were obtained using frozen-thawed sperm, potentially serving as a model for more vulnerable brocket deer species (Duarte et al., 2023). Retrieval of oocytes from super-stimulated ovaries using laparascopic techniques resulted in 65% IVM rates (Rola et al., 2021b). While embryos have not yet been produced in brown brocket deer by conventional IVF methods, iSCNT (using domestic cattle oocytes) resulted in 6% blastocysts (Melo et al., 2022). Notably, progress in wild cervid IVF has been challenging with low blastocyst rates observed in Eld's deer (5%; Thongphakdee et al., 2017), and reindeer (4%; Peippo et al., 2019). In contrast, dedicated efforts in the Japanese sika deer (*Cervus nippon nippon*), a model for the endangered Vietnamese sika deer (*Cervus nippon pseudaxis*), resulted in 30% blastocyst rates once the embryos were co-cultured with ovine epithelial cells (Locatelli et al., 2012). With so many species in need of assistance, prioritizing resources based on ecosystem or food security relevance will be essential for achieving conservation impact with ARTs.

ii) Somatic Cell Nuclear Transfer (SCNT)

Following the report by Dominko et al (1999) demonstrating the bovine ooplasm's capacity as a 'universal recipient', interspecies SCNT (iSCNT) emerged, fueling the rise in somatic cell biobanks in zoological and government institutions, as well as the production of iSCNT embryos in more than 50 species (Mastromonaco et al., 2014). Although blastocyst development has been demonstrated in diverse species, including tiger (*Panthera tigris*), red panda (*Ailurus fulgens*), and minke whale (*Balaenoptera acutorostrata*), only a small number of offspring have been born (Mastromonaco et al., 2014). Challenges stemming from low and aberrant reprogramming have impacted the advancement of iSCNT as a practical tool for offspring production. From a species conservation perspective, the recent birth of the black footed ferret clone using donor cells cryopreserved from a wild female in 1988 and oocytes from domestic ferrets provided an opportunity to re-introduce under-represented genetics, thereby having a significant impact on the diversity of the remaining ferret population (Imbler, 2021). This is a key example of the potential for cloning technologies in the One Plan Approach as it was always known that iSCNT would not become a widespread tool for offspring production, but a powerful tool for the 'resurrection' of key individuals of high genetic value.

Spotlight: Przewalski's Horse (*Equus ferus przewalskii*)

Species with small founder gene pools and high inbreeding coefficients typically require reproductive support as with the Przewalski's horse. Although the first retrievals and interspecies transfers of Przewalski's horse embryos occurred more than 40 years ago (Allen and Wilsher, 2020), there has been only one reported birth of a healthy foal following timed AI and none from IVPET reviewed by Cabeza and Gambini, 2023). Advancements in domestic horse SCNT (approximately 400 foals born; reviewed by Gambini and Maserati, 2017) fostered the opportunity to investigate iSCNT as a tool to produce Przewalski's horse foals. Under the guidance of Oliver Ryder with the San Diego Zoo's Frozen Zoo®, Przewalski's horse stallion cells cryopreserved in 1980 were used to produce 11 transferrable embryos that resulted in 7 pregnancies and 2 live births (Novak et al., 2024). While it may not be a significant scientific advancement, it is a remarkable change in mind-set as the American Association of Zoos and Aquariums previously did not accept nuclear-cytoplasmic hybrids into the captive gene pool. Further, the project was supported by the not-for-profit Revive & Restore (2024), a sponsor of de-extinction projects such as the woolly mammoth, passenger pigeon, great auk and more (Revive & Restore, 2024), which may be academically interesting pursuits but do not serve the conservation community.

iii) In Vitro Gametogenesis (IVG)

The potential to 'convert' adult somatic cells into induced pluripotent stem cells (iPSCs) using human and mouse transcription factors ('Yamanaka factors'; Ogorevc et al., 2016) captivated researchers interested in understanding the cellular and developmental biology of wildlife species. Since then, iPSCs have been generated in >20 wildlife species, including snow leopard (*Panthera uncia*), Sumatran orangutan (*Pongo abelii*), little brown bat (*Myotis lucifugus*), Okinawa rail (*Hypotaenidia okinawae*), Japanese ptarmigan (*Lagopus muta japonica*), to name a few (Katayama et al., 2022; Swegen et al., 2023). Advancements in stem cell induction and differentiation brought IVG closer to reality with the birth of mouse pups following in vitro oogenesis that generated the iPSC-derived oocytes (Hayashi and Saitou, 2013). More recently, in vitro spermatogenesis resulted in iPSC-derived spermatids used to produce living offspring in the rat (Matsumura et al., 2023). Similar to SCNT, iPSC-based IVG requires successful reprogramming of adult somatic cells into a stable pluripotent state, a feat that is not easily achieved (Li et al., 2014). Further, differentiation of the iPSCs into primordial germ cell-like cells (PGCLCs) has been effective in the mouse model but difficult to achieve in farm animals (Strange and Alberio, 2023). This comes with additional safety challenges due to the teratogenic potential that arises from using transgene integrating delivery methods and a highly oncogenic set of genes. There is hope that the significant efforts already underway in both the human and animal fields will propel IVG science forward.

Spotlight: White Rhinoceros (*Ceratotherium simum*)

Since the 1990s, cases of ovarian cycle irregularities and reproductive pathologies have been investigated in captive rhinoceros (reviewed by Roth, 2024). However, the plight of the northern white rhinoceros (rhino), a functionally extinct subspecies with only two living adult females, instigated one of the most lucrative partnerships in conservation ART history between BioRescue and Colossal Biosciences (BusinessWire, 2023). What began as technique development to support white rhino breeding programs has resulted in a race to de-extinct the northern white rhino subspecies. Multiple attempts at live and post-mortem oocyte collection followed by IVM-IVF-IVC using domestic horse protocols have recently resulted in blastocysts (Hildebrandt et al., 2023). While there have been 10 white rhino calves produced by AI with fresh and frozen sperm (Roth, 2024), there are no live calves from the transfer of IVF embryos to date. Currently, there are two research teams that have independently induced a pluripotency state in multiple northern white rhino somatic cell lines, some of which had been cryopreserved for 40 years (Korody et al., 2021; Zywitza et al., 2022). Progress towards IVG was reported in 2022 with the generation of primordial germ cell-like cells northern white rhino iPSCs (Hayashi et al., 2022). While these initiatives don't have immediate application in biodiversity conservation, they are important advancements towards the establishment of somatic cell technologies for genetic management of wildlife species.

Focus for the Future

Forty years of efforts from researchers around the world have resulted in a proportionately small number of cases that have successfully integrated AI, MOET or IVPET into species conservation plans. To date, ARTs have been more readily applied in external fertilizers, specifically fishes and amphibians, since the in vitro environment does not have the complexity of re-creating the oviductal or uterine milieu ex vivo. In contrast, mammals will not experience the same widespread application and abundance in offspring production anytime soon. There are several key research priorities to continue advancing mammalian ARTs: a) the oocyte (culture optimization and cryopreservation), b) the pregnant uterus (recipient management), and c) the environment (emerging pollutants and climate change). Innovative technologies, ranging from microfluidics to 3-D culture systems (Ferraz and Ferronato, 2023), offer untapped possibilities for overcoming the current limitations in producing embryos with optimal developmental capacity pre- and post-implantation. Similarly, the growing literature on nutrition provides important insights for improving pregnancy maintenance and fetal development (Wyse et al., 2022). Unfortunately, reproductive health and ART success will be challenged by the continuously changing natural environments, particularly ubiquitous particles such as micro- and nano-plastics (Aardema et al., 2024).

As species continue to face threats to their long-term survival, ARTs provide some assurance that the genetics carefully stewarded in wildlife biobanks around the globe can be used to keep keystone species thriving and ecosystems intact. For ARTs to become a realistic addition to the One Plan Approach to species conservation, comprehensive programs must include not only the initial research and protocol development phase, but also establishment of partnerships and generation of banked inventory through systematic sample collection. In a field with such limited resources, it is important to remain focused on species or populations that have the greatest chance for self-sustainability in the long term. Thus, projects directing resources and attention towards extinct or functionally extinct species, while making potentially valuable scientific advancements that could be extrapolated to related species, are a distraction from impact-driven species conservation objectives.

Acknowledgements

Thanks to Cristina Lyons for assistance with reference compilation for the manuscript and Virginia Wolfe for preparation of Figure 1. Special thanks to Dr. Ariel Kahrl for providing us with the original files of the sperm images used in Figure 1.

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Author contributions

GFM: Conceptualization, literature review, writing, reviewing, and editing.

Animal Reproduction

Thematic Section: 40th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Bovine embryo production *in vitro*: evolution of culture media and commercial perspectives

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How to cite: Krisher RL, Herrick JR. Bovine embryo production *in vitro*: evolution of culture media and commercial perspectives. Anim Reprod. 2024;21(3):e20240051. https://doi.org/10.1590/1984-3143-AR2024-0051

Abstract

In vitro-produced embryos exhibit lower viability compared to their *in vivo* counterparts. Mammalian preimplantation embryos have the ability to reach the blastocyst stage in diverse culture media, showcasing considerable metabolic adaptability, which complicates the identification of optimal developmental conditions. Despite embryos successfully progressing to the blastocyst stage, adaptation to suboptimal culture environments may jeopardize blastocyst viability, cryotolerance, and implantation potential. Enhancing our capacity to support preimplantation embryonic development *in vitro* requires a deeper understanding of fundamental embryo physiology, including preferred metabolic substrates and pathways utilized by high-quality embryos. Armed with this knowledge, it becomes achievable to optimize culture conditions to support normal, *in vivo*-like embryo physiology, mitigate adaptive stress, and enhance viability. The objective of this review is to summarize the evolution of culture media for bovine embryos, highlighting significant milestones and remaining challenges.

Keywords: embryo culture, *in vitro* embryo production, culture media, embryo metabolism.

Introduction

While advancements have been made in bovine oocyte maturation and embryo culture over the past decades, *in vitro* embryo technologies capable of producing embryos with similar viability as those developing *in vivo* have remained elusive. There is still much to unravel regarding the *in vitro* requirements of oocytes and embryos to facilitate successful development and the production of healthy offspring. Exposure of embryos to suboptimal culture conditions, resulting in altered embryo metabolism, not only leads to decreased blastocyst formation and reduced embryo viability, but also negatively impacts the maintenance of pregnancy, fetal growth, and health of offspring (Farin et al., 2001). The metabolic adaptability of embryos is remarkable, but it comes at considerable cost. Therefore, to mitigate the adaptive stress leading to poor embryo quality, diminished pregnancy potential, and adverse health outcomes in future offspring, it is imperative that embryo culture conditions support normal embryo physiology.

Oocyte maturation *in vitro* is a critical component of *in vitro* embryo production, but it will not be covered here. Development of maturation medium that better supports oocyte developmental competence is worthy of an independent review. The reader is directed to other manuscripts for treatment of this topic (Krisher, 2004, 2013; Labrecque and Sirard, 2014; Dumesic et al., 2015; Lonergan and Fair, 2016; Aguila et al., 2020; Marei and Leroy, 2022; Fair and Lonergan, 2023).

*Corresponding author: rebecca.krisher@genusplc.com
Received: April 21, 2024. Accepted: August 20, 2024.
Financial support: RLK received support from Genus plc; JRH received support from Omaha's Henry Doorly Zoo and Aquarium.
Conflicts of interest: The authors have no conflict of interest to declare.

Commercial application of bovine *in vitro* embryo production

Each year, the Data Retrieval Committee of the International Embryo Technology Society (IETS) gathers, organizes, and publishes statistics describing the embryo industry in farm animal species. The most recent data available for the calendar year 2022 show that the global bovine embryo industry continues to grow (Viana, 2023). However, the actual growth rate between 2021 and 2022 (5.5%) was decreased compared to that observed between 2020 and 2021 (25.6%). For *in vitro* produced (IVP) embryos specifically, from 2020 to 2021 the number of embryos produced grew by 31.5%, whereas between 2021 and 2022, this growth rate dropped to 6.3%. Nonetheless, almost 1.5 million bovine embryos were transferred globally in 2022. Cryopreservation is also a growing trend in cattle embryo technologies, with almost half of all cattle embryo transfers utilizing frozen embryos. In vivo derived embryos are more commonly cryopreserved (65%) than IVP embryos (44%), due in part to reduced cryotolerance of IVP embryos particularly when slow freezing/direct transfer is used versus vitrification. This difference in cryotolerance has implications for how or even if IVP embryos are used for export. In general, embryo technologies are utilized in 28% of countries worldwide, with 95% of the reported embryos being specifically cattle embryos. Notably, more than 80% of all bovine embryos produced globally are *in vitro* derived, and *in vitro* derived embryos continue to drive growth in commercial embryo technologies. Based on the sales of embryo transfer materials, the data retrieval committee suggests that these numbers are in fact an underestimate and that the actual number of embryos produced *in vitro* in 2022 was likely more than 2 million globally. Indeed, for the last decade *in vitro* technologies have been supplanting superovulation and collection of *in vivo* embryos as the preferred method for cattle embryo production. In 2022, seven out of the top 10 embryo producing countries reported a higher number of IVP embryos compared to *in vivo* derived (IVD) embryos.

What accounts for the rapid increase in the adoption of *in vitro* embryo technologies? There is mounting pressure on animal agriculture to produce more protein, both milk and meat, for a growing world population. At the same time, producers must be mindful of sustainability goals and expectations from consumers. Embryo technologies can help to achieve both objectives. Genetically superior animals, identified by genotyping, are more efficient in their production of protein. The use of semen in conventional AI allows bulls of high genetic merit to contribute more to the next generation of production animals, but male genetics are only half the equation. Using embryos accounts for both male and female genetics, making genetic progress much faster (Sirard, 2018). A valuable female can produce significantly more embryos over her lifetime when ovum pick up and *in vitro* fertilization are used to produce embryos, versus superovulation and embryo flushing. Oocytes can even be collected from prepubertal and pregnant females. Importantly, using ovum pick up in calves via laparoscopy and in pre-pubertal heifers can significantly shorten generation interval, resulting in even faster genetic gain. This approach enables the most genetically superior animals to make the greatest contribution to the succeeding generation, leading to swift improvements in production efficiency and ultimately enhancing the sustainability of animal agriculture overall. Currently, the higher cost of embryos relative to semen is the main roadblock to even greater implementation of embryo technologies, given that pregnancy rates are roughly equal between the two technologies. Efforts to automate embryo production to drive down costs while increasing efficiency, quality, and scale are currently being investigated. Relevant to both human and bovine embryo technology, semi-automated vitrification systems have been reported and used in clinical trials (Wang et al., 2024; Roy et al., 2014; Hajek et al., 2021; Barberet et al., 2022).

Challenges of *in vitro* fertilization technology

In vitro embryo production, despite its widespread commercial application, remains an inefficient process. This inefficiency significantly contributes to the high cost per embryo, limiting adoption for many producers. The largest inefficiency in the system is the loss between oocytes recovered at ovum pick up and transferrable quality embryos produced following in vitro fertilization (IVF). Only about 25% of all oocytes collected become good quality embryos,

and this number is even lower if sexed semen is used for fertilization. Another large loss occurs following embryo transfer, as less than 50% of good quality embryos result in pregnancy. Early embryonic losses before day 60, as well as additional losses to abortion or stillborn calves, contribute as well. Overall, fewer than 10 calves are born for every 100 viable oocytes collected. Improvement in the system would mean that oocytes could be collected from fewer, more genetically valuable donors, requiring less farm and laboratory labor to reduce cost and advance genetic gain (Figure 1). However, despite concentrated efforts to improve embryo culture media over the last 20 years, we have seen only minimal improvement in embryo production efficiencies and embryo quality.

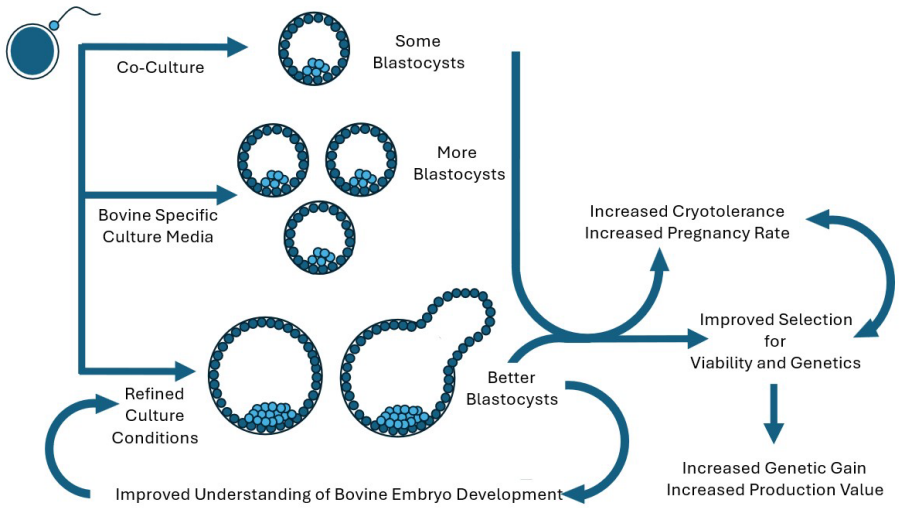

Figure 1. A better understanding of embryo biology informs the development of more effective culture media. Improved media produce embryos that are more *in vivo*-like, which further advance our understanding of embryo biology, improve cryotolerance and the outcome of embryo transfer, and ultimately result in increased genetic gain, increased production value and producer profit, and a more sustainable industry.

Embryo metabolism and the evolution of culture media

The identification of a suitable combination of nutrients, including appropriate concentrations and timing of provision, was pivotal to the formulation of successful embryo culture media. The pioneering investigations into embryo culture utilized murine embryos, which largely rely on carbohydrate metabolism to support development (Brinster, 1965; Biggers et al., 1967). Embryos at the early cleavage stages primarily utilize pyruvate and lactate, whereas in later stages, there is a transition to reliance on glucose metabolism through glycolysis to support formation and expansion of the blastocyst (Gardner and Leese, 1987; Gardner et al., 2001). Murine embryos could develop to the blastocyst stage in simple salt solutions with glucose, lactate, and pyruvate as the only energy sources, usually in the presence of albumin (Whitten and Biggers, 1968). Although the general pattern of carbohydrate metabolism is similar for bovine embryos, with the early cleavage stages primarily reliant on pyruvate oxidation and glycolysis becoming the dominant pathway after the morula stage (Rieger et al., 1992a, 1992b; Kim et al., 1993; Thompson et al., 1996; Krisher et al., 1999), early culture media developed for the mouse were not effective for culturing bovine embryos. Therefore, the first embryo culture systems for bovine embryos utilized media designed for somatic cell culture and often relied heavily on support cells in a co-culture environment. Co-culture was able to overcome the 8-16 cell block in bovine embryos, as well as improve the quantity and quality of blastocysts produced (Ellington et al., 1990; Goto et al., 1992; Voelkel and Hu, 1992; Thomas and Seidel, 1993; Abe and Hoshi, 1997; Krisher et al., 1998). Similar positive effects were observed using cell culture conditioned medium (Eyestone et al., 1991; Hernandez-Ledezma et al., 1993; Vansteenbrugge et al., 1994). It was hypothesized that

somatic cells removed inhibitory substances from the culture media, which at that time was sub-optimal. However, the complexity and undefined nature of co-culture and conditioned media systems made it virtually impossible to study the metabolic requirements of preimplantation bovine embryos *in vitro*.

Although many media have been independently developed, three media are the backbone formulations of most bovine culture media used today: synthetic oviductal fluid (SOF), Charles Rosenkrans medium with amino acids (CR1AA), and potassium simplex optimized medium (KSOM), each developed using slightly different approaches. Synthetic oviductal fluid has been a frequently utilized medium for culturing bovine embryos *in vitro* for more than 20 years. Initially, SOF was developed through the biochemical analysis of ovine oviductal fluid (Tervit et al., 1972). The strategy of mimicking the *in vivo* environment has been described as the 'back to nature' approach (Leese, 1998). Another extension of mimicking the in vivo environment was the concept of sequential media specifically devised to cater to the evolving needs of the embryo during its developmental journey, with one medium for early cleavage stage embryos and another for compaction and blastocyst formation (Gardner and Lane, 1997). A variety of single-step and sequential versions of SOF have been developed, which include the supplementation of amino acids (Gardner et al., 1994; Steeves and Gardner, 1999), incorporation of citrate (Keskintepe et al., 1995), the exclusion of glucose (Takahashi and First, 1992), and the inclusion of EDTA for the first 72 hours of the culture period (Gardner et al., 2000). One of the other primary strategies for culture medium design is to provide the embryo with all the nutrients it may need in a single medium, that may or may not be refreshed during the culture period. The rationale behind this strategy is to let the embryo select which nutrients it utilizes for development from those provided and is often described as 'let the embryo choose' (Biggers, 1998; Biggers and Racowsky, 2002). Empirical evaluations of the effects of specific carbohydrates, amino acids, and vitamins led to the formulation of CR1AA (Rosenkrans et al., 1993; Rosenkrans and First, 1994). A systematic approach known as simplex optimization was used to develop KSOM (potassium simplex optimized medium), originally designed to overcome the 2-cell block in mouse embryos and allow development to the blastocyst stage (Lawitts and Biggers, 1991, 1992; Erbach et al., 1994). This medium was quickly adapted for bovine embryo culture (Liu and Foote, 1995, 1996, 1997; Moreira et al., 2002). There are advantages and disadvantages to each type of system, and one system has not proven to be superior to the other (Macklon et al., 2002; Biggers and Summers, 2008; Machtinger and Racowsky, 2012; Quinn, 2012; Swain et al., 2016; Swain, 2019). Typically, SOF is used as a sequential medium (Matsuyama et al., 1993; Gardner et al., 2000; Gandhi et al., 2000), whereas CR1AA and KSOM are often used as a single step medium. Use of these two different systems has been debated for more than ten years, and choice is often dictated by what is currently in fashion, or what is most convenient for laboratory workflow.

Once media were available that would effectively support the development of bovine blastocysts *in vitro*, an increased emphasis was placed on not just producing blastocysts, but on the quality/viability of those blastocysts and their potential to produce healthy offspring. The conditions under which embryos are cultured impacts their ability to metabolize glucose (Du and Wales, 1993a, b; Rieger et al., 1995). Because the uptake and utilization of glucose are intricately linked to the developmental competency of embryos and likely mirror the energy needs of the embryo (Renard et al., 1980; Gardner and Leese, 1987), culture media choice and optimal laboratory practices are critical to produce high quality, viable blastocysts. Embryos cultured in oviductal cell-conditioned medium exhibited heightened glucose metabolism rates, but lower cell counts and delayed development, indicating that elevated glucose metabolism rates might correlate with diminished embryo viability (Rieger et al., 1995). An increased rate of glucose metabolism at the blastocyst stage has also been associated with reduced embryo viability in mice (Lane and Gardner, 1996).

Once metabolic changes were linked to viability the next step in the evolution of culture media was to provide a specific set of substrates to modulate the metabolism of *in vitro*-produced embryos to closely emulate their more viable counterparts produced *in vivo*. One such strategy is based on the "Quiet Embryo Hypothesis", which states that viable embryos, exhibit reduced oxidative phosphorylation activity and consequently consume less oxygen

because they require less energy to repair stress-induced damage (Leese, 2002; Baumann et al., 2007; Leese et al., 2007, 2008). Another hypothesis suggests that embryo metabolism might resemble that observed in rapidly proliferating cancer cells, a metabolic phenomenon referred to as the Warburg effect (Krisher and Prather, 2012; Smith and Sturmey, 2013). In this model, the most viable embryo is not necessarily the one exhibiting the lowest activity within a particular pathway, but rather the embryo metabolizing specific substrates through the most suitable pathway(s). As the focus has shifted to an evaluation of overall embryo metabolic activity thru multiple pathways, the importance of fatty acids and amino acids as substrates has become apparent.

Somewhat unexpectedly, sheep, pig, and cow embryos can thrive successfully with or without glucose in the culture medium (Tervit et al., 1972; Petters et al., 1990; Takahashi and First, 1992; Thompson et al., 1992; Rosenkrans and First, 1994; Steeves and Gardner, 1999; Gandhi et al., 2000). It is evident that embryos in these species are capable of utilizing endogenous fatty acids and/or amino acids for energy production even in the absence of carbohydrates. Mammalian embryos utilize fatty acids as an energy source (Ferguson and Leese, 2006; Sturmey et al., 2006, 2009b), with Kane (1979) being the first to identify the significance of fatty acids in this context. Interestingly, it appears that fatty acid oxidation in the oocyte and early embryo is closely intertwined with glucose metabolism, likely operating in an interdependent and compensatory manner (Sutton-McDowall et al., 2012; Paczkowski et al., 2014; Herrick et al., 2020). Amino acids also contribute to preimplantation embryo metabolism. The uptake and synthesis of amino acids have been observed in embryos across various species and have been linked to outcomes such as DNA damage, ploidy, embryo sex, and quality (Sturmey et al., 2009a; Picton et al., 2010). Supplementation with a combination of essential and non-essential amino acids in the absence of coculture was found to be advantageous for embryo development (Rosenkrans and First, 1994; Gardner et al., 1994; Steeves and Gardner, 1999), leading to an increase in the cell number of blastocysts cultured *in vitro*.

The effects of metabolic activity on the reduction-oxidation (REDOX) potential of the cell are now known to be as important, if not more so, than simple ATP production. Throughout the preimplantation phase, preserving a normal redox state is crucial for embryo development (Harvey et al., 2002). The cellular redox state is chiefly influenced by the ratios of key redox couples: NAD^+:NADH (largely influenced by lactate dehydrogenase activity) and $NADP^+$:NADPH (partially regulated by the pentose phosphate pathway (PPP)), along with the intracellular balance of reduced glutathione (GSH) to oxidized glutathione (GSSG). These nicotinamide molecules serve as vital cofactors for numerous metabolic reactions or act as their end-products. Thus, fostering optimal embryo metabolism in culture is imperative for preserving redox equilibrium and ensuring developmental competency.

Oxygen

Although the culture medium plays a significant role in the quantity and quality of embryos produced, there are many other factors of the larger culture environment, such as air quality, plastics, oil, etc., that influence outcomes (Wale and Gardner, 2016). One of the most critical of these factors seems to be oxygen in the atmosphere within the incubator. In vivo, oxygen tension in the oviduct and uterus ranges from 1.5% to 8.7%, and is 5.3% at the time of blastocyst formation in hamsters and rabbits (Fischer and Bavister, 1993). Reduced concentrations of oxygen (<10%) relative to normal, atmospheric levels (20%) improve embryo development and viability in all species where it has been evaluated, including cattle (Thompson et al., 1990, 2000). As oxygen is necessary for mitochondrial ATP production, the benefits of reduced oxygen are associated with changes in metabolism, particularly an increase in glucose consumption and production of ATP via glycolysis (Thompson et al., 1996, 2000). However, oxygen can also influence the expression of genes not directly associated with metabolic activity, including anaphase promoting complex and myotrophin, suggesting beneficial effects of oxygen on other aspects of embryo physiology (Harvey et al., 2007).

Growth factors

The fluids of the reproductive tract are known to contain a variety of growth factors, cytokines, and other cell signaling molecules, collectively known as embryokines, that influence embryo development and differentiation (Hansen and Tribulo, 2019). However, most culture media do not contain these embryokines. One reason for this discrepancy is the difficulty in studying the effects of growth factors. There are a large number of candidates whose effects can be inconsistent and dependent on the concentration used, the time of culture when they are included, and the composition of the medium (Herrick et al., 2018; Hansen and Tribulo, 2019; Amaral et al., 2022). In addition, the effects of these embryokines are often very subtle, difficult to interpret, and/or only apparent post-transfer (Hansen and Tribulo, 2019; Sang et al., 2020). For example, the inclusion of interleukin-8 (IL-8) in the culture medium increases the proportion of embryos that hatch but decreases the number of cells allocated to the inner cell mass (Sang et al., 2020). The effects of colony stimulating factor (CSF) 2 are dependent on the sex of the embryo, further complicating the formulation of media for typical (non-sorted sperm) IVF-produced embryos (Siqueira and Hansen, 2016). Although the addition of embryokines to culture media is not well understood, embryos do secrete them into the culture medium and these secreted embryokines are often credited with enhancing development of embryos cultured in groups versus individually.

Fetal bovine serum

Fetal bovine serum remains a common supplement used to compensate for suboptimal embryo culture environments. Serum can buffer stressors and insults in the embryo culture system that may inhibit embryo development. Although the inclusion of serum in the culture medium can enhance development to the blastocyst stage, it may also diminish the ability of resulting embryos to be cryopreserved, and to establish and maintain pregnancy (Rizos et al., 2003; Amaral et al., 2022). Inclusion of serum in culture medium has also been implicated in large, or abnormal, offspring syndrome, although a direct causal link has not been confirmed (Lazzari et al., 2002; Hansen, 2020) nor is the timing of exposure or the threshold concentration of FBS leading to these effects understood. This congenital overgrowth syndrome is observed in ruminants born through assisted reproduction and characterized by significant dysregulation of the epigenome and transcriptome, excessive somatic growth, and various developmental anomalies such as enlarged tongues, umbilical hernias, muscle and skeletal deformities, abnormal organ growth, and aberrant placental development (Li et al., 2019, 2022; Rivera, 2019). The frequency of this syndrome may vary depending on the embryo culture system utilized. Although there are no good data on frequency of occurrence in the ET industry, rough estimates are in the 3-5% range. For producers using embryo transfer of *in vitro* produced bovine embryos on a large scale, this is a significant drawback and has been detrimental to the acceptance of this technology.

Development of next generation embryo culture media

Investigations in our laboratory employed a gas chromatography-mass spectrometry platform to examine the nutrient composition of media following culture of individual embryos to better understand the metabolic profile of embryos *in vitro*. These metabolomic analyses suggested that embryos utilize only a fraction of the nutrients provided to them in the culture environment (Krisher et al., 2015; Herrick et al., 2016). The minimal amount of nutrients that are consumed compared to the abundance of nutrients available in the culture system prompted us to hypothesize that nutrient concentrations in the culture medium could be substantially decreased while still sustaining embryo development. In the mouse, nutrient concentrations (carbohydrates, amino acids, and vitamins) during the culture of murine embryos could be reduced by half with minimal impact on embryo development. However, decreasing nutrients, especially pyruvate and lactate, by more than 50% significantly impaired embryo development and viability (Ermisch et al., 2020). In the bovine embryo, development was largely unaffected

when nutrient concentrations were reduced by as much as 75%, and some embryos were able to develop in medium containing only 6.25% of the original nutrient concentrations (Herrick et al., 2020). Other studies have replicated this work, demonstrating improved blastocyst development and quality when nutrients were reduced by half (Santos et al., 2021). The exceptional resilience of the bovine embryo to significant reductions in nutrient availability is linked to its capacity to utilize endogenous lipids. To further refine our reduced nutrient concentration media for bovine embryos, we supplemented this media with exogenous lipids and L-carnitine to promote lipid metabolism. Under these conditions, blastocyst development was significantly improved, and the expression of embryo quality related genes was increased, although blastocyst cell number was lower (Pasquariello et al., 2023).

Transcriptomic analysis of *in vivo* produced embryos compared to *in vitro* embryos cultured in either standard or reduced nutrient conditions demonstrated that *in vitro* embryos produced in standard conditions were more active metabolically compared to *in vivo* produced embryos, while metabolic processes were in fact downregulated in embryos developed under reduced nutrient conditions (Ming et al., 2023). Embryos developed under reduced nutrient conditions upregulated genes associated with protein hydrolysis and cell survival, a strategy to maintain cellular homeostasis that is reminiscent of the high protein turnover that limits oxidative damage and extends lifespan in caloric restriction. Embryos developed in reduced nutrient conditions also had increased transmembrane transport, likely necessary for nutrient uptake in a restricted environment, again similar to caloric restriction response. Overall, the developmental potential of embryos cultured in reduced nutrient conditions was closer to *in vivo* embryos than that of embryos cultured *in vitro* under standard conditions (Ming et al., 2023). These studies open a new frontier in bovine embryo culture media development and may result in a more developmentally competent *in vitro* embryo. However, additional studies including post-transfer embryo viability and calf health are needed to fully appreciate the capability of this reduced nutrient culture system.

What does the future hold for bovine embryos?

