Optimization of Protocols for In Vitro Embryo Production Using Oocytes Derived from Juvenile Animals

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“You’re going to reap just what you sow”

- Lou Reed

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ABSTRACT

Using prepubertal animals as oocyte donors offers huge potential for increasing the rate of genetic gain by shortening the generational interval in livestock. This can be accomplished combining two technologies: laparoscopic ovum pickup (LOPU) to recover cumulus-oocyte complexes (COCs), and in vitro embryo production (IVEP) to produce embryos from those COCs. However, compared to adult animals, oocytes from prepubertal donors have a low developmental competence following IVEP. Therefore, providing oocytes with optimal conditions to promote the acquisition of developmental competence is crucial for the success of prepubertal LOPU-IVEP programs. This can be accomplished both in vivo, inside the follicle using gonadotropin stimulation, and in vitro during in vitro maturation (IVM). As such, the objective of this thesis was to: 1) optimize in vitro maturation using antioxidants and growth factors in swine; an excellent model for prepubertal oocytes due to the high incidence of polyspermy in this species, 2) optimize a gonadotropin stimulation protocol in Mediterranean water buffalo heifer calves; an excellent candidate for prepubertal reproductive technologies due to their long generational intervals, and 3) assess other exogenous factors that can influence LOPU-IVEP results in prepubertal water buffalo, including season, age, individual variation and bull-effect. In Chapter 4, we found that supplementing porcine IVM medium with follicular fluid and the growth factors: FGF2, IGF1 and LIF, collectively known as FLI, was able to significantly improve embryo development rates (26.6% vs. 12.5%). Second, we found that the supplementation of IVM with cysteine, was essential for embryo development. In Chapter 5, we found that combining FSH stimulation with eCG, 24 hours prior to LOPU was able to significantly improve embryo development rates in water buffalo calves (20.5 vs. 9.0%). Second, we found that using FSH reconstituted in a slow-release formulation of hyaluronan was able to deliver similar results to regular FSH, in addition to being less stressful for the animals and labour intensive. Third, we found that a 4-day stimulation length resulted in the most transferrable embryos per donor per LOPU (2.70 ± 0.5) compared to a 3- and 5-day treatment (1.94 ± 0.6 and 2.25 ± 0.5, respectively). In Chapter 6, we found that despite being a seasonal species, LOPU-IVEP in prepubertal water buffalo was not affected by season. However, both individual variation among donor animals and the choice of bull used for IVF had a significant impact on results. Collectively, experimental results reported in this thesis demonstrated that porcine in vitro embryo production, based on the use of abattoir-derived oocytes collected from

the ovaries of young gilts at near-puberty ages, can be improved by supplementing the IVM medium with growth factors and antioxidants, and enabled identification of factors affecting LOPU-IVEP efficiency in prepubertal water buffalo. With the implementation of the LOPU-IVEP protocols, it was possible to produce transferrable embryos in prepubertal water buffalo at rates comparable to, if not better than the results reported for OPU-IVEP in adult animals, which holds great promise for accelerated genetic gain and assisted breeding programs in this species.

RÉSUMÉ

L'utilisation des animaux prépubères comme des donneurs d'ovocytes offre un potentiel extraordinaire d'augmenter le taux de progrès génétique en raccourcissant l'intervalle générationnelle. Ceci peut être réalisé en combinant deux technologies : le prélèvement d'ovules par laparoscopie (POL) pour récupérer les complexes cumulus-ovocytes (CCOs) et la production d'embryons in vitro (PEIV) pour produire des embryons à partir de ces CCOs. Cependant, par rapport aux animaux adultes, les ovocytes provenant de donneuses prépubères ont une faible compétence de développement après le PEIV. Par conséquent, fournir aux ovocytes des conditions optimales pour favoriser l'acquisition de la compétence de développement est crucial pour le succès des programmes POL-PEIV prépubertaires. Ceci peut être réalisé in vivo dans le follicule en utilisant la stimulation des gonadotrophines et in vitro pendant la maturation in vitro (MIV). L'objectif de cette thèse était ainsi de : 1) optimiser la maturation in vitro en utilisant des antioxydants et des facteurs de croissance chez les cochons; un excellent modèle pour les ovocytes prépubères en raison de l'incidence élevée de la polyspermie de cette espèce, 2) optimiser un protocole de stimulation des gonadotrophines chez les veaux génisses de buffles d'eau méditerranéens; un excellent candidat pour les technologies de reproduction prépubères en raison de leurs longs intervalles générationnels et 3) évaluer d'autres facteurs exogènes comme la saison, l'âge, la variation individuelle et l'effet taurau qui peuvent influencer les résultats de la POL-PEIV chez les buffles d'eau prépubères. Au chapitre 4, nous avons constaté que le fait de compléter le milieu MIV porcin avec du liquide folliculaire et des facteurs de croissance (FGF2, IGF1 et LIF (FLI)), a permis d'améliorer significativement les taux de développement embryonnaire (26,6 % contre 12,5 %). Deuxièmement, nous avons constaté que la supplémentation du MIV avec de la cystéine, était essentielle pour le développement de l'embryon. Au chapitre 5, nous avons constaté que la combinaison de la stimulation de la FSH avec l'eCG 24 heures avant la POL permettait d'améliorer de manière significative les taux de développement embryonnaire chez les veaux buffles d'eau (20,5 contre 9,0 %). Deuxièmement, nous avons constaté que l'utilisation de la FSH reconstituée dans une formulation à libération lente de hyaluronan donnait des résultats similaires à ceux de la FSH ordinaire, en plus d'être moins stressante pour les animaux et moins exigeante en main-d'œuvre. Troisièmement, nous avons constaté qu'une stimulation de 4 jours permettait d'obtenir le plus grand nombre d'embryons par donneur et par POL (2,70 ± 0,5) par rapport à un traitement de 3 et 5 jours (1,94 ± 0,6 et 2,25 ± 0,5, respectivement). Dans le chapitre 6, nous avons

constaté que, bien qu'il s'agisse d'une espèce saisonnière, la POL-PEIV chez le buffle d'eau prépubère n'était pas affectée par la saison. Cependant, les variations individuelles entre les animaux donneurs et le choix du taureau utilisé pour la FIV ont eu un impact significatif sur les résultats. Collectivement, les résultats expérimentaux rapportés dans cette thèse ont démontré que la production in vitro d'embryons porcins, basée sur l'utilisation d'ovocytes issus de l'abattoir et collectés à partir des ovaires de jeunes cochettes à un âge proche de la puberté, peut être améliorée en complétant le milieu MIV avec des facteurs de croissance et des antioxydants et ont permis d'identifier les facteurs affectant l'efficacité de la POL-PEIV chez les buffles d'eau prépubères. Grâce à la mise en œuvre des protocoles POL-PEIV, il a été possible de produire des embryons transférables chez des bufflonnes d'eau prépubères à des taux comparables, voire supérieurs, aux résultats rapportés pour POL-PEIV chez les animaux adultes, ce qui est prometteur pour les programmes de gain génétique accéléré et de reproduction assistée chez cette espèce.

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It has been a wild ride, and there's no way I would have arrived where I am now, putting the finishing touches to my thesis, without the immense support and help of those around me. Nothing presented in this thesis was achieved on my own, it was a team effort. I will forever be grateful to everyone who contributed.

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guidance over the years, for being a part of my committee, and asking the difficult questions. You really did make a difference.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis aimed to investigate and optimize in vitro embryo production using oocytes sourced from juvenile livestock and characterize the exogenous factors that can affect LOPU-IVEP efficiency.

The literature review of this thesis (Chapter 2) entitled "In Vitro Production of Embryos from Prepubertal Holstein Cattle and Mediterranean Water Buffalo: Problems, Progress and Potential" was published in the journal "Animals" in August 2021. It is referenced throughout the thesis and reviews the current state of IVEP in prepubertal ruminants, problems facing the technology going forward, and suggests possible solutions to mitigate those problems. It was nominated for the journal's "2023 best paper award".

Chapter 4 of this thesis entitled "Optimizing Swine In Vitro Embryo Production with Growth Factor and Antioxidant Supplementation During Oocyte Maturation" was published in the journal "Theriogenology" in October 2022. We showed that the addition of the growth factors FGF2, LIF and IGF1 to a non-defined maturation medium containing pFF to be beneficial for early embryonic development. Furthermore, we showed that although cysteine was essential for development, the further addition of melatonin and/or ITS did not yield a significant advantage compared to cysteine alone.

Chapter 5 of this thesis entitled "Optimization of Gonadotropin Stimulation Protocols for In Vitro Embryo Production in Prepubertal Mediterranean Water Buffalo" was published in the journal "Theriogenology" in November 2022. The first large-scale study in Mediterranean water buffalo of its kind, we were able to successfully produce viable embryos at rates which exceed those in adult animals. We also showed that FSH could be reconstituted in hyaluronan and used with prepubertal buffalo for the first time, with a stimulation period of 4 days being optimal.

Chapter 6 of this thesis entitled "Factors Affecting the Efficiency of In Vitro Embryo Production in Prepubertal Mediterranean Water Buffalo" was published in the journal "Animals" in December 2022. In this first of its kind study, we showed that although season and age did not have a large impact on LOPU-IVEP results, individual variation and the choice of bull used for IVF did. We also announced the world's first live buffalo calves born from vitrified embryos produced from prepubertal buffalo.

PREFACE AND CONTRIBUTION OF AUTHORS

This thesis was written in a manuscript-based format following guidelines set forth by the Department of Graduate and Postdoctoral Studies of McGill University. The thesis was written by Luke Currin with editorial support from Vilceu Bordignon and Hernan Baldassarre.

The literature review (Chapter 2) entitled "In Vitro Production of Embryos from Prepubertal Holstein Cattle and Mediterranean Water Buffalo: Problems, Progress and Potential" was published in the journal "Animals" in August 2021 (https://doi.org/10.3390/ani11082275). Luke Currin wrote the review manuscript in collaboration with Vilceu Bordignon and Hernan Baldassarre who contributed to the entire manuscript preparation, including editorial support.

The first research manuscript (Chapter 4) entitled "Optimizing Swine In Vitro Embryo Production with Growth Factor and Antioxidant Supplementation During Oocyte Maturation" was published in the journal "Theriogenology" in October 2022 (https://doi.org/10.1016/j.theriogenology.2022.10.005). Luke Currin was responsible for all experiments, data collection, analysis and writing the manuscript. Werner Giehl Glanzner, Karina Gutierrez, Mariana Priotto de Macedo and Vanessa Guay helped with the investigation. Vilceu Bordignon and Hernan Baldassarre designed the experiments, helped with data analysis and edited the manuscript.

The second research manuscript (Chapter 5) entitled "Optimization of Gonadotropin Stimulation Protocols for In Vitro Embryo Production in Prepubertal Mediterranean Water Buffalo" was published in the journal "Theriogenology" in November 2022 (https://doi.org/10.1016/j.theriogenology.2022.11.043). Luke Currin was responsible for all experiments, gonadotropin stimulation, embryo production, data collection and analysis, as well as writing the manuscript. Mariana Priotto de Macedo, Werner Giehl Glanzner, Karina Gutierrez, Katerina Lazaris, Zigomar da Silva, Vanessa Guay and Maria Elena Carrillo Herrera helped with taking care of the animals, LOPU and data collection. Caitlin Brown, Erin Joron and Ron Herron were responsible for recipient synchronization and pregnancy detection. Hernan Baldassarre and Vilceu Bordignon designed the experiments, helped with the animals, LOPU, data collection and analysis, as well as edited the manuscript.

The third research manuscript (Chapter 6) entitled "Factors Affecting the Efficiency of In Vitro Embryo Production in Prepubertal Mediterranean Water Buffalo" was published in the journal "Animals" in December 2022 (https://doi.org/10.3390/ani12243549). Luke Currin was

responsible for all experiments, gonadotropin stimulation, embryo production and vitrification, data collection and analysis, as well as writing the manuscript. Mariana Priotto de Macedo, Werner Giehl Glanzner, Karina Gutierrez, Katerina Lazaris, Vanessa Guay, Maria Elena Carrillo Herrera and Zigomar da Silva helped with taking care of the animals, LOPU and data collection. Caitlin Brown, Erin Joron and Ron Herron were responsible for recipient synchronization and pregnancy detection. Hernan Baldassarre and Vilceu Bordignon designed the experiments, helped with the animals, LOPU, data collection and analysis, as well as edited the manuscript.

LIST OF ABBREVIATIONS

AFC ..... Antral Follicle Count
AI ..... Artificial Insemination
AMH ..... Anti-Müllerian Hormone
ANOVA ..... Analysis of Variance
AR ..... Androgen Receptor
ART ..... Assisted Reproductive Technology
ATP ..... Adenosine 5'-Triphosphate
Aug ..... August
BSA ..... Bovine Serum Albumin
C ..... Celsius
cAMP ..... 3',5'-cyclic Adenosine Monophosphate
cc ..... Cubic Centimetre
cGMP ..... cyclic Guanosine Monophosphate
CIDR ..... Controlled Internal Drug Release
CNP ..... C-Type Natriuretic Peptide
CO_2 ..... Carbon Dioxide
COC ..... Cumulus Oocyte Complex
CYP19A1 ..... Aromatase
Cyst ..... Cysteine
DAPI ..... 4',6'-diamidino-2-phenylindole
dbcAMP ..... Dibutyryl cyclic Adenosine Monophosphate
Dec ..... December
DHEA ..... Dehydroepiandrosterone
DMSO ..... Dimethyl Sulfoxide
DNA ..... Deoxyribonucleic acid
eCG ..... Equine Chorionic Gonadotropin
EGA ..... Embryonic Genome Activation
EGF ..... Epidermal Growth Factor
EGFR ..... Epidermal Growth Factor Receptor
ER ..... Endoplasmic Reticulum

ET ..... Embryo Transfer
EV ..... Extracellular Vesicle
FBS ..... Fetal Bovine Serum
Feb ..... February
FGF2 ..... Fibroblast Growth Factor 2
FLI ..... FGF2, LIF, IGF1
FSH ..... Follicle Stimulating Hormone
FSH ..... Follicle Stimulating Hormone Receptor
g ..... Gram
G ..... Gravitational Force or Gauge
G1 ..... Grade 1
G2 ..... Grade 2
GnRH ..... Gonadotropin-Releasing Hormone
GPR54 ..... Kisspeptin Receptor
GSH ..... Glutathione
GV ..... Germinal Vesicle
GVBD ..... Germinal Vesicle Breakdown
h ..... Hours
hCG ..... Human Chorionic Gonadotropin
HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPO ..... Hypothalamic-Pituitary-Ovarian
HSD ..... Honestly Significant Difference
HSD3B1 ..... 3β-and steroid δ-isomerase 1
ICSI ..... Intracytoplasmic Sperm Injection
IGF1 ..... Insulin-like Growth Factor 1
IM ..... Intramuscular
IP_3R ..... Inositol Triphosphate Receptor
IT-20 ..... Ionomycin-TPEN-20 minutes
ITS ..... Insulin-Transferrin-Selenium
IU ..... International Units
IVC ..... In Vitro Culture

IVEP ..... In Vitro Embryo Production
IVF ..... In Vitro Fertilisation
IVM ..... In Vitro Maturation
IVM I ..... In Vitro Maturation 1
IVM II ..... In Vitro Maturation 2
JIVET ..... Juvenile In Vitro Embryo Transfer
Jun ..... June
KBW ..... Kilogram of Body Weight
Kp ..... Kisspeptin
LH ..... Luteinizing Hormone
LIF ..... Leukaemia Inhibitory Factor
LOPU ..... Laparoscopic Ovum Pickup
LOPU-IVEP ..... Laparoscopic Ovum Pickup – In Vitro Embryo Production
MAP5 ..... Hyaluronan
Mel ..... Melatonin
mg ..... Milligram
MII ..... Metaphase 2
miRNA ..... Micro Ribonucleic Acid
mL ..... Millilitre
mm ..... Millimetre
mM ..... Millimolar
mmHg ..... Millimetre of Mercury
MOET ..... Multiple Ovulation Embryo Transfer
mRNA ..... Messenger Ribonucleic Acid
mTBM ..... Modified Tris-Buffered Medium
n ..... Number
N_2 ..... Nitrogen Gas
ng ..... Nanogram
nM ..... Nanomolar
Nov ..... November
NPR2 ..... Natriuretic Peptide Receptor 2