We might take a cue from the world of human IVF. Embryo diagnostics, such as embryo sex, genotype, identification of chromosomal abnormalities, presence of desirable production traits, and or prediction of viability would add significant value to a bovine embryo (Figure 1). Today, we can successfully genotype and sex embryos by taking a biopsy of the trophectoderm at the blastocyst stage without significantly compromising subsequent development (Fujii et al., 2019; Oliveira et al., 2023). In fact, biopsy is a recognized emerging technology as the number of embryos being sexed or genotyped is now being tracked by the IETS Data Retrieval Committee and was greater than 23,000 embryos in 2022. Bovine embryos can also be screened for chromosomal abnormalities via trophectoderm biopsy (Turner et al., 2019; Silvestri et al., 2021). Biopsy procedures necessitate advanced technical expertise and expensive equipment, potentially influencing both the precision of genetic testing and implantation potential. Reliable genotype information can be obtained from embryo biopsies, but only a limited number of laboratories are currently using this technology due to these limitations. Prediction of pregnancy success in human embryos using artificial intelligence and machine learning with either photos or time lapse video is in use today (Vermilyea et al., 2020; Enatsu et al., 2022; Diakiw et al., 2022; Salih et al., 2023). These algorithms haven't yet been developed specifically for cattle embryos, partly because of the high cost of time lapse incubators. However, affordable models targeted at the veterinary sector are emerging, suggesting this could soon become a possibility for bovine IVF, and preliminary research supports this assumption (Sugimura et al., 2017). Alternatively, information could be gained about embryo genetics and viability non-invasively from cell free DNA or extracellular vesicles in the culture medium after blastocyst development, or from blastocoel fluid. Noninvasive preimplantation genetic testing (niPGT) of human embryos demonstrates that cell free DNA suitable for genetic analysis can routinely be obtained from these samples, offering an alternative to embryo biopsy that requires less skill, poses less risk to the embryo, and is less expensive (Huang et al., 2019; Rubio et al., 2019; Leaver and Wells, 2020; Rubio et al., 2020; del

Collado et al., 2023). However, reported concordance rates between cell free DNA and biopsy results are variable between studies, and the diagnostic value of noninvasive preimplantation genetic testing remains controversial in human IVF (Huang et al., 2023; Lledo et al., 2023).

Bovine embryos can also be a source of information to discover regulatory pathways important for development. Extracellular vesicles and their microRNA and protein cargo have emerged as pivotal bi-directional messengers between the embryo and its environment at various stages of pre- and post-implantation development (Lange-Consiglio et al., 2020; Salilew-Wondim et al., 2020; Tesfaye et al., 2020; Guzewska et al., 2023). Extracellular vesicles can influence blastocyst development and modulate embryo stress in vitro, potentially by modulating embryo gene expression (Alminana et al., 2017; Lopera-Vasquez et al., 2017; Menjivar et al., 2023). In addition, an embryos' extracellular vesicles and microRNAs may provide clues about the quality and viability of that embryo, possibly leading to non-invasive diagnostic assays (Marin and Scott, 2018; Cimadomo et al., 2019; Hawke et al., 2021). Finally, extended embryo culture (Shahbazi et al., 2016; Isaac et al., 2024) and synthetic embryo models (Kagawa et al., 2022; Kim et al., 2023; Pinzon-Arteaga et al., 2023; Yu et al., 2023) enable us to discover physiological processes occurring in the embryo after the blastocyst stage, a period difficult to study in vivo. In the long run, delivering embryos at a reduced cost (closer to that of a straw of sexed semen, perhaps) while maintaining high value and enhancing outcomes will necessitate concurrent advancements in multiple areas. These include the development of improved culture media supporting normal embryo physiology, enhancements in the culture environment, and the integration of various diagnostic technologies. The forthcoming decade promises to be an exciting era of exploration in bovine *in vitro* embryo production and diagnostics. As these breakthroughs are applied to the commercial bovine embryo transfer industry, they will further bolster the utilization of *in vitro* produced embryos and propel the industry towards a more sustainable approach to feeding the world. However, it's imperative that these advancements are accessible at a price point that ensures a reasonable return on investment for producers, for this technology to be truly transformative.

Conclusion

Rapid adoption of *in vitro* embryo production in the bovine industry highlights the importance of embryo technology for genetic improvement. A growing human population, food insecurity, and climate change put enormous pressure on producers to make protein production more efficient and sustainable. However, large inefficiencies in the system significantly increase cost and may limit full realization of the potential of the technology. The low conversion percentage of oocytes to blastocysts, and the quality of those blastocysts produced, significantly contribute to these inefficiencies. Improvements in embryo culture media resulting in a more viable, more freezable embryo capable of a high level of pregnancy establishment and maintenance, and normal healthy calf production, is essential.

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Author contributions

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SHORT COMMUNICATIONS

STUDENT COMPETITION

The impact of a maternal obesogenic diet on offspring's oocyte mitochondrial biogenesis in the primordial follicles at birth and at weaning. Insights from an outbred mouse model.

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Maternal obesity presents a global health problem, potentially impacting offspring fertility. Research showed that offspring born to obese mothers exhibit mitochondrial dysfunction in the ovulated oocytes at adult age, including markers of mitochondrial biogenesis. However, it remains unknown if these alterations are inborn. Also, lactation from an obese mother can alter postnatal metabolic programming and may impact offspring oocyte quality. Understanding the timings of mitochondrial alterations is crucial as maintaining a healthy primordial follicle pool is vital for sustaining folliculogenesis and guaranteeing oocyte quality upon ovulation. In this study, we aimed to investigate the impact of a maternal obesogenic (OB) diet on offspring's oocyte mitochondrial parameters in primordial follicles, hypothesizing that it affects mitochondrial biogenesis at birth (TP1) and at weaning (TP2).

Female Swiss mice were fed either a control (C)(n=5) or an OB (n=5) diet for 7 weeks and then mated (control males, cross-over design). The females remained on their allocated diets during pregnancy and lactation. Female offspring were sacrificed at TP1 and TP2. Ovaries were fixed in paraformaldehyde. The expression of Mitochondrial Transcription Factor A (TFAM), responsible for mtDNA transcription, and its activator Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) was examined by immunofluorescent staining and confocal microscopy, using one random section/ovary. For PGC-1α, 103 primordial follicles of the C group (TP1:n=55, TP2:n=48) and 100 primordial follicles of the OB group (TP1:n=71, TP2:n=29) were assessed. For TFAM, 80 primordial follicles of the C group (TP1:n=43, TP2:n=37) and 66 primordial follicles of the OB group (TP1:n=34, TP2:n=32) were assessed. Gray scale intensity was quantified in the ooplasm, and in the nucleus for PGC-1α, using three z-positions. First, the main effects of the maternal diet, timepoint (i.e. TP1/TP2) and their interaction were examined using Two-way ANOVA. For PGC-1α, a site effect (i.e. ooplasm/nucleus) was present and Three-way ANOVA was used. If the interaction was significant, treatment effects at each TP were examined by independent student *t*-tests or Mann-Whitney U tests in case of nonparametric testing.

The effect of the maternal OB diet on PGC-1α expression was dependent on the timepoint ($P<0.001$). Primordial oocyte PGC-1α was not altered by maternal OB diet at birth ($P=0.44$) but was increased in both the ooplasm($P=0.04$) and the nucleus ($P=0.046$) at weaning. TFAM was significantly reduced by the maternal OB diet ($P<0.001$) at both timepoints (interaction $P=0.48$).

In conclusion, since PGC-1α was not affected at birth, this indicates no inborn alterations in mitochondrial biogenesis and other PGC-1α-dependent metabolic pathways in oocytes of primordial follicles in offspring born to OB mothers. The effect on TFAM at birth may induce changes in oocyte mtDNA replication that might be adaptive or reparable in nature. Subsequently, nursing from an obese mother induces postnatal changes in PGC-1α which might partially explain the differences in mitochondrial and metabolic functions described in mature F1 oocytes in earlier studies.

Concentration of progesterone, relative mRNA abundance of Interferon-Stimulated Gene-15 and concentration of Pregnancy-Specific Protein B in maternal circulation following timed artificial insemination or embryo transfer in lactating dairy cows

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The objective of this study was to provide early pregnancy diagnosis and examine the relationship between progesterone (P4) concentration, relative mRNA abundance of interferon-stimulated gene-15 (ISG15) and concentration of Pregnancy-Specific Protein B (PSPB) in maternal circulation following timed artificial insemination (AI) or timed embryo transfer (ET). Following estrous synchronization, lactating Holstein-Friesian cows received either AI on d 0 (n = 119) or ET on d 7 (n = 418) with a fresh or frozen in vitro-produced (IVP) blastocyst derived from oocytes collected by transvaginal ovum pick-up from n = 14 Holstein Friesian, n = 8 Jersey and n = 21 Angus donors. Blood samples were collected on d 7 (n = 537), d 18 (n = 524) and d 25 (n = 378) to determine P4, ISG15 and PSPB, respectively, to provide early pregnancy diagnosis and measure associations with subsequent probability of pregnancy or pregnancy loss (PL). Transrectal ultrasonography to determine pregnancy status occurred on d 32, d 62 and d 125 and parturition date was recorded. All available variables where $P < 0.1$ were included in the final model for analysis (PROC GLIMMIX, SAS). Cows with greater P4 on d 7 had greater ISG15 on d 18 ($P < 0.0001$) and PSPB on d 25 ($P = 0.002$). mRNA abundance of ISG15 on d 18 was positively associated with serum PSPB on d 25 ($P < 0.0001$), and both varied by serum P4 quartile ($P = 0.01$ and $P = 0.007$, respectively). There was no overall effect of treatment (AI vs fresh ET vs frozen ET) on mRNA abundance of ISG15 in cows that were pregnant on d 18 ($P = 0.158$), but there was a tendency for cows that received fresh ET to have greater ISG15 than cows that received frozen ET ($P = 0.097$). Conversely, a strong treatment effect was detected for serum PSPB on d 25 in pregnant cows ($P = 0.0002$). Cows that received AI and fresh ET had greater PSPB than cows that received frozen ET ($P = 0.006$ and $P < 0.0001$ respectively). Cows with greater mRNA abundance of ISG15 (d 18) and serum PSPB (d 25) had a greater probability of pregnancy (d 32, d 62, d 125) and of reaching full term parturition (all $P < 0.0001$). Day 18 ISG15 expression was negatively associated with PL after d 18 ($P = 0.0139$). The PL (%) for each quartile of ISG15 was: 47.2%, 39.3%, 43.6% and 22.4% for quartiles 1 to 4, respectively. Serum PSPB on d 25 was associated with the incidence of PL after d 25 ($P < 0.0001$). Cows in the lowest quartile for PSPB had greater PL (58.1%) than cows in PSPB-Q3 (19.2%; $P = 0.023$) and PSPB-Q4 (9.6%, $P = 0.002$). Cows in PSPB-Q2 (35.5%) had greater PL than cows in PSPB-Q4 ($P = 0.0003$) and tended to have greater PL than PSPB-Q3 ($P = 0.051$). In conclusion, cows that had greater P4 on d 7 had greater mRNA abundance of ISG15 on d 18, indicating a stronger maternal response to conceptus-derived interferon-tau. These cows also had a greater PSPB on d 25, which was associated with greater likelihood of reaching full-term parturition. This study highlights the temporal pattern of pregnancy establishment and loss in cows that were bred using AI or IVP-ET, and important areas for additional research to improve IVP procedures.

Funded by Science Foundation Ireland (16/RC/3835) and Department of Agriculture, Food and The Marine (2021R665)

Embryo-induced alterations in the protein profile of bovine uterine extracellular vesicles in vitro

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Effective embryo-maternal communication, crucial for successful pregnancy, is partly mediated via extracellular vesicles (EVs). This study aimed to use uterine explants as an ex vivo model to investigate the communication between blastocysts and the endometrium via EVs, focusing on identifying changes in EV protein profile resulting from this interaction. For this, five synchronized cyclic heifers were slaughtered 7 days after oestrus. From each heifer, four 8 mm circular endometrial explants were obtained from the anterior portion of the ipsilateral uterine horn and cultured individually in 1 mL of protein-free synthetic oviduct fluid (SOF): two were cultured alone (Expl), and two were co-cultured with 5 in vitro-produced bovine blastocysts (Expl+Emb). Also, a group of 50 in vitro-produced bovine blastocysts were cultured alone (Emb) in 500 µL of SOF. Only day 7 blastocysts of excellent or good quality were used. All groups were cultured for six hours at 5% CO2 in humidified air at 38.5°C, after which conditioned medium (CM) was collected for EV isolation. EVs were isolated using size exclusion chromatography, and characterized by detecting CD63, CD81, and CD44 EV markers using flow cytometry. Proteomic analysis was carried out using nanoLC-MS/MS with spectral counting for protein identification and quantification. EVs from five replicates from each group were analyzed. For qualitative analysis, proteins were considered present if detected in ≥3 replicates and were considered exclusive if detected in ≥3 replicates within one group but not detected in any sample within other groups. Bioinformatic analysis was performed with Metascape and Panther tools. We identified 1501 proteins in the CM-EVs from Expl, 1975 in the CM-EVs from Expl+Emb, and 82 in the CM-EVs from Emb. Of these, 66 proteins were detected in the three experimental groups, 1145 were common to Expl and Expl+Emb, none were common to Expl and Emb, none were common to Expl+Emb and Emb, 2 were unique to Expl, and 1 (uncharacterized) was unique to Emb. Moreover, 50 unique proteins were exclusively present when there was an interaction between the endometrium and the embryo (Expl+Emb). These 50 proteins are related to processes such as embryo development (CXADR, MTHFD1L, THOC5, PTK2, MAP7), regulation of stem cell differentiation (EIF2AK2, NELFB, DHX36), and establishment or maintenance of cell polarity (ARF6, PTK2, MAP7, SH3BP1). Additionally, these proteins can potentially modulate endometrial cells, with PTGES2 involved in the eicosanoid metabolic process, EIF2AK2 participating in the interferon tau (IFNT) signaling pathway, and SCRN1 being an IFNT-dependent gene. Our results indicate that the endometrium is responsive to the presence of blastocysts, as evidenced by alterations in the EV protein composition of spent culture medium. Furthermore, these variations may affect embryo development and the IFNT signaling pathway, suggesting EV involvement in embryo-maternal interaction in the first week of pregnancy. Funded by ES-MICIN PID2019-111641RB-I00 & PRE2020-094452.

Oleic Acid prevents the detrimental effect of the combination of saturated NEFAs with Lipopolysaccharide on bovine oocyte competence

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In high-producing dairy cows experiencing Negative Energy Balance (NEB) during the transition period, the mobilization of Non-Esterified Fatty Acids (NEFAs) can coincide with post-partum inflammatory conditions such as metritis, endometritis, and mastitis, resulting in the presence of Lipopolysaccharide (LPS) in both the bloodstream and follicular fluid. This cascade of events may compromise the developmental competence of oocytes. It was previously demonstrated that oleic acid counteracts the toxic effects of saturated fatty acids in vitro (Aardema et al., 2011). This study aimed to investigate whether oleic acid (OA) supplementation during in vitro maturation could mitigate the potential adverse effects of both saturated NEFAs and LPS. For this purpose, abattoir-derived bovine cumulus-oocyte-complexes (COCs) were exposed to different combinations of 100µM of stearic acid (SA), 150 µM of palmitic acid (PA), 10 µg/mL of LPS, and 200 µM of oleic acid for 22 hours of maturation and then fertilized (IVF) and cultured until day 8. A total of 1907 COCs (8 replicates) were divided into 6 experimental groups: Control (n=321), SA+PA (n=319), SA+PA+OA (n=316), LPS (n=310), SA+PA+LPS (n=320), SA+PA+LPS+OA (n=321). Cleavage and blastocyst rates were assessed on respectively days 5 and 8 (Day 0 = IVF). Data were analyzed by ANOVA and results were expressed as mean ± SEM. The cleavage rate of oocytes from the SA+PA (59.6 ± 4.5 %) and SA+PA+LPS (63.7 ± 5.1 %) groups were comparable and both decreased ($P<0.05$) compared to those exposed to SA+PA+OA (77.2 ± 3.0 %). There was no difference between the control, LPS, and SA+PA+LPS+OA (respectively, 71.4 ± 2.4, 66.0 ± 4.7, and 70.3 ± 2.0%). Blastocyst rates were reduced ($P<0.01$) in the SA+PA (17.3 ± 3.9 %) and SA+PA+LPS (19.6 ± 2.9 %) groups compared to the control (29.0±2.3 %). This adverse effect was counteracted by the addition of OA with respectively 31.1 ± 2.2 % for the SA+PA+OA and 29.4 ± 2.1 % for the SA+PA+LPS+OA group. The group of LPS alone was not different from the other groups (23.5 ± 2.7 %). These findings demonstrate that the presence of OA during in vitro maturation (IVM) counteracts the toxic effects of saturated NEFAs (SA+PA) and LPS. However, these results also suggest that oocyte competence is mainly affected by high levels of saturated NEFAs, as no additional toxic effect was observed when LPS was included. Interestingly, LPS alone did not result in a significant reduction in the blastocyst rate of exposed oocytes. In conclusion, the enrichment of the IVM medium with OA is effective at protecting bovine oocytes challenged with high levels of saturated NEFAs and LPS, suggesting a potential application in vivo to alleviate the detrimental effects of NEB.

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The effect of a maternal obesogenic diet on offspring oocyte mitochondrial ultrastructure at birth in an outbred mouse model.

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Obesity induces oocyte mitochondrial dysfunction with ultrastructural damage (e.g. broken membranes, rose-petal structures), predisposing offspring to metabolic disorders (*Marei, Scientific Reports, 10, 2020; Keleher, Plos One, 13, 2018*). Mitochondrial ultrastructural abnormalities (e.g. elongated, increased electron density, mitochondrial degeneration) were also reported in ovulated oocytes of offspring born to diet-induced obese (OB) dams (*Xhonneux, Frontiers in Physiology, 14, 2023*). Recently, we detected similar alterations in oocyte mitochondrial ultrastructure in primordial follicles of adult offspring born to OB mothers (*Xhonneux, Plos One, 2024, under review*). However, it remains unknown if these alterations are inborn, i.e. present in offspring oocytes at birth. Yet, this information is crucial for optimal fertility management in daughters born to obese mothers. Therefore, we hypothesized that pups born to OB mothers already have altered oocyte mitochondrial ultrastructure in the primordial follicle pool at birth. An outbred Swiss mouse model was used to increase the pathophysiological relevance to humans.

Swiss dams (age 3 weeks) were fed control (C, n = 5) or OB (n = 5, high-fat, high-sugar) diets for 7 weeks, then mated with the same Swiss males fed a chow diet in a cross-over design. Dams stayed on the same diet during pregnancy. Pregnancies were monitored and litters were culled within 1h post-partum. One ovary of one female pup per dam was collected for ultrastructural analysis of oocyte mitochondria using transmission electron microscopy. At least 6 primordial follicles per ovary in one random midway ovarian section were examined. Ultrastructure of all oocyte mitochondria in the examined section was categorized, based on shape, density, abnormal membrane structures, vacuoles and clustering, and analyzed with generalized linear mixed models (total mitochondrial count: C = 1587, OB = 1820).

Newborn primordial oocyte mitochondria categorized as homogenous, vacuolated, clustered, or with abnormal membrane structures did not differ between treatment groups. However, maternal OB diet slightly reduced the proportion of oocyte spherical mitochondria (C: 88.28 % ± 1.00, OB: 85.64 % ± 1.11, P = 0.020), and tended to increase the proportion of elongated mitochondria (C: 10.59 % ± 0.96, OB: 12.49 % ± 1.07, P = 0.061). Maternal OB diet also reduced the proportion of newborn oocyte mitochondria with electron dense structures (C: 34.45 % ± 2.18, OB: 22.66 % ± 1.69, P = 0.035).

In outbred mice, pups from OB mothers do show subtle but significant differences in oocyte mitochondrial features in the primordial follicles at birth. However, the abnormalities reported in previous studies in adult mature offspring oocytes were not detected at the primordial stage in newborns. Mitochondrial elongation was limited, and the change in electron density was opposite to that detected at adulthood. Functional consequences of these changes are yet to be determined.

AI / TAI / FTET

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Mapping of ADAMs and ADAMTs expression in response to mating, or seminal plasma infusion in periovulatory sows.

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Boar semen (sperm and seminal plasma (SP)) modulates gene expression, from immune to general metabolism processes, in the preovulatory female genital tract. Some genes can be affected for later events, where the extracellular matrix (ECM) is modified, as during embryo implantation. Desintegrin metalloproteinase (ADAMs), and ADAM with thrombospondin motifs (ADAMTS) are a single-pass transmembrane zinc-dependent family of proteinases, whose biological function mainly depends on protein-protein interactions, becoming key-remodeling enzymes of the ECM. This study aimed to assess if sperm and/or SP triggers changes in *ADAM* and *ADAMTS* mRNA expression in the pig periovulatory genital tract. Mucosal samples (cervix (Cvx), uterus (distal (DistUt) and proximal (ProxUt)), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp) and infundibulum (Inf)) were surgically removed from sows 24 h after natural mating (NM, n=4, to a single Swedish Landrace male each), and, with pooled seminal plasma collected from the same males, cervical infusion with 10 mL of sperm-peak fraction (P1-AI, n=4) or of sperm-free SP from the sperm-peak fraction (SP-P1, n=4) or the whole ejaculate (SP-Total, n=4). Infusions with the protein-free extender Beltsville Thawing Solution (BTS) were used as the control group (n=4). RNA was isolated following a TRIzol-based modified protocol and analyzed for global transcripts using specific microarrays (PORGENE 1.0 ST GeneChip® array, Affymetrix). Normalization of the data (Robust Multiarray Average) and mRNA differential expression (ANOVA: -1> fold change >1, p<0.05) were analyzed with the Transcriptome Analysis Console, and molecular processes were identified by PANTHER. Results, although showing a plethora of differential mRNA expression, clearly marked NM and SP-infusions as downregulators of *ADAMS*. NM induced the **downregulation** of *ADAMTS4* in Cvx, DistUt, and ProxUt, and of *ADAM12* (a gene closely related to the expression of estrogens and progesterone) in DistUt, ProxUt and UTJ. *ADAMTS9* mRNA downregulation was only found in sperm-containing treatments (NM and P1-AI), in ProxUt. ADAMTS9 is an angiogenesis inhibitor, so perhaps our results are related to the preparation of the endometrium for later reproductive events. In addition, SP-infusions also triggered a notable **downregulation of *ADAMs***, but only in the distal segments of the genital tract (Cvx to ProxUt). An isotype (*ADAMTSL4*) was upregulated in Isth, Amp, and Inf and *ADAMTS16* was upregulated in ProxUt after mating, P1-AI and SP-AI, *which* enhances the expression of MMP9 (a tissue inhibitor), degrades ECM, and promote the invasion of trophoblasts. In conclusion, the global downregulation pattern of *ADAMs*and *ADAMTSs* in response to NM might be related to the periovulatory phase, where the ECM remodeling activity is lower, or to SP in the distal parts of the genital tract, which might be related to signaling cascade in the female genital tract. Supported by The Swedish Research Council FORMAS (2017-00946 and 2019-00946), Stockholm, Sweden, and RYC2020-028615-I, RyC2022-036771-I, PID2022-136561OB-I00, and CNS2023-144564, funded by MCIN/AEI/10.13039/501100011033 (Spain) and FEDER funds (EU).

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Factors affecting recipient rejection rate in embryo transfer programs in lactating dairy cows

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Efficiency in dairy cattle farming is essential for maximizing profitability. Incorporating timed embryo transfer (TET) programs into breeding strategies can enhance efficiency by transferring purebred beef embryos in cows not required to generate replacement females, thereby increasing the beef value of surplus calves. Additionally, TET has the potential to improve fertility during warmer seasons and avoid undesired twin pregnancies. Yet, the efficiency of TET programs in high-producing lactating dairy cows must be evaluated based on the rejection rate (RR), pregnancy per ET, embryo loss rate, and cost-benefit ratio achieved. Non-eligible dams for ET among the synchronized cows, as defined by RR, may be influenced by factors such as season, parity, days in milk (DIM), body condition score (BCS), and synchronization protocol. Thus, we aimed to evaluate the effect of such factors in RR in high-producing lactating Holstein cows under confinement systems. The estrus of 331 cows from two farms in southern Spain were synchronized (Double Ovsynch in farm 1 or G6G in farm 2 using D-cloroprostenol [Veteglan, Calier, Spain] and Buserelin [Veterelin, Calier, Spain]) from July 2023 to April 2024. On d 7 after presumptive estrus, reproductive tracts were examined by transrectal ultrasound to assess corpus luteum (CL) status and number (multiple ovulation rate [MOV]: proportion of cows with >1 CL among all ovulated cows), presence or absence of cyst structures, and uterine health status. On the same day, BCS, DIM (89.8 ± 17.8; mean ± SD) and parity (2.2 ± 1.3) were recorded. To calculate RR, the number of unsuitable cows for TET was determined on the basis of having no CL present, abnormal ovarian structures, or small CL (<15 mm). Statistical analyses were conducted using a generalized linear mixed model (GLIMMIX, SAS) to assess the effects of independent variables on the binary response variables (RR and MOV). Fixed effects included farm, season (warmer and cooler), parity (1st, 2nd, and ≥3rd lactation), DIM (<90 d, and ≥90 d), BCS (≤2.50, 2.75, 3 and >3), and synchronization protocol. All effects and two-way interaction were tested and manually removed by backward elimination if non-significant (P>0.05). Overall, RR was 7.2% and was not affected by season, parity, DIM, BCS, or synchronization protocol. As a whole MOV was 13.7%, and it was higher in the warmer (27.1%) than in the cooler season (8.8%; P<0.05). Moreover, ≥3rd lactation cows had a higher MOV (20.8%) compared to 1st and 2nd lactation cows (10.4% and 10.3%, respectively; P<0.05). In conclusion, MOV was higher during the warmer season and in ≥3rd lactation cows, regardless the season, increasing the risk of twin pregnancy with artificial insemination. In such circumstances, TET could potentially be applied widely, as RR is not affected by these factors. Future studies should assess pregnancy per ET, embryo losses, and cost-benefit ratio in these conditions to further confirm the efficiency of this strategy. Funds: MCIN/AEI/10.13039/501100011033 and European Union Next GenerationEU/PRTR (RYC2021-033574-I and TED2021-129764B-I00).

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Long term study of the blood plasma biochemical profile of cattle born by assisted reproductive technologies

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Assisted reproductive technology (ART) is key in cattle breeding as it enables selective mating and improves both reproductive efficiency and genetic improvement. However, there is still limited information on the long-term effects of these methods on the health and development of the offspring. This study aims to investigate the biochemical profile of cattle born through ART, from a previous study (Lopes *et al.*, 2020), over an extended period to understand how these techniques may affect their health and metabolism.

Male and female (not pregnant or lactating) cattle born after artificial insemination (AI, N= 7) were compared to those derived from *in vitro* produced embryos (IVP) using a standard protocol (C-IVP, N= 7), or using oviductal and uterine fluids in the process (RF-IVP, N= 4). Males and females were kept in two diferent open pens, but fed and managed under identical conditions their whole lives. Animals were studied every six months, from 1.5 years old until ≥4 years old, since their first year of life was evaluated by Lopes et al. (2022).Tail vein blood plasma was obtained by centrifugation (1000 G, 10 min) in lithium heparin tubes and stored at -80°C. The biochemical profile included total protein (TP), albumin (ALB), globulin (GLOB), creatinine (CREA), urea (URE), glucose (GLUC), cholesterol (CHOL), triglycerides (TRIG), amylase (AMIL), lipase (LIP), creatinine kinase (CK), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TB). After serial dilutions, inter- and intraassay precision of the methods were lineal and below 15%. Data normally distributed was analysed using ANOVA and Tukey's test; data not normally distributed was analysed using the Kruskal Wallis and Games Howell's test (statistical significance $p<0.05$). The data is shown below as means by group over the years.

The AI group had lower CREA and CHOL levels (1.43±0.05 mg/dL and 109.97±5.27 mg/dL, respectively) than C-IVP (1.75±0.07 mg/dL and 126.57±7.53 mg/dL, respectively) and RF-IVP (1.86±0.08 mg/dL and 140.01±7.19 mg/dL, respectively). The GGT levels were lower in RF-IVP animals (13.00±1.30 UI/L) than both the C-IVP (17.55±1.10 UI/L) and AI groups (16.05±1.23 UI/L). Moreover, the levels of ALT were significantly lower in C-IVP (28.60±1.72 UI/L) than in the AI (34.35±1.81 UI/L) and RF-IVP groups (33.40±2.24 UI/L).

Depending on the age of the animal at the time of sample collection, significant differences were observed for most parameters. The parameters TP, GLOB, CREA, URE, AMIL, AST an increased in their concentration was observed as the animals aged, whilst ALB, GLUC, ALP decreased with age. However, all values can be considered within physiological ranges despite the significant variations.

To conclude, the study showed differences in some biochemical parameters amongst the ART groups and with aging. However, despite the diferences all values were within physiological ranges. These findings are key to study the potential implications of this diferences on the animal's health.

This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa".

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Growth parameters and their hormonal regulation in pigs are differently affected by sex and embryo origin.

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The global pig embryo trade has yet to emerge but replacing live animal transport with *in vitro* produced (IVP) embryos is a future objective. Understanding the potential long-term effects of *in vitro* protocols, especially in commercial traits like growth rates, is crucial. Our study aimed to compare growth parameters (body weight and length) and growth-related hormones (IGF-1 and T4) between IVP and artificially inseminated (AI) animals. Three experimental groups were defined, including 68 crossbred (Landrace x Large White) pigs that were allocated from a colony of animals produced in a previous study. IVP animals produced using BSA (C-IVP; 8 males, 16 females) or reproductive fluids (RF-IVP; 10 males, 7 females) as supplement for *in vitro* culture, were compared to AI animals (AI; 9 males, 18 females). Paternally, all animals were sired by the same boar, while maternally, IVP animals were originated from ovaries obtained at a local abattoir. Growth was measured at birth, 3, 9, 15, every 15 days until 180, 365, 700, 900, 1100, 1250, 1450, 1650 and 1800 days of age. Blood was collected at 90, 180, 365, 700 and 900 days of age. Plasma hormone concentrations were determined using a solid phase, enzyme-labeled competitive chemiluminescent immunoassay. Data were analyzed using a linear mixed-effects model, with group, sex and age as fixed effects. Benjamini-Hochberg procedure was used for p-value correction and differences were considered significant when $P< 0.05$. Weight exhibited an exponential increase up to 6 months of age followed by a linear rise from 12 months of age. Levels of T4 decreased with age, and IGF-1 levels also decreased with age, but only in females and C-IVP males. Males were longer and heavier than females throughout the study. Additionally, males exhibited higher IGF-1 levels than females, while the opposite was observed for T4. Only in males, T4 levels negatively correlated with weight, and no correlation was observed between T4 and body length. IGF-1 positively correlated with weight and length until 6 months of age. IVP animals were significantly heavier (≤d180: 10.6 ± 1.1 kg; ≥365: 35.9 ± 5.4 kg) and longer (≤d180: 8.5 ± 0.8 cm; ≥365: 10 ± 1.9 cm) than those originated from AI throughout the study. In addition, C-IVP animals were heavier (4.3 ± 1.5 kg) and longer (3.2 ± 1.1 cm) than RF-IVP but only up to 6 months of age. Finally, in males, T4 levels of AI were higher than those of their IVP counterparts (1 ± 0.2 µg/dL) and IGF-1 levels of RF-IVP were higher than those of AI (36.1 ± 11.5 ng/mL).

In conclusion, the growth and growth-related hormones of males and females exhibited differences throughout the study. Specifically, males exhibited higher levels of IGF-1, while females exhibited higher levels of T4. While statistical differences were identified between experimental groups, further studies with larger sample sizes are required to determine the clinical significance of these findings, if any.

This study in part of project ID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa"; SH was funded by IJC2019-039404-I and QM by CO-MZ-14.

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Blood plasma biochemical parameters in fully-grown pigs derived from assisted reproductive technologies

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Emerging evidence indicates a long-lasting effect of assisted reproductive technologies (ART) on molecular physiology and metabolic function. Previous studies have reported variations in the metabolic profiles in piglets and growing pigs born from artificial insemination (AI) and born after transfer of in vitro produced embryo (IVP) (París-Oller et al., Res Vet Sci,142:43, 2021) but it is unknown whether these differences persist into adulthood. The aim of this work was to study baseline values of plasma biochemical parameters in ART-derived pigs and to gain insight into the evolution of metabolic profile across their life [young age (45 days), adulthood (365 days), and old age (1250 days)]. Pigs born through AI and IVP [n= 16 and 29 (45 days), 13 and 21 (365 days), and 9 and 13 (1250 days), respectively], produced in a previous study (París-Oller et al., J Anim Sci Biotechnol 12:32, 2021), were kept under same housing, managing and feeding conditions. Plasma was obtained through the centrifugation (1200 *g*, 20 min, 4 ºC, Eppendorf 5810 R) of blood collected in lithium heparin tubes and stored (-80ºC) to determine the biochemical parameters total protein (TP), albumin (ALB), globulin (GLOB), creatinine (CREA), urea (URE), glucose (GLUC), cholesterol (CHOL), triglycerides (TRIG), amylase (AMIL), lipase (LIP), creatinine kinase (CK), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TB) using an automated clinical chemistry analyzer (Olympus AU400, Japan). Inter- and intraassay coefficient of variation were below 15%. Data were analyzed by a mixed ANOVA and Student's t-test for multiple comparisons. In the absence of homoscedasticity, a Student's t-test with Welch's correction was used, and in case of non-normality the Wilcoxon rank-sum test was performed. A P value < 0.05 was considered significant. The analyses of the metabolites showed higher CREA in old-IVP than old-AI (2.32±0.07 vs. 1.925±0.07 mg/dL); higher GLUC in young-IVP than young-AI (107.77±6.10 vs. 83.77±5.41 mg/dl); lower LIP in young-IVP than young-AI (16.74±1.29 vs. 22.91±2.11 IU/L); and lower AMIL, GGT and AST in IVP than AI at all ages. The other metabolites were similar between AI and IVP animals but increased (PT, ALB, GLOB, URE, TBIL), decreased (CHOL, ALP, ALT) or fluctuated up and down (TRIG) with age. The enzyme CK, related to muscle integrity, was the only parameter that was not affected by the group or age of pigs. Reference values for plasma biochemical values provide valuable information for investigators and will help in valid interpretation for health status and for those who use IVP pigs as a research model. In conclusion, these physiological data are useful for veterinarians and livestock producers and show slight persisting differences in some metabolites in pigs naturally and artificially conceived during life although the clinical relevance of such differences is unnoticeable.

This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and “FEDER Una manera de hacer Europa”. SH was funded by IJC2019-039404-I.

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Seasonal fertility variation in dairy cows in two farms in the Mediterranean area

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The ongoing climate changes remarkably affect the distribution of rainfalls and seasonal maximum/minimum temperatures, negatively influencing global agricultural activities. In this context, reproductive performance in dairy cows may decrease. The aim of this retrospective, observational study was to investigate the seasonal fertility variation in Holstein Friesian dairy cows of two commercial farms (F1, F2) located in Sardinia (Italy). A total of 4722 artificial inseminations (AI) in 800 lactating dairy cows (F1 n=300; F2 n=500) were examined over 3 years (2021-23). The monthly conception rate (CR) was calculated as the percentage of inseminated cows diagnosed as pregnant via trans-rectal ultrasound approximately 30 d post-AI. The number of services per conception (S/C) was calculated as the number of AIs needed to conceive per cow. Data were analyzed with R (RStudio Team 2023), by a simple linear regression model with fixed effects in which the two response variables were CR and S/C, and the predictor variables were farm, month, year and their interaction. Average (±SEM) monthly CRs in 2021-22-23 were 58±21%, 60±21% and 43±12% in F1 and 44±15%, 39±8%, and 46±12% in F2, respectively. The mean monthly CR was higher in F1 vs F2 (P<0.01). CRs did not vary within farms among years but were different among months (P<0.05) and farms (P<0.01), while there was no significant interaction between month and farm. Maximum CRs were achieved in winter/autumn (Dec, Jan, Feb, Sep), while minimum CRs were recorded in spring/summer (Apr, Jul, Aug) in both farms. The mean difference between the highest and lowest CR of the two farms in the 3 years was similar (F1=48.7% and F2=40.3% percent points difference). The yearly mean S/C were 2.06±0.36, 2.24±0.52, 2.45±0.34 in F1 and 2.58±0.34, 2.90±0.64, 3±0.52 in F2 in 2021-22-23, respectively. The highest monthly S/C during the three years were achieved during spring/summer (Apr, Jun, Jul) in F1, while the lowest S/C occurred in spring and autumn (Apr, Sep, Oct) in F2. As observed for the CR, the difference between highest and lowest S/C was similar between farms (F1=1.29; F2=1.53). The mean S/C was similar among months, while the variance was significant among years (P<0.02) and farms (P<0.01), but there was no significant interaction between year and farm. In agreement with previous reports, evidence indicates that reproductive performance in dairy cows varies during the year, as the CR decreased in spring/summer compared to the autumn/winter, albeit the S/C did not change seasonally. Since the mechanism by which thermal stress compromises fertility in dairy cattle is multifactorial, additional variables such as parity and daily milk production will be included in this study.

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Testosterone, ano-genital distance, and epididymal sperm quality in bulls derived from artificial insemination and in vitro production.

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Previous studies have documented variances in cattle born from artificial insemination (AI) and in vitro derived embryo transfer (IVP). However, it remains uncertain whether these variances are associated with testosterone levels and bull reproductive function. This study aims to investigate plasma testosterone levels in AI and IVP-derived bulls and to assess physical weight and length and morphological reproductive parameters, including anogenital distance, and epididymal sperm quality. Bulls born via AI (n=5) and IVP (n=9) previously produced (Lopes et al. Animals, 2020) were kept under the same housing, management and feeding conditions. They were evaluated at intervals 75, 150, 360, 600-900 and 1200-1500 days of age. Physical parameters (weight, length, height at withers, thoracic girth) and morphological reproductive parameters such as anogenital distance were measured. Average daily weight gain (ADWG) was calculated from birth. Blood plasma was collected and stored (-80ºC) for testosterone analysis, conducted using an automated clinical chemistry analyzer (Olympus AU400, Japan). Bulls were sacrificed at an age exceeding 1500 days and epididymal spermatozoa were collected for evaluation of motility (using CASA), viability (PNA-FITC and propidium iodide staining, plus flow cytometry), and morphology (contrast phase microscope evaluation). Data were analyzed using a mixed linear model with age and embryo origin (IVP *vs.* AI) as the main factors. Pearson correlations were computed between variables, with significance set at $P < 0.05$.

No significant differences were observed between AI and IVP-derived bulls regarding testosterone levels, physical parameters, or anogenital measurements ($P > 0.05$). Similarly, epididymal sperm parameters, including total motility (AI: 88.8 ± 4.1, IVP: 90.4 ± 5.3; %), progressive motility (AI: 53.2 ± 5.8, IVP: 40.0 ± 5.4; %), viability (viable and non-reacted sperm; AI: 78.9 ± 5.8, IVP: 82.1 ± 1.6; %), and morphology (percentage of abnormal sperm; AI: 93.5 ± 3.0, IVP: 89.0 ± 3.2; %), did not show significant discrepancies. Testosterone levels exhibited an increase with age, ranging from a mean of 13.6 ng/ml at 75 days to 740 ng/ml at 1200-1500 days. Both testosterone levels and age demonstrated direct and significant correlations with all physical parameters (body weight and length parameters) and anogenital distance. Age exhibited an inverse relationship with ADWG. In conclusion, within the constraints of this study's sample size, no disparities were detected in testosterone levels or epididymal sperm parameters among bulls derived from AI and IVP.

This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa". SH was funded by IJC2019-039404-I. AM and AJ were supported by the Investigo Program, funded by the European Union – Next Generation EU (2022-C23.I01.P03.S0020-0000573).

SUPEROVULATION / OPU - IVP / ET

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Factors influencing calving rates after transfer of frozen-thawed in-vivo bovine embryos considering quality of corpus luteum of recipient animals

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The main objective of this study was to evaluate the influence of a cavity in the corpus luteum (CL) of recipients on the success of embryo transfer. Other authors reported no differences in establishment of pregnancy with regard to cavity of the CL (Nogueira et al., 2012; Thomson et al., 2021). In one study (Jaśkowski et al., 2021), significantly better pregnancy results were achieved in recipients with a cavity in the CL than with a compact CL.Calving rates (CR) after the transfer of 330 in-vivo embryos, frozen in ethylene glycol from Holstein donors in North-West Germany between February and December 2022 were evaluated. Stage and quality of the embryos were assessed according to the IETS standard.
The recipients were nulliparous animals (n=330) of the Holstein breed.The transfers took place 7 days after estrus on 64 different farms (1 to 46 transfers per farm).The recipient animals had to have a CL of at least 2 cm diameter on one of the ovaries. The CLs were divided into two categories: 1. Compact CL, 2. CL with a cavity. Size of the cavity was recorded but not accounted for in the analysis. Animals with a large follicle (>15 mm) on one of the ovaries were excluded. The diagnostics were carried out using EasyscanTM (IMV Imaging) ultrasound devices.
The calving was evaluated using the SERVIT database (VIT,Verden).
The statistical analyses were carried out using SPSS. A multivariable mixed logistic regression model was created with calving (Y/N) as the outcome variable and embryo quality (1/2), embryo stage (4-7), quality of CL (1/2) and whether the animal was inseminated before embryo transfer (Y/N) as fixed effects. Interactions of embryo quality and stage with quality of CL were tested in the model but then removed due to non-significant effects. Herd was considered as a random effect (covariance type = variance components). A compact CL was found in 233 (70.6%) animals selected for transfer. A cavity of up to 50% was diagnosed in 88 animals (26.7%) and a cavity of over 50% was found in 9 animals (2.7%). The 330 transfers resulted in a total of 153 calvings (46.4%), in which 142 calves were born alive (92.8%) and 10 were born dead (6.5%).
The mixed logistic regression model showed that neither embryo stage (P = 0.347), embryo quality (P = 0.573) nor CL quality (P = 0.755) had a significant influence on CR. Only prior insemination was significantly associated with CR (P = 0.011). The predicted means for CR were 27.7% (SE = 6.9) in animals that received an insemination before embryo transfer compared to 45.5% (SE = 6.5) in animals that did not receive prior inseminations. Predicted means for CR depending on CL quality was distributed as follows: Compact CL 35.2% (SE = 6.4), CL with cavity 37.0% (SE = 7.2). Transfers with stage 6 had the highest predicted CR with 46.6% (SE = 8.6). Transfers with stages 4 or 5 embryos resulted in predicted CR of 36.8 (SE = 5.7) and 36.5% (SE = 8.4) and stage 7 had a predicted CR of 25.9% (SE = 10.3). Quality 1 embryos had a predicted CR of 38.8% (SE = 4.9) and quality 2 embryos a predicted CR of 33.6% (SE = 9.4). Our analyses were able to confirm other studies in which a luteal cavity did not have a significant effect on calving rates.

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Superovulatory response of Creole ewes treated with autologous platelet-rich plasma (PRP)

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The response to superovulatory treatments is usually low and variable, related to factors such as hormones used, dosage and administration, but also to animal and environmental factors. The application of PRP increases the follicular population ≥2 mm in ewes. However, there is no information on the response to superovulatory treatments. Therefore, attempts to improve responses are justified. The study was carried out on the experimental farm at Universidad Autonoma Chapingo, Mexico with 20 multiparous, healthy Creole ewes with good body condition. The ewes selected at random from a bigger group and without reproductive problems. PRP was obtained according to the procedures defined by Sanchez-Chavez (2023). Platelet concentration was adjusted to 12 500 × 10^6 platelets mL^{-1}. The experimental design was completely random, and the treatments were: T1 = (Control) left ovary received 50 µL of saline solution (n=10) and T2 = right ovary treated with 50 µL of PRP (n=10), in both cases administered through laparoscopy. PRP application on right ovary was because preliminar study did not show significant effect of ovary to PRP administration. The population of ≥2 mm follicles in both ovaries, with an ultrasound (Aloka, Prosound 2, Japan) and a rectal transducer (7.5 MHz) was determined at the beginning of the study and four weeks after PRP administration. The ewes followed a synchronization protocol by inserting an intravaginal releasing progesterone device (CIDR, Zoetis, Mexico) for 12 days, on day 10 they were superovulated by administering 1 250 units of eCG (Novormon 5000, Zoetis, Mexico) as a single intramuscular injection and on day 12 the device was removed. Heat detection was conducted every 12 hours from 24 to 48 hours after CIDR removal with the help of a vasectomized teaser ram. The ewes were inseminated through laparoscopy 18 hours after estrus onset with two doses of fresh semen with 100 × 10^6 spermatozoa per dose, placing every dose in the middle part of each uterine horn, using semen from a proven fertility Creole ram. The superovulatory response was determined seven days after estrus through laparotomy using surgical standard procedures. The variables recorded were: initial number of follicles (INF), number of follicles after PRP application (NFAPRP), number of corpora lutea after estrus (NCL), number of large anovulated follicles (NLAF), and total ovulatory response (TOR= NCL+ NLAF). Data are presented as mean ± standard error. The GLM procedure of SAS was used to analyze the results, considering a significant effect with a $P< 0.05$. The NFAPRP, NCL, and TOR were higher in ovaries receiving PRP compared to ovaries treated with saline solution (3.94 ± 0.31 vs. 2.94 ± 0.31; 4.20 ± 0.60 vs. 2.42 ± 0.60; 5.42 ± 0.69 vs. 3.36 ± 0.69, respectively). However, there were no significant differences between treatments for the INF (3.05 ± 0.33 vs. 3.68 ± 0.33) and NLAF (1.21 ± 0.30 vs. 0.94 ± 0.30), respectively. In conclusion, the intraovarian administration of autologous PRP in Creole ewes allows a significant increase in the number of antral follicles ≥2 mm, the number of corpora lutea following a superovulatory treatment, and the total ovulatory response, under the conditions of this study.

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Semen quality of young bulls does impact success rate of In Vitro Fertilization.

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Shortening the generation interval is one important tool to increase the genetic gain within a breeding program. This can for instance be achieved by using semen of very young bulls (i.e. 10 months old) for In Vitro Fertilization, since it requires only a very limited amount of semen. However, the quality of the semen of young bulls could still be immature and therefore, results with respect to embryo development in In Vitro embryo Production (IVP) can be very variable.

The aim of this study was to see if semen quality is affecting the IVP embryo development and if selection of the semen based on quality is useful to improve IVP results.

Semen quality data (motility and morphology) and embryo development data were available for 3108 different fresh ejaculates of 142HF bulls in the age of 10-12 months. Ejaculates were collected using an artificial vagina and according to the standard CRV protocol. The percentage motile cells was measured by the AndroScope CASA system (Minitube, Tiefenback, Germany) and the percentage morphological abnormal cells was determined by staining a sample using eosin/analin and microscopically counting 100 sperm cells (1000x magnification), differentiating in eight categories (normal cells, three primary and four secondary type of morphological abormalities). In the 3108 OPU sessions on average 10 oocytes were used (min-max; 2-43 oocytes). A total amount of 2,0 x 10^6/ml of semen was used to fertilize oocytes collected from dams in the age of 12-13 months. Embryo development (defined as the % of Q1+2 Day 7 embryos based on number of oocytes in IVC) was monitored using OPU derived oocytes after maturation, fertilization (with the different batches of fresh semen) and culture for 7 days, using the standard CRV protocol. Data were analysed using regression and Chi-square analysis.

The results from the regression analysis between the semen quality parameters and embryo development all showed a very low, non-significant correlation ($P>0.05$). But when both percentage morphological abnormalities and percentage motility were divided in two classes (below 50% and 50% or higher), a clear difference was observed in the embryo development. Using semen samples (n=273) that contained 50% or more morphological abnormal cells results in a significant ($P< 0.001$) lower embryo development rate compared to semen samples (n=2836) with less than 50% morphological abnormalities, i.e. 11% and 22% respectively. Samples with >50% abnormal cells varied in type of abnormalities, but mainly it involved non-compensatable traits like cells with a damaged acrosome, abnormal head shape, or containing proximal droplets. In addition, using semen samples with a motility of less than 50% (n=117) resulted in a significant lower embryo development compared to those (n=2992) with a motility of 50% or higher, i.e., 13% and 22% respectively. Combining both morphology and motility did not further change the results, since most samples with a high percentage of morphological abnormalities also had a low motility.

It is therefore concluded that semen morphology or motility of fresh semen samples is affecting the In Vitro embryo development and that these quality parameters can be used to eliminate semen batches that give a low embryo development.

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Capacitation-IVM system with C-type natriuretic peptide affects mitochondrial distribution, and calcium levels and patterns in lamb oocytes

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In vitro matured oocytes of prepubertal females present a lack of synchronization between cytoplasmic and nuclear maturation (Kochhar et al., Reprod Dom Anim 37:19-25, 2002). Biphasic IVM systems that use C-type natriuretic peptide (CNP) to prevent germinal vesicle breakdown before inducing oocyte in vitro maturation has been called Capacitation-IVM (CAPA-IVM). This study aimed to assess the effect of CAPA-IVM system on lamb oocyte quality. Ovaries from 1-5 months old lambs were recovered at a local slaughterhouse. Cumulus-oocyte complexes (COCs) were collected by slicing and selected using HEPES-buffered TCM-199 medium with the meiotic inhibitor 3-Isobutyl-1-methylxanthine (500µM) and heparin. COCs were cultured in a pre-maturation medium (TCM-199, 4mg/mL BSA, 0.2mM sodium pyruvate, 1mM glutamine, 100 µM cysteamine, and 5µg/mL gentamicin) with 20nM CNP and 10nM 17ß-estradiol (E2), for 6h and in conventional IVM medium (TCM-199 with 0.2mM sodium pyruvate, 1mM glutamine, 100µM cysteamine, 5µg/mL LH, 5µg/mL FSH, 3.7µM E2, 10ng/mL EGF, 10% FBS, and 5µg/mL gentamicin) for further 24 h (CAPA-IVM group). The control group was cultured in a conventional IVM medium for 24 hours. After 24 h (control group) and 30 h (CAPA-IVM group) of culture, 30 COCs per group were denuded and 10 oocytes were stained with Mitotracker Orange CMTMRos (Molecular Probes, Eugene OR, USA) to analyze the mitochondrial distribution following the patterns described by Martin Maestro et al (Animals 10:2414. 2020); 10 oocytes were stained with a commercial Calcium stain kit (Abcam, Cambridge, UK) to check the distribution and the level of calcium by measuring the intensity of fluorescent signal; and 10 oocytes were used to measure the early apoptotic using the annexin V-FITC apoptosis staining kit (Abcam). Three replicates were made. Images were taken using a optical microscope camera and analysed with ImageJ program. Data were statistically analyzed by Fisher's exact test (cualitative data) and multiple T-test (cuantitative data). The data obtained from the Mitotracker stain showed 1.5 times more intensity ($p<0.0001$) and a higher percentage of normal distribution pattern in CAPA-IVM group than in control group (76.7% and 60.0%, respectively; $p=0.0149$). The calcium stain showed that calcium levels were 2.9 times higher ($p<0.0001$) in CAPA-IVM group than in control group and 73.33% of oocytes from CAPA-IVM group presented a peripheric distribution (under the plasmatic cell membrane) while only 30% of oocytes from control group showed this distribution ($p<0.0001$). Finally, CAPA-IVM system and conventional IVM protocol provided similar percentages of early apoptotic (10% and 13%, respectively; $p=0.687$) and dead (3% and 17%, respectively; $p=0.097$) oocytes.

In conclusion, the results from this study show that CAPA-IVM system increases the quality of the oocyte from prepubertal donors via increasing the number of mitochondria and its normal distribution, increasing the intracytoplasmic calcium and showing a peripherical distribution that can influence avoiding polyspermy by the activation of the mechanism involved in the cortical reaction.

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Cumulus-oocyte communication in prepubertal goats according to GV chromatin configuration and nuclear stage during in vitro maturation

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Oocyte competence for embryo development relies on bidirectional communication with cumulus cells (CCs) via gap-junctions located at the tip of transzonal projections (TZP). In cattle, functional gap junction-mediated communication decreased in oocytes with a high chromatin condensation in the germinal vesicle (GV), and it was related to early signs of atresia (Lodde, Mol Reprod Dev, 74:740-749, 2007). Sirard (Anim Reprod, 16:449-454, 2019) suggested that bovine oocytes with an intermediate level of chromatin condensation could be related to high developmental competence. Our aim was to analyze cumulus-oocyte communication, assessed by TZP density, according to GV chromatin configuration at the beginning of IVM and nuclear stage during IVM. Cumulus-oocyte complexes (COCs) were collected by ovary slicing and matured in TCM-199 with FSH, LH, estradiol, EGF and cysteamine during 24h at 38.5ºC with 5% CO2. Oocytes with two or more layers of compact cumulus cells and homogeneous cytoplasm were selected. A sample of 10 COCs/replicate (3 replicates) were recovered at several times during IVM (0h, 6h, 12h and 24h) for TZP density and nuclear assessment. COCs were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.25% triton for 30 min and actin filaments were stained with 5 µg/mL phalloidin-FITC for 1h. Then, COCs were counterstained with 1 µg/mL Hoescht 33258 for 10 min and analyzed with confocal laser microscopy. One image was taken per oocyte in the equatorial plane and TZP density was quantified as phalloidin-FITC average fluorescence intensity in the whole zona pellucida area. Oocyte GV chromatin configuration and nuclear stage was classified according to Sui et al. (Mol Reprod Dev, 71:227-236, 2005) as: GV1 (diffuse filamentous chromatin), GVn (condensed net-like chromatin), GVc (condensed clumped chromatin), GV breakdown (GVBD), metaphase I (MI) and metaphase II (MII). Twenty-five oocytes in GV at 0h, 11 oocytes in GVDB at 6h, 24 oocytes in MI at 12h and 21 oocytes in MII at 24h were analyzed. Data were statistically analyzed by two-way ANOVA followed by Tukey's correction. Results showed that, at 0h, GVn-oocytes presented a higher TZP density ($p<0.05$) compared to GV1 and GVc (20.65, 13.07 and 12.84 arbitrary units; n= 8, 9 and 8; respectively), which did not differ significantly from each other. At 6h, the TZP density of GVBD-oocytes (15.05 arbitrary units) did not differ from any GV configuration. MI-oocytes at 12h and MII-oocytes at 24h had a similar TZP density (7.98 and 4.03 arbitrary units; respectively), which was lower ($p<0.05$) compared to GVBD. In conclusion, TZP density in prepubertal goats increases with GV chromatin condensation up to an intermediate stage (GVn), then it declines when the oocyte reaches a high GV chromatin condensation. Following nuclear maturation, the oocyte and CCs progressively lost communication.

Study funded by Spanish Ministry of Science and Innovation (PID2020-113266RB-100) and Generalitat of Catalunya (AGAUR, 2020FI-B00025).

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The positive association between maternal body condition score and antral follicle count does not affect the number of produced blastocysts in OPU mares

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Little is known regarding the association between a mare's body condition score (BCS) and the ovum pick-up (OPU)-intracytoplasmic sperm injection (ICSI) outcomes. This study aimed to study the effect of maternal BCS on oocyte developmental competence and to detect if the BCS can determine the mare's ability to produce embryos *in vitro*. Overall, 107 OPU-ICSI sessions were performed in Warmblood and Quarter mares (*n*= 59), with a BCS of 2-8 out of 9. These mares were admitted to the routine commercial OPU-ICSI services at the Faculty of Veterinary Medicine, Ghent University. The OPU-ICSI outcomes, including the antral follicle count, the number of recovered oocytes, the number of mature oocytes, the number of presumptive cleaved zygotes, and the number of blastocysts were recorded per session. Spearman's correlation coefficient was checked between BCS and antral follicle count. For the blastocyst-producing sessions, the OPU-ICSI outcomes of thin (BCS= 2-4; *n*= 27), fit (BCS= 5-6; *n*= 33), and obese (BCS= 7-8; *n*= 5) mares were compared using Welch-One way ANOVA followed by the Games-Howell or Kruskal-Wallis test. For all mares, the BCS was significantly associated with the antral follicle count (*r*= 0.234, *P*= 0.019). For the blastocyst-producing sessions, the antral follicle count between thin, fit, and obese mares was significantly different (16.73 ±1.39, 22.58 ±1.61, and 22.60 ±8.63, respectively, *P*= 0.022), which did not significantly change the number of produced blastocysts (1.73 ±0.27, 2.39 ±0.36, and 2.20 ±0.97, respectively, *P*= 0.547). However, there were no differences (*P*> 0.05) in the numbers of recovered oocytes, mature oocytes, and cleaved zygotes between thin (11.23 ±1.07, 7.15 ±0.75, and 4.81 ±0.45, respectively), fit (14.58 ±1.46, 8.55 ±0.89, and 5.84 ±0.70, respectively), and obese (14.80 ±6.61, 9.80 ±3.72, and 4.20 ±1.32, respectively) mares. Taken together, the maternal BCS cannot determine the mare's ability to produce blastocysts after OPU-ICSI. The blastocyst-producing thin mares (BCS= 2-4 out of 9) exhibited a lower antral follicle count, which did not affect the final number of produced blastocysts between thin, fit, and obese mares.

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Interleukin-6 supplementation during in vitro maturation improves the spindle and chromosome organization of pig oocytes.

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Interleukin-6 (IL-6) is a multifunctional cytokine which, present in follicular fluid, is involved in oocyte maturation. Since this effect on pig oocytes in vitro matured (IVM) is uncertain, this study evaluated the impact of adding IL-6 to the IVM medium on the maturation rate and the organization of chromosomes and spindle during meiotic maturation. For this purpose, oocytes collected from ovaries of prepuberal gilts of 6 months of age were matured for 44 hours in IVM medium consisting of TCM-199 with 0.57mM cysteine, 0.1% PVA and 10 ng/mL epidermal growth factor and supplemented with 0 (control; N=182), 10 (N=152), 50 (N=141), 100 (N=136) and 200 (N=164) ng/mL of IL-6. Following the IVM, oocytes were denuded, fixed, and stained with Hoechst 33342 and anti-α-tubulin antibody conjugated with FITC to assess maturation and the percentage of aligned chromosomes and well-formed spindle. Subsequently, the oocytes were examined under fluorescence microscopy (Hoechst 33342: blue fluorescence; α-tubulin-FITC: green fluorescence). The maturation rate was calculated as the percentage of oocytes with a metaphase plate and a polar body over the total number of oocytes. The proportion of well-aligned chromosomes was determined as the ratio of oocytes with an organized structure (forming a line or ring) to the total number of oocytes evaluated. Conversely, the proportion of well-formed spindles i.e, the ratio of oocytes displaying the spindle with a bipolar morphology (barrel-like shape) or a well-structured network of microtubules (star-like shape), was considered over the total number of oocytes. The experiment was replicated 4 times. Maturation rate data were analyzed by a mixed ANOVA model followed by the Bonferroni post hoc test, and the results presented as means ± SD. Data on aligned chromosomes and well-formed spindle were analyzed using the Chi-square test, and the results presented as percentages. While no differences were observed among maturation rates of oocytes treated with IL-6 compared to controls (range from 83.2±31.5% to 89.3 ± 8.6%), oocytes treated with 100 ng/mL of IL-6 showed a significant increase ($P< 0.05$) in the percentage of aligned chromosomes (65.6%) and well-conformed meiotic spindle (66.4%) compared to controls (53.2% and 52.4%, respectively). These findings indicate that the addition of IL-6 to the IVM medium of oocytes does not increase maturation rate. However, exogenous 100 ng/mL of IL-6 significantly improves the morphology of metaphase plates and spindles of IVM-prepubertal pig oocytes, a crucial aspect for producing viable oocytes and influencing their future development.

Supported by MCIN/AEI/10.13039/501100011033; PID2022-138666OB-I00), Spain, and FORMAS (Project 2019-00288), Sweden.

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Effect of embryo breed and type of semen used for IVF on gestation length and calving characteristics following fresh transfer of IVP embryos.

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The aim of this study was to compare gestation length (GL), calving difficulty (CD) and birth weight (BW) of calves derived from in vitro produced (IVP) embryos from dairy and beef breeds. Oocytes were collected once per week for up to four successive weeks from Holstein-Friesian dairy donors (HF, n = 51) and Angus beef donors (AA, n = 34) using transvaginal ovum pick-up. Following in vitro maturation, in vitro fertilization was undertaken using either conventional (CV) or X-sorted (SS) sperm. On day 7, embryo recipients (n = 471) were randomly assigned to receive a single Grade 1 blastocyst (HF or AA) that had been fertilized with CV or SS. The embryo recipients were lactating dairy cows in seven herds that had been synchronized with a progesterone-Ovsynch protocol. Embryos were transferred fresh to the uterine horn ipsilateral to the corpus luteum. At the end of the subsequent gestation, dams were induced at day 280 of gestation if parturition was not imminent (n = 31). Parturition data were captured for up to 156 calves on 6 herds (HF, n = 58, AA, n = 98). Individual calf data was available for GL (n = 156), CD (n = 130) and BW (n = 148). BW and CD were recorded immediately after calving, with CD being scored on a scale of 1 to 4: 1 = normal unassisted calving (n = 87), 2 = some assistance (n = 32), 3 = considerable assistance (n = 9) and 4 = veterinary assistance (n = 2). Treatment effects on BW, GL and CD were determined using generalised linear mixed models in SAS. The first analysis included calves sired by 7 HF bulls and 6 AA bulls, and a second analysis was restricted to bulls that had calves derived from both CV and SS semen (HF, n = 5, AA, n = 4). In the first analysis, embryo breed, semen type, and induction status were included as fixed effects. The second analysis also included the effects of sire and sire x semen type interaction. BW was greater for AA calves compared with HF calves (42.9 kg vs 37.6 kg, $P < 0.001$) but tended to be less in calves generated using CV compared with SS (39.2 kg vs 41.4 kg, $P = 0.073$). GL was not different for AA compared with HF calves (280.9 ± 0.5 days vs 280.6 ± 0.6 days, $P = 0.57$) or between calves generated using CV or SS sperm (280.9 days vs 280.6 ± 0.5 days, $P = 0.65$). The odds of a difficult calving was not affected by breed (HF vs AA, OR = 0.94, 95% CI = 0.40, 2.20, $P = 0.88$) or semen type (CV vs SS, OR = 1.32, 95% CI 0.57, 3.05, $P = 0.50$). Cows that were induced had increased odds of calving difficulty (Induced vs Spontaneous OR = 8.45, 95% CI 3.47, 20.57, $P < 0.001$). In the second analysis, the interaction between sire and semen type was not significant for GL, BW or CD (all $P > 0.12$). Sire had an effect on BW ($P = 0.015$) and CD ($P = 0.002$), but not GL ($P = 0.22$). In conclusion, calf BW was affected by breed but not semen type, GL was not affected by breed or semen type and CD was not affected by breed or semen type but was affected by induction status.

Supported by the Department of Agriculture, Food and the Marine Research Stimulus Fund (Grant 2021R665).

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Vitrification by two different protocols does not affect pregnancy outcomes in in vitro-produced equine embryos.

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Despite the fast evolution of in vitro production in horses, there is still limited understanding of the impact of the effect of different vitrification protocols and the transfer of older embryos (i.e. > 10 days to become embryos) on pregnancy outcomes. Here, we aim to evaluate the effect of the vitrification method and the developmental kinetics on pregnancy rates and embryonic loss. To do so, equine embryos (n=139) were produced after ovum pick-up and intra-cytoplasmatic sperm injection in a commercial program. Day of blastocyst formation was evaluated and categorized as early (day 7 and 8; n=52), mid (day 9 and 10; n=65) and late (day 11 to 13; n=22), and transferred fresh (n=28) or vitrified on a Cryolock (Irvine Scientific) by one of the following protocols: (1) holding of embryos in base solution (BS; DMEM-F12 with 20% FCS), vitrification for 5 min in solution 1 (VS1; BS+ 1.5M ethylene glycol) followed by 40 s in solution 2 (BS + 7M ethylene glycol and 0.6M galactose); (2) vitrification at 23°C with a commercial VIT-Kit (Minitube, Germany) in VS1 (5 min), VS2 (5 min) and VS3 (40 s), containing increasing concentrations of glycerol and/or ethylene glycol, based on (Eldridge-Panuska et al. Theriogenology. 2005; 63:1308-1319). Subsequent warming was performed in three-steps for protocol 1: thawing medium (TM; DPBS 0.1% glucose, 36 mg/L pyruvate, 0.4% BSA) with 0.3M sucrose (1 min), 0.15M sucrose TM (5 min), and holding in TM (5 min) at 38.2°C (Choi and Hinrichs. Theriogenology. 2017; 87:48-54); or directly at 38°C for 5 min in Emcare holding medium (Spervital, The Netherlands) in protocol 2. All embryos were washed four times in Emcare and transferred immediately to a day 4 recipient mare. Early pregnancy (7d post transfer), and embryonic loss (loss of pregnancy between first positive control until 42d of gestation) were registered. Generalized mixed-effects models were used to test the effect of the vitrification method and embryo developmental category on pregnancy outcomes. Similar early pregnancy was obtained with fresh (71.4±8.5%) or vitrified embryos (both methods; 68.9±4.24%; p=0.8), so was between fresh and the three-step warming (69.5±6.0%) or the direct warming (68.3±6.0%). Likewise, similar embryo loss rates were observed by comparing fresh (20.0±8.9%) with three-step (31.7±7.3%; p=0.6) or direct warming (12.2±5.1%; p=0.7). However, there was a tendency in favor of direct against the three-steep warming (p=0.09). As vitrification did not affect pregnancy outcomes, the effect of blastocyst kinetics was evaluated over all embryos (fresh and vitrified). No differences were found in pregnancy rates or embryo loss between early (76.9±5.8% and 12.5±5.2%, respectively) and mid embryos (72.3±5.5% and 17.0±5.4%; p=0.9).However, late embryos tended to reduce pregnancy rate (45.5±10.6%) against early (p =0.05) and mid (p=0.1), and exhibited higher embryo loss (30.0±14.4%, p<0.05) compare to both groups. In conclusion, vitrification is a safe method to preserve equine embryos with flexibility using protocols with direct warming, and the transfer of old embryos (>d10) should be analyzed carefully to avoid pregnancy losses.