O_2 ..... Oxygen Gas
OBMS ..... Out of Breeding Mating Strategies
OPU ..... (ultrasound-guided transvaginal) Ovum Pickup
OPU-IVEP ..... Ovum Pickup – In Vitro Embryo Production
PA ..... Parthenogenetically Activated
PBS ..... Phosphate-Buffered Saline
pFF ..... Porcine Follicular Fluid
PVA ..... Polyvinyl Alcohol
PZM-3 ..... Porcine Zygote Medium 3
RNA ..... Ribonucleic Acid
ROS ..... Reactive Oxygen Species
rpm ..... Revolutions per Minute
SCNT ..... Somatic Cell Nuclear Transfer
SD ..... Standard Deviation
SERCA ..... Serca/endoplasmic Reticulum Calcium ATPase
Sept ..... September
SNP ..... Single Nucleotide Polymorphism
StAR ..... Steroidogenic Acute Regulatory Protein
std ..... Standard
T ..... Temperature
TAI ..... Timed Artificial Insemination
TCM-199 ..... Tissue Culture Medium 199
TPEN ..... N, N, N', N'-tetrakis(2-pyridinylmethyl)-1, 2-ethanediamine
TUDCA ..... Tauroursodeoxycholic Acid
TZP ..... Transzonal Projection
µg ..... Microgram
µL ..... Microlitre
µm ..... Micrometre
UPR ..... Unfolded Protein Response
USP ..... United States Pharmacopeia
v/v ..... Volume/Volume

VS1 ..... Vitrification Solution 1

VS2 ..... Vitrification Solution 2

w/v ..... Weight/Volume

y.o. ..... Years old

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CHAPTER 1
INTRODUCTION

The wonderful thing about reproductive science is that it represents a fascinating intersection between many different scientific disciplines that do not normally overlap, covering everything from medicine, to conservation, to agriculture. For example, with rates of human infertility doubling since the 1980s, more couples are seeking assisted reproductive medicine than ever before [1]. In fact, approximately 2% of all infants born in the United States annually are now conceived using assisted reproductive technologies (ARTs) [2]. In conservation, Black-Footed Ferrets (*Mustela nigripes*) are a prime example of a species which has strongly benefitted from reproductive science [3]. Despite once occupying a large range covering a broad swath of Western North America, black-footed ferrets were thought to be extinct in the 1970s, until a small residual population was discovered in Wyoming in the 1980s [4]. The remaining 18 wild individuals were trapped and, thanks to a strong captive breeding program, including the development of artificial insemination (AI) and semen cryopreservation techniques for this species, black-footed ferrets have now been reintroduced to sites across Canada, the United States, and Mexico with multiple self-sustaining populations now present in the wild [3, 4]. This is an encouraging story considering their ecological importance in the food web, acting as an important predator of prairie dogs, an agricultural pest in some regions [5]. Finally, in agricultural science, despite in vitro embryo production (IVEP) barely being used commercially as recently as the 1990s, the majority of the more than 1.5 million cattle embryos transferred annually are now produced in vitro [6]. Thus, the use of reproductive biotechnologies in agriculture has become a particularly fascinating field, especially the biology of oocytes from prepubertal animals, as they serve as an excellent model for infertility research [7].

With the world population continuing to grow, and the impacts of global warming becoming more evident, the global food supply chain must become more sustainable and efficient. The demand for agricultural output will need to increase around 70% by 2050, compared to 2005 levels [8]. Therefore, assisted reproductive technologies (ARTs), including in vitro embryo production (IVEP), will play a pivotal role across many different agricultural sectors in the future. This thesis specifically focuses on swine and water buffalo reproduction, two critically important livestock species (Figure 1.1).

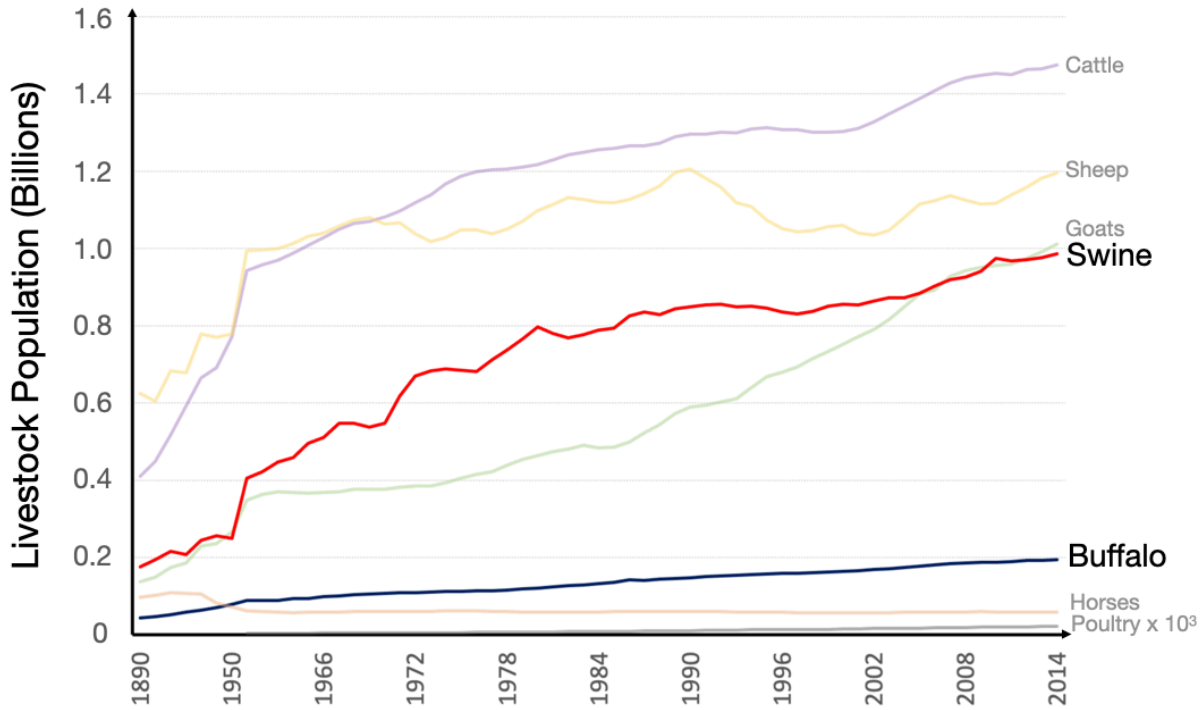

Figure 1.1. Global Population of the Main Livestock Species between 1890 and 2014. Total number of livestock measured as the number of live animals at a single point in any given year. Poultry are expressed as chickens + turkeys at a factor of 1000. Adapted using publicly available data from [9].

Swine (*Sus scrofa*) are a cornerstone of agricultural livestock production, especially in Quebec, Canada's largest swine producer and major international pork exporter [10]. In addition to their importance as a protein source in the human diet, domestic pigs have become an incredibly important model for biomedical research [11, 12]. Pigs are considered important animal models due to their anatomical, physiological, metabolic and genetic similarities to humans compared to mice [13]. For example, pigs are the leading candidate for the generation of organs suitable for xenotransplantation [14, 15]. Consequently, there are multiple breeds and transgenic strains of pigs available for research purposes across a wide array of applications [11]. For these reasons, the development of efficient and reliable protocols for IVEP of porcine embryos are crucial. However, compared to other livestock species, successful implementation of porcine in vitro fertilization (IVF) has been challenging [16, 17].

Despite their similar names and appearance, water buffalo (*Bubalus bubalis*) are a completely different species and only distantly related to African Cape Buffalo (*Synceris caffer caffer*) and American Bison (*Bison bison*), the latter of which is sometimes called buffalo. Water buffalo on the other hand, are fully domesticated and an incredibly important livestock species globally, with a world population of more than 200 million, more than 96% of which is concentrated in Asia [18]. However, the popularity of buffalo has grown outside of Asia in recent years, especially in South America, and to a lesser extent the United States and Canada, where dairies have recently been established [18]. Collectively, water buffalo produce around 10-15% of the world's milk production. Like dairy cattle, there are multiple breeds of water buffalo, with different breeds displaying different traits; there are three principal categories: Swamp, Riverine and Mediterranean buffalo (Table 1.1). Swamp buffalo are popular in Southeast Asia, partly due to their large hooves, which are ideal for draught in flooded rice paddies. Riverine buffalo are a diverse group of buffalo, comprising many different breeds, and are popular in equatorial regions due to their ability to thrive in hot climates. Finally, Mediterranean water buffalo are a distinct breed of Riverine buffalo that have been heavily selected for dairy production, most notably for the mozzarella cheese industry in Italy [18]. As such, buffalo products occupy unique niches at both ends of the economic spectrum, not only acting as an affordable protein source in developing countries, but also as expensive specialty products like *Mozzarella di Bufala Campana*.

Table 1.1. Comparison of Different Water Buffalo Types

	Swamp	**Riverine**	**Mediterranean**
Appearance	Lighter coloured compared to others, often with lots of grey.	Varied appearance	Dark black, moderate horns compared to Swamp
Chromosomal Number (2n)	48	50	50
Main Geographic Distribution	Southeast Asia, China	India, Pakistan, Brazil	Italy, Balkans, Europe
Important Uses	Draught and meat	Dairy and meat	Dairy, most notably for mozzarella cheese.
Characteristics	Fairly uniform	Multiple breeds, including Murrah	Distinct breed, heavy selection pressure

Despite the socio-economic importance of water buffalo herds worldwide, buffalo exhibit poor fertility, and successful implementation of assisted reproductive technologies has been challenging [19]. Reproductive efficiency in water buffalo is hampered by a variety of factors including the late onset of puberty and age at first calving, seasonality, extended post-partum anoestrous and calving intervals, as well as subdued estrus expression [20]. Compared to cattle, where a variety of ARTs are routinely used, most techniques yield poor results to their cattle counterparts [21, 22]. Although technologies designed to maximize the genetic potential of males are currently used commercially, tools designed for the propagation of female gene-lines are limited (Figure 1.2). For example, timed artificial insemination (TAI) using frozen conventional or sex-sorted semen is routinely used and a proven technology to improve genetic progress in buffalo [23]. In this way, the top genetics from elite bulls in Italy are available worldwide. However, technologies designed to maximize the proliferation of female genetics, including multiple-ovulation embryo transfer (MOET) and ultrasound-guided transvaginal ovum pickup (OPU) combined with in vitro embryo production (IVEP), have yielded poor results compared to cattle [23]. This is especially frustrating since buffalo mature very slowly, making rates of genetic gain very poor.

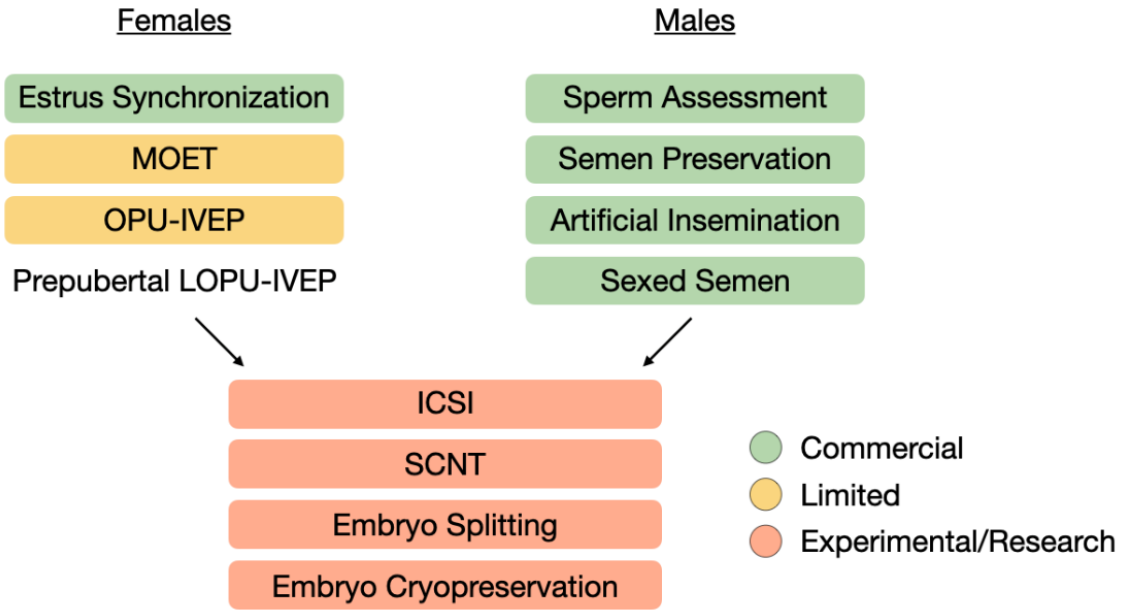

Figure 1.2. The use of assisted reproductive technologies in Water Buffalo. Note the limited options available for the propagation of female genetics. MOET and OPU-IVEP only has very limited use.

Producers always want to breed their best animals to create the best progeny. This has been done for thousands of years and is called selective breeding. Hence, over time, livestock can be bred to produce more milk or meat, be more resistant to diseases or convert feed more efficiently. For example, modern broiler chickens reach market weight in 1/3 the amount of time, all while consuming less feed compared to their historical counterparts [24, 25]. The same is true for Mediterranean water buffalo, with selective breeding used over many generations resulting in their unique phenotype and milk composition compared to other breeds of buffalo. The rate at which this progress is made is called the rate of genetic gain and is calculated using the following formula:

$$\frac{\Delta G}{t} = \frac{h^2 \; x \; S}{[GI]}$$

Where:

$\frac{\Delta G}{t}$ is the rate of genetic gain

h^2 is the heritability

S is the selection differential

$[GI]$ is the generational interval

In order to maximize the rate of genetic gain, as the generational interval (the elapsed time between generations) is a denominator, the shorter this interval is, the better. However, water buffalo mature very slowly, and although highly variable and dependant on many factors including breed, season, climate, and nutrition [19, 26-28], water buffalo generally reach puberty around two years of age, meaning heifers will typically only give birth to their first calf around 3 years of age [27, 29]. In fact, globally the average age at first calving in buffalo is around 36 months, although in Italy, for example, where buffalo are intensively managed, the average age at first calving has been reduced to less than 28 months [30, 31]. Consequently, although some improvements have been made through management with carefully curated nutrition and artificial selection, rates of genetic gain in buffalo remain slow. Therefore, prepubertal reproductive technologies, where embryos can be produced from animals as young as two months of age, hold great potential in water buffalo (Figure 1.3). As the specifics of how this is done is discussed in greater detail in Chapter 2, briefly, laparoscopic ovum pickup (LOPU) can be used to recover oocytes from gonadotropin-stimulated calves. These oocytes are then subjected to IVEP to produce embryos, which can then be transferred into adult recipient females [32, 33]. However, multiple studies in

multiple species have documented low embryo development rates using oocytes from prepubertal donor animals following IVEP [23, 34-42].