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Comparative analysis of in vitro production efficiency between adult sows and prepubertal gilt ovaries

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The in vitro production of porcine embryos has become a pivotal technique in reproductive biology and biotechnology laboratories, serving not only in animal breeding programs, but also in the generation of models for human disease research, due to the genetic and physiological similarities between pigs and humans. For these reasons, establishing a standardized protocol to enhance the IVP of porcine embryos is of paramount importance. This goal can be attained by standardizing the origin of gametes, the culture media and the techniques utilized for IVM, IVF, and embryo culture.

In this sense, we have avoided the source of variation between males and ejaculates by employing frozen-thawed boar semen straws. We have also developed a complete set of ready-to-use, lyophilized culture media for pig IVP embryos, which can be stored for long periods, minimizing batch-to-batch variation and mitigating the risk of formulation errors associated with homemade media preparations. Nevertheless, achieving an optimal source of oocytes remains a challenge, including limited access to reproductive material at local abattoirs and the reduced developmental potential of immature oocytes from prepubertal gilts. To identify the optimal oocyte source, we analysed the variation in the IVP yield between ovaries sourced from prepubertal gilts and adult sows collected simultaneously from the same abattoir.

Ovaries were collected from adult sows (S) and prepubertal gilts (G), the latter selected based on ovarian morphology (absence of corpora lutea or albicantia), at a local abattoir. Following collection, COCs were aspirated from follicles of 2-8mm, selected based on morphology, and cultured for 22h in NaturARTs-PIG-IVM1-LYO medium (EmbryoCloud, Spain), and 22h in NaturARTs-PIG-IVM2-LYO medium. Following IVM, frozen-thawed boar sperm, same boar and ejaculate, was selected by swim up in PIG-SUM-LYO medium, and oocytes and spermatozoa were coincubated for 22h in PIG-IVF-LYO medium, after which embryos were transferred to PIG-IVC1-LYO medium for 24h, when cleavage was evaluated. After this, 2-cell embryos were transferred to PIG-IVC2-LYO medium until day (d) 7 of culture. Embryo stage was observed on d6 and d7 after insemination. A total of five replicates were conducted, with 100 oocytes per group per replicate. Statistical analysis was performed using Student's t-test ($P < 0.05$).

The cleavage rate (%) on d2 was significantly higher in S (77.5±1.9) compared to G (67.4± 2.1). Similarly, blastocyst formation rate (from cleaved embryos, %) on d6 was higher in S (55.6±2.6) than in G (28.7±2.5), with a consistent trend observed on d7 (S: 50.6 ±2.6; G: 29.9±2.5). There were no significant differences in blastocyst expansion rates between groups (16.0± 3.8 and 14.0± 2.5 on d6; 34.7±4.8 and 33.5± 3.5 on d7; for G and S, respectively).

Our findings suggest that ovaries from adult sows yield significantly higher numbers of embryos with similar developmental kinetics compared to prepubertal gilts. Thus, for both biomedical applications and genetic improvement in swine breeding programs, adult sow ovaries may be preferable for in vitro embryo production.

Funded by Fundación Séneca (22247/PDC/23), PLEC2022-009246/AEI/10.13039/501100011033 and NextGenerationEU/PRTR.

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Relationship between fetal morphometrics and birth weight of calves following the transfer of in vitro-produced embryos derived from conventional or sex-sorted sperm.

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The ability to accurately predict calf birth weight during gestation would be of significant benefit to dairy farmers, improving both cow and calf welfare. Data on fetal development and calf birth weight (BW) following in vitro fertilization with sex-sorted semen remain scarce, however. The aim of this study was to investigate the effect of semen type and embryo genotype on fetal size at Day 63 of gestation and calf BW of female calves following timed embryo transfer with fresh in vitro produced (IVP) embryos. These embryos were produced using oocytes collected from either beef or dairy donors and fertilized in vitro using either conventional (CV) or X-sorted (SS) sperm. Single Grade 1 blastocysts were transferred fresh to recipient lactating dairy cows (n=510) in 7 herds. For all cows that had not returned to estrus, pregnancy status was diagnosed on Day 32-38 by transrectal ultrasonography. All cows diagnosed pregnant (n=277) were re-examined on Day 63; in pregnant cows (n=243, CV-Dairy=45, SS-Dairy=58, CV-Beef=67, SS-Beef=73), fetal sex (males, n=70, females, n=173) was determined based on the position of the genital tubercle. The sex ratio was 117:14 (F:M) when SS sperm was used, reflecting the accuracy of the sorting process. Only female fetuses/calves were considered in this study. A video of each fetus was recorded, and thoracic diameter (TD, n=106) and biparietal diameter (BPD, n=93) were subsequently measured using ImageJ's freehand line function. Calf BW (n=119) was recorded at parturition. ANOVA was conducted using PROC GLM in SAS to determine the effects of semen type (CV vs. SS), embryo genotype (beef vs. dairy), and two-way interactions on Day 63 TD and BPD, and calf BW. The relationship between BPD and TD and between each BPD or TD and calf BW was determined by Pearson correlation with PROC CORR. At Day 63, the mean (± standard deviation) TD and BPD for female fetuses was 18.8 ± 1.03 and 13.5 ± 1.03 mm, respectively, and it was not affected by semen type or embryo genotype. Beef heifers were heavier than dairy heifers (42.9 ± 7.78 vs 36.7 ± 7.05 kg; respectively, $P<0.0001$). There was a tendency for an interaction between embryo genotype and semen type ($P=0.06$), since beef heifers conceived with SS were heavier ($p<0.01$) than those conceived with CV, or dairy heifers conceived with both types of semen (43.8 ± 7.95, 39.0 ± 6.08, 37.0 ± 7.28 and 37.8 ± 7.73 kg, for SS-Beef, CV-Beef, SS-Dairy and CV-Dairy, respectively). BPD was not correlated neither with TD nor calf BW. However, TD was associated with calf BW ($R^2=0.25$, $P=0.02$). In conclusion, under the conditions of this study, TD is a better predictive measurement for the BW of female calves compared with BPD. Future studies will correlate Day 63 fetal measures and calf BW with maternal blood transcriptome analysis.

Supported by the Department of Agriculture, Food and the Marine Research Stimulus Fund (Grant 2021R665).

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Bovine donors infected with Epizootic Hemorrhagic Disease: Impact on in vitro embryo production and risk of transmission through in vitro embryos production

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Epizootic Hemorrhagic Disease Virus (EHDV) is an Orbivirus transmitted by insects (*Culicoïdes* spp). Although infected animals may remain asymptomatic, the most characteristic symptoms include ulcers and inflammation of the muzzle, ptyalism (stomatitis), lameness, weight loss, anorexia, and fever. This viral disease emerged in Europe in late 2022 (Spain, Italy) and more recently in France (autumn 2023) (ANSES, 2023). The aim of this study was to characterize the *in vitro* embryo production efficiency of asymptomatic EHDV-infected donors and to assess the risk of virus transmission during the *in vitro* embryo production procedures.

Four asymptomatic EHD infected cow donors from two herds that had a clinical episode of EHD 3 to 4 months before OPU session and six negative EHD cow donors were included in this study. The EHD infected donors were positive for EHD virus in blood samples, 10 days before the OPU session, by RT-PCR test (IDGene™ EHDV Duplex) with a Ct (Number of PCR cycles to detect DNA virus) between 33-35. Donors were stimulated with decreasing pFSH doses (Stimufol; Reprobiol®, Belgium) twice daily for 3 days (dose: 300 µg). Cumulus oocyte complexes (COCs) were collected by OPU 12 to 24 h after the last FSH injection and *in vitro* matured using a standard IVM protocol. COCs were fertilized with frozen-thawed X-sorted sperm in modified Tyrode's bicarbonate buffered solution medium using different non-pre-tested bulls. Presumptive zygotes were cultured in SOF medium up to Day 7 at 38.5 °C in 5% CO2 and 5% O2 with maximum humidity. Grade 1 expanded blastocysts (IETS classification) were recorded on days 6.5 and 7. Embryo production was analyzed with ANOVA ($p<0.05$ was significant).

A PCR test (ID Gene™ EHDV Duplex) was used to detect a conservée séquence of all EHD virus serotypes in OPU collected medium (CM), maturation medium (MAT), Fertilization medium (FERT), Denudation medium (DEN), culture medium (CULT), the last three embryo washing media (LAV) and unfertilized and degenerated embryos (EMB) of EHDV positive donors.

For EHDV-positive donors, viral RNA was not detected by RT-PCR in association with any of the media collected during IVP (CM, MAT, FERT, DEN, CULT, LAV) or in unfertilized and degenerated embryos (EMB).

The total number of selected COCs for IVM did not significantly differ between EHD and Control groups (8.7±2.5 vs. 9.5±3.5). At D7, Grade 1 expanded blastocyst rates were not different between EHD and Control groups: 38.5±17.4 vs 50.9±17.7.

In conclusion, asymptomatic EHDV donors still viropositive in blood by RT-PCR with a Ct between 33-35 do not exhibit reduced efficiency in embryo production. It seems that the EHD virus is not likely to be transmitted by *in vitro* embryo production, 3 to 4 months post-infection. Further studies are needed to assess the effect of infection of donors by the EHDV on embryo production and the risk of transmission through *in vitro* embryo production procedures at earlier stages of the infection.

Folliculogenesis, Oogenesis, and Superovulation

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Follicular response to the intraovarian administration of autologous platelet-rich plasma (PRP) in Katahdin ewes

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In the ewe, the growth of antral ovarian follicles as waves depends on the action of FSH and the number of such follicles affects the response to superovulatory treatments. 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 number of antral follicles in the ovaries after the intraovarian application of PRP. The study was conducted at the Experimental farm of Universidad Autonoma Chapingo, Mexico. Eleven multiparous (2.1 ± 0.8) Katahdin ewes, healthy, and in good body condition (3.2 ± 0.3) were used during the breeding season of 2023. The study was conducted according to the guidelines of the Ethical Committee (No. CECBS23-13) of Universidad Autonoma Metropolitana, Mexico. Blood samples were collected from the jugular vein in 2.7 mL vacutainer tubes containing sodium citrate and were maintained at 4 ^{o}C during the procedure. The samples were centrifuged at 1 000 *g* for 30 min and in order to obtain the platelets, the serum obtained was centrifuged for a second time at 1 500 *g* for 10 min. The upper two-thirds of the tubes were discarded, keeping the lower third part with a higher platelet concentration (12 500 × 10^6 platelets mL^{-1}). Using a laparoscope (Rigid laparoscope, Storz, Germany), 50 µL of autologous PRP were administered inside the ovarian stroma of the right ovary of each ewe, while the left ovarian stroma received 50 µL of saline solution, without pinching follicles ≥4 mm or corpus luteum. The ewes were fasted from food and water for 18 h and anesthetized by intravenous administration of 2.3 mg of 10% xylazine (Procin, Pisa, Mexico) and 7.7 mg of ketamine (Anesket, Pisa, Mexico) per 10 kg^{-1} of body weight. The experimental design and the treatments were: T1 (Control) = ovary with an application of saline solution and T2 = ovary with an application of PRP. Next, the number of antral follicles ≥2 mm in size in both ovaries was determined weekly, for six weeks, using an ultrasound (US) device (Aloka, Prosound 2, Japan) equipped with a rectal transducer (7.5 MHz). Before each US session, the ewes were fasted. The number of antral follicles was analyzed with GLM of SAS. The effect of treatment was considered significant with a $P< 0.01$. The number of antral follicles in the ovaries was similar ($P> 0.05$) between PRP and control in weeks 0, 1, and 2 (6.36 ± 0.43 vs. 6.27 ± 0.30; 5.81 ± 0.53 vs. 5.81 ± 0.42, and 4.72 ± 0.23 vs. 4.90 ± 0.28, respectively). However, there were significant differences ($P< 0.01$) for weeks 3 (8.45 ± 0.41 vs. 6.09 ± 0.34), 4 (7.90 ± 0.62 vs. 5.63 ± 0.38), 5 (9.00 ± 0.52 vs. 5.72 ± 0.42), and 6 (8.45 ± 0.37 vs. 6.09 ± 0.46). The results suggest that the use of autologous PRP might be an alternative to complement superovulatory treatments in ewes, increasing the number of antral ovarian follicles. In conclusion, under the conditions of this study, the intraovarian application of autologous PRP increased the number of antral follicles in the ovaries of Katahdin ewes from week 3 to 6 post-treatment.

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Acute and chronic effects of a high-fat high-sugar diet on the granulosa cell transcriptome. Insights from an outbred mouse model

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Continuous consumption of western-type diets (high in fat and sugar, HFHS diets) results in systemic metabolic changes including low-grade inflammation, lipotoxicity and oxidative stress. This impairs different tissue functions, and eventually leads to obesity and increased risk of metabolic disorders. Consumption of HFHS diet and the development of obesity have also been linked with reduced fertility, partially by impacting ovarian follicle development and oocyte quality. Previous studies have shown that diet-induced alterations in metabolism and tissue functions are dependent on the exposure time and occur in a multiphasic pattern [1]. Similar patterns were reported in the alterations in oviductal cell functions [2, 3], but the dynamics of diet-induced alterations in the ovarian follicles have not been previously described. The aim of this study was to determine the onset and progression of changes in the granulosa cell transcriptomic profile after starting HFHS diet feeding.

For that, 5 week (wk) old female outbred Swiss mice were fed a control (CTRL, 10% fat) or HFHS (60% fat, 20% fructose) diet for up to 16wk. Mice (n=3-4 per treatment per timepoint) were sacrificed at 3 days, 1wk, 4wk, 8wk, 12wk and 16wk. This was done during the follicular phase of the estrous cycle by applying the Whitten effect for 24h. Granulosa cells were collected by puncturing the antral follicles in L15 medium, then the cells were washed with PBS by centrifugation, snap-frozen and used for RNA sequencing. Differential gene expression analysis was performed as a series of pairwise comparisons at all time points using edgeR. Gene Set Enrichment Analysis was then done with GAGE and significant GO terms ($q<0.1$) were summarised using the “Reduce and Visualize GO” tool (REVIGO).

A few significant GO term annotations were detected already at day 3 (21 GO terms), the majority of which were also detected at wk1 (but not afterwards), together with the dysregulation of another 228 GO terms (245 in total at wk1). These acute changes were mainly related to endoplasmic reticulum and mitochondrial functions, translation, cell differentiation and cell signaling. No significant GO terms could be detected at wk4, which may indicate transient metabolic adaptations. Subsequently, the highest number of significant GO term annotations was detected at wk8 (495, including 103 of those detected at wk1). The majority of these annotations were also detected at wk12 (255 GO terms) and wk16 (126 GO terms). These chronic changes are related to cell metabolism, steroid biosynthesis, immune responses, autophagy, biogenesis, and mRNA processing.

The results show that the ovarian follicle microenvironment is sensitive to very short-term changes in the diet composition, and that the alterations in ovarian cell biological functions follow a time-dependent multiphasic cascade. Defining and understanding these progressive changes should increase the awareness needed to protect reproductive health and improve efficiency of preconception care interventions.

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Reconstruction of immature bovine cumulus oocyte complexes for IVM

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The majority of retrieval human cumulus oocyte complexes (COCs) are subjected to the ICSI for fertilization in the infertility treatment program by assisted reproductive technologies (ART). However, approximately 15% of human oocytes are immature after oocyte denudation when induction of superovulation was applied. Human germinal vesicle oocytes are usually discarded from the ART program due to their low maturation rate and competence. It is known that cumulus cells (CCs) play an important role in maintaining the meiotic arrest of oocytes and achieving their competence by communication through the gap junctions (connexins 37 and 43). The restoration of lost contacts between CCs and denuded oocytes *in vitro* could improve oocyte IVM outcomes and increase the available gamete number for fertilization.

The study aimed to evaluate the effect of different culture systems on the restoration of gap junctions between CCs and oocytes and IVM outcomes.

The study utilized bovine CCs and oocytes as a model. Ovaries were obtained from the local slaughterhouse and 4-8 mm follicles were aspirated. Retrieval immature COCs (n=747) with more than 2-3 layers of CCs were randomly divided into groups: 0, immature COCs; 1, COCs matured in a drop under mineral oil; 2, denuded oocytes (DOs) matured in a drop under mineral oil; 3, DOs with CCs matured in a drop under mineral oil; 4, DOs with CCs matured in round-bottomed plates with cell-repelling surface; and 5, DOs with CCs matured in a hanging drop. All oocytes were matured in IVM medium (Stroebech, Denmark) during 22 hours, then maturation rate and RNA expression level of the *Gja1* (connexin 43) and *Gja4* (connexin 37) genes were assessed in the study groups. Three biological replicates were conducted for assessment of the genes' expression in each group.

Our study has shown that CCs was expanded in group 1 while in groups 3 and 5 CCs made partial aggregates with oocytes. In group 4, all CCs formed a whole rounded aggregate with oocytes in the center. The *Gja4* RNA expression level was higher in oocytes in all groups after IVM with the highest value in group 2 ($p<0.05$). The *Gja4* RNA expression level in CCs was increased in groups 3-5 after IVM, while in group 1 it did not have any changes ($p<0.05$). The *Gja1* expression in oocytes was upregulated after IVM only in group 2 while in CCs it was downregulated in all the groups where CCs were presented regardless culture system ($p<0.05$). The highest maturation rate was recorded in group 1 (76.2%), while in groups 2-5 it was 29.4; 62.3; 35.1 and 56.1%, respectively.

These results confirm that CCs are very important for successful oocyte maturation, and premature denudation may provoke changes in expression profiles of connexin 37 and 43 in oocytes. Perhaps such changes were a kind of compensatory effect in order to restore lost connections between oocytes and CCs. Adding CCs to DOs in a drop or hanging drop co-culture system improves the maturation rate. The use of plates with a round bottom and a cell-repelling surface leads to restoring the round shape of reconstructed COCs and may keep oocytes under meiotic arrest longer.

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Single-cell DNA methylation sequencing reveals epigenetic alterations induced by bovine oocyte in vitro maturation

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Oocyte *in vitro* maturation (IVM) is a key step for the *in vitro* production of bovine embryos. However, there is a concern that this process may induce suboptimal developmental competence of bovine oocytes including epigenetic alterations. Currently, epigenetic profile of bovine oocyte IVM is relatively limited and inconsistent, probably arising from differences in the detection approach and experimental design used between studies. The aim of this work was to determine whether DNA methylome alterations are present in bovine oocytes that were *in vitro* matured and to identify conserved biomarkers across species. To achieve this, the results of this study were compared to those obtained in a similar study with *in vitro* matured porcine oocytes (unpublished data). The study was performed on 18 *in vitro* matured oocytes recovered from 2-8 mm follicles of abattoir-derived bovine ovaries and 28 *in vivo* matured oocytes collected by ovum-pick-up. Transvaginal aspiration was performed at 96–98 h after GnRH administration. The analysis of DNA methylation was performed by single-cell whole-genome bisulphite sequencing. Then, differentially methylated regions (DMRs, FDR < 0.05, FC > 0.1) were determined using the R package limma. Results showed that global DNA methylation profiles differed between *in vitro* and *in vivo* groups. Individual oocytes were clustered using Uniform Manifold Approximation and Projection analysis, which showed a clear separation within the *in vivo* group according to breed and age. The analysis of DMR identified a lower number of hypermethylated and hypomethylated regions in the IVM group, which were more frequent in variably methylated regions (VMRs), promoters, transcripts and imprinted genes. No differences were found in methylation of CpG islands of genes previously related to large offspring syndrome between groups. Regarding the effect on genomic imprinting, methylation was lower for IVM oocytes in the imprinted gene *CDKN1C* and higher in the *BEGAIN* gene when compared to the *in vivo* group. In addition, the analysis of genes that have been previously predicted for their possible function in the imprinting process showed a number of differences between the *in vivo* and *in vitro* group. For example, we identified lower methylation in *in vitro* matured oocytes in CpG islands of 5 "candidate" genes *(SEMA7A, ZNF575, ATP4B, PDGFA, COMP)* while only one was hypermethylated in the coding region of the *PLCL2* gene. Finally, we identified conserved differences in methylation related to IVM between bovine and porcine oocytes for 14, 8 and 3 genomic features in the transcripts, VMRs and promoters, respectively. The findings indicate that some of the epigenetic alterations are associated with suboptimal developmental competence of IVM oocytes. In conclusion, these results could help to improve this technique when employing *in vitro* production procedures in cattle.

The authors acknowledge the financial support from MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa", grant number PID2020-113366RB-I00. L. Abril-Parreño

is funded by MICINN (Spain) through the programme Juan de la Cierva-Formación (FJC-2021-047738-J37703).

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Evaluation of the effect of three drugs on follicular development in water buffaloes (Bubalus bubalis) from an in vitro embryo production program.

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One limitation of the buffalo *in vitro* production of embryos (IVP) is the number of oocytes and their developmental potential. Contradictory results have been reported regarding the effect of different sources of drugs, natural or recombinant, on the induction of follicular development. This work aimed to evaluate the effect of the administration of three different drugs on follicular development in an IVP buffalo program. Twenty healthy adult parous females located in Itatí (Corrientes, Argentina) were used. Ovarian mapping was performed using an ultrasound scanner (Mindray, DP30-Vet). Groups of five animals were divided in 4 treatments (TRT).On day 0, all animals received an intravaginal device (IVD) with progesterone + 2 mg of estradiol benzoate intramuscularly (IM). The IVD was removed on the day of Ovum Pick-Up (OPU). In TRT-1 (Control), OPU was performed on day 7. In TRT-2, 1050 IU of recombinant equine chorionic gonadotropin (eCG, FoliRec®) was administered IM on day 4 and OPU was performed on day 7. In TRT-3, 2500 IU of serum eCG (Ecegon®) was administered IM on day 4 and OPU was performed on day 7. In TRT4, recombinant follicle-stimulating hormone (FSH) (Cebitropin B®) was administered IM starting on day 4, 105 µg distributed in decreasing doses every 24 hours for two days (60 µg and 45 µg). Forty-four hours after the last application, the OPU was performed. Before OPU the numer of follicles was counted and classified according to their size: minor (≤3 mmØ), medium (4-8 mm Ø) or large (>8 mmØ). Oocytes were evaluated within the follicular fluid, graded I, II, III, IV and expanded according to the International Embryo Transfer Society (IETS, IL), and pooled for each TRTs. Oocytes were matured, fertilized and obtained embryos were cultured for 6 days. Data was compared using the Mann–Whitney or comparisons of proportions tests; a p-value < 0.05 was considered as statistically significant. The average number of follicles per animal before and after treatment was 13±3 and 9.6±3, respectively, with no significant differences between TRTs. There were significant differences in the number of follicles from 4 to 8 mm between TRT 1 (p=0.02), TRT2 (p=0.03), TRT3 (p=0.05) and TRT4 (p=0.03), showing an increase of 2.4 times compared to the number of follicles at the day of IVD insertion. No significant difference was found between the drugs in the number of oocytes and embryos between TRTs. A range of 20 to 24 oocytes were obtained, with 15.7%, 30%, 50.5%, 3,8% corresponding to GI-GII, GIII, IV and expanded oocytes respectively. There was an increase in the number of medium sized follicles in all TRTs as reported in the literature. But it is paradoxical from this study that the control group had an increase in the number of follicles, and a decrease in the total follicular population. It was not possible to demonstrate an effect of the drugs or protocols on the quality of oocytes and the number of embryos which in this species remain low. The authors believe that the groups are too small to compare the 4 different treatments. Future studies need bigger groups or fewer treatments in the same study to obtain more robust results.

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M6a RNA methylation during in vitro maturation of sheep cumulus-oocyte complexes with different development competence

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M6a is an abundant epigenetic modification of mammalian mRNAs and is largely undescribed in mammalian oocytes and embryos. It regulates mRNA metabolism and affects maternal transcript decay prior to embryo genome activation, thereby potentially affecting developmental competence. RNA methylation dynamics are controlled by three groups of modulators that add (writers), remove (erasers) or bind to (readers) the methyl group to the N6 position of adenosine (Qin et al. Int J Mol Med. 2020). We used a well-characterized differential model of developmental competence consisting of cumulus-oocyte complexes (COCs) of adult (2-4 years old) and prepubertal (30-40 days old) Sarda sheep, that show respectively high and low development competence. The differential model was previously studied in terms of in vitro developmental capabilities and morphological, cellular, biochemical and molecular aspects. Aim of this work was to evaluate m6a methylation in the differential model of developmental competence in terms of gene expression of m6a modulators and m6a abundance. COCs were recovered from ovaries of adult and prepubertal sheep. Oocytes and granulosa cells (GCs) were separately stored in CellProtect Reagent (Qiagen, Hilden, Germany) at germinal vesicle stage (GV) or after 24h in vitro maturation (IVM). Total RNA was isolated from pools of 10 oocytes and GCs from pools of 25 COCs, with RNeasy MicroKit (Qiagen, Hilden, Germany), reverse transcribed and used for gene-specific relative quantification by Real Time-PCR. RNA isolated from GCs was subjected to m6A quantification with EpiQuik™ m6A RNA Methylation Quantification Kit (EpigenTeck, Farmingdale, NY, USA). Data were analyzed with the General Linear Model ANOVA, considering the two factors "competence" and "maturation stage" and their interaction. Differences were considered significant when $p<0.05$.

Relative transcript abundance was similar in oocytes and GCs of different developmental competence at the GV stage. Conversely, after IVM, low competence COCs showed altered gene expression compared to high competence COCs, and specifically downregulation of *METTL3*, *METTL14*, *VIRMA*, *YTHDF3*, *YTHDC1* and *ALKBH5* ($p<0.05$) in oocytes and, upregulation of *METTL14,VIRMA* ($p<0.05$) and*YTHDF3* ($p=0.05$) in GCs. In accordance with a higher expression of the methyltransferases, colorimetric assay showed higher m6A levels in total RNA of GCs derived from low competence COCs after IVM ($p<0.05$). Data show that m6a RNA methylation is altered in low competence sheep COCs after IVM and suggest an involvement of the epitranscriptome in oocyte developmental potential.

This study contributes to the growing understanding of the oocyte epitranscriptome which is crucial for unravelling the mechanisms involved in the oocyte-embryo transition, shedding light on the impact of epigenetic RNA modification in mammalian reproduction and unexplored aspect of female infertility.

Funded by: “European Union – Next Generation EU”; “PON Research and Innovation 2014-2020”, “Finanziato a valere sulle risorse del bando Fondazione di Sardegna – annualità 2017”.

Physiology of Male Reproduction and Semen Technology

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Phosphatidylserine on sperm head interacts with Annexin A5 on oviduct luminal cilia to form a sperm reservoir in pigs

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After insemination in mammals, a sperm subpopulation reaches the oviducts, where they bind to luminal epithelial cells in the isthmus to form a "sperm reservoir". Sperm binding to isthmic epithelial cells (IECs) is assumed to select sperm of high fertilizing ability and lengthen their lifespan until ovulation time. Expression of annexins (ANX) was reported on the surface of the porcine oviduct epithelium (Teijeiro *et al*. Mol Reprod Dev 76:334, 2009). However, the mechanisms of sperm reservoir formation are still largely unknown. The objective of this study was to explore the role of ANXA5, a protein that binds with high affinity to phosphatidylserine (PS; Jing J. Int J Mol Sci 25(5)2865, 2024), on formation of sperm reservoir in pigs.

Fresh ejaculated semen from different pools of 3-5 Pietrain boars (6-24 months) of proven fertility were used for all experiments. Sperm motility was analyzed by computer-assisted sperm analysis (IVOS II). Sperm acrosome status (PNA) and membrane integrity (propidium iodide) were analyzed by flow cytometry. Isthmic mucosa fragments (IMF) were collected from pre-pubertal gilts at slaughterhouse and epithelial spheroids (ES) produced *in vitro* as previously described (Schmaltz *et al*. Theriogenology, 219:116, 2024). A Tyrode-based non-capacitating medium (NCM) or a capacitating medium containing caffeine and albumin were used for ES co-incubation with sperm (1.10^6/mL, 30 min). Bound sperm density on ES (in number of sperm/mm^2) was determined using confocal microscopy after Hoechst staining. Recombinant porcine (rp) ANXA5 (RP1796S-100; Kingfisher Biotech) and soluble PS (P7769; Sigma) were added to co-incubation media. Mouse anti-ANXA5 and anti-PS antibodies (Sigma) were used for immunodetection. Three to six biological replicates with different pools of ES and semen were performed for each experiment. One-way ANOVA followed by Tukey post-hoc tests were used to analyze the data.

ANXA5 was immunodetected exclusively on cilia at the surface of IMF and derived ES. In addition, rpANXA5 from 0.01 to 10 µg/mL and PS from 0.01 to 1 µg/mL inhibited boar sperm binding to ES in a dose-dependent manner without decreasing sperm motility compared to controls. Pre-incubation of sperm but not ES with 10 µg/mL rpANXA5 was sufficient to inhibit sperm binding to ES. The rpANXA5 and PS were immunodetected on boar sperm heads in the acrosomal region. Compared with NCM, the capacitating medium increased within 30 min the proportions of live sperm positive for PS detection, bound to fluorescent rpANXA5, and the bound sperm density on ES. On the opposite, acrosome reaction decreased the proportion of PS+ sperm, their ability to bind to rpANXA5 and prevent their binding to ES.

Our data indicate that PS on boar sperm acrosomal region and ANXA5 on isthmic cilia play a role in the formation of the sperm reservoir. Sperm membrane remodeling at the time of capacitation enhanced sperm head PS exposure and may facilitate interactions with ciliary ANXA5 in short time. This is the first report of a role of PS-ANXA5 interaction and sperm capacitation in the formation of the oviduct sperm reservoir.

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Enhancement of boar epididymal sperm motility through seminal plasma extracellular vesicles

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Extracellular vesicles (EVs) from sexual accessory glands have been implicated in various aspects of sperm function, including motility, acrosomal integrity and calcium mobilization. While epididymal vesicles interact directly with sperm in the male reproductive tract, EVs from sexual accessory glands are thought to influence sperm function in the female reproductive tract (reviewed by Foot and Kumar, 2021). However, studies on the role of accessory sexual gland EVs in sperm function and preservation have yielded conflicting results (Pons-Rejraji et al., 2011; Park et al., 2011). This study aims to investigate the effect of EVs from seminal plasma on the viability, acrosomal integrity and mitochondrial activity of epididymal spermatozoa during 72 h storage (time to preserve the insemination dose in porcine).

Seminal plasma from boars with proven fertility was used for isolation of EVs by ultracentrifugation from five different ejaculates. The EVs were characterised by means of DLS, TEM and EV-specific protein markers (CD63, CD81, HSP70) as previously described Toledo-Guardiola et al. 2024. To assess sample purity, high-resolution flow cytometry was performed to quantify the amount of albumin, a contaminant present in very small EVs. The same pool of EVs was used for all replicates (n=7). Epididymal spermatozoa from randomly selected boars from the abattoir were co-incubated with EVs or not (control group) and stored at 15ºC for up to 72 hours. Total and progressive motility parameters were assessed by Computer-Assisted Sperm Analysis (CASA), while flow cytometry was used to evaluate sperm viability (propidium iodide), acrosomal integrity (peanut lectin) and mitochondrial metabolism (rhodamine 123) at 0, 24, 48 and 72 hours.

The results showed that epididymal spermatozoa supplemented with EVs exhibited an increase in total motility (24 and 48 hours) and an increase in progressive motility (48 hours) compared to the control group. However, there was a deterioration in acrosomal integrity with vesicle supplementation at all time points. Viability and mitochondrial activity remained unaffected by the presence of vesicles but declined over the preservation period.

This study demonstrates the effect of EV supplementation of epididymal sperm on motility and acrosome integrity, which may be related to calcium mobility by ATPase contained in EVs. However, no work with

epididymal spermatozoa has been found in the literature to support the results of this study.