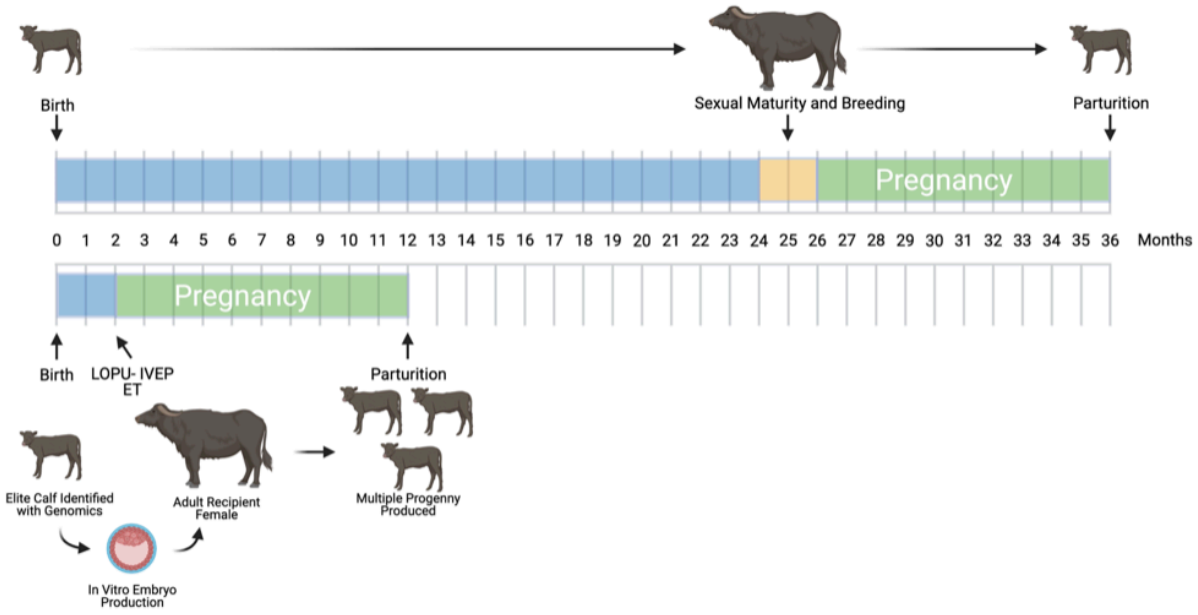

Figure 1.3. Shortening the generational interval in Mediterranean water buffalo using prepubertal LOPU-IVEP. Using this technique, multiple progenies from elite animals can be born before the donor animal reaches puberty. Figure created using BioRender.com.

In summary, water buffalo are an important livestock species; however, due to their slow-maturing nature, rates of genetic gain in this species are extremely low. The use of oocytes sourced from prepubertal ruminants for IVEP would significantly accelerate the rates of genetic gain. LOPU enables the recovery of these oocytes, however, multiple studies have demonstrated low embryo development rates following IVEP. Since oocyte competence is acquired sequentially in vivo, with full competence attained just prior to ovulation [43, 44], we believe oocytes from prepubertal oocytes require a specially tailored maturation process both in vivo inside the follicle prior to LOPU, and in vitro during maturation prior to IVF. Furthermore, given the novelty of prepubertal LOPU-IVEP in water buffalo, the impact that exogenous factors may have on its success is unknown. Therefore, the general objective of this thesis was to investigate the impact of pre-oocyte collection treatments, in vitro maturation conditions, and exogenous factors, on the development of embryos produced using oocytes derived from juvenile animals.

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CHAPTER 2
LITERATURE REVIEW

This literature review was published in the journal "Animals" in August 2021 (https://doi.org/10.3390/ani11082275)

In Vitro Production of Embryos from Prepubertal Holstein Cattle and Mediterranean Water Buffalo: Problems, Progress and Potential

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2.1. Simple Summary: In vitro embryo production using oocytes from prepubertal cattle and buffalo collected by laparoscopy can be used to produce embryos from genetically superior females. Following transfer of these embryos into adult recipient animals, multiple offspring can be produced from these elite animals in a very short timeframe, long before they reach sexual maturity, thereby reducing the generation interval and accelerating genetic gain. This review

article summarizes recent advances in this technology, outlines the current limitations, and suggests possible avenues to further improve this emerging biotechnology.

2.2. Abstract: Laparoscopic ovum pick-up (LOPU) coupled with in vitro embryo production (IVEP) in prepubertal cattle and buffalo accelerates genetic gain. This article reviews LOPU-IVEP technology in prepubertal Holstein Cattle and Mediterranean Water Buffalo. The recent expansion of genomic-assisted selection has renewed interest and demand for prepubertal LOPU-IVEP schemes; however, low blastocyst development rates has constrained its widespread implementation. Here, we present an overview of the current state of the technology, limitations that persist and suggest possible solutions to improve its efficiency, with a focus on gonadotropin stimulations strategies to prime oocytes prior to follicular aspiration, and IVEP procedures promoting growth factor metabolism and limiting oxidative and endoplasmic reticulum stress.

Keywords: Holstein; Mediterranean Water Buffalo; in vitro embryo production; laparoscopic ovum pickup; accelerated genetic gain; prepubertal; embryo development

2.3. Introduction

In vitro embryo production (IVEP) and embryo transfer (ET) technologies have had a momentous impact on livestock production, with their use growing substantially in recent years. Despite barely being used on a commercial scale as recently as the late 1990s, IVEP has increased at an average annual rate of 12%, according to data provided by the International Embryo Transfer Society [1, 2]. Moreover, it has been applied in most important livestock species, as reviewed in previous publications, e.g., cattle [2], buffalo [3], camelids [4], swine [5], goat and sheep [6, 7], and cervids [8]. In cattle, where IVEP is broadly used, the majority of embryos transferred worldwide have been produced in vitro since 2016 [1]. Although no single factor can be attributed as the sole cause of this major milestone, improved media composition, the introduction of sexed semen, faster turnover compared to conventional multiple ovulation embryo transfer (MOET), and the ability to use semen from multiple bulls on oocytes from a single donor at the same time are all believed to be contributing factors [9].

Another key factor that explains IVEP expansion is the refinement of technologies to enable safe and practical collection of oocytes from live females. In large adult animals, most

oocytes used for commercial embryo production are collected via ultrasound-guided trans-vaginal ovum pickup (OPU). However, in species that are too small for oocyte collection via OPU (e.g., sheep, goat, deer), a laparoscopic ovum pick-up (LOPU) procedure was developed in the early 90s [10]. Since then, it has been refined and adapted for use in a wide range of both domestic and wild species [10-18]. The LOPU approach has several advantages over OPU, including that the ovary is viewed directly with a depth of field, rather than on a two-dimensional sonogram, enabling superficial follicles to be aspirated accurately without risking injury to the ovarian stroma [19]. This minimizes ovarian trauma, and hence the risk of sequels including tissue adhesions. As such, LOPU can be repeated on a regular basis while minimizing long-term reproductive concerns [11, 20].

Of particular interest is the application of LOPU to conduct IVEP in very young animals. LOPU allows the recovery of oocytes from animals as young as two months of age, long before they are sexually mature or large enough for ultrasound guided OPU. Subsequently, IVEP allows for these oocytes to be fertilized in vitro to produce blastocysts, which are then transferred into adult recipient females, as shown in Figure 1. Using this approach, multiple offspring from the donor animal can be born before it reaches sexual maturity. Using LOPU-IVEP, it is now possible to exploit the large ovarian pool of oocytes present at young ages to rapidly proliferate genetically superior, valuable, or endangered animals [19, 20]. It also provides a faster mechanism for the proliferation of animal lineages of particularly valuable genotypes [21]. Additionally, from a more basic-science perspective, prepubertal animals are also excellent negative models for the acquisition of developmental competence, leading to a better understanding of infertility and the development of new fertility treatments [22].

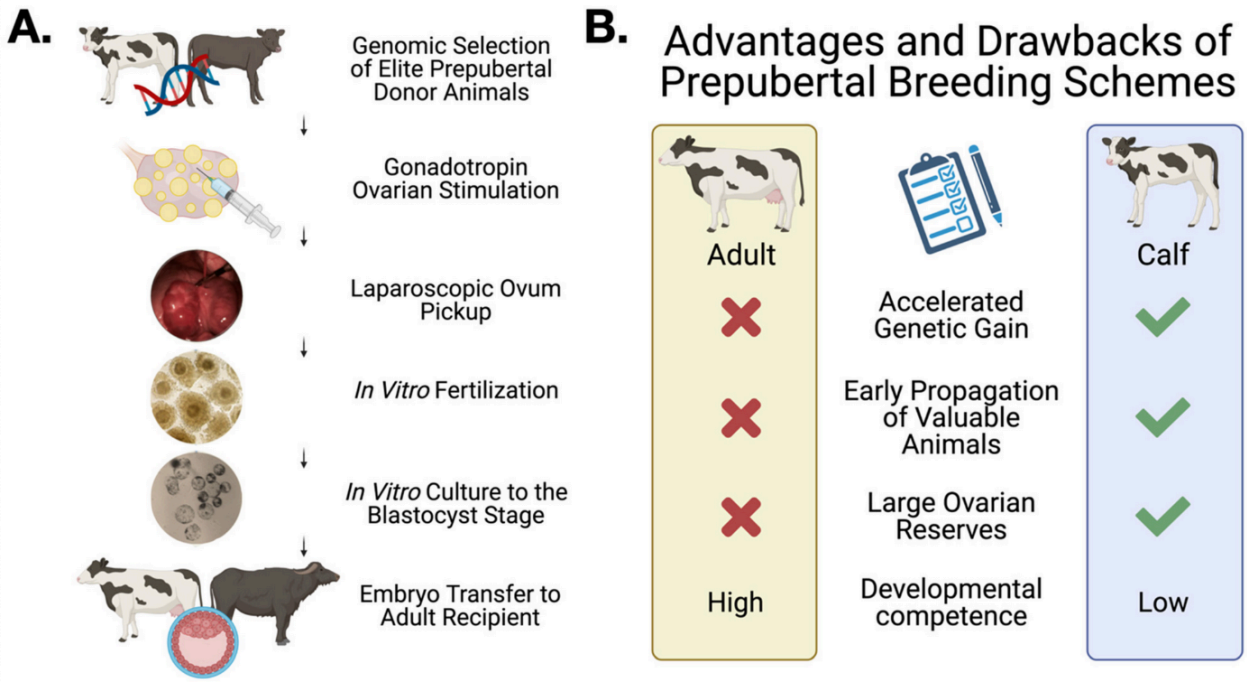

Figure 2.1. Overview of LOPU-IVEP in Prepubertal Buffalo and Cattle. (**A**): Flow chart showing the typical steps involved in prepubertal LOPU-IVEP programs. (**B**): Comparison between adult and prepubertal breeding schemes, showing the advantages and drawbacks of each. Figure created with BioRender.com, accessed on 30 July 2021.

There are two main reasons for the interest in using prepubertal animals as oocyte donors. First, the ovarian pool of available oocytes is vast; prepubertal animals consistently yield large numbers of cumulus-oocyte complexes (COCs) compared to their adult counterparts [19, 23]. Second, early propagation of elite animals results in shorter intervals between generations, thereby increasing the rate of genetic gain [24] and enabling faster access to the latest genetic lineages. However, multiple studies have consistently shown that, although large number of COCs can be recovered, poor embryo development rates result in few blastocysts from prepubertal-derived oocytes in many domestic livestock species including cattle [25, 26], buffalo [27] goat [28, 29], sheep [30, 31], and pig [32, 33]. Although differences in oocyte competence vary among species, in cattle, prepubertal oocytes typically yield a 10–15% blastocyst rate compared to ~30% using oocytes from adult animals [23]. While the exact reasons for the impaired competence are unknown and are most likely a combination of multiple factors, various differences have been

noted such as smaller oocyte size, incomplete cytoplasmic maturation, variations in gene expression, and alterations in protein synthesis and metabolism [26, 34, 35].

This review will focus on prepubertal reproductive technologies, sometimes dubbed 'juvenile in vitro embryo transfer' (JIVET), in Holstein–Friesian cattle (Bos taurus taurus) and Mediterranean water buffalo (Bubalus bubalis). Together, these species serve as complementary animal models to investigate prepubertal oocyte competence and improve prepubertal reproductive technologies since Holsteins mature relatively quickly while water buffalo mature much more slowly. In normal breeding practices using artificial insemination, Holstein heifers typically give birth to their first calf around two years of age, while, on average, water buffalo heifers are not expected to calf until around three years of age. As such, the goals of this review are to outline the current state of the technology, identify research gaps and suggest possible future avenues of research.

2.4. Increasing the Rate of Genetic Gain by Shortening Generation Intervals

Selective breeding, or artificial selection, is the practice where individuals are bred based on specific merits in order to proliferate a desirable trait. Broadly speaking, exceptional animals are bred to produce superior offspring. Recently, genomics has revolutionized selective breeding strategies and reliable single-nucleotide polymorphisms for various traits have been identified in both cattle and water buffalo [36]. The rate at which these genetic gains (the difference in genetic value between parent and offspring) take place is inversely correlated with the generation interval [37]. Therefore, it is beneficial to breed the best animals at the youngest age possible in order to maximize the rate of genetic gain. Using buffalo as an example, if a calf undergoes LOPU/IVEP/ET at two months of age, offspring would be born at around the time the donor animal is one year old, effectively decreasing the generation interval by up to two years.

2.5. History of LOPU-IVEP in Prepubertal Calves

It was identified early on that using prepubertal animals in breeding programs would lead to dramatic increases in the rate of genetic gain. However, most early attempts at using MOET in prepubertal cattle predominantly failed. Some of the first attempts in the early 1970s noted that, when embryos sourced from prepubertal animals were placed in culture, development arrested

before reaching the morula stage [38-40]. It should be noted that IVEP technology was still in its infancy at that time. Nevertheless, these pioneering studies showed that prepubertal animals could respond to exogenous gonadotropin stimulation [41]. Although animals responded well to follicle-stimulating hormone (FSH) treatment, they did not ovulate reliably in response to injections of pituitary extracts high in luteinizing hormone (LH) [41, 42], resulting in low recovery rates and poor embryonic development [38, 39]. Based on those observations, assumptions were made that the prepubertal reproductive tract was detrimental and not conducive to normal fertilization and early embryo development [38, 41]. Ultimately, this resulted in MOET strategies being abandoned and attention instead turned to LOPU-IVEP.

Substantial research was done in the 1990s to develop reliable LOPU and IVEP techniques for prepubertal animals in several species. Studies during this period showed that the LOPU component was largely successful, but IVEP was not. Following LOPU, multiple authors reported high oocyte yields in young animals, often more than what is typically recovered from adult animals [19, 23, 43]. Following IVEP, the oocytes from young animals resulted in blastocyst development rates that were consistently lower than rates in mature animals [25, 44-47]. For example, Revel and associates found similar fertilization and cleavage rates between oocytes from three-month-old heifer calves and adult cattle, but prepubertal oocytes failed to produce similar blastocyst rates [23]. These poor results, combined with the inability to identify genetically superior animals at such young ages at that time, led to the loss of interest in prepubertal LOPU-IVEP research projects for around 20 years.

Since these studies in the 1990s, significant advancements in marker-assisted selection, genomics, and IVEP have renewed interest and demonstrated the potential to circumvent many of the initial shortfalls. Genomic marker selection in particular is having a huge impact on the dairy industry where the production phenotype can be accurately predicted as soon as the animal is born through screening of single-nucleotide polymorphisms [48, 49]. Along with the progress in genome selection, significant improvements in IVEP practices have been accomplished in recent years, resulting in the ability to produce high quality embryos in vitro, comparable to their in vivo derived counterparts [9]. Recent innovations such as sequential media compositions and advanced low-oxygen tension incubators have enabled production of embryos in vitro possessing cryotolerance capabilities similar to embryos produced in vivo [2, 50]. As genomic selection and

modern IVEP technology become more and more cost-effective in the future, their application and use are expected to continue growing.

Despite significant progress in recent years, problems with prepubertal IVEP technologies still exist. As evidenced from studies in different species, prepubertal oocytes have a reduced developmental competence compared to adult oocytes, with fewer IVEP embryos reaching the blastocyst stage, as observed in bovine [23], buffalo [27], ovine [30], caprine [51], and swine [52]. It has been shown that calves respond well to FSH stimulation and produce many follicles, often producing more than cows [19, 23, 43]. Consequently, prepubertal donors typically produce more COCs and 2-cell stage embryos than adult donors. Blastocysts derived from prepubertal oocytes are competent to support full-term development and normal offspring have been produced in multiple species, including buffalo [7, 27] and cattle [23, 53, 54]. Hence, the primary challenge remains the improvement of oocyte competence to enable higher embryo development rates to the blastocyst stage. In this regard, learning how to prime and prepare prepubertal oocytes, both in vivo inside the follicle and in vitro during maturation and culture, seems the most logical and promising path to consolidate LOPU-IVEP uses in prepubertal breeding schemes, as shown in Figure 2.

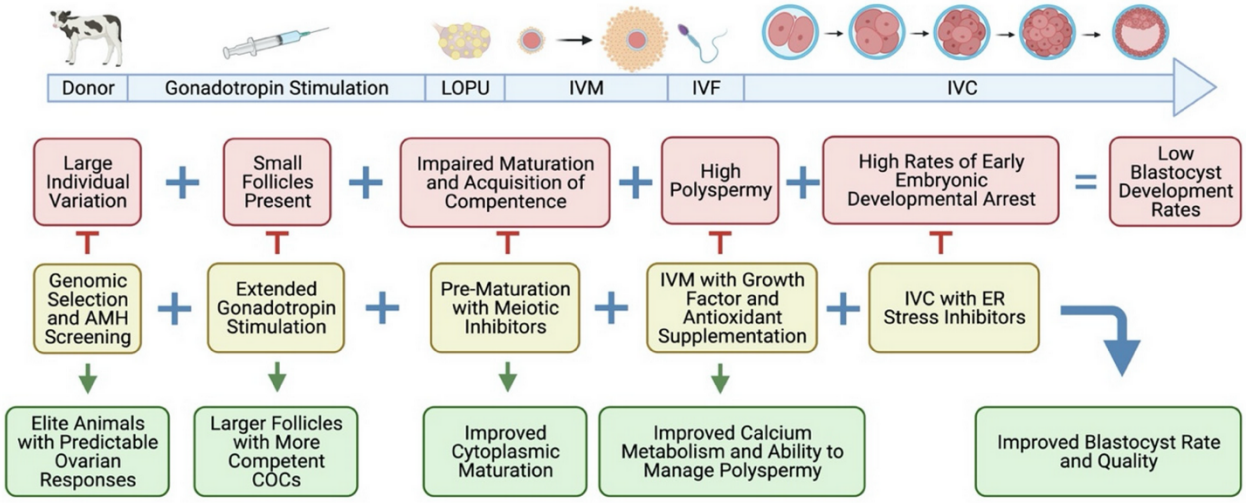

Figure 2.2. Overview of LOPU-IVEP in prepubertal cattle and buffalo, showing potential targeted approaches to address the main problems holding the technology back. Figure created with BioRender.com, accessed on 30 July 2021.

2.6. Understanding Developmental Competence of Oocytes

One of the greatest challenges in overcoming the impaired developmental competence of prepubertal oocytes is that the underlying reasons are not fully understood, and the cause is most likely a combination of multiple factors. For example, the hypothalamic-pituitary-ovarian axis in prepubertal animals is immature, which could lead to defective signalling and steroidogenesis in ovarian follicles. In turn, an improper follicular micro-environment could affect metabolism within the oocyte itself or the crosstalk between the oocyte and granulosa cells, ultimately resulting in oocytes unable to reach full developmental competence.

2.6.1. The Hypothalamic–Pituitary–Ovarian (HPO) Axis

The HPO axis is essential for the management of the oestrous cycle and, consequently, fertility. Kisspeptins (Kp) are a family of neuropeptides in the hypothalamus, which were discovered in 2003 to operate upstream of gonadotropin-releasing hormone (GnRH) signalling [55]. GnRH neurons express the receptor for kisspeptin, GPR54, and consequently have been implicated in many critical roles including timing the onset of puberty, secretion of gonadotropins, transmission of the negative and positive feedback loops, and generation of the LH surge [56, 57]. This upstream hypothalamus signalling is believed to be the last component of the HPO axis to mature in juvenile heifers, and is the limiting factor determining the HPO functionality prior to puberty [58]. Specifically, the number of Kp-positive cells in the arcuate nucleus and pre-optic area are believed to be responsible for the negative and positive feedback loops, respectively, and have been shown to increase during prepubertal development in the ewe [59]. Downstream, in the pituitary, GnRH receptors do not change with age, and secrete gonadotropins in response to GnRH at a very young age [60, 61]. In the ovary, the relative mRNA abundance of FSH receptor in granulosa cells is significantly lower in prepubertal Holsteins compared to adult cows, possibly explaining the smaller average follicle size in prepubertal animals, and consequently the reduced developmental competence of oocytes [62].

2.6.2. Follicular Microenvironment

The lower developmental potential of calf oocytes may be due to environmental deficiencies in vivo prior to retrieval [23, 63]. Hence, a clear understanding of the follicle and its

follicular fluid is important. Calf follicular fluid contains approximately half the LH concentration compared to cow follicular fluid (2.0 ± 0.2 ng/mL vs. 4.0 ± 0.3 ng/mL) [64]. This is in accordance with the plasma concentration of LH, which is also lower in younger animals [65]. Although changes in LH concentration may have no direct impact on the oocyte itself due to a lack of LH receptors, it would affect steroidogenesis and androgen production in granulosa and theca cells [66]. A disruption in estrogen production would affect the transcription of genes regulated by estrogen response elements. Alternately, impaired androgen metabolism could also affect fertility, as androgen-receptor knock-out mice are sub-fertile [67]. In a similar manner to LH, calf follicular fluid has also been shown to contain approximately half the estradiol content compared to adults (6.3 ± 2.1 ng/mL vs. 12.7 ± 5.5 ng/mL) [64]. Collectively, it can be speculated that these differences in the follicular micro-environment may negatively impact the acquisition of developmental competence, and may partially explain the low IVEP outcomes observed in calves [63]. This further emphasizes the importance of suitable gonadotropin stimulation regimes to emulate a follicular microenvironment that will promote oocyte competence prior to LOPU.

2.6.3. Oocyte and Granulosa Cell Crosstalk

Oocyte competence is dependent on intercellular communication within the ovarian follicle during follicular growth and development, and is regulated by endocrine, paracrine, and autocrine factors [68]. While direct inter-cellular connections are mediated via gap junctions and transzonal projections (TZPs) [69], indirect intercellular communication can occur through extracellular vesicles (EVs) secreted into the follicular fluid [70]. Collectively, these pathways facilitate bi-directional communication, signaling and transport of molecules between the oocyte, granulosa, and theca cells [70, 71].

Developmental competence increases gradually and sequentially as oocytes increase in size due to transcriptional activity during follicular and oocyte growth [72, 73]. This is vital as oocytes from prepubertal animals are smaller and have a thinner zona pellucida than those from adults, despite originating from follicles of the same size [74]. For example, calf oocytes have a mean diameter of 118.04 ± 1.15 μm compared to a mean diameter of 122.83 ± 0.74 μm for mature cows [24, 74]. Since a small variation in diameter represents a larger variation in volume, small variations in diameter may have important impacts on developmental competence. As such, the capacity of bovine oocytes to mature to metaphase II during IVM is positively correlated with their

diameter [75]. Aside from diameter, several cytoplasmic differences have also been observed between oocytes from prepubertal and adult animals. For example, oocytes from adult cows have more lipid droplets in their cytoplasm compared to those from heifers, both before and after IVM [63]. Other differences include incomplete cytoplasmic maturation, altered gene expression and protein synthesis, as well as defective metabolism in oocytes from young animals [26, 34, 35].

More recently, the intimate relationship between the oocyte and cumulus cells has been investigated to better define the role of TZPs [76, 77]. Although more research needs to be done to determine how the physiology, distribution and retraction of TZPs impacts IVEP outcomes in both prepubertal and adult oocytes, TZPs are known to facilitate communication and the transport of essential molecules between granulosa cells and the oocyte [76, 78]. Despite differences observed in the organization of TZPs in COCs from lambs compared to adult ewes, the impact on embryo development remains unclear [79].

In addition to intercellular communication via TZPs, the roles of EVs on intra-follicular cell communication has also become of particular interest [70]. EVs are small lipid bilayer particles secreted by cells into the extracellular space, which then diffuse and act on secondary target cells, transporting various molecules including proteins, lipids, messenger RNA (mRNA), and microRNA (miRNA) [80, 81]. Since the initial discovery of EVs in equine follicular fluid in 2012 [82], they have since been described in bovine [83] and porcine follicular fluid [84, 85] and were shown to play multiple roles inside the follicle, including granulosa cell proliferation and cumulus expansion [86, 87]. Notably, studies have found variability in EV and miRNA profiles when comparing follicular fluid from follicles of different sizes and young vs. old animals [82, 86-88]. For example, da Silveira found significant differences in the number and profiles of miRNAs present when comparing follicular fluid from young (3–13 y.o.) and old (>20 y.o.) mares [82, 89]. Others have found similar results when comparing younger (<31 y.o.) and older (> 38 y.o.) women [90]. How these findings may translate into prepubertal vs. adult cattle and buffalo remains unknown. However, it has been shown that supplementation with EVs in vitro was able to increase blastocyst rates in cattle to 37%, compared to 26% using IVM with EV-free fetal calf serum [91]. Thus, it is possible that supplementation with adult EVs in prepubertal IVEP programs may help improve oocyte competence.

2.7. Hormonal Stimulation

Due to the impaired HPO axis in prepubertal animals, an efficient hormonal stimulation protocol is critical to provide the COCs with a conducive intra-follicular milieu prior to LOPU. Previous work in our laboratory showed that FSH stimulation in prepubertal calves was able to mimic a functional HPO axis by increasing mRNA expression of FSH receptor (FSHR) and cytochrome P450 family 19 subfamily A member 1 (CYP19A1), while decreasing levels of steroidogenic acute regulatory protein (StAR) and hydroxy-δ-5-steroid dehydrogenase, 3β-and steroid δ-isomerase 1 (HSD3B1) in calf granulosa cells [62]. The molecular changes that occur during follicular and oocyte growth involving molecules synthesized within the oocyte or imported from granulosa cells are critical for the acquisition of an oocyte's developmental competence and support the theory that "the history of the follicle determines the future of its oocyte" [92]. In support of this, several studies have shown a positive correlation between the follicular diameter and developmental competence of the oocyte in many species, including sheep [93], goat [94, 95], cattle [35, 72, 92], buffalo [96], and pig [97, 98]. For example, in adult cattle, oocytes from follicles 2–6 mm in diameter produced an average blastocyst rate of 34.3%, while oocytes from follicles > 6 mm in diameter produced an average blastocyst rate of 65.9% [72]. A similar pattern was observed in adult buffalo, with oocytes originating from follicles < 3 mm in size resulting in a blastocyst rate of 2.4 ± 1.5% while oocytes originating from follicles > 8 mm in diameter resulted in a blastocyst rate of 16.9 ± 1.7% [99]. This same trend was observed in prepubertal animals, with blastocyst rate per oocyte increasing from 6.8% to 13.8%, comparing oocytes from small (<5 mm) and large (≥5 mm) follicles in Holstein calves [100].

In prepubertal animals, LOPU-IVEP has been performed following hormonal stimulation protocols that were adapted from those used for adult animals. The goals of gonadotropin stimulation are not only to increase the size of follicles, and consequently oocyte competence, but also to increase the number of follicles suitable for aspiration [21]. Follicle stimulating protocols have consisted of multiple injections of FSH, single injections of compounds with a longer half-life such as equine chorionic gonadotropin (eCG), or a combination of both FSH and eCG [101, 102, 103]. Due to its short metabolic half-life, FSH is typically re-administered every 12 h for 3–4 days. Studies in the 1990s found that calves had a significantly better follicular response when subjected to multiple injections rather than a single injection of a large dose of FSH [102, 103]. However, combining a single FSH injection with one of eCG resulted in a similar ovarian response to multiple FSH injections, suggesting a single dose of FSH is able to recruit but not sustain

development of a follicle cohort [102, 103]. These data seem to be supported by the fact that combining a single injection of FSH with a low dose of eCG can result in a similar ovarian response to multiple-injection regimes, with the FSH bolus able to recruit a follicle cohort, and the eCG able to sustain continued development [45]. It could be possible that eCG aids in follicle development from its inherent LH activity, which could act synergistically with FSH [103, 104]. When comparing the interval between FSH, with and without eCG, we found that FSH injections every 8 h starting 72 h before LOPU, until a single dose of 400 IU of eCG 36 h prior to LOPU, yielded better blastocyst rates compared to FSH injections every 12 h without eCG (17.5 ± 8% vs. 8.9 ± 5%) [100].

2.8. LOPU and COC Quality

As the LOPU procedure is essentially the same for all ruminants and has been described in detail in other manuscripts [7, 10, 53], this review will not focus on the technical aspects of the procedure itself. However, it is worth highlighting that LOPU has been shown to be extremely safe and can be repeated on a regular basis. For example, LOPU has been repeated ~10 times in goats [20], and we repeated the procedure every two weeks in prepubertal Holsteins and buffalo between 6 and 9 times over a 3–4 month period [105]. Following this, none of the animals had reproductive problems later in life, as they were used to produce more embryos by trans-vaginal OPU and had normal fertility following artificial insemination. In our experience with prepubertal calves and buffalo, oocyte recovery rate (the proportion of follicles from which COCs were recovered) following LOPU is usually very good. Indeed, the average recovery rate was 77.1 ± 27% in Holstein calves (n = 109 LOPUs) [53], and 84.3 ± 29.3% in buffalo calves (n = 56 LOPUs, unpublished). Concerning COC quality, 87.4 ± 19% were deemed usable including 67% grade 1 and 20.4% grade 2 [53]. In addition, we observed that the gonadotropin stimulation regime used affected COC quality, with a longer stimulation protocol (≥72 h) resulting in a viability rate of 95.3% ± 18%, compared to 85.4% ± 22% for a shorter protocol (36–42 h) [53].

2.9. Individual Variation

In adult cows, the ovarian response upon gonadotropin stimulation is widely variable among animals [106]. The same variation was observed in calves [21, 106], with research in our

laboratory revealing similar results in both Holsteins [100] and buffalo [105] calves as shown in Table 1. The large individual variation is problematic in selecting the best calves to be used in a prepubertal LOPU-IVEP scheme, which may be mitigated by determining the serum concentrations of anti-Müllerian hormone (AMH) given its correlation with an individual animal's response following gonadotropin stimulation observed in adult cattle and buffalo [107, 108, 109]. Although more work needs to be done to confirm this remains true in prepubertal buffalo calves, data suggest that AMH concentration remains a credible marker for LOPU-IVEP performance in prepubertal Bos taurus and indicus calves [110]. This is particularly useful since the follicular population is difficult to assess using ultrasound at such a young age.

Table 2.1. Individual variation of usable COCs recovered from calves over six LOPU sessions.

Species	Number of Animals	Number of COCs Recovered			
		Total	Mean ± SD All Calves (Total Per Calf)	Mean ± SD Bottom Calf (Total)	Mean ± SD Top Calf (Total)
Holstein	11	1393	22.2 ± 14 (126.6)	12.7 ± 4 (72)	38.2 ± 11 (229)
Buffalo	8	774	16.2 ± 9 (81)	10.1 ± 3 (50)	26.6 ± 6 (130)

SD = standard deviation. Data adapted from [100, 105].

2.9.1. Seasonality

In the specific case of buffalo, another factor potentially contributing to variation in results is season. Buffalo are sensitive to long photoperiods, with reproductive efficiency improving in the autumn and winter as daylight decreases, similar to sheep & goat [111-114]. Season has been reported to influence the age at puberty [115]. Moreover, in adult Mediterranean buffalo undergoing repeated OPU, embryo yield improved significantly in the autumn [116], but there are yet no studies on the impact of season on prepubertal oocyte quality. Additionally, heat stress is well-researched and known to impact the estrous cycle, follicular development, oocyte quality and embryonic development rates in ruminants [117-119].

2.10. In Vitro Embryo Production

Following LOPU, oocytes undergo in vitro maturation, fertilization, and culture. Although variations exist in cattle and buffalo, these usually last for 22 h, 18 h, and 7 days, respectively. Most protocols have followed media compositions and procedures consistent with those used for adult animals with minimal derivations [23, 101, 120]. As such, commercially available media can be used. However, prepubertal oocytes may benefit from specially tailored IVEP protocols supplemented with various factors, which is discussed below.

2.10.1. Oocyte In Vitro Maturation (IVM)

Although in vivo maturation was the norm for many years, and the first Holstein calf born in the world from IVF was a product of in vivo maturation [121], in vitro maturation has yielded more reliable and consistent results in recent years. The objectives of IVM are both nuclear and cytoplasmic maturation. Nuclear maturation is the transition from germinal vesicle (prophase I) to metaphase II, while cytoplasmic maturation allows morphological, functional and biochemical changes to take place in the cytoplasm.