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Graphene oxide increases the sperm fertilizing ability by modifying the sperm proteome

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Over the last 30 years, our society has witnessed to an impressive increase in the adoption of in vitro fertilization (IVF) techniques due to the continuous rising incidence of infertility rates. To surpass the limits related to sperm selection, the use of non-physiological materials has emerged as one of the most promising strategies for advanced sperm preparation. Indeed, recent studies have reported that the addition of graphene oxide (GO) at specific concentrations during capacitation is able to increase the fertilization ability of spermatozoa in swine, bovine and mice models.
On this basis, this study aims to elucidate the complex events occurring during sperm capacitation in the presence of GO, using a proteomic approach in a swine model. To this end, spermatozoa were analyzed at different capacitation times (0 and 2 h, T0 and T2 respectively) in the presence and absence of GO at 0.5 µg/mL (GO and CTRL groups, respectively). Proteomic analyses were performed on spermatozoa pooled from three different animals using Filter Aided Sample Preparation (FASP) protocol. Tryptic peptides were analysed in triplicate by LC-MS/MS using the UltiMateTM 3000 UPLC chromatographic system coupled to the Orbitrap FusionTM TribridTM (Thermo Fisher Scientific) mass spectrometer. Data were processed using MaxQuant and Perseus, matching spectra against the UniProt database (taxonomy Sus scrofa) to obtain LFQ Intensity values used for functional analysis. Finally, protein ratios (GO/CTRL T0 and GO/CTRL T2) were used for functional analysis by Ingenuity Pathway Analysis (IPA software, Qiagen, Hilden, Germany).
In particular, 755 and 626 proteins were quantified in the GO and CTRL groups at T2, respectively, obtaining different protein sets with a total of 274 proteins with different values in terms of abundance ($p<0.05$). For instance, different values were obtained for proteins with a crucial role in sperm capacitation, as the heat shock protein 1-like, 26S proteasome regulatory subunit 7, cAMP-dependent protein kinase type II-alpha regulatory subunit and A-kinase anchoring protein 4. Furthermore, the comparison analysis carried out with IPA showed that some biofunctions have opposite trends in the two comparisons (GO/CTRL T0 and GO/CTRL T2), for instance, infertility, fertility, transport of molecule, metabolism of carbohydrate, infection of embryonic cell lines. To our knowledge, this is the first study comparing protein levels in sperm capacitated with or without GO using proteomics approach. The differences observed among the study groups suggest that the significant changes occurring in the sperm proteome during capacitation in the presence of GO might be one of the contributing factors to the increased fertilizing ability of spermatozoa.

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An optimized method for epigenetic histone 3 modifications (H3K27me3 and H3K27ac) immunodetection in pig spermatozoa

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Improving the selection of boars before entering artificial insemination programs is a priority for breeders, since subfertile boars generate economic losses. Sperm chromatin assessment could be a decisive tool for boar selection. Most of sperm histones are replaced by protamines during spermatogenesis, except for a subset of them demonstrated to have critical epigenetic roles and influence fertilization and gene expression in the early embryo. The acetylated and trimethylated forms of histone 3 (H3K27ac/H3K27me3) are related to DNA transcription activation and repression, respectively, being potential epigenetic targets. Our objective was to optimize the immunostaining detection of H3K27ac and H3K27me3 and determining their location in the boar spermatozoon.

Frozen sperms samples in straws (3 per boar), of 3 boars (20-24 months of age) of Large White breed with a fertility and sperm quality proved, were thawed (37 °C, 30 s), centrifuged and fixed in 4% paraformaldehyde (PFA). The samples were washed and resuspended in PBS, spread on slides and dried. After 20 min, samples were permeabilized for 30 min with Triton X-100 (0.1% or 0.5%) in PBS, washed and decondensed with dithiothreitol (DTT) 10 mM, 20 mM, or 25 mM (15 min, 37 °C), then blocked with 3% BSA for 1,5 h. Primary antibodies (Abcam) were tested at 1/100, 1/200, 1/500, or 1/1000 for H3K27ac (ab177178) and 1/100 or 1/200 for H3K27me3 (ab6002), incubating overnight at 4 °C. After washing, the slides were incubated with the secondary antibody (Abcam;1/400: ab150081 for H3K27ac and ab150120 for H3K27me3) for 2 h, washed and mounted. In addition, negative controls using only secondary antibody were used. Samples were assessed by fluorescence microscopy, using ImageJ v. 1.54 to estimate fluorescence intensity at least in 200 spermatozoa per slide. The experiment was triplicated using different males, and statistical analyses were performed using the R statistical software and ANOVA. The best protocol was then used to analyze the fluorescence localization patterns.

The optimal fluorescence intensity and definition were obtained using Triton X-100 0.1% and DTT 20 mM at 1/100 both primary antibodies: 69330,9±11315,8 UA for H3K27me3 ($p<0,01$ respect the rest of treatments) and 104298,7±4520,8 UA for H3K27ac ($p<0,001$ respect the rest of treatments). H3K27ac signal varied between boars and was mainly located on the acrosomal region in two boars (>50% of sperm) and on the postacrosomal region in one boar (>90% of sperm). However, H3K27me3 signal appears as a crown around the sperm head or almost throughout the periphery of the sperm head in all boars (>70% of sperm taking into account both signals).

Future research will aim at testing if these epigenetic marks are related to chromatin status and boar fertility. The main interest is contributing to the early detection of subfertile boars, reducing the subsequent economic losses.

Supported by RTI2018-095183-B-I00 and DIN2020-011558 funded by MCIN/AEI/10.13039/501100011033, by "ERDF A way of making Europe" and "European Union NextGenerationEU/PRTR" and LE023P20 funded by Junta de Castilla y León (Consejería de Educación/ERDF, EU). Thanks to AIM/Topigs-Norsvin, M. de Prado and G. Rivas.

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Bull spermatozoa tolerance to natural extracts from grape marc with antimicrobial properties

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The cattle breeding industry uses semen extenders containing wide-spectra antibiotics, which are of concern because of the risk of contributing to antimicrobial resistance (AMR). Polyphenol-rich plant extracts have been tested as supplements in semen extenders because of their antioxidant properties, but their antimicrobial effects have been little explored. Sustainable and environmentally friendly methods allow the revalorization of grape marc as agricultural waste, obtaining extracts with antimicrobial properties (doi: 10.1007/s11356-019-07472-1). We tested the tolerance of bull spermatozoa to the extender supplementation with two grape marc extracts (Pex and Eex differing on solvent extraction, proprietary from i-Grape, Spain). Ejaculates (artificial vagina) from five adult Holstein bulls were pooled, extended in OPTIXcell (IMV), and supplemented with 5 to 30 µl of extract per ml sample. Sperm motility was assessed after 30 min at 37 °C after supplementation (D0) and after 24 h at 5 °C (D1). Motility analysis was carried out with a Nikon E600 microscope (×10 negative phase contrast, Basler acA1920-155uc at 200 fps), processing 1-s videos with OpenCASA v. 2 (https://github.com/calquezar/OpenCASA). The experiment was triplicated, and data were analyzed using linear mixed-effect models (control with no extract as reference).

Eex 30 µl/ml and 20 µl/ml (D0 and D1, respectively) and Pex 20–30 µl/ml showed a negative significant effect in total motility. However, Eex (10–30 µl/ml) and Pex (only at 30 µl/ml) decreased progressive motility in D0, but at D1, only the Pex effect remained. The kinematic parameters were little affected and always showed minor effects. Velocity (VCL, VAP, VSL) significantly increased in D0 with Pex 20 µl/ml and in D1 with Eex 10 µl/ml; linearity (LIN) decreasing in D0 with 30 µl/ml, with no significant effects in D1; head beat cross frequency (BCF) increasing in D0 with Pex 20 µl/ml; and the fractal dimension increasing (hyperactivated-like movement) in D0 for Eex 30 µl/ml and P 20–30 µl/ml, and only Eex 30 µl/ml in D1. No significant effects were detected for other kinematic parameters (STR, WOB, ALH).

Considering the overall motility evaluation, 5–10 µl/ml formulations could be used to extend bull semen. Our findings open the possibility of replacing or reducing antibiotics in bull semen extenders using these sustainably obtained natural extracts, contributing to the circular economy and fighting AMR. Subsequent steps will refine this range for cryopreservation, including antimicrobial efficiency in practical conditions.

Supported by NeoGiANT 101036768 (H2020-LC-GD-2020-4) and PID2022-137640OB-I00 and DIN2020-011558 (MCIN/AEI/10.13039/501100011033, by "ERDF A way of making Europe" and “European Union NextGenerationEU/PRTR”). Thanks to Xenética Fontao and Á. López.

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Could Estrogen and Progesterone be used as biomarkers in swine saliva?

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Estradiol (17-beta-estradiol, E_2) and progesterone (P_4) play a key role in the regulation of the estrous cycle, and their determination is performed in blood plasma (BP) or serum. Blood collection in pigs is stressful for the animals and requires immobilization for an optimal collection. Saliva is also useful for monitoring ovulation, assessing ovarian function, or testing for pregnancy. Furthermore, its collection is more convenient and less invasive for frequent sampling. Despite these advantages, studies on sex hormones in swine saliva are very scarce, which limits their practical application. In the present study, the levels of E_2 and P_4 in saliva and BP were obtained to determine the potential association between them, and to verify if saliva could be used to monitor the reproductive status in sows. Twenty-seven hormonally synchronized ($PgF_{2\alpha}$, eCG and hCG) sows were sampled 24 hours after the onset of estrus and artificially inseminated. For saliva sampling, sows chewed a zip tied sponge for 15 to 30 seconds; then the sponge was removed from the zip tie and placed in a tube designed for human saliva collection (Salivette®). In the laboratory, the saliva was centrifuged at 1000 rpm for 5 min and stored in 1.5 ml cryovials at -80ºC until analysis. Blood sample was collected from the external jugular vein under general anesthesia. Hormone concentrations in saliva and BP were determined using a solid-phase, enzyme-labeled competitive chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Healthineers). E2 and P4 were detected in all plasma samples, but P4 was only detected in 12/27 (44.4%) saliva samples and 100% of plasma samples (minimum detection level >0.20 ng/ml). P4 levels were higher in plasma than in saliva (8.67±1.94 vs. 1.16±0.74 ng/ml, Wilcoxon non-parametric test, p=0.004), while E2 levels (54.09±6.52 vs. 78.73±8.24 pg/ml, p=0.003) and E2/P4 ratio (25.71±9.76 vs. 251.31±48.39, p=0.002) were higher in saliva than in plasma. The use of Bland-Altman plot to compare plasma and saliva values for E2, P4 and ratio E2/P4 confirmed the inconsistency of the results. This fact confirms that saliva is not a suitable fluid to determine P4 and E2 in pigs with this methodology, a fact that was later confirmed when saliva and BP were obtained from 2 additional pregnant sows (> 60 days of gestation), obtaining P4 values in saliva with a very low value (2.11 ng/ml) when in plasma was 23.38 ng/ml, while E2 was higher in saliva than in plasma (86.1 vs. 15.70 pg/ml). Further studies are needed to clarify the inconsistency of these results in order to use a less invasive technique such as saliva sampling to determine reproductive hormones and assess the reproductive status of gilts and sows.

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Effects of curcumin extract on bull semen samples in in vitro fertilization

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Curcumin extract has demonstrated antioxidant and antibacterial properties at the concentration of 5% in bull semen samples suggesting that it could be used as an alternative to antibiotic[1]; however, fertilization outcomes remain largely unknown. This study aimed to assess the impact of treating bull semen with curcumin extract on *in vitro* fertilization (IVF) results. Bovine ovaries were collected from the abattoir, reaching the IVF laboratory within 4 hours. Upon arrival, cumulus oocytes complexes (COCs) were aspirated and COCs of good grades were selected and randomly distributed between treatment groups, and subsequently matured. All media used was from Stroebech Media ApS, Hundested, Denmark and procedures except *in vitro* fertilization were according to the manufacturer´s protocol. The COCs were matured for 22 hours before fertilization. In total, five batches of ovaries were used, each divided in two replicates containing an average of 30 oocytes. Fertilisation was done with an equal number of untreated control replicates present. Semen samples from one bull with verified *in vivo* and *in vitro* fertility, was diluted to a concentration of 0.5 x 10^6 spermatozoa per mL and treated with curcumin extract at a concentration of 5% for 30 minutes. A lower spermatozoa concentration than recommended was used for IVF, aiming to assess the curcumin effect. Cleavage was assessed 44 hours after onset of fertilization. The parameters that were evaluated were total number of COCs in maturation (OMAT), percentage of cleaved oocytes (CLEAV), percentage of oocytes with more than 2 cells from cleaved (ABOV2) and percentage of blastocysts on day 8 from cleaved (D8). The results (Mean and standard deviation; SD) were as follows for treated and control group respectively: OMAT (311 ± 2.55 vs 317 ± 1.79), CLEAV (50.25 ± 10.89 vs 51.97 ± 13.58), ABOV2 (50.94 ± 19.46 vs 41.88 ± 22.18) and D8 (19.87 ± 15.77 vs 18.23 ± 7.80). In conclusion, sperm treated with curcumin can successfully fertilize oocytes, promote further cell divsions, and had blastocyst formation in IVF.

Acknowledgements: Thanks to HK Scan Linköping abattoir for samples, and the Seydlitz Foundation for funding.

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A longitudinal study of changes in body condition score, testicular morphometry and testicular tissue tone in Sarda rams under traditional management system

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Rams play a crucial role in commercial flocks. During the breeding season, a fertile adult ram can serve at least 5 ewes per day. However, ram infertility is one of the major problems, which is not always given the attention it deserves. In the breeding season of the Sarda dairy sheep (May-November), rams are exposed to high environmental temperatures, and they are often sidelined until the following mating season without the proper care. We can obtain important information on the rams' reproductive condition through the recurrent reproductive evaluations of the ram, including scrotal ultrasonography. The specific aim of this study was to examine the body conformation and testicular parameters of the Sarda rams throughout the breeding and non-breeding seasons. As an initial approach, we recorded the body condition score (BCS), testicular width and tone from March to December. Body condition scoring assessed the muscling and subcutaneous fat development on a scale from 1 to 5 [1-emaciated, 2-thin, 3-average, 4-fat, and 5-obese] and testicular tone was evaluated by palpation using a 3-point scale [1-soft, 2-intermediate, and 3-rigid]. Twenty-eight Sarda rams (2-6 years of age) from 4 commercial farms located in the province of Sassari, Sardinia (3-14 rams per farm) were examined at approximately -2, 2, 4, 6 and 7 month from the introduction to a flock (TFI-between late April and early june); all rams remained in the flock untill December. All rams received 3 s.c., slow-release melatonin implants (18 mg; MELOVINE® Ceva Salute Animale SPA) 20 days before TFI to advance mating and increase their fertility rate. The male/female ratio ranged from 1/40 to 1/50. Preliminary statistical analyses utilized R software to determine the main effects of consecutive examination days (or TFI), ram age, and the interaction of these terms using two-way repeated measures analysis of variance (ANOVA) and Holm-Sidak post-ANOVA test. $P \leq 0.05$ was considered statistically significant. There was a significant main effect of TFI and ram age for the three parameters studied. Mean BCS declined ($P<0.05$) from 2 to 6 months after TFI (3.3±0.09 vs. 2.9±0.05; mean SEM), and it was significantly greater in the 6-year-old compared with the 2-year-old Sarda rams(3.4±0.1 vs. 2.9±0.05). Mean testicular tone was greater ($P<0.05$) 2 mo before TFI (2.4±0.1) compared with 2 (1.6±0.1) and 4 mo (1.8±0.1) after TFI. Lastly, the mean testicular width was greater ($P<0.05$) 2 mo before (5.2±0.09 cm) than 4 mo after TFI (4.8±0.1 cm), and it was greater ($P<0.05$) in 4-year-old (5.3±0.1 cm) compared with 2-year-old rams(4.9±0.08 cm). Our present observations indicate that both the breeding activity and age of Sarda rams can affect their BCS and testicular width, with the most significant declines in those variables occurring by 4 mo (testicular parameters) or from 2 to 4 mo after ram introduction to a herd (BCS), and a rise in testicular size and BCS by 4 and 6 years of age, respectively.

Developed within the framework of the project “Ecosystem of Innovation for Next Generation Sardinia” (ECS 00000038) supported by the Italian Ministry of Research and Education under the National Recovery and Resilience Plan.

Embryology, Developmental Biology

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Age related decline in ovarian hyaluronan; impact on oocyte maturation and embryo development

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It is known that the fertility of females and the chance of producing viable and developmentally competent embryos decreases with age. Recent reports have shown an age-related decline in ovarian hyaluronan (HA), associated with increased stiffness of the ovary texture due to changes in ovary extracellular matrix components, collagen replacement of HA. Aneuploidy during meiosis is prevalent in aged oocytes and is the prime contributor to decreased gamete quality with age due to incorrect chromosome segregation. We proposed that the HA content of oocytes is crucial for oocyte nuclear maturation and its decline will have negative effects on meiotic progression of the oocyte and mitotic cell division in cleaved embryos.

Using ovaries of young and aged mice (3 months v. 22 months) and dogs (3-4 months v. 5-6 years) we explored how ageing affects collagen and HA levels. Collagen was stained using picrosirius red stain, and HA was stained using Hyaluronan Binding Protein immunohistochemistry.

In dog ovaries, there was a dramatic increase in collagen deposition with age throughout the ovarian stroma (7.34% vs 25.82%, $p<0.0005$). Within primordial follicles, the pre-granulosa cell layer of young dogs had less collagen deposition than in old dogs (0.65% vs 2.18% respectively). Collagen deposition in both the theca and granulosa cell layers increased with age in secondary follicles (theca: 14.59% vs 27.86%, $p=0.0185$; granulosa: 0.37% vs 1.24% $p<0.05$). No difference in collagen deposition within the granulosa or theca cell layer of tertiary follicles between young and old ovaries. Unlike in the dog ovaries, there was a significant increase in collagen deposition in the stroma surrounding the vasculature in ovaries of aged mice (16.95% vs 50.46%, $p< 0.005$). In mouse ovaries, collagen deposition patterns were similar to that found in dog ovaries; all three follicle types, collagen deposition showed an increasing trend with age in the theca cell layer (primary: 0.76% vs 12.51%, secondary: 1.15% vs 7.04%, tertiary: 1.49 vs 36.96% in young and old mice respectively).

Whilst there was no significant difference in HA identification within the stroma between the ages in dog ovaries, there was a decrease in HA present in the blood vessels with increasing age (91.98% vs 15.33%, $p<0.0001$). A higher amount of HA was found in the theca cell layer in the younger dog ovaries (59.57% vs 38.01%, $p=0.08$). Similarly, HA expression decreased with age in the granulosa cell layers of secondary and tertiary follicles (secondary: 17.69% vs 4.07%, $p< 0.05$) (tertiary: 7.39% vs 0.58%, $p< 0.05$ respectively). In mouse ovaries, HA staining increased with age in the granulosa cell layer (15.90% vs 36.86%, $p<0.05$) of secondary follicles, but showed no difference in the theca cell layer (16.65% vs 23.12%) between the ages. However, in tertiary follicles, the amount of HA did not differ in the granulosa cell layer between the ages (2.06% vs 3.67%) yet increased with age in the theca cell layer (10.82% vs 77.46%, $p<0.001$). Finally, blood vessels contained a significantly higher HA amount in younger mice when compared to the older (59.57% vs 21.34%, $p<0.005$).

Further, we carried out in vitro culture of bovine cumulus oocyte complexes in the absence or presence of a HA synthase inhibitor (4-methylumbelloferone; 4-MU). The inhibition of HA synthesis during oocyte maturation reduced nuclear maturation to MII stage ($p<0.05$), and cleavage rate after in vitro fertilisation (49 ± 4.8% v. 76 ± 9.8% in control). Importantly, no blastocyst could be produced from the cleaved oocytes (31 ±4.9% in control). Supplementation of HA to 4-MU treated oocytes during in vitro maturation reversed some of the effects (56 ±6.7%, $p>0.05$). Inhibition of HA synthesis post-cleavage by 4-MU blocked embryo development in a dose-dependent manner ($p<0.5$). Similarly, Inhibition of HA receptors RHAMM and CD44 in post-cleavage embryos, resulted in arrest of embryos at early stages (RHAMM, $p<0.001$), or reduction of blastocyst rate (CD44, 11.5% v 19% in control).

In summary, using these multi-species model we have characterised the effect of ageing on ovarian structure, and the potential impact of reduced HA on oocyte and embryo development. Such information may help advancing development of therapeutic techniques to reduce the pathological effects of ageing on female fertility.

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Isolation and characterization of extracellular vesicles of oviductal and uterine fluid of receptive rabbit does

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Extracellular vesicles (EVs) are structures released by various cell types and detected in numerous body fluids. EVs mediate cell-to-cell communication by transferring biomolecules (i.e. mRNAs, miRNAs, proteins) that can modulate the activities of recipient cells. Within the female reproductive system, EVs have been observed in the oviducts and uterus of various species. However, the EVs concentration in the oviductal and uterine fluids of rabbits, an induced ovulatory species, remains to be determined. This study aimed to isolate and characterize the EVs from oviductal and uterine fluid of receptive rabbit does. All rabbit does (n=15 animals) were synchronized with 25 I.U. of equine chorionic gonadotropin (Serigán, Lab. Ovejero, León, Spain) i.m., and after 48 h, 20 µg of gonadorelin i.m. (Cystoreline, Ceva, Spain) was applied to induce ovulation. After at least 14-15 h, all animals were euthanized (with barbituric overdose), and after a laparotomy, ovaries and the rest of the reproductive tract were extracted. Oviducts and uterine horns from each doe were flushed separately with PBS (1.5 mL and 2 mL, respectively) and each sample was subjected to 3 series of centrifugation at 4 ºC to discard the pellet, while the resulting supernatant was frozen at -80 ºC. Subsequently, EVs from oviducts and uterine horns were isolated by size exclusion chromatography (SEC) and concentrated using an Amicon Ultra-15 filter (Merck-Millipore Ltd., Ireland) to a final volume of 100 µL per sample. Nanotracking (NTA) analysis was conducted to characterize the size and concentration of particles present in the oviductal fluid (OF) and uterine fluid (UF) pools (5 pools of 3 animals/fluid) and morphology was evaluated by transmission electron microscopy (TEM). The EVs concentration and size were evaluated by student´s T test. The NTA results revealed that the concentration of particles was lower ($P<0.05$) in OF (2.74×10^9 particles/mL) compared with UF (6.06×10^9 particles/mL). In addition, the mean size was lower ($P<0.05$) in OF (210.5 nm) compared with UF (232.9 nm) while in modal size no differences were found (169.2 vs 181.7 nm, respectively). Additionally, TEM analysis confirmed the presence and morphology of EVs in oviductal and uterine samples. In addition, EVs in both fluids were positive for EVs proteins (CD9, HSP70 and ALIX), and negative for CANX (negative control). In conclusion, the analysis confirmed the presence of EVs in oviductal and uterine fluids from receptive rabbits, with particle concentrations and sizes consistent with EVs. However, more studies are needed to determine whether the differences found have a specific biological role in reproductive health and the promising potential of EVs as biomarkers for comprehensively understanding and monitoring female fertility.

KCB by Maria Zambrano; YNC by Margarita Salas; Spanish Ministry of Science and Innovation: PID2019-111641RB-I00

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Intergenerational inheritance of large offspring syndrome in IVP dairy cows

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The number of bovine IVP embryos is increasing worldwide, but ~10% of IVP-derived calves are born too heavy (compared to ~2% with AI or MOET). The reason for this large offspring syndrome (LOS) is not yet clearly defined, but it seems to be more prominent in female IVP calves [1]. In addition, little is known about the inheritance of birth weights among IVP-derived progeny.

This study aimed to monitor the birth weight of IVP dairy calves over multiple generations.

Birth weight data of 9112 IVP calves (Holstein Friesian), comprising up to four generations, was analysed. A LOS calf was defined as being larger than the 97th percentile birth weight of 1081 male and 1329 female AI controls, equivalent to ≥47 kg for females and ≥52 kg for male calves. Both mean birth weight and percentage of LOS animals was calculated. Intergenerational birth weight data were analyzed using a General Linear Mixed Model (GLMM) with normal distribution, applying fixed effects for LOS (mother), sex (calf) and recipient parity (heifer/cow), and random IVF bull effects. The proportion of LOS was analysed by chi-square analysis. Statistical significance was accepted at $p<0.05$.

We did not observe a cumulative increase in birth weights over four successive generations of IVP animals. The mean birth weights of 18 great-grandmothers, 20 grandmothers, 22 mothers and 49 calves were 39.6 ± 0.9, 42.0 ± 0.8, 41.6 ± 0.8 and 40.4 ± 0.5 kg, respectively ($p=0.128$ GLMM).

Comparing the last two generations from this data set, we identified 12 LOS and 567 non-LOS mothers with an average birth weight of 50.8 kg and 39.9 kg, respectively. The average birth weight of the IVP offspring from LOS mothers was 42.2 ± 0.6 and 44.3 ± 0.6 kg for females and males, respectively. For non-LOS animals, it was 40.9 ± 0.2 and 43.6 ± 0.2 kg. The difference in birthweight between the LOS male and normal male calves was not significant, but there was a significant difference ($p=0.043$ GLMM) between the birth weights of LOS female and normal female calves.

A similar pattern was observed when looking at the proportion of LOS calves from both mother groups. For the male calves, the percentage of LOS calves did not differ significantly (6/82=7 vs 81/1564=5% for LOS vs non-LOS mothers, respectively), while there was a significant (p =0.008 Chi-square) increase in female LOS calves (12/70=17% vs 103/1240=8% for LOS vs non-LOS, respectively).

We conclude that successive IVP rounds over at least four generations did not significantly increase calf birth weight. However, LOS mothers produced disproportionately more LOS daughters than non-LOS mothers. This compounding effect was not observed in sons of LOS mothers, which were normal. This intergenerational skewing of LOS inheritance across the maternal lineage may point to epigenetic errors related to incorrect maternal imprinting or X-chromosome inactivation dynamics.

Reference

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Lipotoxicity during bovine in vitro oocyte maturation induces genome-wide DNA hypermethylation in post-hatching day 14 embryos

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Maternal metabolic disorders are associated with elevated free fatty acids in the follicular fluid (FF), predominantly palmitic acid (PA). Mimicking this by adding lipotoxic PA concentrations during bovine in vitro maturation (IVM) can induce persistent DNA methylation alterations in the resulting blastocysts, despite being morphologically normal. After the blastocyst stage, the epigenome undergoes a re-establishment of DNA methylation patterns. Therefore, we aimed to examine if DNA methylation patterns were still altered in day 14 extra-embryonic tissue (EXT) originating from oocytes matured in vitro under elevated PA conditions.

This is a follow-up study of Desmet et al. (2020, Human Reprod. 35:293-307). Bovine cumulus-oocyte complexes (COCs) were in vitro matured (24h) under two conditions: 1) BASAL: physiological concentrations of PA, stearic (SA), and oleic (OA) acid (28, 21, and 23 µM); and 2) High PA (HPA): 150 µM PA, and physiological SA and OA. COCs were in vitro fertilized and cultured in control conditions until day 7. Blastocyst rates were significantly decreased in HIGH PA (4.6% decrease; $P<0.05$). Normal and expanded blastocysts (equal proportions) were transferred to healthy cows (8 blastocysts/cow, 8 cows, 5 replicates). Embryos were recovered at day 14 (n=46) and assessed under a stereomicroscope. HIGH PA significantly reduced the proportion of tubular embryos compared to BASAL ($P<0.05$), but not the length ($P>0.1$). Embryo sex was determined using the amelogenin sequence length polymorphism. For the present study, EXT sections were snap-frozen for Methyl-MiniSeq® (Zymo Research, Irvine, CA, USA) to measure genome-wide DNA methylation patterns. Only male tubular embryos were analysed (6 BASAL and 4 HPA) as they were the most abundant and to minimize variation. Sequence reads were aligned to the bosTau9 genome using Bismark 0.19.0, with a minimum of 5x coverage for considered methylation sites. Data were analyzed using *MethylKit* in R to determine differentially methylated probes (DMPs) with FDR 0.05 and 25% differential methylation cut-off. Genomic features were annotated using the *genomation* package.

When comparing HPA to BASAL, 43,864 differentially methylated probes (DMPs) were counted (on 1,048,574 analysed positions), of which 35,389 were hypermethylated (80.68% of total). This relative hypermethylation was evident in all chromosomes (range: 75.12% - 83.46%). The total number of unique genes (accession numbers) that were differentially methylated (>5 DMPs/gene) in HPA compared to BASAL was 2369.

In conclusion, we show that lipotoxicity only during IVM can induce long-term effects on day 14 EXT DNA methylation patterns despite the epigenetic reprogramming during early embryo development and post-hatching growth in a healthy uterine environment. In general, the majority of the DMPs in the HPA group were hypermethylated compared to BASAL. Since DNA hypermethylation is associated with silencing of gene transcription, this might have functional implications. The previous RNA-sequencing analysis of these samples revealed transcriptomic alterations. We are currently integrating the epigenetic and transcriptomic data and annotating the affected genes.

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Follicular fluid extracellular vesicles supplementation during in vitro maturation of bovine cumulus-oocyte complexes: preliminary results for effects on oocyte competence and cumulus cells gene expression.

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In vivo oocytes develop in follicular fluid (FF) containing extracellular vesices (fEVs), which carry bioactive molecules that affect cellular function. The present study aimed to examine effects of fEV during IVM on oocyte (OO) maturation and subsequent embryo development, and their mRNA contents related with lipid metabolism and epigenetic regulation. Expression of the same transcripts in cumulus cells (CC) was also determined. Bovine cumulus oocyte complexes (COC) and FF were separately collected by aspiration of 3-6mm follicles from abattoir ovaries. fEV were isolated by size exclusion chromatography from 1ml FF (SEC, qEV1 columns 35 nm Gen 2, Izon) and ultracentrifugation (100,000 x g for 70 min at 4°C, Beckman Coulter), and transcripts within fEVs analyzed in part of the samples by RT-qPCR. The other part of fEV samples was used for IVM medium supplementation. COC were matured in medium (TCM199 with 0.4 mM glutamine, 0.2 mM pyruvate, 50 mg/mL gentamicin, 20 ng/ml EGF) supplemented with 10% fetal calf serum (FCS, control), 10% FCS depleted of its own EV (dFCS) or 10% dFCS+fEV (fEV), at 38.5ºC and 5% CO_2 in air, for 24h. After IVM, CC were removed from a subset of the COC, and denuded OO evaluated for first polar body extrusion (1st PBE) and mitochondrial activity (FI=fluorescence intensity, MitoTracker Orange CMTMRos, Thermo Fischer Scientific), while CC were assessed for lipid metabolism and epigenetic regulation transcripts by RT-PCR. The remaining COC were submitted to IVF and assessed for day 7 (D7) blastocyst rates and mitochondrial activity in embryos. The Kruskal-Wallis test was used to analyze the rates of maturation, blastocyst, and ΔCt values (at least 3 replicates) and Mann-Whitney test for mitochondrial activity; significance was 5%. Maturation rates (1st PBE) were not affected by treatments (75.9 to 78.9 %, n=220-222 per group). Mitochondrial activity in oocytes was higher in fEV [28814 (n=22) *vs* 21566 (n=23), and 23397 FI (n=23), for FCS and dFCS, $P<0.05$]. D7 blastocyst rates were unaffected [~ 40%, n= 347-364 per group, $P>0.05$), as well as their mitochondrial activity (11232 to 13610, n=12-16 per group, $P>0.05$). Transcripts for DNMT1, DNMT3A, MAT2A, SHMT2 (epigenetic regulators) and PLIN2, LDLR, CD36 and FABP5 (lipid metabolism) were present in fEV and were also expressed in CC. Transcripts abundance in CC was not affected by treatments ($P>0.05$). In conclusion, maturation and embryo development were not affected by fEV, but mitochondrial activity in oocytes treated with fEVs during IVM was increased, suggesting they may transfer molecules affecting mitochondrial function. Although lipid metabolism and epigenetic regulators were detected in fEV, their abundance was not changed in treated CC, indicating lack of transfer of studied mRNA from fEV to cells. However, as fEV carry other molecules, effects on other functions cannot be ruled out. As these are preliminary results, analysis of lipid contents and of more replicates for mitochondrial activity are ongoing. Financial support: FAPESP (SPEC Grant # 2021/09886-8; AR Grant #2021/06760-3); FS - DS Scholarship (Capes 88887.694635/2022-00); AB - PD Scholarship (FAPESP 2023/01524-5); JRQO - ScI Scholarship (FAPESP 2023/12424-1); LCZJ DS Scholarship (Capes 88887.836321/2023-00); LCM - ScI Scholarship (PUB-USP 2023/83-1).