Multiple studies have shown that, although prepubertal oocytes are able to complete nuclear maturation, their ability to manage cytoplasmic maturation is more ambiguous. For nuclear maturation, it has been shown that oocytes can undergo germinal vesicle breakdown and successfully arrest at metaphase II [43, 46, 47, 103]. It has been suggested that this process may be delayed in lamb oocytes compared to ewes [79]. However, our findings with oocytes collected from Holstein [53, 100] and buffalo (unpublished) calves revealed that ~80% were able to mature to the metaphase II stage and successfully extrude the first polar body after 24 h of IVM. In terms of cytoplasmic maturation, electron microscopy studies have shown that organization of the oocyte organelles, such as the number and distribution of cortical granules as well as the population of mitochondria, are different in prepubertal compared to adult oocytes [47, 79, 122]. Damiani and colleagues compared cortical granule migration in calf and cow oocytes and found that cortical granules did not migrate as efficiently in calf oocytes as only 19% (17/90) of calf oocytes exhibited migration compared to 71% (83/117) in cow oocytes. This may impact normal fertilization and the initiation of the block to polyspermy, since 81% (73/90) of calf oocytes still possessed clusters of cortical granules following IVM [47]. Furthermore, cortical granule migration was delayed in 70% (19/27) of calf oocytes compared to 28% (7/25) in cow oocytes [47]. In addition to cortical granule migration, other cytoplasmic differences have been noted, including the distribution of

mitochondria and lipid droplets [47]. These cytoplasmic deficiencies may be associated with the impaired competence of prepubertal oocytes. In support of this, it has been shown that transferring the nuclei of adult oocytes into enucleated calf oocytes resulted in similarly low development rates to those observed in control calf oocytes [123].

2.10.2. In Vitro Fertilization (IVF)

The ability of calf oocytes to properly manage fertilization, oocyte activation and the block to polyspermy appears to be impaired. Research in the 1990s showed that, although fertilization rates (as measured by sperm penetration) were the same between prepubertal and adult donors, there was a significantly higher rate of abnormal fertilization in prepubertal (16%) than adult (7%) oocytes [24, 47]. Work in our laboratory provided additional evidence that polyspermy is a significant problem for IVF in calf oocytes. Working with Holstein calf oocytes and using the industry standard concentration of 1 million motile sperm/mL, polyspermy rates were over 40% [53]. However, when the sperm concentration was reduced to 500,000 motile sperm/mL, the incidence of polyspermy decreased to 19.7% [53]. In addition, the normal fertilization rate, as evidenced by the presence of two polar bodies and two pronuclei, increased from 59.4% to 69.7% [53]. Interestingly, we observed a steady decrease in polyspermy rates with age, declining from 45.5% in animals < 100 days old, to 12.8% in animals >130 days old [53]. We also observed similar results working with buffalo calves, with age and semen dose affecting polyspermy rates [105].

2.10.3. Embryo In Vitro Culture (IVC) and Transfer

Following fertilization, cell division appears to be delayed, with a low proportion of calf-derived embryos reaching the 4 and 8-cell stages of development at standardized time points [35, 120]. In addition, embryo development to the blastocyst stage is significantly lower than what is achieved with adult Holsteins and buffalo oocytes [22, 23, 27, 35, 120]. In our experience with Holstein calves, cleavage rates varied between 60–70% and blastocyst rates were around 20%. However, both embryo yield and quality were significantly affected by the gonadotropin stimulation protocol and age of the calves [53]. In buffalo, this is potentially compounded by the fact that both the oocyte donor and sire used during IVF have a large influence on IVEP outcome,

with only around 10% of males suitable for IVF [27, 124]. Despite limited information in the published literature on the timing and causes of embryonic development arrest, it has been shown in 6–8-month-old heifers that 67% (40/60) of cleaved embryos that failed to reach the blastocyst stage arrested between the 2 and 8-cell stage, which was significantly higher than the 18% (5/28) observed in embryos from adult animals [120]. This suggests that prepubertal oocytes are unable to transition from oocyte to embryo and properly regulate embryonic genome activation, as the stage of developmental arrest coincides at around this time [125].

Recently, the possible impact of ARTs on the embryonic epigenome has garnered attention, with studies suggesting offspring produced by IVEP may be at higher risk of various disease [126, 127]. For example, large offspring syndrome has been associated with epigenomic differences in imprinted genes [128, 129]. Furthermore, the extent of cellular reprogramming and epigenetic inheritance of both parental methylomes on the embryo is currently being investigated [130]. Whether prepubertal LOPU-IVEP programs may affect epigenetic inheritance is unclear, however. Evidence in bulls suggest that the age of the sire influences the transcriptome and epigenome of blastocysts produced by IVF [131]. In females, transcriptomic comparison of blastocysts produced from the same heifers between 8–14 months old revealed that genes related to mitochondrial function were impacted in younger heifers [132]. How these differences may affect future embryo development of offspring is unknown.

Despite the lower development to the blastocyst stage, prepubertal embryos can reach this stage in a similar timeframe and have normal characteristics including a visible inner cell mass [120]. In terms of cell numbers, as an indicator of embryo quality, there were no differences in the trophectoderm-inner cell mass ratio between hatched and unhatched blastocysts from cows and 6–8-month-old heifers [120]. However, the total cell count in day 8 blastocysts was slightly lower but not statistically different between embryos of heifers (89 ± 20) and adult cows (100 ± 30) [120]. Additionally, heifer-derived and cow-derived blastocysts seem to have similar lipid metabolism, with day 8 blastocysts containing comparable triglyceride concentrations [120].

The ultimate and essential test for blastocyst quality is the ability to establish pregnancy and result in healthy offspring following embryo transfer. Pregnancies and live births with full-term offspring following LOPU-IVEP and embryo transfer have been reported by multiple authors using calf-derived oocytes in both Holsteins [23, 43, 44, 53, 101] and buffalo [7, 27]. Although earlier studies have suggested lower rates of establishing pregnancy with prepubertal-sourced

embryos, our findings revealed more encouraging results. Indeed, we obtained a 62% (13/21) pregnancy rate after transferring LOPU-IVEP blastocysts from Holstein calf oocytes. Of the 13 confirmed pregnancies, 4 were interrupted for experimental reasons and 100% of the 9 that were allowed to continue carried their pregnancy to term [53]. In buffalo, of 10 embryo transfers, 3 became pregnant, all of which delivered healthy calves [7, 105]. Other authors reported similar results in prepubertal buffalo by confirming 3 pregnancies and delivery of healthy calves after the transfer of 8 IVEP embryos [133, 134]. With the knowledge that these prepubertal LOPU-IVEP-ET schemes do work, animal breeding companies are now starting to offer these programs on a commercial basis. However, further research is needed to improve and ensure the long-term financial viability of these programs going forward.

2.10.4. Embryo Cryopreservation

In addition to yielding similar rates of embryos and pregnancies following transfer, another goal is for prepubertal-derived embryos to have cryotolerance similar to that of adult-derived embryos. It is well documented that in vivo produced embryos are more cryotolerant than their in vitro produced counterparts [135, 136, 137]. As such, embryo quality plays a major role in post-thaw survivability, with the cytoplasmic lipid content, i.e., the number and size of lipid droplets, shown to affect cryotolerance significantly, with more lipids being detrimental [135]. This presents a unique challenge for buffalo embryos, as they have high levels of lipids [3]. To address this problem, L-carnitine supplementation in vitro has been shown to aid in the lipid metabolism, as well as providing antioxidant protection, which improved post-thaw survivability in both Holsteins [138, 139] and Buffalo [140, 141]. However, this strategy remains to be tested in prepubertal-derived embryos.

2.11. Future Perspectives: What Can We Do Better?

With the knowledge that prepubertal LOPU-IVEP technologies do work, as evidenced by healthy calves born following embryo transfer, the current challenge is improving efficiency. As such, attention should focus on conditions both in vivo, before LOPU, and in vitro, following LOPU. In vivo approaches should include innovative gonadotropin stimulation protocols for young donor animals in order to enhance the intra-follicular environment and maximize oocyte

development inside the follicle. In vitro approaches should focus on amending IVEP procedures to better accommodate the requirements of prepubertal oocytes to maximize meiotic maturation, normal fertilization and embryo development to the blastocyst stage.

2.11.1. Optimized Gonadotropin Stimulation

Efficient gonadotropin stimulation regimes should increase the size of follicles available for aspiration, as embryo development rates are directly associated with follicular size [35, 72, 92, 96]. As such, gonadotropin stimulation over a longer period of time has been shown to be beneficial in calves. Work in our laboratory compared short (3 FSH injections, 12 h apart, starting 36 h prior to LOPU, total FSH 100 mg) vs. long gonadotropin treatments (6 FSH injections, 12 h apart starting 72 h prior to LOPU, total FSH 96–140 mg) and revealed that not only did the proportion of large follicles aspirated increase (11.2% vs. 34.0%), but cleavage rate (59.0 ± 23% vs. 72.7 ± 21%) and blastocyst rate (18.3 ± 15% vs. 36.7 ± 26%) were also significantly increased in the longer treatment [53]. Other studies have shown that an even longer stimulation duration of 7 days, compared to 4 days, resulted in a larger proportion (56.4 ± 8.3% vs. 27.8 ± 7.5%) and number (13.3 ± 1.8 vs. 9.0 ± 1.3) of large follicles (≥9 mm) [142, 143]. However, the study focused only on the dynamics of follicular populations by serial ultrasound scanning, and the effects of such a prolonged protocol on oocyte competence and embryo development rates remains to be tested. Similarly, gonadotropin stimulation significantly increased the proportion of medium (4–8 mm) and large follicles (≥9 mm) in buffalo aged between 5 and 9 months [144].

2.11.2. Oxidative Stress and the Importance of Antioxidants

Oxidative stress caused by reactive oxygen species (ROS) can damage cells by disrupting homeostasis and leading to apoptosis. Glutathione (GSH) is considered the major line of defence against oxidative injury by helping to maintain the redox state within the cell. In addition to its role in preventing oxidative stress, GSH has been shown to play an important role in the transport of amino acids, as well as in DNA and protein synthesis [145]. The tripeptide thiol compound has been shown to be synthesised during oocyte maturation in bovine [146], bubaline [147], caprine [148], and porcine [149] oocytes. GSH is also known to play important roles in the formation of the male pronucleus and early embryonic development [150]. As oxidative stress is known to be

pervasive during in vitro manipulation, compared to conditions in vivo, most IVEP protocols use antioxidants aimed at either promoting GSH synthesis (e.g., cysteine), or scavenging ROS (e.g., melatonin) [151]. Since oxidative stress is known to play a significant role in vitro and prepubertal oocytes may be deficient in their ability to combat ROS, it is plausible that they are more susceptible to oxidative stress [151, 152]. As such, prepubertal IVEP may require specialized antioxidant treatments tailored to their needs. This may be especially important in buffalo because of the high concentration of lipids within the oocyte and therefore the increased risk of lipid peroxidation.

Although many different antioxidants have been tested and used over the years in adult IVEP schemes, there are fewer studies assessing the efficacy in prepubertal animals, especially in cattle and buffalo. Working with 1–2-month-old goats, Rodriguez-Gonzalez and colleagues found that IVM supplemented with cysteamine increased the GSH concentration, and improved blastocyst yield and total cell number per blastocyst [148]. Similar results were found in adult buffalo by Gasparrini and colleagues [153]. In a subsequent paper by the same group, they showed that supplementation with cysteamine combined with cystine, was even more advantageous than cysteamine alone, increasing the transferrable embryo rate from 23.8 ± 3.9% to 30.9 ± 5.8% [154]. Whether these findings can be applied to prepubertal animals remains to be determined.

Another antioxidant used in many IVEP schemes across multiple species is melatonin, which has been shown to reduce oxidative damage in the oocyte [155, 156]. Melatonin is produced throughout the body, including the ovary, and has been detected in follicular fluid of bovine [157], porcine [158], bubaline [159], and caprine [155] follicles. In prepubertal goats, higher concentrations of melatonin were detected in large follicles (> 5 mm) compared to small follicles (<3 mm) [155]. The same trend was found in adult Murrah buffalo [159]. In prepubertal goats, melatonin supplementation during IVM increased the blastocyst rate [155], decreased intracytoplasmic ROS, improved ATP content, and enhanced mitochondrial activity [156]. Similar results were found in adult Holstein cows [160] and water buffalo [161]. While melatonin supplementation during IVM of COCs from 4–5-week-old lambs was found to have no effect on development rates [162], in 6–10-month-old Holsteins, it was shown to increase blastocyst rates from 11.1 ± 3.5% to 23.1 ± 5.1% [163].

2.11.3. Endoplasmic Reticulum Stress

Endoplasmic reticulum (ER) stress is a major contributor to embryonic death because physiological and exogenous stressors typically lead to disruptions in protein folding and ROS production in the ER [164]. Induction of ER stress has been shown to impair embryo development rates in multiple species [165, 166], while ER stress inhibitors have been shown to improve IVEP development rates [165, 167, 168]. Tauroursodeoxycholic acid (TUDCA), a bile acid, was shown to inhibit ER stress and improve in vitro embryo development and blastocyst quality in different species [168-172]. TUDCA supplementation was shown to decrease the incidence of DNA double strand breaks in porcine blastocysts [168] and decrease intracellular ROS concentrations in oocytes from adult cattle [173]. In buffalo, treatment with TUDCA decreased cell apoptosis in embryos under ER stress induced by tunicamycin [166]. In prepubertal Holsteins, IVC supplementation with 50 μM TUDCA tended to increase blastocyst rates (30.9 ± 12% vs. 25.7 ± 2%) compared to the control [100]. More studies are needed to better evaluate the impact of TUDCA in prepubertal oocytes, such as testing higher concentrations during IVC. It is also possible that supplementing both IVM and IVC with TUDCA could further impact prepubertal IVEP because of its role in the regulation of calcium metabolism [174, 175], which could also favor normal fertilization and embryo cleavage.

2.11.4. Cytokines and Growth Factors

Cytokines and growth factors are small peptide proteins involved in cellular signalling and communication. Fibroblast growth factor 2 (FGF2), leukaemia inhibitory factor (LIF), and insulin-like growth factor (IGF1) are among the growth factors found in follicular fluid that have regulatory effects on COCs. Working with porcine oocytes, Yuan and colleagues (2017) assessed the impact of adding these growth factors (in a cocktail coined 'FLI') to IVM media and observed a significant increase in oocyte maturation, embryo development and quality, and litter size following embryo transfer [176]. Working with lambs, Tian and colleagues found that combining FLI with insulin-transferrin-selenium (ITS) during IVM increased the blastocyst rate more than two-fold (44.2 ± 5.7% vs. 21.6 ± 4.6%) compared to the control group [162]. How these findings may benefit IVEP systems for prepubertal cattle and buffalo remains unknown.

2.11.5. Oocyte Pre-Maturation In Vitro

There is evidence that a short 'pre-maturation' period in presence of meiotic inhibitors such as c-type natriuretic peptide (CNP), epidermal growth factor receptor (EGFR) inhibitor, and cAMP prior to IVM may improve oocyte competence. During LOPU, separation of COCs from their follicles causes cAMP concentrations to decrease, resulting in spontaneous resumption of meiosis [177]. During pre-maturation, oocytes are temporarily arrested at the GV stage, to allow more time for cytoplasmic maturation to occur and promote synchrony among aberrant nuclear and cytoplasmic maturation [177, 178]. Several studies have shown pre-maturation protocols able to increase blastocyst rate and quality [179, 180]. CNP increases cGMP concentrations in COCs, which inhibits the cAMP hydrolyzing enzyme phosphodiesterase 3A, maintaining meiotic arrest [181]. Pre-maturation of prepubertal goats COCs for 6 h with CNP maintained TZP density, which is essential for cGMP transport into the oocyte and, consequently, meiotic arrest [182]. This treatment significantly increased blastocyst development rates compared to controls (30.2% vs. 17.2%), possibly due to an improved ability of the oocyte to manage oxidative stress, as CNP pre-maturation resulted in increased intra-oocyte glutathione concentrations and decreased ROS [182]. EGFR inhibition can also be used to reversibly arrest bovine COCs at the GV stage [183]. These pre-maturation protocols may represent a new alternative for use in combination with growth factors, antioxidants and inhibitors of ER stress to further improve prepubertal IVEP efficiency. However, these approaches require further validation.

2.12. Conclusions

Although several obstacles remain to be overcome, the use of prepubertal breeding schemes based on LOPU-IVEP is a powerful method for accelerating genetic gain. In Holsteins, the technology has reached a level of commercial viability, with several large biotechnology companies currently using this technology. Although the potential reward in applying this technology in buffalo is larger due to their prolonged sexual maturity, more work needs to be done for further efficiency optimization. Enhanced stimulation protocols yielding more competent oocytes at collection, coupled with in vitro procedures that will improve cytoplasmic maturation and the oocyte's machinery to fight oxidative and ER stress, are among the improvements that will likely increase the proportion of competent oocytes recovered from prepubertal compared with post-pubertal animals.

2.13. Author Contributions

Conceptualization, L.C., H.B., V.B.; methodology, L.C., H.B., V.B.; writing—original draft preparation, L.C.; writing—review and editing, H.B., V.B.; visualization, L.C., H.B., V.B.; supervision, H.B., V.B.; project administration, H.B., V.B.; funding acquisition, H.B., V.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

No new data were created or analyzed in this study. Data sharing is not applicable.

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Conflicts of Interest

The authors declare no conflict of interest.

2.14. References

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CHAPTER 3
HYPOTHESIS AND OBJECTIVES

3.1. Rationale

The use of oocytes from prepubertal donor livestock animals in IVEP programs would significantly increase the rate of genetic gain and enable faster access to the latest genetic lineages. Therefore, the technology has great potential for improving the profitability and efficiency of livestock farming operations, especially in species which have extended generational intervals like water buffalo. However, multiple studies have demonstrated that oocytes sourced from prepubertal donors exhibit low developmental competence, significantly hindering the viability of prepubertal LOPU-IVEP programs. In this context, this thesis is focused on improving the developmental competence of oocytes from juvenile pig and buffalo females and understanding the factors which can affect results. Collectively, we hope to elucidate an efficient protocol for LOPU-IVEP, and establish information needed to guide future implementation.

3.2. General Hypothesis

- Oocytes from prepubertal donor animals will benefit from tailored programs designed to promote the acquisition of oocyte competence, both in vivo inside the follicle prior to LOPU and in vitro during oocyte maturation and prior to IVF.
- Multiple exogenous factors will affect the efficiency of LOPU-IVEP including season at the time of LOPU.

3.3. Objectives

Objective 1: Optimize in vitro maturation conditions using a combination of growth factors and antioxidants to promote in vitro acquisition of oocyte competence, using porcine oocytes due to their easy abattoir availability and oocyte similarities with buffalo.

Objective 2: Optimize gonadotropin stimulation protocols for prepubertal water buffalo calves to promote in vivo acquisition of oocyte competence.

Objective 3: Describe and characterize the exogenous factors that can affect LOPU-IVEP efficiency in prepubertal water buffalo and assess the cryotolerance of the resulting embryos.

CONNECTING STATEMENT 1

As highlighted in the literature review, using prepubertal animals in LOPU-IVEP would dramatically improve rates of genetic gain and the long-term viability of buffalo farming. However, low rates of in vitro embryo production have hindered the widespread commercial use of this technology. Therefore, we believe that oocytes from prepubertal animals require a specially tailored process in order to prime the oocytes ready for fertilization and early embryonic development.

Procedures to promote the acquisition of oocyte competence should be two-pronged, occurring both in vivo inside the follicle prior to LOPU, and in vitro during in vitro maturation post-LOPU. Considering the best use of resources, we decided it was preferable to focus on the in vitro parameters first, with the optimization of in vitro maturation media the topic of Chapter 4. Performing LOPU is expensive and stressful for the animals; therefore, it was best to conduct this stage of research using slaughterhouse-derived oocytes. Ovaries from prepubertal water buffalo calves are not available at slaughterhouses in Canada. However, porcine oocytes are an excellent model for the study of prepubertal oocytes and widely available from abattoirs. When sent to market, gilts are still of a peripubertal age, and polyspermy rates are high in porcine IVF compared to other livestock species. Hence, they are an easier, low-cost strategy, while also not having to subject animals to LOPU. Furthermore, porcine oocytes are also physiologically similar to buffalo oocytes due to their very high lipid content, making them especially prone to lipid peroxidation and oxidative stress.

Therefore, the goal of our first research manuscript, and the first objective of this thesis, was to optimize in vitro maturation protocols for porcine oocytes used in IVF. Specifically, we tested the impact of combining a cocktail of three growth factors: FGF2, IGF1 and LIF (collectively known as FLI) with follicular fluid, as well as a series of three different antioxidants: cysteine, melatonin and insulin-transferrin-selenium (ITS) used individually, and in combination. Improvement assessment was based on their impact on cumulus expansion, meiotic maturation, fertilization and embryo development.

CHAPTER 4

Original Research Article 1

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Optimizing Swine in vitro Embryo Production with Growth Factor and Antioxidant Supplementation During Oocyte Maturation

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Porcine oocytes, IVM, IVF, Growth Factors, Antioxidants, Embryo Development

4.1. Abstract

Porcine in vitro fertilization often results in low embryo development rates compared to other livestock species, which is often associated with either a low fertilization rate or high incidence of polyspermy. Since the quality of oocyte maturation is known to play a significant role in oocyte competence, we investigated the impact of supplementing in vitro maturation (IVM)

medium containing porcine follicular fluid (pFF) with the growth factors FGF2, LIF and IGF1 (FLI), along with different combinations of cysteine, melatonin and ITS, on cumulus cell expansion, oocyte meiotic maturation, fertilization outcome, embryo development and blastocyst cell numbers. Maturation medium containing pFF yielded the greatest cumulus expansion. Compared to pFF and FLI individually, using pFF and FLI together resulted in the best embryo development rates over total oocyte placed in IVF (12.5% vs. 15.0% vs. 26.6%, respectively). Supplementation of IVM medium containing pFF and FLI with either cysteine, melatonin or insulin-transferrin-selenium, revealed that cysteine was essential to improve embryo development, while melatonin and ITS had a limited impact on improving blastocyst rates. Finally, we observed that pig oocytes matured in medium supplemented with pFF, FLI, cysteine and melatonin had a high proportion of monospermic zygotes (68.2%) and low proportion of polyspermic zygotes (15.9%) following IVF and yielded superior cleavage (78.2%) and blastocyst (32.0%) rates.

4.2. Introduction

The porcine model is of keystone importance due to its paramount status in livestock production and food security, as well as its extensive use in biomedical research [1, 2]. In vitro fertilization (IVF), an important tool for the study of embryo development and for the creation of porcine models, remains a significant challenge in pigs compared to other livestock species mainly due to inconsistent fertilization rates and high incidences of polyspermy leading to low embryo development rates [3-5]. Although the capricious nature of porcine IVF has been widely reported in the literature, the exact mechanisms underlying these results remain poorly understood [6, 7]. It has been noted however, that a high percentage of in vitro matured porcine oocytes fail to complete cytoplasmic maturation, with the developmental competence of oocytes matured in vitro low compared to those matured in vivo [5, 8] Therefore, it has been postulated that IVM conditions may be insufficient to promote complete cytoplasmic maturation and confer cumulus oocyte complexes (COCs) the necessary means to properly manage oocyte activation, the block to polyspermy and regulate early embryonic development [9, 10].

A wide array of growth factors have been identified to influence oocyte maturation. Among the important findings for pig oocytes is that supplementing a defined IVM medium with fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF) and insulin-like growth factor 1 (IGF1), collectively known as '*FLI*', improved cumulus cell expansion, oocyte nuclear maturation, as well

as blastocyst rate and embryo quality following IVF [11, 12]. Furthermore, FLI supplementation during IVM was shown to modulate the dissociation of transzonal projections (TZPs) during maturation in both bovine [13] and porcine oocytes [11]. Thus, potentially allowing oocytes to better manage the movement of molecules to and from granulosa cells, ergo, promoting cytoplasmic maturation [14]. However, as all these growth factors are naturally present in follicular fluid [15-17], it remains unclear whether an IVM medium containing follicular fluid would provide sufficient growth factors required for the complete nuclear and cytoplasmic maturation of porcine oocytes, or whether combining a pFF-supplemented medium with FLI would offer any advantage.

In vitro-produced embryos are known to bear higher oxidative stress burdens compared to in vivo-produced embryos, which are associated with multiple factors that include organelle changes [18], impaired meiotic maturation [19] and cell death [20-22]. Indeed, oxidative stress occurs when cells are exposed to either an over-abundance of reactive oxygen species (ROS) or possess insufficient machinery required to neutralize them [20], causing damage to a wide array of cellular components including lipid peroxidation of cellular membranes and nucleic acid fragmentation in mitochondria [18, 20]. Furthermore, environmental factors can also induce excess ROS production including atmospheric oxygen, visible light and temperature changes [21]. Therefore, proper oxidative stress management using antioxidants is critical for in vitro embryo production. Glutathione (GSH), present in both sperm and oocytes, is the cell's major line of defence against oxidative stress, helping to maintain the redox state within the cell [23]. GSH has been shown to be synthesized during oocyte maturation in a variety of species including cattle [24], buffaloes [25], sheep [26], goats [27] and pigs [28]. Antioxidants can either scavenge reactive free radicals directly (e.g., melatonin) or promote GSH synthesis with thiol containing compounds such as cysteine or cysteamine [29]. For example, cysteine is a sulphur containing amino acid and GSH pre-cursor, which must be biologically available for GSH synthesis to occur [23, 30].

Melatonin and insulin-transferrin-selenium (ITS) are other antioxidant compounds present in the ovary and follicular fluid that can mitigate ROS loads. Melatonin (N-acetyl-5-methoxytryptamine) is a hormone principally produced in the pineal gland, but is also produced in other tissues throughout the body including the ovary [31]. As such, melatonin has been found in the follicular fluid of multiple species including cattle [32], pigs [33], buffaloes [34], sheep [35] and goats [36]. Although well known to be a major hormone regulating the reproductive axis of seasonal breeding animals [37], melatonin has also been described as a broad-spectrum antioxidant

and effective free radical scavenger [38-41]. Due to its amphiphilic nature, melatonin can readily translocate through cellular membranes into nuclei or mitochondria, as well as scavenge free radicals directly in the cytosol [38]. For these reasons, melatonin supplementation during oocyte IVM has been used in multiple species, including cattle [39, 42], pigs [33], sheep [43] and goats [36]. On the other hand, ITS, which was originally used in cell culture systems to reduce dependency on fetal bovine serum, is another antioxidant that has been tested during IVM of bovine [44, 45], caprine [46], ovine [47], bubaline [48] and porcine [49, 50] oocytes. In pigs, ITS supplementation during IVM has also been shown to improve cortical granule distribution [50]. Within the ovary, insulin is known to promote the proliferation of granulosa cells, production of progesterone and regulation of steroidogenesis [51]. Transferrin is a major glycoprotein found in follicular fluid [52] that binds to iron and may help reduce ROS concentrations, while selenium is an essential trace element known to have antioxidant properties, as selenite is a co-factor for the reduction of glutathione peroxidase [53]. However, it remains unclear if combining antioxidants with different properties, i.e. direct scavengers and pro-GSH thiol donors, would further benefit porcine IVM in medium supplemented with pFF.

In this manuscript, we explore whether porcine COCs benefit from growth factor and antioxidant supplementation during in vitro maturation in a medium containing 20% pFF. The specific objectives were to improve cumulus cell expansion, oocyte meiotic maturation, normal fertilization and embryonic development, and consisted of: i) assessing the impact of pFF, FLI or pFF + FLI supplementation on IVM; ii) comparing the effect of supplementing cysteine, melatonin or ITS during IVM in presence of pFF+FLI; and iii) investigating if cysteine, melatonin and ITS have additive effects during IVM in presence of pFF+FLI.

4.3. Materials and Methods

4.3.1. Chemicals

Unless otherwise stated, all chemicals and reagents were purchased from Sigma Chemical Company (Milllipore Sigma; Oakville, Ontario, Canada).

4.3.2. Oocyte Collection and In Vitro Maturation (IVM)

Porcine ovaries from prepubertal gilts were collected at a local abattoir (CBCo Alliance, Les Cèdres, Quebec, Canada) and transported to the laboratory at 32°C in a 0.9% saline solution

containing penicillin (100 UI/mL) and streptomycin (10 mg/mL). Cumulus-oocyte complexes (COCs) were aspirated from 3 to 6 mm follicles using a 10 mL syringe and 20-gauge needle. Follicle aspirate was centrifuged (300 rpm for 3 minutes) to isolate cellular material from the supernatant. COCs were washed in manipulation media (TCM-199 HEPES-buffered medium supplemented with 1% pFF) and suitable COCs with a minimum of at least three compact layers of cumulus cells and homogenous cytoplasm were selected for IVM. In order to prepare pFF, following COC isolation, follicle aspirate supernatant was further centrifuged (5000 X g for 20 minutes), the supernatant was then removed and centrifuged a second time, before being filtered through a 0.22 μm filter.

Groups of 30 COCs were matured at 38.5°C in 5% CO_2 and 95% air for a total of 44h in 90 μL drops of maturation medium, plated under mineral oil (Fisher Scientific, Ottawa, Ontario, Canada) according to the experiment, described below. The base IVM medium used in all experiments consisted of TCM-199 (Life Technologies, Burlington, Ontario, Canada) supplemented with 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 10 ng/mL epidermal growth factor (EGF; Life Technologies), 0.91 mM sodium pyruvate, 3.05 mM D-Glucose, 0.5 UI/mL hCG (Chorulon®; Merck Animal Health, Kirkland, Quebec, Canada), 10 μg Armour std./mL FSH (Folltropin-V®; Vétoquinol, Lavaltrie, Quebec, Canada) and 20 μg/mL gentamicin. Following 22h of incubation in this maturation medium (IVM 1), oocytes were transferred into a new drop of the same medium, but without hCG, FSH and dbcAMP (IVM 2), for an additional 22h under the same conditions. Depending on the experiment, both IVM media 1 and 2 were supplemented with pFF, FLI, cysteine, melatonin and ITS as described below.

4.3.3. Cumulus Expansion

COCs were photographed prior to maturation (0h), and again following IVM II (44h), using a Leica Infinity1 camera attached to a Leica M205 C stereomicroscope at a standardized zoom. Images were then analyzed using ImageJ software (NIH, Bethesda, MD) to measure the area of individual COCs, which was then used to determine the cumulus expansion rate relative to their initial size prior to maturation.

4.3.4. Parthenogenetic Activation (PA)

Following maturation, cumulus cells were removed by vortexing for two minutes in HEPES-buffered TCM-199 supplemented with 0.1% hyaluronidase. Oocytes were activated using the IT-20 protocol [54]. Briefly, denuded oocytes were washed twice in TCM199 supplemented with 2 mg/mL bovine serum albumin (BSA, fatty acid free). Oocytes were then exposed to 15 μM ionomycin for 5 minutes, washed in TCM199 supplemented with 3 mg/mL BSA, followed by exposure to 200 μM N,N,N',N'-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) for 15 minutes. Finally, oocytes were incubated in PZM-3 medium supplemented with 7.5 μg/mL cytochalasin B and 3 mg/mL BSA for 4 hours to prevent the extrusion of the second polar body; followed by transfer into IVC drops.

4.3.5. In Vitro Fertilization (IVF)

Following maturation, cumulus cells were removed by vortexing for two minutes in HEPES-buffered TCM-199 medium supplemented with 0.1% hyaluronidase. Denuded oocytes were washed three times in modified Tris-Buffered Medium (mTBM) [55], containing 2 mM caffeine and 0.2% BSA. Fresh, cooled boar semen (CIPQ, Roxton Falls, Quebec, Canada) was washed in mTBM without caffeine before being resuspended in mTBM with caffeine. Sperm concentration was counted, and motility assessed. Finally, the denuded oocytes were fertilized in groups of 60-80 using 2 x 10^5 motile sperm/mL in a four-well NUNC plate with 500 μL of mTBM for 5h. After IVF, presumptive zygotes were washed and vortexed in mTBM medium to remove sperm stuck to their zona pellucida; followed by transfer into IVC drops.