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A single-cell transcriptomic atlas of sheep gastrulation and conceptus elongation

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Gastrulation involves the differentiation of three germ layers (ectoderm, mesoderm and endoderm) from the epiblast. In ungulates, gastrulation occurs in the embryonic disc (ED) during conceptus elongation, upon the formation of the primitive streak and concomitant to the massive proliferation of the extraembryonic membranes. Our aim was to analyse the development of embryonic and extraembryonic lineages during gastrulation and conceptus elongation by single-cell RNA sequencing in the sheep. Using the Evercode™ WT Mini kit (Parse Biosciences), we obtained scRNAseq profiles from 80 embryos collected *in vivo* from superovulated ewes at embryonic days (E) 11 (spherical; n = 15), E11.5 (spherical and ovoid; n = 25), E12.5 (tubular and filamentous; n = 25 isolated EDs + extraembryonic membranes [EEMs]) and E13.5 (filamentous; n = 15 isolated EDs + EEMs), and from 216 day (D) 14 *in vitro* embryos (from 3 IVF replicates), cultured in N2B27 medium supplemented with activin A and ROCK inhibitor from D6/7 (Ramos-Ibeas *et al.*, 2022; Development). Transcriptomes of 21,551 cells passed quality controls, with a median of 4,584 genes detected per cell. Unbiased cluster of all *in vivo* embryos (UMAP) and known cell-type markers allowed us to identify 15 clusters, as well as novel sheep cell-type marker genes. Trophectoderm (TE) cells expressed *DAB2*, *GATA2*, *CDX2*, *TFAP2A* and *TFAP2C*, among other specific markers. A TE sub-population with high interferon-tau (*TP-1P8*), *PAG11, FURIN* and *PTGS2*, and low *CDX2* and *PTGES* expression, proliferated from E11.5 and showed enriched GO terms in lipid metabolism and transport, and PPAR signalling pathway. All hypoblast cells expressed *GATA4*, *FN1*, *HNF1B* and *APOA1*, while visceral and parietal hypoblast specifically expressed *PRDM1* and *TDGF1*, respectively. Anterior visceral hypoblast (AVH), expressing *CER1*, *NODAL*, *EOMES* and *OTX2*, was identified in E11 and E11.5 embryos. Epiblast cells expressed *SOX2*, *SALL2*, *DNMT3B, GPC4* and *PHC1*, among other markers. *TBXT*, *WNT3* and *CDX1*-positive primitive streak cells emerged separating from the epiblast cluster at E11.5, while anterior primitive streak cells, identified by *GSC*, *CHRD, NODAL* and *CER1*, appeared from E12.5. Mesoderm cells, expressing *BMP4*, *HAND1, COL3A1* and *SNAI2*, were detected from E11.5, and definitive endoderm (DE) cells, expressing *FOXA2*, *PRDM1*, *SOX17, BMP7* and *POU5F1*, appeared at E12.5. Both populations proliferated rapidly from E12.5 to E13.5. Finally, primordial germ cells (PGCs), expressing *POU5F1*, *NANOG*, *KIT*, *SOX17* and *TFAP2C*, emerged at E12.5, and *GABRP*, *TFAP2A*, *GRHL2* and GATA3-positive amnion cells were detected at E13.5. UMAP of D14 *in vitro* embryos separated clear trophectoderm, hypoblast and epiblast clusters, as well as few AVH and mesoderm cells, being more similar to E11.5 than to more advanced *in vivo* embryos. This single-cell molecular map of lineages differentiation during sheep gastrulation and conceptus elongation uncovers the timing of formation and cellular origin of critical structures within the sheep conceptus and provides clues to achieve gastrulation and conceptus elongation *in vitro*.

Work supported by PID2021-122153NA-I00 and StG 757886-ELONGAN.

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3D-DNA-FISH localization of DUXC genes during bovine early development: a pioneer factor silenced by heterochromatin vicinity after EGA?

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In bovine embryos, the transition from maternal to zygotic transcription occurs during the 8-cell stage. This activation of the embryonic genome (EGA) is divided into two steps: a low-level wave of transcription (minor EGA) occurring just after fertilization followed by a major burst of transcription (major EGA). This initial onset of transcription activation is regulated by pioneer transcription factors (TFs) like *DUX4* (Double homeobox 4 in humans) which activate the transcription of a cascade of other TFs and chromatin modifiers, increasing the chromatin accessibility to transcription machinery, like RNA Pol II (*Vuoristo et al., iScience 25, 104137, 2022*). In post-EGA mouse embryos, the murine ortholog of *DUX4*, *Dux*, localizes close to heterochromatin (either near the nucleolus or the nuclear envelope), which is linked to repression of its transcription, and in 2-cell-like embryonic stem cells (ESCs) *Dux* is sequestered at the nucleolus periphery to maintain its repression (*Xie et al, Genes&Dev, 36:331-347, 2022*). In bovine, our preliminary results demonstrated that the bovine ortholog, *DUXC*, is expressed as early as the 1-cell stage and that *DUXC* knock-down (KD) with RNA interference induces developmental arrest at the 8-cell stage. Our study aims to determine the localization of the *DUXC* locus in bovine embryos before and after EGA compared to heterochromatin, the nucleolus, and the nuclear envelope. Using DNA-FISH, we analyzed the shape (assessment of volume and sphericity) and position of *DUXC* DNA-FISH signal (with a mixture of 13 fluorescent probes covering the *DUXC* locus) in nuclei (n >30) from the 2-cell to the morula stages. We find that before EGA, *DUXC* sequences are less compact and at a higher distance from DAPI-dense regions. After EGA (16-cell and morula stages) *DUXC* loci are located at the periphery of the nucleus and *DUXC* signal is associated with the telomeres. In agreement with the results in murine ESCs, we demonstrate that *DUXC* in bovine early embryos is located at the nuclear periphery after EGA, which is concomitant with its repression. To delve further into the putative involvement of *DUXC* in the activation of repeated element transcription and chromatin remodeling, we will compare RNA-sequencing data between normal and *DUXC* KD embryos during early development (from the 2-cell to 8-cell stages).

Acknowledgements: ANR-10-LABX-73-01, ANR-23-CE20-0015-01

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Haptoglobin supplementation to the in vitro culture improves embryo development and quality in bovine

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Haptoglobin (HG) is a protein synthesized in the liver, which binds free hemoglobin to inhibit oxidative activity. Although traditionally associated with inflammation due to its presence during such processes, it is also a regular component of healthy mammalian reproductive tissues and fluids. Its occurrence in reproductive organs appears to be specific to certain cell types or stages of the reproductive cycle, suggesting a potential role for HG in mammalian reproductive events. Notably, in pigs, HG has been used to promote *in vitro* early embryo development (García-Vázquez et al., Scie. Rep., 2021). This study aimed to determine the influence of HG on the developmental kinetics and quality of *in vitro*-produced bovine embryos. Presumptive zygotes were cultured in 25 µL of in vitro culture medium alone (Stroebech Media®) (Control: n=516) or supplemented with 5 µg of HG (H5: n=519) during the entire culture period (Day 1-Day 7/D1-D7) or during two developmental periods: D1-D4: from presumptive zygotes to 16-cells stage (depicting HG effect in the oviduct; $H5_{OV}$: n=446); or D4-D7: from 16-cells to blastocyst (BD7) stage (depicting HG effect in the uterus; $H5_{UT}$: n=438). Embryo development was evaluated at 96 hpi and at D7-8, while the quality of BD7 was assessed by i) mitochondrial activity with MitoTracker DeepRed, ii) lipid content by Bodipy 493/503 and iii) differential staining of inner cell mass (ICM) and trophectoderm (TE) by anti-CDX2 antibody (Biogenix, Fremont) and Hoechst (n~20 per group). Data obtained from 5 replicates were analysed using One-way ANOVA. No differences were observed in the proportion of embryos that reached the 16-cells stage at 96 hpi, which ranged from 70.6±0.4 to 71.7±0.5%. Consequently, a similar proportion of embryos with a delayed development (< 16 cells), which ranged from 15.7±0.6 to 17.8±0.6 %, was observed. Blastocyst yield at D7 - 8 was significantly higher ($P < 0.001$) for H5 (28.3±0.4 - 32.8±0.6%, respectively) and $H5_{UT}$ (27.4±0.6 - 32.2±0.9%), compared to Control (23.0±0.5 - 27.9±0.6%) and $H5_{OV}$ (23.7±0.6 - 28.3±0.7%). The mitochondrial activity was lower ($P<0.001$) in BD7 from H5 and $H5_{UT}$ groups, compared with Control and $H5_{OV}$ groups. When analyzing the lipid content, we observed that the total area of lipid droplets in BD7 resulting from H5 during the entire culture period (D1-D7) or from D1-D4 ($H5_{OV}$) was significantly reduced ($P<0.001$) compared with the control and $H5_{UT}$ groups. The total cells, TE, and ICM did not exhibit differences among BD7 produced in all groups. In conclusion, this study indicates that blastocyst development was significantly improved when HG was present with remarkable effects on their quality in terms of mitochondrial activity, and lipid content. These findings underscore the important role of HG in modulating critical facets of bovine embryonic development and quality.

KCB by Maria Zambrano; YNC by Margarita Salas; Spanish Ministry of Science and Innovation: PID2019-111641RB-I00 and PID2021-123091NBC21: MCIN/AEI /10.13039/501100011033/ and FEDER Una manera de hacer Europa.

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Interaction of culture media and oocyte quality affects in vitro embryo production of Egyptian local goats

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The development of embryos produced in vitro is influenced by culture conditions and oocyte quality. Therefore, this research aimed to study the effect of oocyte quality and types of culture media on the embryonic development of goat. According to Wieczorek *et al.,* 2020 the first experiment was done using 920 good COCs retrieved (6 replicates) from slaughterhouse ovaries (a local abattoir in Cairo, Egypt, October, 2023). In vitro maturation (IVM) was done using the standard procedure of goat IVM (Wieczorek et al., 2020; AbdElkhalek et al., 2024). In vitro fertilization (IVF) of matured oocytes was conducted using epididymal spermatozoa. The presumptive zygotes were cultured in three different media known as basic medium (G1), SOF medium (G2), and GT-L™ medium (G3). In addition, the embryonic development was monitored for seven days. In the second experiment, 730 COCs (6 replicates) were divided morphologically into two groups: (G1) good-quality COCs and (G2) low-quality COCs. The COCs were in vitro mature and fertilized, as done in the first experiment. The presumptive zygotes were vitro cultured in the best medium selected from the first experiment. Cumulus expansion score and nuclear maturation rate (GV; GVBD; MI and MII) were evaluated using Hoechst staining (Ghanem *et al*. 2021). In the two experiments, embryonic development was assessed in terms of cleavage, morula and blastocyst formation rates (No. of blastocysts embryo No. of culture oocytes). The quality of produced embryos was evaluated by estimating the total cell number using Hoechst staining. Moreover, samples (embryo and oocytes) were used for measuring cytoplasmic mitochondrial activity and lipid content by applying staining of Mito-Tracker green and Nile red stains, respectively. Data of this study indicated that the embryonic development rate (cleavage and blastocyst rates) was higher in the group cultured with G-TL™ (50.56 and 42.83% respectively) than in basic (5.96 and 2.64% respectively) and SOF media (23.1 and 12.25% respectively). G1 had the lowest ($p \leq 0.01$) mitochondrial fluorescent intensity, lipid fluorescent intensity, and total number of embryonic cells, whereas G3 had the highest intensities (59.25, 18.73, and 32.63 vs. 106.1, 45.33, and 62.86, A.U respectively).In contrast, the G2 group's results for the same parameters were 83.69, 28.93, and 43.13 A.U, respectively. In the second experiment the nuclear maturation rate (extrusion of first polar body (non-invasive assessment; morphology) and Metaphase II % (invasive assessment by Hoechst staining) was significantly increased ($P \leq 0.05$) in good (29.79 and 46.67%) compared to bad COCs (9.33 and 13.33%). A higher proportion ($P \leq 0.01$) of oocytes with diffuse mitochondria distribution (increased level of mitochondrial aggregation around the nucleus (central) indicates oocyte maturation) was observed in good (66.67 %) than low-quality COCs (6.64%). In conclusion, selecting good quality oocytes and culturing presumptive zygotes in G-TL TM medium improved goat in vitro embryo production (IVP).

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NODAL is not required for pre-gastrulation embryo development in sheep

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NODAL signalling is essential for mammalian embryo patterning before and during gastrulation. In mice, *Nodal* is initially expressed at the egg cylinder stage and its ablation impairs the formation of anterior visceral endoderm and mesoderm. In ungulates, *NODAL* expression starts earlier, in the inner cell mass at the blastocyst stage, and is later expressed by the epiblast and visceral hypoblast in the embryonic disc (ED), but its role is unclear due to the lack of a knock-out (KO) model. The objective of this study was to analyse the role of *NODAL* in ovine embryo development through CRISPR-mediated genome editing. *NODAL* KO embryos were generated by introducing a stop codon at the first exon using cytosine base editor (BE3). *In vitro* matured oocytes were microinjected with BE3-encoding mRNA and sgRNA (BE+G, containing KO embryos), or with BE3-encoding mRNA alone as microinjection control (BE, only formed by wild-type -WT- embryos). Microinjected oocytes were fertilized and embryo development was assessed at Day (D) 8 (blastocyst stage, cultured in Stroebech IVC medium) or at D12 (right before gastrulation, following an extended culture in N2B27 medium from D6/7). D8 and D12 embryos were imaged, fixed and immunostained to detect SOX2 (epiblast marker), SOX17 (hypoblast marker), and CDX2 (trophectoderm marker). Embryo genotyping in BE+G group was performed by Sanger sequencing. Embryos were considered as KO when all alleles showed a stop codon in the target region, heterozygous (Hz) when a non-edited allele was present, or WT if all alleles were unmodified. Blastocyst rate was similar between the group containing *NODAL* KO embryos (BE+G) and the control group (BE) (42.2±3.7 vs. 47.2±3.3 %; mean ± s.e.m; 4 replicates; t-test, $p>0.05$). In BE+G group, 12/25 (48 %) D8 blastocysts were KO. No significant differences were detected in the number of SOX2+ (16.9±4 vs. 12.2±4.6 vs. 9.8±2), SOX17+ (29.2±5.9 vs. 25.7±6.7 vs. 27.3±9.9), CDX2+ (78.2±13.7 vs. 114.2±17.5 vs. 64.3±22.7) or total cells (133.9±15 vs. 159.6±22.8 vs. 153±24.7) between KO, Hz and WT D8 blastocysts (mean±s.e.m; one-way ANOVA, $p>0.05$). Embryo survival from D6/7 to D12 *in vitro* was similar between BE+G and BE groups (82.5±3.8 vs. 93.8±3.1 %; mean±s.e.m; 3 replicates; t-test, $p>0.05$). In BE+G group, 46/85 (54.1 %) D12 embryos were KO. No differences were detected in embryo area (393.1±35.4 vs. 335±40 vs. 386.6±33 μm^2; mean±s.e.m; one-way ANOVA, $p>0.05$), in the percentage of embryo surface covered by hypoblast cells (63.6±4.5 vs. 51±5.5 vs. 64.5±4.3 %; one-way ANOVA, $p>0.05$), in the number of embryos showing surviving epiblast cells (26/41 [63.4 %] vs. 28/33 [84.8 %] vs. 34/55 [61.8 %]; Chi-square test, $p>0.05$), in the number of SOX2+ epiblast cells (21.7±4.9 vs. 27.8±4.2 vs. 20.6±4.6; one-way ANOVA, $p>0.05$), or in the number of embryos developing an ED (6/26 [23 %] vs. 11/28 [39.3 %] vs. 8/34 [23.6 %]) between KO, HZ and WT embryos at D12. In conclusion, *NODAL* is dispensable for ovine embryo development up to pre-gastrulating stages.

Work supported by PID2021-122153NA-I00 and StG 757886-ELONGAN.

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Resilience of preimplantation bovine embryos to the availability of energy substrates

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Exposure to metabolic stress during fetal life increases the susceptibility to metabolic diseases in adulthood. This notion is supported by epidemiological and experimental evidence that led to the theory of Developmental Origins of Health and Disease. The adaptive mechanisms the embryo/fetus puts in place to cope with intra-uterine stressors are defined developmental plasticity and entail the capacity of one genotype to generate several phenotypes in response to different environments. To capture the main molecular events of developmental plasticity, we monitored gene expression of bovine blastocysts exposed in vitro to a mild metabolic challenge. With preliminary experiments we standardized the experimental model to remove serum and fertilize by X-sorted semen, to control sources of variability. Then metabolic challenges were given by varying the content in energetic substrates (pyruvate, lactate, glucose, citrate, amino acids) of the culture medium. Three energetic levels, containing 0.5, 1.0, and 1.5-fold increase in energetic substrates, were selected based on the absence of apparent changes in preimplantation embryo development, evaluated by blastocyst rate, distribution of blastocyst stage (early, expanded, hatch-ed/ing), cell number, and pattern of cell lineage specification (N>3; n>31). Genome-wide analysis conducted on 3 independent replicates of 5 expanded blastocysts per treatment revealed minimal differences in gene expression, likely exposing key regulatory genes whose differential expression allowed the adaptation to the changing metabolic environment. Two distinct expression patterns were observed for these genes: progressive upregulation and progressive downregulation along with the increasing energetic availability. The lack of substantial differences seems in line with the general observation that offspring born after a mild intra-uterine exposure to metabolic stress have normal physiological and biochemical parameters until later in life, while a generalized disruption of gene expression would probably impact embryo/fetal/early post-natal life rather than induce a late onset of the disease. Nevertheless, if metabolic stress experienced during preimplantation development were to commit a late phenotype, some kind of mark shall be established at this stage. A possible answer to this question came from the analysis of transcript isoforms. Using a specific bioinformatic pipeline, the presence of two or more transcript isoforms of genes related to epigenetic changes and nuclear reprogramming were detected, indicating that, even in absence of obvious changes in gene expression, the metabolic challenge induced biological effects that can be epigenetically encoded in the embryo. As a proof of concept, acetylation of histone proteins increased when the energetic substrates were higher. These findings shed light on the mechanisms at the onset of developmental plasticity, whereby the activation/repression of few key genes and usage of transcript isoforms confer resilience to metabolic stressors and provide a direct link between changes in the availability of energetic substrates and epigenetic reprogramming.

Funded by: MUR PRIN2017, No. 20172N2WL3_002; Piano di Sostegno alla Ricerca UNIMI: Linea 2 – Azione A

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High negative energy balance and post-calving period alters the molecular profile of epithelial uterine cells in dairy cows

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Negative energy balance (NEB) during the post-calving period can have a significant economic impact due an overall reduced fertility in dairy cows. This reduction is due, in part, to the impact on uterine biology. The aim of this study was to evaluate if NEB intensity modifies the molecular profile of epithelial uterine cells (EUC) when the uterus is recovering from calving and during the predicted period for starting artificial insemination (AI) in dairy cows, at 30 and 60 days post-calving (DPC), respectively. To characterize NEB intensity, serum samples collected from individual dairy cows at 14 DPC were subjected to metabolite analysis (Low NEB: non-esterified fatty acids, NEFA, 0.3 to 0.8 mmol/L and beta-hydroxybutyrate, BHB, 0.55 to 1.1 mmol/L; High NEB: NEFA ≥ 0.9 mmol/L and BHB ≥ 1.2 mmol/L). At 30 and 60 DPC, the estrous cycles of healthy cows (without clinical or subclinical diseases; 30 DPC n=7, 3 Low and 4 High NEB; 60 DPC n= 6, 3 Low and 3 High NEB) were synchronized using a progesterone-releasing intravaginal device (IVD, 1.9g), estradiol benzoate, and PGF2α analogue. The IVD was removed after 7 days, and estradiol cypionate and PGF2α analogue were administered intramuscularly. On day 9, cows received GnRH analogue and were fixed-time inseminated without oestrous detection. On Day 5 after AI uterine fluid was collected by nonsurgical lavage with 50 ml PBS and EUC were isolated by centrifugation. Total RNA was extracted from EUC and subjected to sequencing analysis. The Nextera XT DNA Library Prep (Illumina) was used to prepare the libraries and sequencing were performed on the NextSeq 2000 (Illumina, USA). Differential gene expression analysis was performed using DESEQ2 R package, considering adjusted P values < 0.10 and an absolute log2 fold change > 0.5. At 30 DPC, we identified 7 genes exclusive to Low NEB, 4 genes exclusive to High NEB and 73 DEGs between the groups, with 24 more expressed in Low NEB and 49 more expressed in High NEB group. At 60 DPC, we found 3 genes exclusive to Low NEB, 8 exclusive to High NEB and 24 DEGs, with 15 more expressed in Low NEB and 9 more expressed in High NEB. Functional enrichment analysis of genes that were exclusive or upregulated in the EUC demonstrated that biological processes are modulated differently in these cells. Genes related to cell proliferation and differentiation were upregulated in EUC from Low NEB cows, while genes related to inflammation and metabolism were increased in EUC from High NEB cows at 30 DPC. At 60 DPC, genes related to immune response, lipid accumulation and conceptus development were upregulated in EUC cells from dairy cows in Low NEB. In contrast, in the High NEB group, genes related to metabolism and cell proliferation were more expressed, suggesting the uterine cells are recovering from this metabolic insult and still present an environment compromised to supporting embryo growth and development at 60 DPC. In conclusion, using RNA-seq approach and dairy cows with different metabolic status during two important periods after calving, this work demonstrates that NEB intensity and post-calving period alter gene expression in EUC. **Support:** FAPESP grants 2020/13075-2, 2023/15072-9 and 2021/06645-0.

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Evidence that undernutrition in early gestation reduces maternal leptin and impairs AMH in juvenile offspring in dairy cattle

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In beef heifers, undernutrition up to 110 days of gestation (DG) increased maternal testosterone (T) peripheral concentration and diminished the number of healthy growing ovarian follicles in female offspring, as assessed by reduced total number of antral follicles and peripheral concentration of anti-Mullerian hormone (AMH). Leptin (L) production by adipose tissue decreases during weight loss and L can alter androgen production. We hypothesized that maternal nutritional restriction from shortly before conception to two different stages of early gestation would cause 1) an increase in maternal L and T peripheral concentrations and 2) a decrease in AMH circulating concentration in dairy female calves.

Holstein-Friesian heifers (n=42) homogenous for age (14-17mo) and weight (366±41kg) were randomly assigned to three experimental groups and, starting 10d before artificial insemination (AI), were individually fed at: (i) 0.6 of their maintenance energy requirements (M) up to 80DG (Nutrient Restricted, NR80; n=16) or (ii) 120DG (NR120, n=16), and (iii) 1.8M until 120DG (Control, C; n=10). Estrous cycles were synchronized, and heifers were inseminated with sex-sorted semen from a single sire. Pregnancy was diagnosed and confirmed via ultrasound 28 and 55DG, respectively. After the end of the differential diet, heifers were group fed ad libitum until calving. Peripheral maternal L and T concentrations were measured in heifers pregnant with a single female calf (NR80, n=8; NR120, n=9; C, n=5). Twenty-two single female calves were born (NR80, n=8; NR120, n=9; C, n=5) and peripheral AMH concentration were measured regularly from birth to 120 days of age (d). Data normality was tested with the Shapiro-Wilk test. Hormonal concentrations were analyzed as repeated measures within treatments using the multivariable linear regression model.

Maternal L concentration was influenced by diet ($p<0.001$) and DG ($p<0.05$), but their interaction tended to be significant ($p=0.075$). Leptin concentration was similar among groups before the start of the differential diet, it was lower in both NR80 and NR120 vs C from 30 to 120DG ($p<0.05$) and was similar among groups from 150DG to calving. Peripheral T concentration in pregnant dams increased as gestation progressed ($p<0.001$) but was not affected by diet. Circulating AMH concentration in female calves was influenced by maternal diet ($p<0.001$) and decreased as calves grew older ($p<0.001$) but was not conditioned by the interaction of maternal diet and age. NR80 and NR120 calves had lower AMH than C from birth to 60d, whereas no difference was detected among groups when calves were 90 and 120d.

In conclusion, maternal undernutrition from preconception to either 80 or 120DG reduced peripheral AMH in female progeny in dairy calves, indicating a potential impairment of ovarian reserve. In the dams, nutritional restriction reduced peripheral L, yet did not influence T concentrations.

Acknowledgements: *DESTINE*, MIUR-PRIN 2017

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Nanoplastic exposure during bovine oocyte maturation affects mitochondrial and developmental process pathways and delays embryo development

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The ubiquity of micro- and nanoplastics (MNPs) has recently gained increasing attention due to their potential effects on health and reproduction. MNPs have been observed to pass biological barriers, including the reproductive tract in rodents, and are able to enter the ovary (Liu, Z. J Hazard Mater. 2022;424(Pt C):127629.) To unravel the effects of nanoplastics (NP; < 1 µm) on oocyte developmental competence, we exposed maturing bovine cumulus-oocyte complexes (COCs) to NPs. This research is also of interest for human reproduction, as bovine early development until implantation is largely comparable to human (Menezo, YJ. Reprod Biomed Online. 2002;4(2):170-175.).

Previously, we discovered that during the in vitro maturation (IVM), 50 nm polystyrene (PS) NPs can enter the oocytes and hamper the oocyte nuclear maturation rate at a concentration of 3 µg/mL. To investigate the underlying mechanisms, RNA-seq was performed on RNA isolated from bovine COCs after the 23h IVM in the medium (NaHCO3-buffered M199 supplemented, with 100 IU/ml Penicillin-streptomycin, 0.05 IU/mL FSH, 0.1 µM cysteamine, and 10 ng/mL EGF) with or without 50 nm PS-NPs (Polysciences Europe GmbH, Hirschberg an der Bergstrasse) at 3 µg/mL at 39°C and 5% CO_2 in air, with the coverage of 20 million reads and 6 gigabases per sample, followed by Gene Set Enrichment Analysis (GSEA) using Kyoto Encyclopedia of Genes and Genomes pathway and gene ontology database. Subsequently, to determine whether the formerly demonstrated decreased oocyte nuclear maturation in response to NP exposure has a sustained effect on embryo development, the day 7 and 8 blastocyst rates were scored after standard IVF and embryo culture in synthetic oviductal fluid with 0.1% BSA (w/v) at 39°C with 7% O_2 5% CO_2 (298 COCs per treatment in 4 replicates). Generalized Linear Model binomial link-logit followed by Bonferroni adjustment for multiple comparisons was used for statistical analysis. A p-value < 0.05 was considered statistically significant.

Following GSEA, pathways related to mitochondrial functions such as oxidative phosphorylation, and mitochondrion organization were downregulated in 50 nm NP-exposed COCs during IVM. Additionally, the analysis indicated a negative regulation of developmental process. Notably, on day 7 of IVP, a significant decrease (p=0.017) was observed in blastocyst rate in the exposure group (36.2±5.8%) compared to the control group (29.2±4.4%), while the cleavage rates on day 5 and blastocyst rates on day 8 were not significantly different between the two groups.

In summary, exposure to NPs during IVM might affect mitochondrial functions, and appears to result in a delayed early embryo development. Further investigations are ongoing to unravel the role of mitochondrial function in the observed toxicity of NPs.

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Novel insights into the bull effect on in vitro embryo production: a proteomics approach

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Variations in in vitro embryo production (IVP) depend not only on the quality of the oocytes but also on the sperm used for fertilization. Penetration of multiple spermatozoa into the oocyte, or polyspermy, might be one of the factors contributing to the variation of in vitro fertilization efficiency. This study examines the role of the bull in the process of IVP, from fertilization to embryo development and quality. To do so, bovine cumulus-oocyte complexes were collected from slaughterhouse-derived ovaries, and routine IVP was performed (Wydooghe et al., 2014, Reprod. Fertil. Dev.; 26) with frozen-thawed semen from four bulls. Polyspermy rates from each bull were examined in 13 replicates as previously described (Fernández-Montoro et al. 2024, Reprod. biol. 24, 2). On day 8, embryos at similar stages were collected individually to perform proteomics analyses (n = 16 in high; 23 in low). Generalized mixed-effects models were used to evaluate fertilization and developmental parameters, while MSqRob2 was used for differential expression analysis (FDR of 0.05 and 1.3 $\log_2$ by Benjamini-Hochberg technique). One bull averaged 57% polyspermy rates (high), whereas the other three averaged 20% (low). Although the cleavage and day 7 blastocyst rates were higher in the high polyspermy bull (87 ± 2.5%, $p < 0.01$ and 37.7 ± 3.3%, $p = 0.01$, respectively) compared to the low polyspermy group (76.4 ± 2.9 and 27.4 ± 2.3%), the day 8 blastocyst rate did not show differences among the bull with high polyspermy (41.7 ± 3.4%, $p = 0.26$) and the low polyspermy bulls (37.9 ± 2.5%). Also, no significant differences ($p > 0.05$) were found between bulls for the rate of early, normal, expanded, hatching, or hatched day 8 embryos. Proteome analysis of the blastocysts using label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) allowed the quantification of 6878 proteins. Quantitative analysis showed a total of 942 differentially expressed proteins between the high polyspermy bull and the low polyspermy group, from which 84 were up-regulated and 858 down-regulated. Functional analysis indicated an enrichment of pathways related to oxidative phosphorylation, chromatin organization, transcription, translation, and post-translational modification in low polyspermy bulls. No differences were observed in the proteome profile of blastocyst among the low polyspermy bulls. Our results suggest that polyspermic fertilization can be the cause of embryonic arrest in the bull with high polyspermy rates, as it had the highest cleavage but exhibited similar blastocyst rates to the other bulls. Despite still representing a competitive blastocyst rate, embryos from this bull showed a lower abundance of proteins involved in important metabolic processes, suggesting imbalances that could reduce their developmental potential. In conclusion, our findings demonstrate that a bull with high polyspermy rates can produce embryos at satisfactory rates albeit with diminished quality, potentially affecting further development. Although individual specificity might be present, it is imperative to evaluate polyspermy rates when selecting bulls to be introduced into IVP programs.

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Periparturient oleic acid supplementation increases oocyte yield in dairy cows

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The periparturient negative energy balance (NEB) in dairy cows, results in bodyfat mobilization and high levels of non-esterified fatty acid (NEFA), which has been related to the observed reduced fertility performance. In particular, saturated NEFAs are associated with lipotoxic effects on cells, including the oocyte. These effects are counteracted by high levels of oleic acid (C18:1) in follicular fluid and cumulus cells and protect the oocyte against saturated NEFA (Aardema et al., Biol of Reprod 96:982-992,2017). Oocytes that are presumed to be fertilized after a voluntary waiting period of on average 60-100 days postpartum, start their development during the NEB and may be hampered in quality. Around the periparturient period, follicles are exposed to high levels of saturated palmitic (C16:0) and stearic acid (C18:0) via blood (Aardema et al., Biol of Reprod 88:164, 2013). The current study investigated whether periparturient C18:1 fat supplementation counteracts the potential negative effects of NEB on oocytes during the over 120-day follicular growth phase, based on the Britt hypothesis (Britt et al., Bov Proc 24:39-43, 1992).

Pregnant HF heifers (age two years), were pseudo-at-random divided based on BCS and received a fat supplement rich in either C16:0 (78.8%-PA, n=5), which is the standard supplement given in The Netherlands, or rumen-protected C18:1-rich supplement (62,9%-OA, n=6) from 4 weeks before until 4 weeks after calving. Serum samples of -4 weeks (t=0), -2, +2, and +6 weeks post-calving (pc), were analyzed for fatty acids in triacylglyceride (TAG) by HPLC-Mass Spectrometry. At days 50-60, 80-90, and 120-130 pc COCs were two times, with a 5-day interval, collected from 3-12 mm antral follicles by transvaginal ovum-pick-up (OPU). Collected COCs were *in vitro* matured, followed by IVF and embryo culture until day 8, according to our standard protocol. Number of oocytes was analyzed with a Poisson distribution and developmental competence with a logistic regression model, Akaike's information criterion was used for model reduction, to calculate 95% profile (log) likelihood confidence intervals. The study was approved by the ethical committee.

The fatty acid composition in blood of control heifers was dominated by C16:0 at -2 (nearly 60% of total) and + 2 weeks (45% of total), around 30% higher than in the OA group. In the OA group, the C18:1 in blood (25% of total), was 10% higher than in the PA group. At +6 weeks fatty acid compositions were comparable in the groups. In the OA group, a higher number of follicles (21.9±7.9 vs 14.5±6.0) and COCs (13.1±6.8 vs 8.2±4.0) was recorded compared to the PA group. In the OA group, the number of the collected COCs was overall 1.6 times higher in comparison to the PA group. Developmental competence of oocytes was not different between the groups.

Fat supplementation resulted in a change in the fatty acid composition in blood, the OA condition demonstrated a profile richer in C18:1 and lower in C16:0 in comparison to the PA group. Interestingly, OA supplementation resulted in a significantly higher amount of collected oocytes at OPU. These data suggest that periparturient OA supplementation may increase oocyte yield during periparturient NEB.

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Isolation and characterization of extracellular vesicles from sheep reproductive fluids

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Extracellular vesicles (EVs) are nanoparticles involved in cellular communication releasing miRNAs, proteins and lipids into target cells modulating cellular response, and are present in biological fluids. Recent works have noticed the role of the EVs from female reproductive fluids in guiding sperm towards the fertilization site and promoting capacitation signaling pathways. The aim of this study was the isolation and characterization of ovine EVs from follicular, oviductal and uterine fluids, comparing two isolation methods (ultracentrifugation (UC) and size exclusion chromatography (SEC)) and fractions (pellet and supernatants). For this end, reproductive fluids from six sheep were pooled after flushing extraction, with each pool comprising samples from two individuals. Concentration, population, and size were evaluated by nanoparticle tracking analysis, and the detection of EVs markers as the CD9, CD81, and CD63 tetraspanins by flow cytometry allowed for their identification. Commercial exosomes expressing green fluorescent protein and CFSE-labelling were used for intact EVs gating in flow cytometry evaluations. Differences between samples were evaluated by one-way ANOVA analysis and Bonferroni test was used for multiple comparations. Our results showed no significant differences ($p > 0.05$) in EVs concentration between fluids. However, the percentage of EVs positive for CD63 was higher ($p < 0.05$) in follicular fluid compared to oviductal and uterine fluids (67.40 ± 1.39 *vs* 62.11 ± 1.33 and 61.58 ± 1.33, respectively). Significative differences ($p < 0.05$) were found in the percentage of CD9-positive EVs present in follicular fluid compared to that in uterine fluid (26.53 ± 1.05 *vs* 22.46 ± 1.00), while no differences ($p > 0.05$) between fluids for CD81 were found. Regarding the isolation method, the EVs concentration obtained was higher ($p < 0.05$) by UC than by SEC ($7.27x10^8 \pm 6.21x10^7$ *vs* $5.09x10^8 \pm 6.21x10^7$ EV/mL). Moreover, the percentage of CD63-positive EVs obtained by UC was higher ($p < 0.05$) than that by SEC (65.38 ± 1.12 *vs* 62.01 ± 1.08), with no differences ($p > 0.05$) for CD9 and CD81 positive populations. Regarding fractions, there were no differences ($p > 0.05$) in concentration, but CD63 positivity was significantly higher ($p < 0.05$) in the pellet than in the supernatant (66.60 ± 1.22 *vs* 60.79 ± 1.02). No differences ($p > 0.05$) were found in CD9 and CD81 populations. Our findings confirmed the superior efficiency of UC method for EVs isolation, revealing differences in the tetraspanin composition of EVs, compared to those obtained by SEC, as well as among fractions. Moreover, we demonstrated the differential EVs populations among female reproductive fluids suggesting specific roles on both oocyte and sperm physiology. Further investigation into the effects of these distinct EVs populations on oocyte competence, sperm fertilization ability and embryo development will provide valuable insights.

Work supported by the Spanish Ministry of Science, Innovation and Universities under the project: PID2021-126988OB-IOO.

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Exploring mitophagy dynamics in bovine oocytes during in vitro maturation in response to mitochondrial membrane uncoupling

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Oocytes mitochondria have unique dynamics, morphology, and reactive oxygen species (ROS) production compared to somatic cells, potentially leading to mitochondrial quality control mechanisms that may differ from the typical activation of mitophagy in response to mitochondrial dysfunction. Previous research in inbred C57BL/6 mice has shown that oocytes do not induce mitophagy in response to increased oocyte dysfunction (Boudoures *et al*. 2017, Dev Biol. 426(1):126-138). However, since significant differences in mitochondrial functions in oocytes between outbred and inbred models have been reported, here we aimed to explore the ability of bovine oocytes to induce mitophagy as a response to mitochondrial membrane uncoupling by adding carbonyl cyanide m-chlorophenyl hydrazone (CCCP) during in vitro maturation (IVM).

Cumulus oocyte complexes (COCs) were aspirated from bovine ovaries collected from a local slaughterhouse. Three replicates were conducted with COCs assigned to five experimental groups (80 COCs per group): control (CTR), solvent control (SC) with 0.05% v:v DMSO, BafilomycinA1 (50nM) (BafA1), CCCP (CP)(10µM), and CCCP+BafA1. BafA1 inhibits autophagy by preventing lysosomal acidification, resulting in autophagosome accumulation. After a 2-hour exposure to CTR or CCCP-containing medium, with or without BafA1, COCs were matured for 22 hours. Finally, denuded mature oocytes were used for live-cell staining (JC-1 and CellROX Red) and Western Blotting (PINK1 and LC3 expression levels). Additionally, pools of 50 oocytes of the CTR, SC and CP groups were fertilized and routinely group cultured for 7 days to evaluate developmental capacity.

JC-1 staining confirmed the mitochondrial membrane uncoupling effect of CCCP treatment compared to CTR (P=0.02), accompanied by a decrease in ROS production measured by CellROX signal intensity for CP vs CTR (P=0.001), which may be due to a reduced proton gradient and electron transport. Cleaved (ΔN-PINK1) and full-length PINK1 (FL-PINK1) were quantified using Western Blotting, with the ΔN-PINK1/FL-PINK1 ratio being a measure of PINK1 cleavage, while FL-PINK1 stabilization in dysfunctional mitochondria may indicate activation of the PINK1-PARKIN pathway. Interestingly, CCCP treatment did not lead to lower PINK1 cleavage rates (0.76±0.52) compared to CTR (1.82±1.69) and SC (1.01±0.65)(P=0.62). Comparing the presence of autophagosomal protein LC3 between CCCP versus CCCP+BafA1 and SC versus BafA1, representative for CCCP-induced and DMSO-induced autophagy, revealed that CCCP did not significantly increase autophagy flux compared to DMSO (P=0.93). Remarkably, CCCP-treated oocytes showed similar blastocyst rates to CTR and SC groups after IVP on day 8 (CTR 0.36±0.06, SC 0.34±0.03, CP 0.43±0.01)(P>0.1).

Our findings suggest that bovine oocytes do not activate PINK1/Parkin-mediated mitophagy in response to CCCP-induced mitochondrial uncoupling at the onset of maturation. The reduction in ROS and potential other mechanisms might contribute to normal development to blastocysts. Also, further exploration with additional replicates and examination of other outcome parameters are needed to fully understand the complexities of mitophagy induction in oocytes.

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In vitro procedures deregulate the embryonic disc and extraembryonic membranes transcriptome of day 15 elongated bovine embryos

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In vitro embryo production is intensively applied worldwide for cattle production because of several advantages. Indeed, more than 1.1 million in vitro produced (IVP) embryos were transferred in 2022 compared to 360.000 in vivo produced (IVV) embryos (Viana, EmbryoTransfer Newsletter, 41, 20-38, 2022). However, IVP embryos yield around 25% lower pregnancy rates compared with IVV embryos, with most of the losses occurring at ~day 20 of pregnancy (Ealy et al., J Anim Sci, 97, 2555-2568, 2019), probably because of impaired elongation from ~day 13 of pregnancy (Clemente et al., Biol Reprod, 85, 285-95, 2011). This period involves critical events, such as gastrulation and formation of the embryonic disc (ED), as well as the development of the extraembryonic membranes (EEM). Therefore, we hypothesize that in vitro procedures negatively affect the molecular signatures of both embryonic regions. The goal was to quantify the effect of the in vitro procedures on the transcriptome of ED and EMM of the same length day 15 bovine conceptuses compared to in vivo ones. IVP embryos were derived from oocytes aspirated from slaughterhouse ovaries after maturation, fertilization, and culture in serum-free medium until transfer at day 7, while IVV embryos were generated after ovarian super-stimulation and artificial insemination, following standard protocols for both procedures. Animals were flushed at day 15 of gestation to recover the elongated embryos. Sections of the EMM and ED from tubular same-length IVP and IVV embryos (5.9 ± 0.2 vs 5.6 ± 0.2 mm, respectively; n=4 per group) were submitted to paired-end RNAseq. Raw data were aligned to the ARS-UCD1.3 bovine genome, and the processed files were analysed using the DESeq2 package for the R software to determine differentially expressed genes (DEG) at a false discovery rate (FDR) < 0.05. Enriched biological processes (FDR < 0.05) associated with DEG in the EMM or ED between IVP and IVV embryos were determined using the DAVID database. There were 801 and 1516 DEG in the EMM and ED, respectively, between IVP and IVV embryos. The functional analysis showed that DEG more expressed in the EMM of IVP embryos (429) enriched cell migration, while down-regulated genes (372) were associated with transcription and ontological terms involved in chromatin structure, such as core histones, DNA binding and nucleosome. DEG upregulated in the ED of IVP embryos (564) were involved in glycerophospholipid and fatty acid metabolism. Notably, downregulated (or inhibited) DEG (952) enriched anatomical morphogenesis processes and pathways playing critical roles in embryonic development, such as canonical Wnt, bone morphogenetic protein and transforming growth factor beta signalling pathways. In conclusion, these results demonstrated that the in vitro procedure deregulated the molecular signatures of EMM and ED regions during early gastrulation and negatively impacted the developmental process of IVP embryos, even when they were of similar length to the IVV counterparts, which can explain why most IVP conceptuses are lost after this period.

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SOX17 ablation impairs hypoblast formation and reduces trophectoderm proliferation in bovine embryos

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Following blastocyst hatching, cattle embryos are composed by three lineages: the epiblast –which will form the embryonic disc and subsequently the fetus- and two extra-embryonic lineages –the trophectoderm and the hypoblast-. Post-hatching embryo development accounts for most embryonic losses in cattle and is remarkably divergent from that of mice, the only mammal where it has been thoroughly studied by loss-of-function approaches. In mice, post-hatching development relies on the reciprocal signalling between the three lineages, but the role of inter-lineages signalling remains unknown in other mammals. The objective of this work has been to determine the developmental ability of cattle embryos lacking hypoblast. To generate hypoblast-devoid embryos, the hypoblast-specific transcription factor SOX17 was ablated by CRISPR technology. In vitro matured bovine oocytes (n=378, 4 replicates) were divided in two groups: one was microinjected with Cas9-encoding mRNA and a sgRNA against SOX17 (n=276, C+G group, containing KO embryos), and the other was microinjected with Cas9-encoding mRNA alone, serving as microinjection control (n=102, C group, formed by wild-type WT embryos). Microinjected oocytes were fertilized in vitro and developed to Day (D) 12, by conventional culture in SOF medium to D7 followed by culture in N2B27 medium. D12 embryos were fixed and subjected to immunohistochemistry (IHC) to detect the development of specific lineages and genotyped by miSeq. No significant differences were observed in developmental rates between both microinjection groups (Cleavage rate: 82±3.8 vs. 83.6±1.5 %; blastocyst rate: 35.0±9.2 vs. 23.9±4.9 %; D7 to D12 survival rate: 73±4.6 vs. 70.2±5.4 %; for C vs. C+G, mean±s.e.m., t-test $p>0.05$). 34 out of 39 D12 embryos genotyped in C+G group (87 %) were edited, and 15 were KO (38.4 %). SOX17 expression was not detected by IHC in SOX17 KO embryos, which failed to undergo hypoblast differentiation as evidenced by the lack of an inner cellular layer beneath the CDX2+ trophectoderm and by the absence of FOXA2+ cells. In contrast, WT and edited non-KO embryos developed hypoblast, but while complete hypoblast migration was observed in 12/20 (60 %) WT embryos, no edited-non-KO embryo (0/19) achieved complete hypoblast migration. D12 embryo diameter was significantly reduced in SOX17 KO and edited non-KO embryos compared to WT (0.93±0.09 vs. 0.60±0.04 vs. 0.59±0.07 mm, for WT, edited and KO embryos, respectively, ANOVA, $p<0.05$). No significant differences were observed in embryonic disc formation rate (10/20, 5/19 and 5/15, for WT, edited non-KO and KO embryos, respectively). In conclusion, SOX17 is required for hypoblast differentiation and hypoblast devoid embryos show a reduced trophectoderm proliferation by D12.

Work supported by StG 757886-ELONGAN and PID2020-117501RB-I00.

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Isolation and characterization of extracellular vesicles from bovine granulosa cells under oxidative stress conditions

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Recent studies have demonstrated the potential of extracellular vesicles (EVs) derived from stressed granulosa cells to induce an adaptive response to cellular stress in recipient cells (Gebremedhn et al. Sci Rep, 10, 15824, 2020). This study aimed to isolate and characterize EVs derived from granulosa cells subjected to oxidative stress. Bovine granulosa cells were obtained by aspirating 3-6 mm follicles and cultured in TCM-199 medium + 10% exosome-depleted fetal bovine serum (FBS). Sub-confluent cells were exposed to 5 µM H2O2 for 40 min to induce oxidative stress followed by 24h of cell culture in TCM-199 medium + 10% exosome-depleted FBS. Non treated cells served as Control. Oxidative stress was confirmed by assessment of mitochondrial activity (Mitotracker) and Reactive Oxygen Species (ROS) production (DCFH-DA) assessing fluorescence intensity in arbitrary units. EVs were isolated from spent media by size exclusion chromatography (SEC; Hansa BioMed) and concentrated using an Amicon Ultra-15 filter (Merck-Millipore Ltd., Ireland) to a final volume of 100 µL per sample. Nanotracking (NTA) analysis was conducted to characterize the size and concentration of particles present in Control and Oxidative Stress (OS) groups (4 replicates/treatment) and morphology was evaluated by transmission electron microscopy (TEM). The induction of oxidative stress in granulosa cells was confirmed by a two-tailed student's T test, revealing higher ($P < 0.05$) levels of ROS in the OS group (17.02 ± 0.19) compared to the Control group (12.15 ± 0.09). Additionally, there was a significant ($P < 0.05$) decrease in mitochondrial activity in the OS group (94.83 ± 1.93) compared to the Control group (140.00 ± 2.91). NTA analysis revealed that particle concentration in the Control group was $2.07 \times 10^{11} \pm 1.6 \times 10^{10}$ particles/mL, with a mean size of 237.3 ± 4.9 nm and a modal size of 170.1 ± 8.3 nm. Particle concentration in the OS group was $1.98 \times 10^{11} \pm 2.2 \times 10^{10}$particles/mL, with mean and modal sizes of 223.2 ± 7.0 nm and 160.0 ± 13.4 nm, respectively. The student's T test did not show significant differences among groups in terms of particle concentration, mean and modal sizes among Ccontrol and OS groups. Additionally, TEM analysis confirmed the presence and morphology of isolated EVs. In conclusion, the induction of oxidative stress in granulosa cells leads to increased levels of ROS and reduced mitochondrial activity. Furthermore, our selected methodology demonstrated effectiveness in isolating oxidative stress-induced EVs from bovine granulosa cells, exhibiting particle concentration and size consistent with known EVs characteristics although no differences were observed in terms of EVs concentration, size or morphology between the two experimental groups. The successful isolation and characterization of extracellular vesicles from oxidative stressed granulosa cells establish an effective methodology for further research, specifically in analyzing the cargo of these EVs to investigate their potential to modify oocyte composition.

This study was supported by projects PID2020-116531RB & 2021SGR00900; JDM by FPI PRE2021-098675; KCB by Maria Zambrano; YNC by Margarita Salas.

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ZFN10 is dispensable for first and second lineage differentiation events in bovine embryos

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The transcription factors regulating first lineage differentiation, which give rise to the trophectoderm and inner cell mass (ICM), have been thoroughly studied in the mouse model but the roles of key regulators such as OCT4, CDX2 or TEAD4 are not conserved in other mammals, including ungulates. Zinc finger (ZNF) proteins are transcription factors that are involved in gene silencing during mouse embryonic stem cell differentiation and one of the family members (ZFN10) is predominantly expressed by the bovine ICM, thereby constituting a plausible candidate to mediate first lineage differentiation in ungulates. The objective of this study has been to elucidate ZNF10 role during bovine early embryogenesis by assessing the developmental ability of *ZFN10* KO embryos generated by introducing a stop codon by base editing technology. *In vitro* matured bovine oocytes (n=379, 4 replicates) were divided in two groups: one was microinjected with cytosine base editor (CBE) encoding mRNA and a sgRNA against *ZNF10* (n=224, C+G group, containing KO embryos), and the other was microinjected with CBE encoding mRNA alone, serving as microinjection control (n=155, C group, formed by wild-type WT embryos). To assess if *ZFN10* embryos were able to complete first and second lineage differentiation events, microinjected oocytes were fertilized *in vitro* and developed to Day (D) 12 by conventional culture in SOF medium to D7 followed by culture in N2B27 medium. D12 embryos were fixed and subjected to immunohistochemistry to detect trophectoderm (CDX2+) and the two lineages derived from the ICM in a second differentiation event: epiblast (SOX2+) and hypoblast (SOX17+). Following image acquisition, embryos from C+G group were genotyped by Sanger. No significant differences were observed in developmental rates between both microinjection groups (Cleavage rate: 70.4±9.8 vs. 77.5±4.4 %; blastocyst rate: 18.0±4.0 vs. 23.6±3.9 %; D7 to D12 survival rate: 74.5±2.7 vs. 74.4±5.2 %; for C vs. C+G, mean±s.e.m., t-test $p>0.05$). 33 out of 35 D12 embryos genotyped in C+G group (94 %) were edited; 5 were heterozygous (Hz), and 28 were KO (80 %). *ZNF10* KO embryos were able to develop up to D12 normally. D12 embryo diameter was similar between WT, Hz or KO embryos (1.0±0.07 mm vs. 1.22±0.09 vs. 0.96±0.09 mm, for WT vs. Hz vs. KO, respectively, mean±s.e.m, ANOVA, $p>0.05$). Embryonic disc formation rate was also unaffected by ZNF10 ablation (11/25 vs. 3/5 vs. 14/28, for WT vs. Hz vs. KO, respectively, Chi-Square, $p>0.05$), and the number of epiblast (SOX2+) cells in the embryonic disc was similar between the three genotypes (39±7 vs. 57±17 vs. 51±7, for WT vs. Hz vs. KO, respectively, mean±s.e.m, ANOVA, $p>0.05$). No significant differences were observed either in the rate of complete hypoblast migration between all three genotypes (14/22 vs. 5/5 vs. 21/28, for WT vs. Hz vs. KO, respectively, Chi-Square, $p>0.05$). In conclusion, ZNF10 is not required for first and second lineage differentiation events.

Work supported by StG 757886-ELONGAN, PID2020-117501RB-I00 and PID2021-122153NA-I00.

Cloning, Transgenesis and Stem Cells

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A Decade of health and genomic stability: the cattle produced by transposon-mediated transgenesis and embryo transfer

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In previous study, we produced one fluorescent transgenic female and one fluorescent transgenic male cattle (SNU-SB-1, SNU-PB-1) using transposon system. Although the transgenic animals has led to concerns about their long-term health and genomic stability, there are a few studies. In this study, we assessed these factors in the transgenic cattle over a decade by employing a transposon-mediated transgenesis and embryo transfer technique. Our longitudinal study included health evaluations and complete genome re-sequencing. Using blood analysis, we observed no significant changes in physiological parameters in the cattle, which are now over ten years old. To assess the effect of transposon-mediated transgene insertion on genome stability over a 10 year period, whole-genome DNA sequencing was performed using blood sample from the transgenic cattle and one age-matched wild-type cattle. As a result, in the 10-year-old transgenic cattle, the number of genomic variants detected was comparable to that in wild-type cattle. SNU-SB-1 and SNU-PB-1 contained 6,155 and 7,990 somatic SNPs and 3,367 and 3,652 somatic Indels, respectively. Among these variants, 17 and 9 non-reference homozygous (NonRefHom) SNPs were found in SNU-SB-1 and SNU-PB-1 cattle, respectively, along with 132 and 111 NonRefHom Indels. In somatic structure variants(SVs), six and two somatic SVs were detected in SNU-SB-1 and SNU-PB-1 cattle, respectively, all of which were heterozygous. The copy number variations (CNVs) in 10-year-old transgenic cattle mirrored those detected in 1-year-old transgenic cattle, indicating the absence of somatic copy number alterations over 10 years in transgenic cattle. Taken together, our whole-genome DNA resequencing data indicated that transposon-mediated transgene insertion in transgenic cattle did not perturb genome stability over the course of a decade. These results highlight the feasibility of using transposon systems for creating transgenic livestock, with potential broader applications in agriculture and biotechnology. This research greatly enhances our understanding of the long-term effects of transgenesis in large animals, affirming the safety and stability of the method.

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Effects of holding immature porcine oocytes on in vitro maturation and parthenogenetic embryo development

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The growing significance of pigs as both animal models and potential organ donors in biomedical and biotechnological research has sparked interest in *in vitro* porcine embryos production. However, challenges persist in achieving oocyte complete developmental competence. Delaying meiotic progression before *in vitro* maturation (IVM) by holding immature oocytes has been shown to have potential benefits in horses, but its effect on porcine oocytes is unclear (Lazzari G., JEVS, 89, 103097, 2020). This study aimed to investigate if pre-IVM holding and modulation of the IVM timings could improve porcine oocyte maturation and parthenogenetic (PGA) embryo development. Swine cumulus-oocyte complexes (COCs, 30/group) were directly subjected to IVM and cultured in TCM199 with 10% fetal calf serum, additives, and hormones under 5% CO2 at 38 °C (a). Alternatively, they were kept in H-SOF in the dark at room temperature for 24 hours (h) before IVM (b: 24hHOLD). For each group 4 timing were considered: (1) a. COCs subjected to 26h-IVM; b. 24hHOLD + 26h-IVM; (2) a. COCs subjected to 42h-IVM; b. 24hHOLD + 42h-IVM; (3) a. COCs subjected to 46h-IVM; b. 24hHOLD + 46h-IVM (4) a. COCs subjected to 50h-IVM; b. 24hHOLD + 50h-IVM. All experiments were conducted in at least 3 biological replicates and the data were analyzed using one-way ANOVA with Tukey's multiple comparison test. Following IVM, cumulus cells expansion was evaluated, COCs were denuded, and maturation was assessed by identifying first polar body extrusion of metaphase II (MII) oocytes. Matured oocytes were PGA activated by a double electric pulse of 1kV/cm in activation medium (0.3M mannitol and 1mM Ca^{++}). Our results revealed that oocytes exposed to 26h-IVM, with or without 24h pre-IVM holding, did not reached the MII stage, as expected. Preliminary data indicated that maturation rates did not differ between the 42h, the 46h and 50h-IVM timings and their respective 24hHOLD groups, ranging from 70.15 ± 4.66% to 79.14 ± 7.16%. By contrast, significantly lower ($P \leq 0.05$) cleavage rates were identified for 42h-IVM group (59.85 ± 11.44%) and its 24hHOLD counterpart (52.42 ± 10.94%) compared with 46h (73.77 ± 7.21%) and 50h (86.65 ± 4.26%) IVM groups and their 24hHOLD counterparts (70.69 ± 2.37% and 85.56 ± 13.47% respectively). Finally, oocytes directly matured 50h in IVM and their respective 24hHOLD+ 50h-IVM group indicated significantly higher ($P \leq 0.01$) PGA blastocyst development rates (16.37 ± 4.07% and 17.04% ± 2.25%) in comparison with the 42h (3.77 ± 2.78%) and 24hHOLD+ 42h-IVM counterpart (2.22% ± 3.85%). Both 50h-IVM groups showed a tendency toward improved PGA blastocyst development compared to the 46h-IVM (9.78 ± 5.24%) and 24hHOLD + 46h-IVM (10.83% ± 1.44%) groups, though the difference was not significant. These findings show that 42h-IVM reduces developmental capacity in PGA pig embryos. Additionally, holding oocytes in H-SOF for 24h before 42h, 46h, or 50h of IVM does not affect developmental rates compared to non-held groups.

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Optimizing time of lipofection for improved CRISPR/Cas9-mediated genome editing in porcine embryos

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Lipofection has been widely used to introduce external molecules into cells since its development in 1987 by Felgner *et al* (Proc Natl Acad Sci USA, 84, 7413, 1987). We have generated gene edited embryos by this method using the CRISPR/Cas9 system without removing the *zona pellucida* of the oocyte (Piñeiro-Silva, Animals, 13, 342, 2023), but the optimal time of the lipofection treatment has not been studied. Therefore, our objective was to compare three different times of lipofection to generate Calpain3 mutant embryos. *In vitro* matured oocytes were lipofected with Lipofectamine CRISPRMAX Cas9 with sgRNA and protein Cas9 for 4h (L4h), 8h (L8h) and 24h (L24h), fertilized and cultured *in vitro* for up to 6 days. The IVF and the lipofection treatment were performed simultaneously. An untreated group was used as control (C) with an IVF time of 24h. Penetration and monospermy rates were evaluated at 24h post-insemination (pi) by Hoechst staining and evaluation under fluorescence microscope. Cleavage and blastocyst rates were evaluated at day 2 and 6 pi. Mutation and mosaicism rates were analyzed by fluorescent PCR-capillary gel electrophoresis. Overall efficiency was also evaluated (mutant embryos/total oocytes). 619 oocytes were analyzed for IVF (C:149, L4h:153, L8h:155, L24h:162) and 700 for embryo development and gene mutation (C:169, L4h:176, L8h:175, L24h:180). 3 replicates were performed. Data were analyzed by a Kruskal–Wallis test and compared by a Conover–Inman test.

The penetration rate was higher in C group in comparison with L4h group (95.5±1.8% vs. 82.4±3.3%, $p<0.05$), with intermediate values in the other groups (L8h:88.2±2.8%, L24h:89.2±2.6%). Monospermy was similar in all groups (C:52.3±4.4%, L4h:47.3±4.7%, L8h:41.2±4.5%, L24h:49.2±4.4%, $p=0.35$). The cleavage rate was lower in the L4h and L24h groups compared to C group (42.6±3.7%, 49.4±3.7% vs. 61.5±3.8%, $p<0.05$), but blastocyst rate was similar in all groups (C: 27.8±3.5%, L4h: 21.0±3.1%, L8h: 29.7±3.5%, L24h: 21.7±3.1%, $p=0.15$). The mutation rate was lower in the L24h group compared to the L8h group (27.0±7.4% vs. 49.0±7.1%, $p<0.05$), with intermediate values for L4h group (43.2±8.3%). The overall efficiency followed the same pattern, with higher values for L8h in comparison with L24h (14.4±2.7% vs. 5.6±1.7%, $p<0.05$) and intermediate values for L4h (9.1±2.2%). The mosaicism rate was similar for all groups (L4h: 50.0±12.9% n=8/16, L8h: 40.0±10% n=10/25, L24h: 20.0±1.3% n=2/10, $p=0.32$). Considering these results, the CRISPR/Cas9 system is able to enter the oocyte in the first 4 hours of coincubation with lipofectamine but reaches the highest efficiency at 8 hours of coincubation. L4h had lower penetration and cleavage rates due to the shorter sperm-oocyte coincubation time. In addition, lipofectamine may have a toxic effect, as the cleavage and mutation rates decreased with longer lipofection time. For these reasons, lipofection can be an effective method to produce genetically modified animals and embryos, but optimization of the process is still needed to explore the potential application of this method.

Supported by Fundación Seneca 22065/PI/22 and Universidad de Murcia predoctoral fellowship R-496/2022.

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Successful generation of mitotically stable lines of chicken blastoderm-derived embryonic stem cells

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Pluripotent embryonic stem cell (ESC) lines established from the blastoderm, which had been isolated from embryonic discs of the domestic fowl (*Gallus gallus domesticus*) embryos at the stage X not only serve as an innovative model for studying avian embryonic development but are also gaining increasing importance, both in the context of safeguarding various populations outside their natural environment and in preserving the genetic diversity of poultry species and breeds. The purpose of the current study was to develop an efficient strategy for: (1) isolating chicken blastodermal cells (BCs); (2) generating stable BC-derived ESC lines under *ex ovo* conditions; and (3) identifying their pluripotency-related proteomic profiles based on the expression of such biomarkers as Oct-3/4 (octamer-binding transcription factor-3/4, also designated as POU5F1; a member of the family of POU (Pit-Oct-Unc)-domain- and homeodomain-containing transcription factors) and Sox2 (sex-determining region Y (SRY)-box 2; a member of the high mobility group (HMG)-box family of DNA-binding transcription factors). ESCs, which had been established from BCs isolated from a total of 45 fertilized eggs at the stage X (n = 3), were cultured for a minimum of 15 passages under *ex vivo* conditions. During the *ex-ovo* expansion of blastoderm-derived ESCs, their adhesive and proliferative capabilities were compared depending on the type of vessels used for *in vitro* culture (8-well Ibidi glass-bottomed microplates coated with synthetic polymeric substrate/poly-*L*-lysine and cell culture dishes comprised of polystyrene plastic and coated with type I collagen - SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes). By using Western-blot analysis and immunofluorescence staining, the protein expression profiles have been investigated for the pluripotency-related biomarkers that have been represented by the members of a family of homeobox (Hox) transcription factors (Oct-3/4 and Sox2), which display the presence of homeodomain, i.e., a conserved 60-amino acid helix-turn-helix motif-containing and DNA-binding domain. Throughout the extended *in vitro* culture, chicken B-ESCs have been found to maintain the typical morphology of embryonic stem cells. Moreover, the expression of selected pluripotency-related markers of stemness was confirmed at the protein levels based on the detecting the presence of such homeotic (homeodomain/homeobox-containing) transcription factors as Oct-3/4 and Sox2. Conclusively, the use of 8-well Ibidi glass-bottomed microplates coated with poly-*L*-lysine and SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes exerted a comparable advantageous impact on the proliferative capabilities of chicken BC-derived ESC lines.

ACKNOWLEDGEMENTS: This work was funded by research grant No. 01-19-12-21 from the National Research Institute of Animal Production in Balice near Kraków, Poland, to Marcin Samiec.

Cryopreservation and Cryobiology

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The potency of Moringa oleifera leaf extract inclusion in tris egg yolk extender of bull epididymal semen during cryopreservation

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The post-slaughtered bull semen has been described to be extremely susceptible to cold shock and sensitive to peroxidative damage as a result of the high content of polyunsaturated fatty acids found on the sperm membrane. Owing to this, the reactive oxygen species (ROS) and free radicals stress affects cryopreservation outcomes. During cryopreservation, semen extender is required to enhance the improvement of semen qualities. Semen extender can be supplemented with Moringa oleifera leaf extract (MOLE) as the source of natural antioxidant which is required to reverse the development of ROS caused by imbalances between the ROS activities and amounts of antioxidants in the semen. The extract from MOLE is used because of its accessibility to many farmers is South Africa. The study evaluated the effect of different MOLE concentrations levels of 0; 4; 8 and 12% supplemented in the tris-egg yolk semen extender during cryopreservation of epididymal bull semen. Bull testicles (n=50) were randomly collected from the local slaughterhouse and transported (5°C) to the laboratory. The recovery of epididymis and flushing of sperm was done within an hour of collection. Furthermore, semen was diluted with tris-egg yolk extender (TEY- fraction A) and equilibrated for 120 min, and later supplemented with TEYE- fraction B containing different level of MOLE: TEYE+MOLE 0% (control), TEYE+MOLE 4%, TEYE+MOLE 8% and TEYE+MOLE 12%) and then loaded into 0.25 mL straws before freezing. Thawing of frozen semen straws was done for 10 sec in air and 1 minute inside the warm water (37°C), and the semen samples were evaluated for sperm viability and abnormalities% per treatment groups using a computer-aided sperm analyser system. Data was analysed using the General Linear Model procedures of the Minitab statistical package of 2019. There was a significant effect of TEYE+MOLE levels (0; 4, 8 and 12%) on epididymal sperm rapid motility (50.31±1.4; 54.06±2.3; 57.94±2.7; and 53.63±1.8; respectively) and sperm viability% (45.25±3.4; 53.63±4.0; 58.63±4.4 and 56.44±2.5, respectively; $P<0.05$). The TEYE+MOLE8% had a better sperm motility and viability% as compared to other treatments groups. No significant difference was recorded on sperm morphology% amongst all levels of TEYE+MOLE. In conclusion, supplementing of MOLE extracts in the tris-egg yolk semen extender was able to maintain better epididymal sperm motility and viability recovery following thawing.