4.3.6. In Vitro Culture (IVC)

Presumptive zygotes were cultured in groups of approximately 20 in 60 μL drops of PZM-3 medium supplemented with 3 mg/mL BSA, under mineral oil, in a humidified atmosphere of 5% CO_2 and 95% air at 38.5°C. Culture medium was supplemented with 10% fetal bovine serum (FBS) on day 5. Embryo cleavage rates were recorded 48-h following IVF or PA, while blastocyst rates were recorded after 168h (day 7). Embryos that developed to the blastocyst stage were fixed and stained for cell counting.

4.3.7. Staining and Fluorescent Microscopy

Subsets of oocytes, zygotes and blastocysts were fixed in 4% paraformaldehyde for 15 minutes and then permeabilized in PBS containing 0.3% BSA and 0.1% Triton X-100. Samples were later exposed to 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) for 20 minutes before being washed three times in the same PBS-BSA-Triton solution. Samples were then mounted on microscope slides using mowiol 40-88 mounting media, covered with a cover slip, and evaluated using a fluorescent microscope. To assess nuclear maturation, oocytes were denuded and fixed after IVM, and those at the MII stage were considered matured. To assess fertilization rate, presumptive zygotes were fixed 15h after the end of the fertilization period and were classified as normally fertilized (2 pronuclei), polyspermic (>2 pronuclei), non-fertilized (MII stage), or immature (GV/GVBD/Metaphase I stages). Fragmented or dead oocytes were removed from consideration. The number of cells per blastocyst was determined by counting the number of nuclei.

4.3.8. Experimental Design

Following aspiration, COCs were pooled and randomly distributed to different treatments. Each experiment was replicated at least 3 times.

4.3.8.1. Experiment 1: Growth Factors and Follicular Fluid

In this experiment a total of 2 682 COCs (IVF: n= 1511, 5 replicates; PA: n=1171, 4 replicates) were used to test the impact of: (i) the base IVM medium with 0.1% w/v polyvinyl alcohol (PVA) and FLI (20 ng/mL recombinant human LIF (Peprotech, Cranbury, NJ, USA), 20 ng/mL recombinant human IGF 1 (Peprotech) and 40 ng/mL recombinant human FGF2 (Gold Biotechnology, St. Louis, MO, USA)); (ii) the base IVM medium with 20% pFF (without PVA and FLI); and (iii) the base IVM medium with both 20% pFF and FLI (without PVA). All treatments contained 100 µg/mL cysteine.

4.3.8.2. Experiment 2: Melatonin Concentration

In this experiment, a total of 1 217 COCs (3 replicates) were used to determine the optimal melatonin concentration. The base IVM medium supplemented with 20% pFF and FLI was used in this experiment. Melatonin concentrations tested were 0, 0.1, 1, 10 and 100 nM in the absence of other antioxidants.

4.3.8.3.Experiment 3: Individual Antioxidants

In this experiment, a total of 1 005 COCs (3 replicates) were used to test antioxidants individually. The base IVM medium supplemented with 20% pFF and FLI growth factors was used in this experiment. Selected COCs were matured either in the absence of antioxidants (control) or the presence of cysteine (100 μg/mL), melatonin (100 nM), or ITS (5 μL/mL = 0.5 mg/mL recombinant human insulin, 0.275 mg/mL human transferrin and 0.25 μg/mL sodium selenite).

4.3.8.4.Experiment 4: Antioxidant Combinations

In this experiment, a total of 954 COCs (3 replicates) were used to test antioxidants concurrently. The base IVM medium supplemented with 20% pFF and FLI growth factors was used in this experiment. Selected COCs were matured with either cysteine alone (100 μg/mL), cysteine (100 μg/mL) + melatonin (100 nM), cysteine (100 μg/mL) + ITS (5 μL/mL), or cysteine (100 μg/mL) + melatonin (100 nM) + ITS (5 μL/mL).

4.3.9. Statistical Analyses

All data was analyzed using the JMP software (SAS institute Inc., Cary, NC). In each experiment, normality of data was tested using the Shapiro-Wilk W test and normalized when necessary. A one-way ANOVA followed by Tukey-Kramer HSD test was then performed. Fertilization data was analyzed using the chi-square test in a contingency table. Differences were considered statistically significant at the 95% confidence interval ($P < 0.05$).

4.4. Results

4.4.1. Impact of IVM supplementation with FLI

In the first experiment, the impact of supplementing IVM medium with either pFF or FLI individually, or pFF and FLI together was assessed. Cumulus expansion following IVM was significantly ($P<0.05$) improved when COCs were matured with pFF or pFF + FLI compared to the FLI group without pFF (Table 1; Supplementary Figure 1). This difference in cumulus expansion was not reflected in nuclear maturation (MII stage) however, as all 3 treatments yielded similar maturation rates (Table 2). Following parthenogenetic activation, embryo cleavage rates were not statistically different across treatments. However, embryo development rates

(blastocyst/cleaved) were significantly better ($P<0.05$, $n = 946$) in the pFF + FLI group (73.1 ± 12.4%) compared to FLI alone (53.4 ± 11.0%) (Fig. 1A). Blastocyst cell counts were similar ($P>0.05$) among treatments (pFF: 60.9 ± 29.4, FLI: 59.5 ± 22.9, pFF + FLI: 69.9 ± 31.4; $n = 64$). When IVF was performed, normal fertilization rates and polyspermy rates were not statistically different ($P>0.05$; Fig. 1B). However, the non-fertilization rate was significantly higher ($P<0.05$, $n = 279$) in oocytes matured with FLI (40.0%) than pFF (17.2%). Oocytes matured with pFF + FLI had significantly lower immaturity rate (3.2%) compared to pFF alone (11.1%) (Fig. 1B). The mean number of pronuclei in polyspermic zygotes was similar among treatments (pFF: 3.6 ± 0.8, FLI: 3.8 ± 0.8, and pFF + FLI: 3.7 ± 1.2; $P>0.05$; $n=26$). Total embryo cleavage rates at 48h post IVF did not differ significantly ($P>0.05$) among pFF, FLI and pFF + FLI treatments. IVM in pFF + FLI resulted in a significantly ($P<0.05$) higher blastocyst rate over cleaved compared to FLI (43.3 ± 8.0% vs. 23.7 ± 13.5%) and over oocyte compared to pFF (26.2 ± 10.1% vs. 12.5 ± 7.7%, $n = 831$) (Fig. 1C). The percentage of hatched blastocysts was numerically higher in the pFF + FLI treatment (34.8 ± 9.7%) than pFF (29.7 ± 21.4%), and FLI (19.0 ± 15.6%), but was not statistically different between treatments. However, the blastocyst cell number was significantly higher in the pFF + FLI treatment (73.6 ± 31.4) compared to the FLI group (59.9 ± 29.4), the pFF treatment (66.2 ± 27.7) was in between ($P<0.05$, $n = 158$). As supplementation with both follicular fluid and FLI (pFF + FLI) resulted in a higher blastocyst rate and cell number than pFF or FLI individually, all subsequent experiments were performed using IVM supplemented with both pFF and FLI.

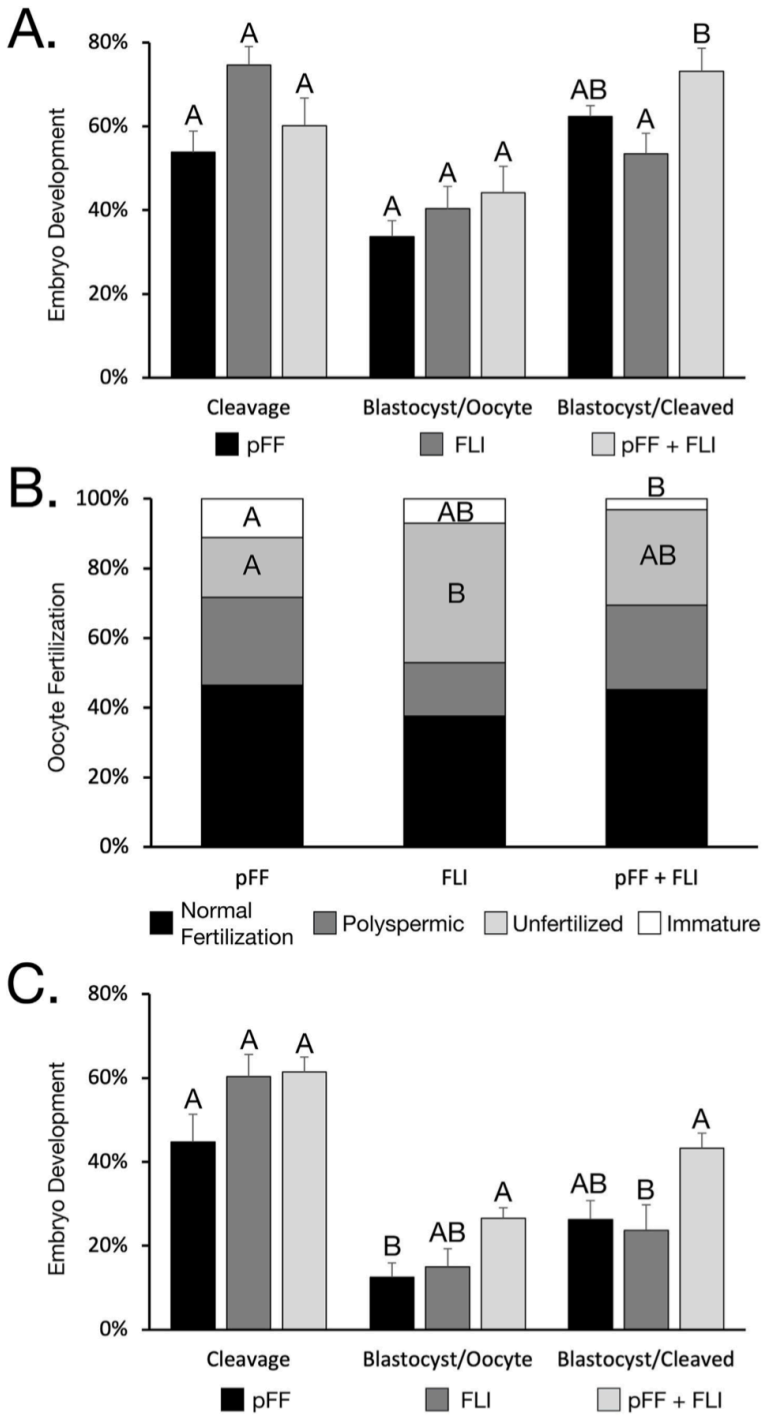


Figure 4.1. The impact of follicular fluid and FLI during IVM on fertilization outcome and embryo development. A: Embryo development rates for parthenogenically activated oocytes. B: Proportion of normally fertilized, polyspermic, unfertilized and immature oocytes among treatments. C: Embryo cleavage and blastocyst rates among treatments in IVF produced embryos. Values within the same chart with different script (A, B) differ significantly ($P<0.05$).

4.4.2. Impact of IVM supplementation with antioxidants

As different concentrations of melatonin have been used in previous IVM studies, we first tested the impact of supplementing IVM medium containing pFF and FLI with 0, 0.1, 1, 10 and 100 nM melatonin. Cumulus expansion was significantly increased ($P<0.05$) when COCs were matured in the presence of 100 nM melatonin compared to the other concentrations (Table 1; Supplementary Figure 1). Meiotic maturation to MII stage also tended to increase ($P=0.055$) as the melatonin concentration increased (Table 2). Total fertilization rates were similar among all five concentrations; however, normal (monospermic) fertilization was higher, and polyspermy was lower ($P<0.05$) in oocytes matured in the presence of 100nM (52.9% and 11.8%) compared to 0 nM (20.4% and 33.3%), 0.1nM (21.4% and 38.1%), and 1nM (25.0% and 36.1.8%) melatonin (Fig. 2A). The mean number of pronuclei of polyspermic zygotes, embryo cleavage, blastocyst rates, hatching rates and blastocyst cell counts were not significantly affected by melatonin concentration. However, blastocyst rates were numerically increased as melatonin concentration in IVM was increased (Fig. 2B). Both hatching rate and blastocyst cell counts were not significantly affected ($P<0.05$) by the melatonin concentration during IVM. Based on these findings, 100nM melatonin was used in the subsequent experiments.

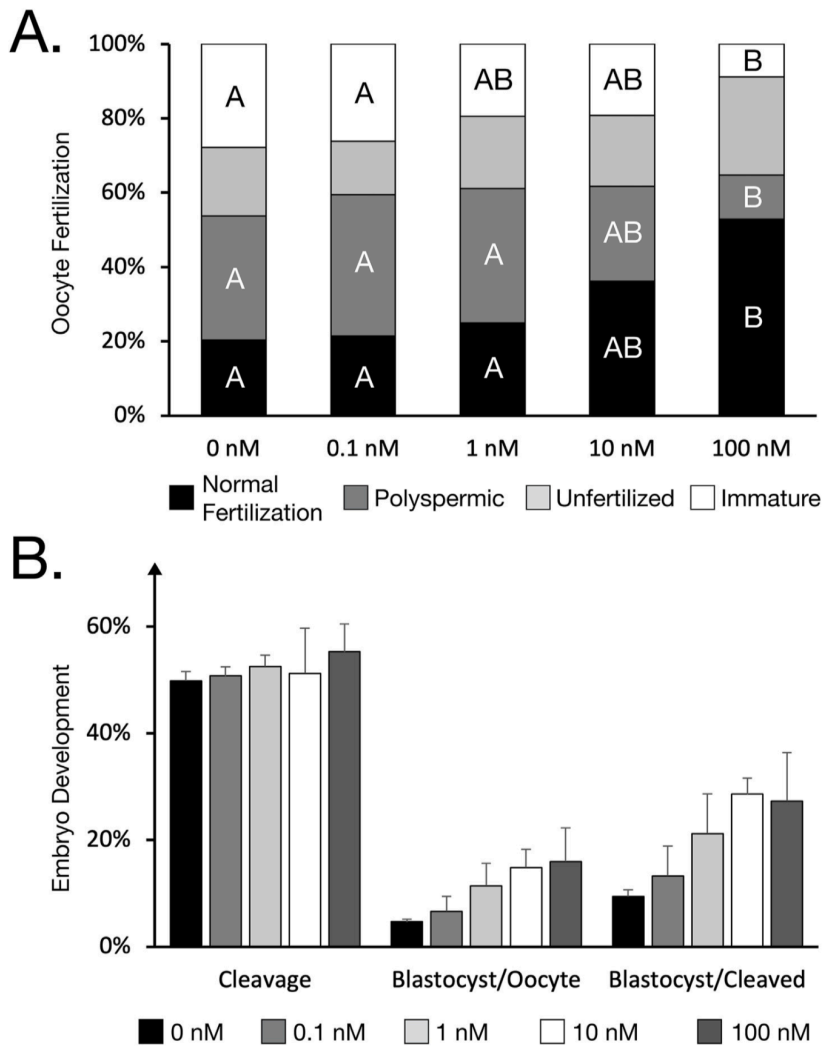


Figure 4.2. Impact of melatonin concentration during IVM on oocyte fertilization outcome and embryo development. A. Fertilization outcome in response to melatonin concentration. B. Embryo development as a function of melatonin concentration. Values within the same chart with different script (A, B, C) differ significantly ($P<0.05$).

The effects of cysteine, melatonin and ITS were evaluated against IVM without antioxidant supplementation in the presence of pFF and FLI. Cumulus expansion was significantly less prominent ($P<0.05$) in COCs matured in absence of antioxidants than in presence of cysteine, melatonin, or ITS. However, cumulus expansion of COCs matured with ITS was significantly higher than COCs matured with melatonin (Table 1; Supplementary Figure 1). Nuclear maturation rates were similar in oocytes matured in the presence of cysteine, melatonin or ITS, while the absence of an antioxidant resulted in significantly lower ($P<0.05$) maturation (Table 2). Following

fertilization, there was a significant difference in normal fertilization rate between the melatonin (51.4%) and no antioxidant treatments (26.3%), while cysteine and ITS yielded similar rates (Fig. 3A). Although there were no significant differences among treatments on unfertilized and polyspermy rates, the absence of any antioxidant yielded significantly more immature oocytes compared to the cysteine and melatonin (no antioxidant: 28.9%, cysteine: 8.3%, melatonin: 10.8%, ITS: 12.1%; n=144) (Fig. 3A). Embryo cleavage rates were not significantly different between treatments (Fig. 4B). The blastocyst rate was significantly higher ($P<0.05$) when COCs were matured in presence of cysteine (23.5 ± 7.7%) than no antioxidant (5.3 ± 2.6%), but supplementation of IVM medium with melatonin (14.4 ± 8.4%) or ITS (10.3 ± 4.1%) did not improve blastocyst rate compared to the control (Fig. 3B). Embryo hatch rates (no antioxidant: 23.3 ± 25.2%, cysteine: 23.7 ± 21.4%, melatonin 28.1 ± 2.9%, ITS: 22.5 ± 9.8%), were not statistically different among treatments ($P>0.05$). Blastocyst cell numbers were numerically higher in melatonin (83.2 ± 40.1) and cysteine (81.3 ± 34.4) treatments than ITS (58.4 ± 36.3) and no antioxidant (67.0 ± 26.4), but were not statistically different ($P>0.05$, $n = 50$).