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Relationship between oocyte mitochondrial activity and female age in mares

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In animal reproduction, dams are not usually required to produce offspring at advanced age. However, for mares of high genetic value, owners are increasingly asking for obtaining embryos from older animals. A decrease in mitochondrial activity, in oocytes recovered from advanced maternal age women, led to spindle assembly alterations, reduced levels of proteins responsible for chromosomes pairing and segregation, and telomere shortening. This study aimed to understand the relationship between female age and mitochondrial activity in the horse. Oocytes were recovered by follicular fluid aspiration from ovaries of young (<15 years; N° mares = 43) and aged (>15 years; N° mares = 46) slaughtered mares. All ovaries have been recovered only from healthy mares. Media, supplements, antibodies were purchased from Sigma Aldrich (Milan, Italy) unless otherwise stated. Oocyte were matured in DMEM-F12+10%FBS (Gibco, ThermoFisher Scientific, Waltham, USA) +50 ng/ml EGF+100 ng/ml IGF-1+0.1 IU/mL FSH-LH (Pluset, Calier, Como, Italy) at 38.5°C and 5% CO_2 for 30 hrs. After IVM, oocytes were fixed in 4% paraformaldehyde, then the zona pellucida was removed for immunofluorescence (IF). Blocking was done with goat serum (GS) in Tween 20+BSA (PBS-TB) followed by an overnight incubation at 4°C in PBS-TB+GS+DLAT antibody (to detect mitochondrial activity) +TOMM20 antibody (to identify mitochondrial distribution), and a second incubation with anti-mouse AF488 and anti-rabbit AF568 antibodies in PBS-TB. Oocytes were examined by confocal microscopy (Dragonfly High Speed Confocal Microscope System equipped with Fusion program) and images were processed by FIJI ImageJ. CTCF (corrected total cell fluorescence) formula (CTCF (pixel) = Integrated Density – [Area of selected cell X Mean fluorescence of background readings]) was used to calculate cell fluorescence. Data, expressed as mean± standard deviation, were analyzed for normal distribution, using a Shapiro Wilk test, and processed by a two tailed T-test using Wizard 2 (Version 2.0.16). Significance was assessed for $p<0.05$. A total of 78 matured oocytes were used, 34 from young mares and 44 from aged ones. Some of them served to adapt a human IF protocol to the horse, thus 23 (young) and 38 (aged) oocytes were finally analyzed. The average fluorescence registered for DLAT, was significantly higher in oocytes recovered from young (254086.8 ±385910.8) than aged mares (103494.1±34065.3; $p<0.05$). However, despite the apparently higher number of mitochondria revealed by TOMM20 in oocytes from older females, the values were not statistically different (young 15944.7±17785.7 vs aged 34065.3±57579; $p>0.05$). These findings open exciting prospects for future studies and clinical applications. Deepening the molecular mechanisms underlying the decrease in mitochondrial activity in oocytes from aged mares might be useful to identify new strategies to improve oocyte quality and increase the chances of success in assisted fertilization techniques.

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Development of bovine vitrified oocytes can be improved by antioxidants added during post-warm recovery period

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Vitrification of bovine oocytes can impair further embryo development mostly due to oxidative stress. Various exogenous antioxidants (ascorbic acid, N-acetyl cysteine, melatonin, resveratrol, niacin, coenzyme Q10 and others), added either during the maturation, or closely prior to vitrification, have been shown to ameliorate the post-warm oxidative stress in mouse, sheep and bovine oocytes with a variable success. The aim of this study was to verify whether a relatively short recovery culture of oocytes post-warming in presence of chosen antioxidants (glutathione and astaxanthin) is enough to improve their post-warm survival and development. Bovine *in vitro* matured oocytes (n= 905) were washed in the vitrification medium (M199, 10% foetal bovine serum (FBS), 30 % ethylene glycol, 1 M sucrose), placed onto electron microscopy grade grids and vitrified using ultra-rapid cooling technique. Following warming, the oocytes were incubated for 3 hours (post-warm recovery) in the maturation medium (M199, 10 % FBS, 0.25 mM sodium pyruvate, 50 µg/mL gentamicin, 1 I.U FSH/LH (Pluset) supplemented with either glutathione (GSH; 5 mM; n=223) or astaxanthin (AX; 2.5 µM; n=226) or none (Control; n=456). Vitrification significantly induced the formation of ROS (CellROX fluorescent staining) in oocytes, while GSH reduced this value ($p<0.05$) in post-warmed oocytes. GSH, although did not increase the total blastocyst rate (Day 6-Day 8: 20.20% vs. 17.49 % in control), but it increased the proportion of faster developing blastocysts (Day 6: 23.07% vs. 13.5%; Day 7: 48.7% vs. 42.5%, resp.), reduced the apoptosis incidence (TUNEL assay) up to the control level and reversed harmful impact of vitrification on actin cytoskeleton structure (phalloidine-TRITC staining). AX reduced ROS formation and lipid peroxidation (BODIPY staining) in vitrified oocytes. Development to the blastocyst stage in vitrified oocytes (D6-D8 blastocysts: 15.34%) was not improved by AX (17.3%). Nevertheless, AX promoted blastocyst proliferation (DAPI staining; total cell number - 105.28± 4.45) compared to vitrified group (94.03±5.08) and showed trend of improving the actin cytoskeleton quality. RT-qPCR assay revealed that AX stimulated expression of development-related genes (*GJB5*, *CAT* and *GPX4*) and suppressed pro-apoptotic *CAS9* gene expression. In conclusion, glutathione confirmed its protective action against vitrification-induced damages. Astaxanthin during post-warm recovery period reduced oxidative stress (ROS) in vitrified oocytes and improved quality of blastocysts. These results suggest that even a short recovery culture of bovine oocytes post-warming in presence of antioxidant(s) can improve their development.

Grant support: Slovak Research and Development Agency, grants no. APVV-19-0111, APVV-23-0203.

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Conservation of bovine ovarian cortex. Impact of cryopreservation procedure and sample size on the morphology of preantral follicles.

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The cryopreservation (CP) of ovarian cortical tissue (OcT) offers an alternative to overcome the lack of an effective oocyte CP method. Isolation and subsequent *in vitro* culture of preantral follicles (PaF) from CP OcT could allow to obtain viable oocytes for *in vitro* embryo production or conservation programs. Slow freezing (SF) of OcT is a more established protocol. Over the last decade vitrification has also developed for OcT, however the need of an effective standardized protocol remains unsolved. In this work we analysed the effects of SF and vitrification on the PaF morphology. Thus, bovine OcT pieces of 10mm x 10mm x 1mm (large) or 10mm x 5mm x 1mm (small) from 3 animals were frozen-thawed (FT) or vitrified-warmed following two protocols (VW1 & VW2). SF up to -70ºC was performed in PBS+4g/L BSA+12% DMSO following a three-step protocol at room temperature (RT). Then cryovials were stored in liquid nitrogen (LN). For thawing, the cryovials were thawed in a water bath at 42ºC, placed in ice and cryoprotectant was removed by serial dilution with PBS+4g/L BSA. OcT samples (VW1 & VW2) were vitrified in 1.8 ml open cryovials. VW1 samples were incubated at RT in PBS + 20% FCS (HM) + 5% ethylene glycol (EG) + 5% DMSO + 0,125M sucrose (V1S1; 5 min) and then in HM + 10% EG + 10% DMSO + 0,25M sucrose (V1S2; 2 min). Finally, samples were moved to HM + 20% EG + 20% DMSO + 0.5 M sucrose (V1S3; 3 min) and vitrified in LN. For warming, samples were incubated at RT in V1S2 (5 min) and in V1S1 (5 min), and washed in HM (5 min).

VW2 samples were incubated in in HM + 7.5% EG + 7.5% DMSO + 0.5 M sucrose at RT (25 min) and vitrified in HM + 20% EG + 20% DMSO + 1 M sucrose (15 min). For warming, samples were incubated in HM + 1M sucrose (1 min; 37ºC), in HM + 0.5M sucrose (3 min; RT) and in HM (5 min; RT). Fresh control (Ctrl), FT, VW1 and VW2 OcT pieces were fixed, dehydrated, embedded in paraffin, and serially sectioned (5 µm). Follicle morphology and distribution was examined in H/E slides. PaF were classified as collapsed (showing ooplasm shrinkage or detachment of the basal lamina) or morphologically normal (MNF) (Herraiz *et al.*, Fertility and Sterility 113:609, 2020). A total of 1,454 PaF were counted (Ctrl: 377; FT: 340; VW1: 400; VW2: 337). Data were analysed by a Pearson's Chi square. VW2 significantly improved the percentage of MNF compared to VW1 (66.37 *vs* 29.83 and 65.79 *vs* 26.94, for large and small samples respectively; $p<0.001$) with any used sample size.

When the percentage of MNF in large FT samples was compared with Ctrl, it was significantly reduced (51.27 *vs* 85.71, respectively; $p<0.001$). Interestingly, FT OcT and Ctrl small samples showed similar percentages of MNF (88.11 *vs* 78.89, respectively; $p>0.05$). The total percentages of MNF were similar among groups and when specific follicular subpopulations (primordial, primary and secondary) were examined. Although CP negatively affected follicular integrity, further studies are needed for evaluating the functionality of the surviving PaFs.

PID2022-138600OR-I00; PREP2022-000436.

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Effects of cold storage and different cryopreservation methods on canine epididymal sperm

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Recent years have seen a drastic decline in biodiversity. In addition to the traditional in vitro conservation of genetic material, there is a need to establish a well-functioning protocol to cryopreserve the genetic material of a male of an endangered species or with valuable genetics. Storage of epididymal sperm is a feasible method to achieve this goal, however, time of sample delivery and freezing technique are key factors affecting the success rate. Our aim was to investigate the freezing ability of canine epididymal spermatozoa in fresh stage and after 24 hours storage at 4°C with two different freezing protocols (ultra-rapid freezing [UR] and vitrification [VF]). Testes were collected from 15 castrated mixed-breed dogs. Sperms were obtained from cauda epididymis immediately after collection or after 24h storage, using the incision method. Collected sperms were cryopreserved with UR and VF. The following parameters were evaluated: total and progressive motility, normal morphology rate, rate of acrosome defects, rate of detached heads, rate of tail defects and proAKAP4 concentration. Concerning the total motility, no significant difference was found between the UR and fresh groups. There was no significant difference in the progressive motility between the UR 24h group (32.8% ± 22.8) compared to the fresh groups (Fresh 0h [49.7% ± 17.2] and Fresh 24h [56.3% ± 22.7]), but significantly lower progressive motility was found in the UR 0h group [14% ± 10.4] compared to both Fresh 0h and 24h. However, significantly lower total and progressive motility in VF groups (<10%) were found compared to both fresh and UR groups. Regarding the morphology analysis, UR and VF resulted in significantly lower rate of normal morphology than that of Fresh (19.4 ± 7.5, 29.4 ± 9.8 and 44 ± 11.8, respectively). A significant effect of the freezing method was also observed in acrosome defect rate (13% ± 7.8; 26% ± 12.8 and 29.7 ± 16.4 in Fresh, UR and VF, respectively). In case of detached head and tail defects, in addition to incubation, the freezing method had no effect. Assessing the proAKAP4 level, higher overall concentration was found in fresh and VF groups than that of UR group, which resulted in higher proportion of excellent (100%, 17% and 63.6% in Fresh, UR and VF, respectively) quality samples. We found that total motility after 24 hours storage and ultrarapid freezing was not statistically different from fresh samples. Despite the low motility,vitrification can preserve the high proAKAP4 concentration, which is a key factor in semen quality. However, more studies are needed to clarify this finding. These data indicate that one day storage and cryopreservation of epididymal sperm of a suddenly dead male can be a feasible method for fertility preservation, providing suitable samples for genetic conservation.

Project no. 134887 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the FK_20 funding scheme. This study was supported by the strategic research fund of the University of Veterinary Medicine Budapest (Grant No. SRF-001.).

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Canine preantral follicle cryopreservation: comparison of slow freezing, open and closed vitrification.

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There is an increased interest and practical potential in the application of ovarian preantral follicles (PAF). Follicle in vitro culture (FIVC) could provide access to high numbers of oocytes which can be matured and fertilized in vitro. Moreover, cryopreservation (CP) of PAFs can be efficient in mammalian gene preservation, regardless of the age or reproductive phase. The aim of our study was to find the best method to cryopreserve canine preantral follicles.

Ovaries were collected from ovariohysterectomized bitches (N= 20; undefined crossbreds) being in different ages. Ovariohysterectomies were carried out on multiple days (10 independent culture trials). After collection, ovaries were placed in sterile 50 ml centrifuge tubes, containing PBS + 10% bovine serum. Samples were stored at room temperature and delivered to the laboratory within 2 hours. The ovarian cortex of each ovary was sliced to approximately 1 mm^2 pieces with a surgical blade, then placed in digestive solution (HEPES-modified Medium 199 + 3 mg/ml collagenase) and incubated for 90 minutes at 37 °C. Following the enzymatic digestion, preantral follicles were isolated manually with 28G needles attached to 1 ml syringes. After isolation, morphologically normal secondary follicles were selected and randomly divided into four groups: fresh control (FR), slow freezing (SF), open vitrification (with open pulled straw; OPS) and closed vitrification (with cryotube; CT).

Live cell rate of follicles was analyzed immediately after isolation (FR) or thawing (SF, OPS and CT). Then, PAFs of each group were cultured in vitro for 10 days in 20 µl drops of FSH-supplemented medium (OptiMEM) at 38.5 °C and 6.5% CO2. Survival rate, area change, and estradiol production were examined. Live cell rate (determined by calculating calcein-AM positive cells) of follicles was similar to FR (83.6%±17.6) in OPS (80.3%±23.5) and SF (93%±3) groups, instead of CT, where significantly lower rate was found (58.7%±28). PAF survival rate during the IVC was lower in all of the cryopreserved groups than in fresh (82.2%, 85.2% and 37.5% in CT, OPS and SF, respectively; vs. 98.4% in FR). Fresh follicles showed continuously increasing area and estradiol production from Day 2 to 10 of IVC, while PAFs of OPS and SF increased their size until Day5. CT stopped their growth after Day2. Estradiol production was elevated continuously in FR and OPS throughout the IVC period (41.6 to 105 pg/ml, and 37.4 to 42.4 pg/ml, respectively; from Day2-10, median values), while remained unchanged in CT and SF follicles (28.2 to 27.9 pg/ml, and 27.49 to 31.9 pg/ml, respectively).

Our data show that open vitrification is superior to other cryopreservation methods to preserve canine isolated PAFs, however, refinement of the system is needed.

Project no. 134887 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the FK_20 funding scheme. This study was supported by the strategic research fund of the University of Veterinary Medicine Budapest (Grant No. SRF-001.).

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Comparison of dimethyl sulfoxide and propylene glycol for vitrifying in vitro matured bovine oocytes: effects on meiotic spindle and ROS levels

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Ethylene glycol (EG) and dimethyl sulfoxide (DMSO) have been established as cryoprotectants (CPAs) for vitrification/warming of bovine *in vitro* matured (IVM) oocytes (García-Martínez *et al*, Theriogenology **184**, 110-23, 2022). Given DMSO's cytotoxicity, propylene-glycol (PG) has been proposed as an alternative CPA. This study examined the effects of vitrifying IVM bovine oocytes with PG or DMSO combined with EG on spindle morphology and reactive oxygen species (ROS) production after warming. Oocytes were IVM for 21h and vitrified using *in silico* designed protocols: 7.5%DMSO-7.5%EG for 2min30sec and 15%DMSO-15%EG+0,5M sucrose for <1min (VIT-DMSO) or 7.5%PG-7.5%EG for 1min35sec and 15%PG-15%EG+0.5M sucrose <1min (VIT-PG). Oocytes were warmed and allowed to recover for 3 additional hours. Fresh, non-vitrified IVMoocytes served as control and IVM oocytes exposed to vitrification/warming solutions but non-vitrified/warmed served as controls for CPA cytotoxicity (CPA-DMSO or CPA-PG). Spindle morphology was assessed at 24h of IVM. Intracellular ROS levels were quantified by labeling with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and assessed after warming (0h) and at 24 h of IVM (3h). Relative ROS levels was used as a variable measure to classify intensity levels. All procedures were performed as described in García-Martínez *et al*. (Int J Mol Sci, **21**, 7547, 2020). Data were statistically analyzed with GraphPad Prism; Shapiro–Wilk was used for testing normality, Levene's test for homogeneity of variance followed by one-way ANOVA or a Kruskal-Wallis test ($p<0,05$). Oocytes vitrified with EG+PG showed similar percentages of oocytes reaching the MII stage (VIT-PG:78.6%±1.7 n=70) than the control fresh group (Control: 81.3%±1 n=123) while the other groups showed significantly lower percentages .(CPA-PG:62.7%±2.3 n=59; CPA-DMSO: 71.9%±1.9 n=57; VIT-DMSO: 77.4%±1.2 n=62). Although lower ($P<0.05$) than the Control fresh group (80%±0.7 n=123), no differences in percentages of normal spindle configuration were observed among vitrified groups (VIT-DMSO: 75%±1.8 n=62; VIT-PG:74.6%±2.1 n=70). Exposure to EG+DMSO resulted in lower ($p<0.05$) percentages of normal spindle configuration (CPA-DMSO: 73.2% ±2.3% n=57) compared to the control group, but significantly higher percentages were observed after exposure to EG+PG (CPA-PG: 89.2%±0.8 n=59). At 0h post-warming, vitrification with EG+DMSO resulted in higher relative ROS levels (12±0.9 n=56) than at 3h post-warming (6.1±0.1 n=52). No differences in relative ROS levels were observed in the VIT-PG group (0h: 6.5±0.2 n=42; 3h: 6.8±0.2 n=43) or CPAs groups (CPA-DMSO 0h:7.2±0.3 n=39; 3h:6.4±0.1 n=44; CPA-PG 0h:6.6±0.2 n=27; 3h:5.9±0.1 n=35). Results showed that vitrifying IVM bovine oocytes with EG+PG had no effect on spindle morphology when compared to the use of EG+DMSO, but it did reduce ROS levels after warming. These findings highlight PG's potential as a CPA, but further research on embryo development after vitrification of IVM bovine oocytes with EG+PG is required to confirm the efficiency of this CPA.

Study supported by projects PID2020-116531RB-I00 and 2021 SGR 00900.

Support Biotechnologies: Diagnosis through Imaging, Molecular Biology, and "OMICS"

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Utilizing ultrasonographic echotexture as a diagnostic tool to assess postpartum uterine endometrial involution

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The present study aims to utilize computer-assisted ultrasonographic echotexture analysis (BS200 Pro® Image processing and analyses software, BAB, Ankara, Turkey) as a diagnostic method to evaluate the histological involution of the uterus in postpartum cows. The study included a total of 27 cows from three different breeds (Holstein, Brown Swiss, and Simmental) and varying parities (1st, 2nd, and 3rd). The breed and parity factors were added to the statistical design. The study timeline was divided into eight distinct periods ranging from day -15 (pre-partum) to day 42 (postpartum). The researchers employed ultrasound measurements to assess the cervical (CD) and uterine horn diameters (UHD), and they collected ultrasonographic images of the uterine endometrium on different days for echotexture analysis. These images were analyzed using a computer-assisted echotexture program, which measured various parameters including mean gray level (MGL), gradient (GR), homogeneity (HOM), and contrast (CON) values. Throughout the study, clinical assessments and pH measurements of vaginal discharges were conducted on the same day intervals. Additionally, on days 21, 28, 35, and 42, samples from the endometrium were taken for cytological examination. Blood samples were collected from the cows on all study days to determine serum calcium, phosphorus, and magnesium levels. Blood beta-hydroxybutyric acid (β-HBA) levels were measured on days 7 and 21. The study found that MGL, GR, HOM, CON, CD, and UHD of the cows exhibited significant variations on different study days. In healthy cows, MGL and GR levels decreased from day 1 to day 21, and then increased until day 42. Problematic cows showed differences in MGL, GR, HOM, and CON values on various examination days. Comparing the overall average of all days, healthy cows had lower HOM, CD, and UHD, while they had higher MGL, GR, and CON values when compared to problematic cows. The study established ROC cut-off values of GR (12.01), HOM (0.055), and CON (37.19) on day 35 of postpartum for this purpose. In conclusion, the research indicates that computer-assisted ultrasonographic echotexture analysis may serve as a valuable tool for evaluating endometrial uterine involution in postpartum cows. This could help distinguish between a healthy and problematic uterine endometrium, ultimately impacting the success rates of first insemination in postpartum cows.

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Effect of heat stress on proteome and transcriptome of in vitro cultured bovine oviductal epithelial cells.

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Heat stress is known to reduce fertility of cattle. Increased body temperature affects oocyte and embryo development directly or indirectly through alterations of the maternal reproductive tract. Heat stress-induced modifications of the oviductal microenvironment may disturb embryonic development and cause embryonic death. This study aimed to identify short-term heat stress-induced changes in bovine oviductal epithelial cells (BOECs) and their secretory profiles under *in vitro* conditions. Oviducts ipsilateral to the corpus luteum at Day 1 to 3 of the oestrous cycle were collected at a slaughterhouse and transported to the laboratory on ice. The oviducts were gently squeezed using tweezers and recovered BOECs of each oviduct were divided into three groups at a concentration of 10^6 cells/ml and cultured for 24 hours in DMEM/F-12 medium (Gibco 21041-025) supplemented with 0.1% FCS. The first group was incubated for 12 hours at 40.5°C followed by 12 hours at 38.5°C (HS12 group), the second group was incubated for 24 hours at 40.5°C (HS24 group) and the third group was incubated at 38.5°C for 24 hours (control group). After 24 hours, BOECs ($2x10^6$) and conditioned media (2 ml) were collected separately and frozen. In total, 9 samples of BOECs (3 of each group) were submitted for RNA sequencing to detect changes in their gene expression profile. In each group (n=3), the protein of the conditioned media (n=7) was purified, and 10 µg were analysed using nanoLC-MS/MS to identify secreted proteins. Approximately 20,000 genes were analysed with RNA-Seq and the expression profiles were compared between groups. Heat-treated groups HS12 and HS24 showed differences in the mRNA expression of eleven genes compared to the control group, each after Bonferroni correction. Subsequently, genes which were highly significantly differentially expressed in RNA-Seq were selected for validation by qPCR. Various heat shock proteins, e.g., HSPA6, HSPA1A, and HSPH1, were found up-regulated in the HS24 group. Using mass spectrometry, 600 to 900 proteins could be identified in the conditioned media. The protein composition was analysed using ANOVA. Overall, only a few variations were found in the HS24 group and even less in the HS12 group. These proteins with tendentially different expressions can be assigned to the functional groups chaperone binding, stress response, growth factor binding and protein folding. In conclusion, BOECs cultured *in vitro* showed signs of heat stress in the transcriptome with clearly upregulated heat stress genes in the HS24 group, but less clear results in the HS12 group. In the protein secretome profile, few effects of heat treatment were evident. Further analysis of data is planned to identify proteins with potential influence on early embryo development.

This study was funded by the Gesellschaft für Forschungsförderung NÖ, FTI-Call 2020 (FTI20-012).

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Integrative analysis of microRNA and mRNA transcriptomes revealed dysregulation of TGF-beta signaling pathway in SOPS-vitrified porcine blastocysts

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Vitrification is the only effective method to cryopreserve pig embryos. The superfine open pulled straw (SOPS) method provides excellent in vitro viability post warming with blastocysts derived in vivo. However, higher pregnancy loss has been observed after transfer of vitrified embryos when compared to transfers with fresh embryos. The aim of this study was to investigate how SOPS vitrification of pig blastocysts affects the expression profile of microRNA (miRNA) transcriptome, as well as its relation to changes in the expression of target genes (TGs) in the vitrified blastocysts. In vivo derived porcine blastocysts were collected surgically and vitrified using the SOPS method (n = 60) as described before (Cuello et al., Sci Rep 6:33915, 2016). Embryos were cultured in vitro for 24 h after warming. Fresh blastocysts (n = 60) cultured for 24 hours served as controls. After in vitro culture, five pools of eight viable blastocysts from each group were prepared for analysis based on a microarray approach for miRNA (GeneChip miRNA 4.1 Thermo Fisher Scientific) and mRNA (GeneChip® Porcine Genome Array, Affymetrix) expression. Then, an integrative analysis of miRNA and mRNA transcriptomes data were performed with the Transcriptome Analysis Console 4.0.2 software. Biological interpretation of data was investigated using the Partek Genomics Suite and Pathways software. A threshold of 1.5-fold change and $p<0.05$ was used to identify differentially expressed (DE) miRNAs and DE TGs. Survival after 24 h of in vitro culture was similar for vitrified blastocysts (96.7%) and the controls (100%). The vitrified blastocysts had 94 (one upregulated and 93 downregulated) DE miRNAs compared with the controls, one of them (miR-503) was annotated for Sus scrofa. The altered miRNAs identified in this study were related mainly to cell proliferation, apoptosis, and the response to cell stress. Microarray analysis showed 210 (44 downregulated and 166 upregulated) DE genes in vitrified blastocysts compared to the control group. A total of 27 DE genes were found to be TGs regulated by the DE miRNAs identified in this study. Gene Ontology term analysis revealed that the DE TGs were associated mainly with biological processes such as reproductive process, cell population proliferation and growth. Some of these TGs were significantly involved in the TGF-beta signaling pathway, which is essential for embryo development, implantation and placentation. Dysregulation of miR-548a-3p and miR-4685-5p was also observed in the vitrified blastocysts. These miRNAs were associated with the overexpression of the ZFP36L1. This DE TG also plays a key role in embryonic development. In summary, vitrification via the SOPS system dysregulates miRNAs. Further studies are needed to clarify the consequences of dysregulation of miRNAs and TGs involved in the TGF-beta and the potential impact of this dysregulation in implantation and pregnancy. Supported by MCIN/AEI/10.13039/501100011033 and ERDF (RTI2018-093525-B-I00), Spain; Seneca Fundation (19892/GERM/15), Spain.

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Imaging mitochondrial Hydrogen Peroxide in bovine oocytes and early embryos using a novel ratiometric sensor: a preliminary study

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Reactive oxygen species (ROS) are a wide range of molecules known to trigger oxidative stress (OS). OS is one of the main factors impairing embryo quality when oocyte maturation, fertilization, and early embryogenesis are performed in vitro. Nevertheless, the role of ROS as key regulators of physiological mechanisms is also widely accepted, leading to the general assumption that excessive ROS suppression could alter cellular functions (Tsunoda, Reprod Med Biol 2014;13:71). A better understanding of the role of each ROS is needed to modulate culture systems to improve reproductive efficiency. An issue in this type of studies is the limited ability to track and quantify fluctuations of each ROS with high resolution in space and time (Sies, Nat Rev Mol Cell Biol, 2022; 23:499). To start to address this issue, we tested the suitability of a novel ultrasensitive fluorescent ratiometric sensor (Hyper7), capable of monitoring H_2O_2 fluctuations in vivo (Pak, Cell Metabolism 2020; 31:642), to detect changes in H_2O_2 levels in bovine oocytes and zygotes, as previously done in the Xenopus (Han, Cell Rep 2018; 22:21). In our experiments, mRNAs encoding the mithocondria (mt)-targeted Hyper7 was microinjected into immature oocytes or in vitro matured ones. The microinjected oocytes were then in vitro matured or fertilized. Preliminary trials revealed that micromanipulation per se did not severely impair the ability of the oocyte to mature, or to be fertilized and reach the blastocyst stage. To validate the sensor, a total of 172 oocytes and 154 zygotes were included in the study. Samples were imaged under control conditions or in the presence of a prooxidant challenge (tert-Butyl hydroperoxide). Imaging was conducted at the NOLIMITS microscopy facility of the University of Milan, using spinning disk microscopy equipped with a temperature-controlled CO_2 chamber and appropriate lasers and filters every 30 seconds. Image analysis was conducted after background subtraction to calculate the ratio value of the oxidized versus the reduced form of the sensor as previously described (Han, Cell Rep 2018; 22:21). Data analysis showed that the sensor can detect H_2O_2 in oocytes and zygotes. However, under control conditions, a significant increase was observed after 20 minutes (Friedman test), suggesting phototoxicity as a causal factor during imaging. Nevertheless, both oocytes and zygotes treated with prooxidants exhibited higher values than those in the control group, confirming the sensors' ability to quantify elevated H_2O_2 levels (Two-way ANOVA). On the other hand, additional studies conducted on 75 zygotes revealed that high concentration of a mix of antioxidants (acetyl-L-carnitine, N-acetyl-L-cysteine, and α-lipoic) did not reduce mt-H2O2 but significantly increased its production, suggesting some paradoxical effects. Our study marks the first application of Hyper7 in mammalian oocytes and zygotes, while posing the critical need to manage phototoxicity in subsequent research. Funded by H2020 MSCA-ITN-ETN n.860960 (EUROVA); SEED2019 UNIMI N.1250 (cROSs-Talk) & Piano di Sostegno alla Ricerca: Linea 2 – Azione A (Molecular and structural responses to stressors in different cells and tissue models).

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Colostrum as an indicator for better reproductive performance in dairy cow

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Effective donor cow selection is essential in implementing assisted reproduction in the herd. There is much debate about how to measure cow immunity because it is closely related to cow productivity, health and reproductive performance. The work aimed to analyze the immunoglobulin G (IgG) level in cow colostrum in relation to the parameters of subsequent lactation. The study involved the analysis of various parameters such as 305 days (305P) and total lactation productivity (TP), milk fat (MF), milk protein (MP), somatic cell count (SCC), service (SP) and inter-calving period (ICP), artificial insemination times per pregnancy (AIPP). The IgG level was measured immediately after calving using a Digital Brix Refractometer (*Spectrum Technologies Inc*.). Recording data from the farm management system was used. The research was conducted on a herd of 600 dairy cows with an average milk yield of approximately 10 thousand per cow per year. It was a free stall-keeping system where cows received a well-calculated total mixed ration twice a day, and water was always available. The data was analyzed using *SPSS 21*, and statistical significance was determined using the Mann-Whitney test with a *p*-value less than 0.05. All cows were divided depending on IgG level in the colostrum: minimal level (23% and less, n=58), optimal (more than 23%). The study's results revealed that the IgG level in milk was 22-30%, and it was not affected by seasonal variations or twins ($p>0.05$). IgG in colostrum was not an essential factor regarding 305P, TP, MF, MP and SCC ($p>0.05$). The SP (110.2±40.39 vs 92.5±31.56 days) and ICP (395.1±41.16 vs 373.7±32.01 days) were longer ($p>0.05$) in cows with minimal (23% and less) IgG, and these cows had more AIPP (2.4±1.17 vs 1.4±0.67, $p<0.05$). A statistically significant positive correlation was detected between IgG in milk and the number of cow lactation ($r=0.42$, $p<0.05$), but a mild negative correlation between IgG level and AIPP ($r=-0.45$, $p<0.05$). In conclusion, the level of IgG in colostrum could help reveal cows with a more stable immune system, higher fertility, shorter service and inter-calving period. These cows might be more suitable for the role of donor cows, but the correlation of colostrum IgG levels with ovarian functionality indicators remains to be investigated.

This work was supported by the EAFRD project No. 22-00-A01612-000008.

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Case report - producing embryos from euthanised or slaughtered cattle

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In vitro production (IVP) of bovine embryos does not necessarily need to be started with cumulus-oocyte-complexes (COCs) of living animals. Especially for research, lots of oocytes are mandatory. Therefore, IVP with COCs of slaughtered cattle has been used for decades. In some cases, cattle with a high individual value to their owners (economic or emotional) have to be euthanized or slaughtered due to illness or declining production. If a "final" reproduction of these animals is planned, ovaries can be gained after slaughter or before euthanasia.

Ten cases of post-mortem IVP are reported here in a German IVP lab for cattle from 2020 to 2024. The animals belonged to the following breeds: Brown-Swiss (2), Galloway (3), Holstein Friesian (3), Simmental (1) and Wagyu (1), aged 2 up to 17 years. The last calving was between 2 and 18 months before IVP. The reason for slaughtering or euthanasia were fertility problems in 6 of 10 cases, for example oophoritis, endometritis or subfertility. Orthopedic diseases like septic arthritis, pelvis fracture and tearing of the adductors happened to the other four animals. The diseases endured between three days and one year. Six animals were slaughtered and four ovariectomies were performed before euthanasia. Transport of the ovaries in saline (0.9 % sodium-chloride or phosphate buffered saline with heparin) took 72 minutes in average (5 up to 210 min). In most cases, seven out of ten, the ovaries showed few (at most 2) or no follicles and/or corpora lutea on the surface compared to ovaries of healthy, cyclic animals. After slicing between 13 and 120 COCs could be collected, quality grades 1-2, considered as IVF suitable (5th Edition IETS Manual), and 3-4 were on average equally distributed with 28.4 and 25.0 COCs, respectively. A standard IVP-protocol was performed up to day 7 of IVC (IVF = day 0), and cleavage and developmental rates were recorded. Between 3.8 and 84.4% of the presumptive zygotes cleaved to embryos (average 41.5%) and developmental rates were between 0 and 18.5% (average 5.9%). On average 2.5 transferable embryos were produced per animal, but this success varied because in four out of the ten cases no embryo and in one case 12 embryos were obtained. By grading the embryos, stages of morulae as well as early, blastocysts and expanded blastocysts were detected. In the four non-serving cases reproductive diseases (oophoritis, subfertility), a very long calving interval (18 months) as well as a short one (2 month) might have had an impact on the result, possibly related to a low COCs count and/or being of poor quality (grade 3 and 4). In two cases transport of the ovaries to the lab endured longer than 180 min. Duration of the diseases as well as severity of clinical signs were not clearly related to IVP failure in our observations.

In summary, reproductive diseases were the main reason for slaughtering or euthanasia of the animals presented here and this, as well as too long or short calving interval and/or long transport could be the reason for unsuccessful IVP cycles. On the other hand, in six out of ten cases transferable Embryos were produced as a final chance of reproduction for these animals.

Workshop:

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