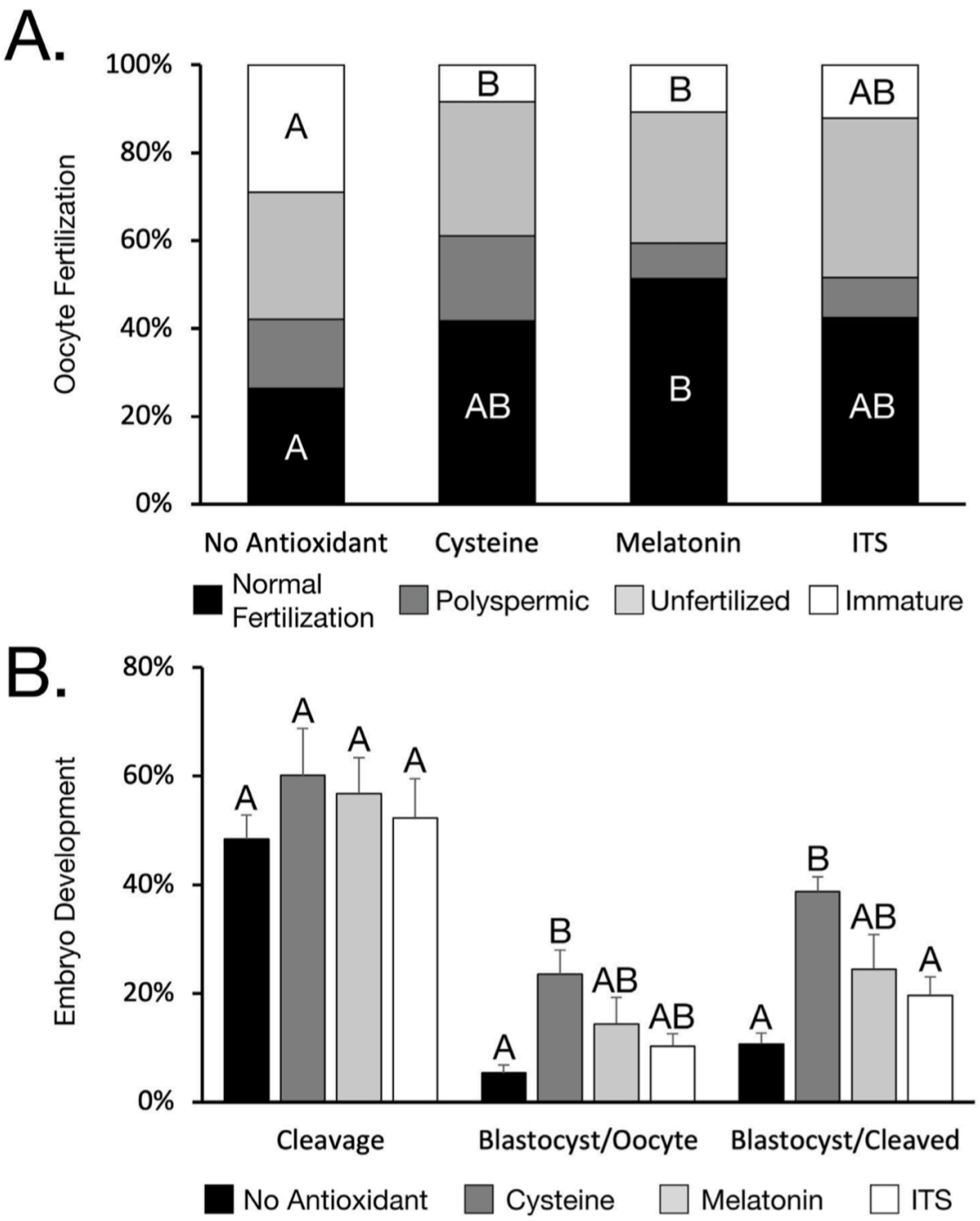


Figure 4.3. The effect of individual antioxidants on oocyte fertilization and embryo development. A: Oocyte fertilization outcome per treatment following IVF. B: Embryo development rate by antioxidant. Values within the same column with different script (A, B) differ significantly ($P<0.05$).

4.4.3. Impact of IVM supplementation with antioxidants in combination

The final experiment was conducted to assess if there was an additive effect of antioxidants during IVM by supplementing the maturation medium containing pFF, FLI and cysteine (Cyst) with melatonin (Cyst + Mel), ITS (Cyst + ITS), or both melatonin and ITS (Cyst + Mel + ITS). COCs matured in the two treatments containing ITS (Cyst + ITS and Cyst + Mel + ITS) led to the greatest cumulus expansion compared to cysteine or melatonin (Table 1; Supplementary Figure 1). Nuclear maturation rates were not affected ($P>0.05$) by antioxidant treatments (Table 2). Following IVF, there were no significant differences in normal fertilization, polyspermy, unfertilized oocytes, and immaturity rates among treatments ($P>0.05$, n = 172, Fig. 4A). In polyspermic zygotes, there was no significant difference in the mean number of pronuclei present (Cyst: 3.1 ± 0.4, Cyst + Mel: 3.1 ± 0.4, Cyst + ITS: 3.3 ± 0.5, Cyst + Mel + ITS: 3.4 ± 0.5, $P>0.05$, n = 30). Cleavage rates were similar across treatments. Blastocyst rates per oocyte were numerically higher for COCs matured with Cyst + Mel but were not statistically different than other treatments (n = 561; Fig. 4B). There was no significant difference among treatments ($P>0.05$) for embryo hatching rates (Cyst: 19.8 ± 21.6, Cyst + Mel: 21.5 ± 15.5, Cyst + ITS: 24.5 ± 8.1, Cyst + Mel + ITS: 27.5 ± 7.6). Blastocyst cell counts for COCs matured with Cyst + Mel (74.0 ± 34.0) was numerically higher, but not statistically significant than Cyst (69.5 ± 41.9), Cyst + ITS (58.7 ± 31.2), or Cyst + Mel + ITS (56.2 ± 28.6).

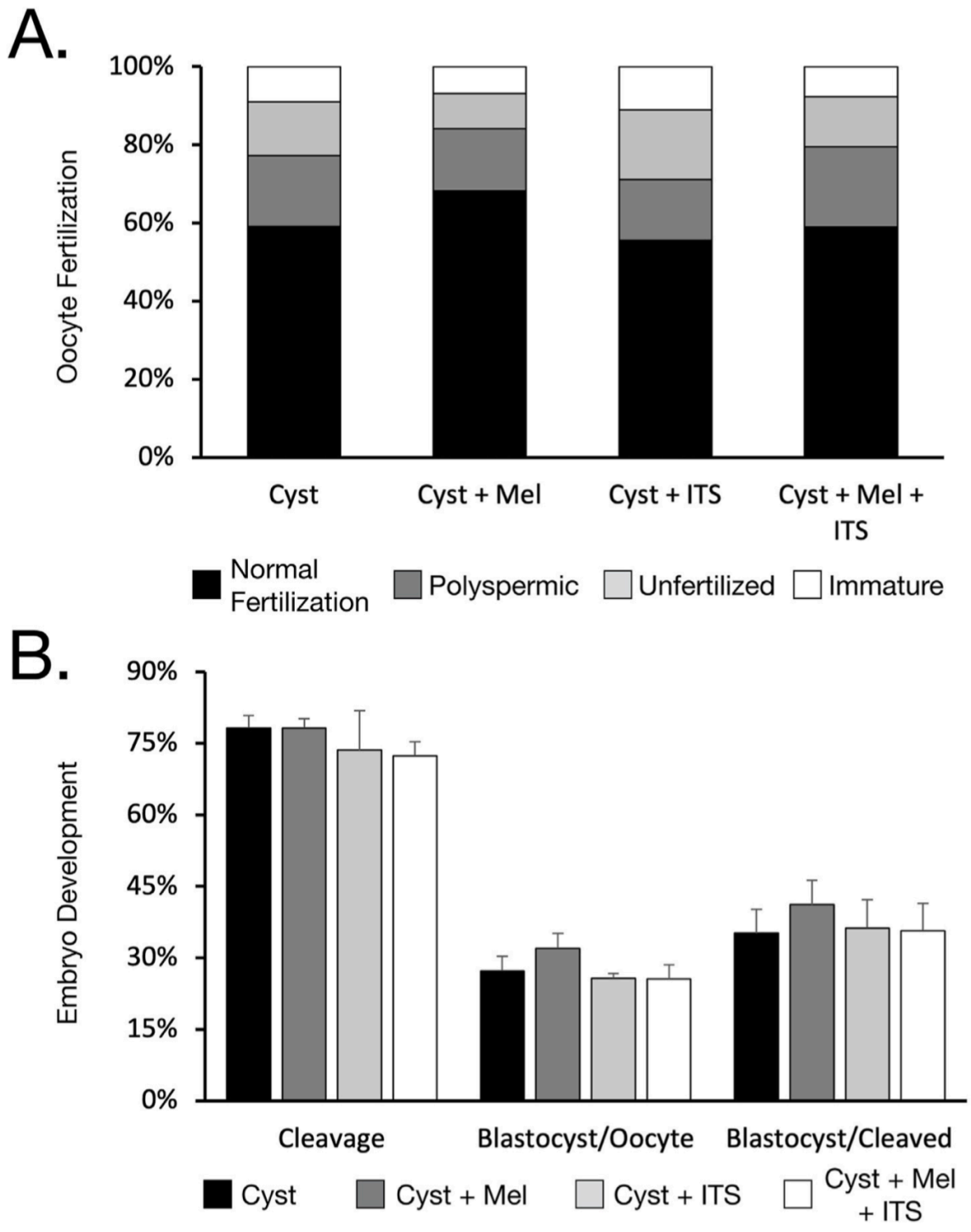


Figure 4.4. The impact of using antioxidants in combination on oocyte maturation, fertilization outcome and embryo development rate. A: The effect of antioxidant combinations on fertilization outcome. B: The effect of antioxidant combinations on embryo development rates.

Table 4.1. The impact of supplementing the IVM medium with different compounds on the expansion of cumulus cells.

Experiment	Treatment	Number of COCs	Cumulus expansion (fold increase during IVM ± SD)
Exp. 1: Growth factors	pFF	144	8.71 ± 11.3^{A}
	FLI	152	3.68 ± 4.7^{B}
	pFF + FLI	135	7.16 ± 6.6^{A}
Exp. 2: Melatonin concentration	0	118	5.63 ± 4.5^{A}
	0.1 nM	169	5.63 ± 4.8^{A}
	1 nM	141	6.02 ± 5.8^{A}
	10 nM	149	6.79 ± 6.2^{A}
	100 nM	130	8.16 ± 3.9^{B}
Exp. 3: Individual antioxidants	No antioxidant	117	6.10 ± 9.2^{C}
	Cysteine	109	7.92 ± 7.2^{AB}
	Melatonin	148	6.90 ± 6.4^{B}
	ITS	131	8.99 ± 7.2^{A}
Exp. 4: Antioxidant combinations	Cyst	145	9.14 ± 9.4^{A}
	Cyst + Mel	148	9.31 ± 7.6^{AB}
	Cyst + ITS	137	11.19 ± 8.5^{B}
	Cyst + Mel + ITS	127	11.12 ± 11.6^{AB}

Cumulus expansion is represented as the average fold increase in the cumulus cell area at the end of IVM (± standard deviation) compared to the cumulus cell area at the beginning of IVM. Values within each experiment with different script (A, B, C) differ significantly ($P<0.05$).

Table 4.2. The impact of supplementing IVM medium with different compounds on the meiotic maturation of porcine oocytes

Experiment	Treatment	Number of Oocytes	Meiotic Maturation Rate (Mean ± SD)
Exp. 1: Growth factors	pFF	88	90.6 ± 8.0
	FLI	109	89.6 ± 6.2
	pFF + FLI	108	95.2 ± 2.0
Exp. 2: Melatonin concentration	0	33	59.4 ± 3.6
	0.1 nM	46	63.0 ± 25.4
	1 nM	45	78.5 ± 5.7
	10 nM	47	77.8 ± 10.2
	100 nM	44	93.3 ± 6.0
Exp. 3: Individual antioxidants	No antioxidant	30	53.0 ± 17.2^{B}
	Cysteine	36	89.2 ± 3.0^{A}
	Melatonin	31	89.0 ± 9.8^{A}
	ITS	29	83.8 ± 6.2^{A}
Exp. 4: Antioxidant combinations	Cyst	42	92.9 ± 7.1
	Cyst + Mel	40	92.5 ± 7.7
	Cyst + ITS	38	84.4 ± 7.4
	Cyst + Mel + ITS	41	92.6 ± 8.6

Meiotic maturation is represented as the percentage of oocytes at metaphase II at the end of IVM (± standard deviation). Values within each experiment with different script (A, B, C) differ significantly ($P<0.05$).

4.5. Discussion

In vitro embryo production is a core technology for a variety of research applications in the swine species including animal selection and breeding to meet increasing global food demands, and the production of genome-edited animals for use in biomedicine and agriculture [56, 57]. However, in vitro production of porcine embryos often results in inconsistent and poor development rates, which are associated with the quality of the oocytes and poor IVF efficiency [5-7]. Attempts to improve porcine IVF efficiency have included adjusting sperm concentration [58, 59], gamete preparation and co-incubation time [59-61], and IVF media composition [60, 62]. However, none of these approaches have conclusively solved the problem. Oocyte competence is known to impact IVF efficiency and subsequent embryo development. For example, oocytes derived from larger follicles, which are in a more advanced stage of their cytoplasmic maturation, compared to those derived from smaller follicles, yield superior blastocyst rates and embryo quality than oocytes derived from small follicles [63-67]. Thus, numerous strategies and attempts have been tested to improve porcine oocyte competence in vitro. For example, previous studies revealed that supplementation of IVM medium with pFF along with cAMP improved normal fertilization rates and embryo development [68-70]. More recent evidence also indicated that porcine oocyte competence can be further improved by supplementing growth factors [11, 71] and antioxidants [49, 50, 72-76] during oocyte maturation. Although follicular fluid contains both growth factors and antioxidants, it remained unclear if pFF can provide enough of these compounds for the full acquisition of porcine oocyte competence during IVM.

Findings in this study demonstrated that IVM medium supplemented with both pFF and FLI improved blastocyst development rates compared to pFF or FLI alone. When used separately, IVM in pFF and FLI resulted in similar embryo development, which indicate they have an additive effect in the acquisition of porcine oocyte competence. We found that cumulus expansion was greatly improved when COCs were matured in the presence of pFF, either alone or combined with FLI, compared to FLI alone. Additionally, we observed that oocyte nuclear maturation improved when oocytes were matured in the presence of pFF and FLI together rather than pFF alone. Together, these findings suggest that pFF and FLI act additively to enhance porcine oocyte competence by improving cell expansion and nuclear maturation. The fact that we observed a decreased rate of unfertilized oocytes that were matured in pFF compared to those matured in FLI suggests that cumulus expansion promoted by pFF may benefit porcine IVF efficiency. However,

cumulus expansion can affect other aspects of oocyte physiology such as the retraction of cell transzonal projections that occur independently of nuclear maturation and is regulated by other growth factors, most notably epidermal growth factor (EGF) [77]. As such, IVF efficiency in pigs is probably the result of several different factors in addition to the oocyte maturation process, including excess acrosomal reaction in the sperm [78] and an inappropriate microenvironment during fertilization itself [7].

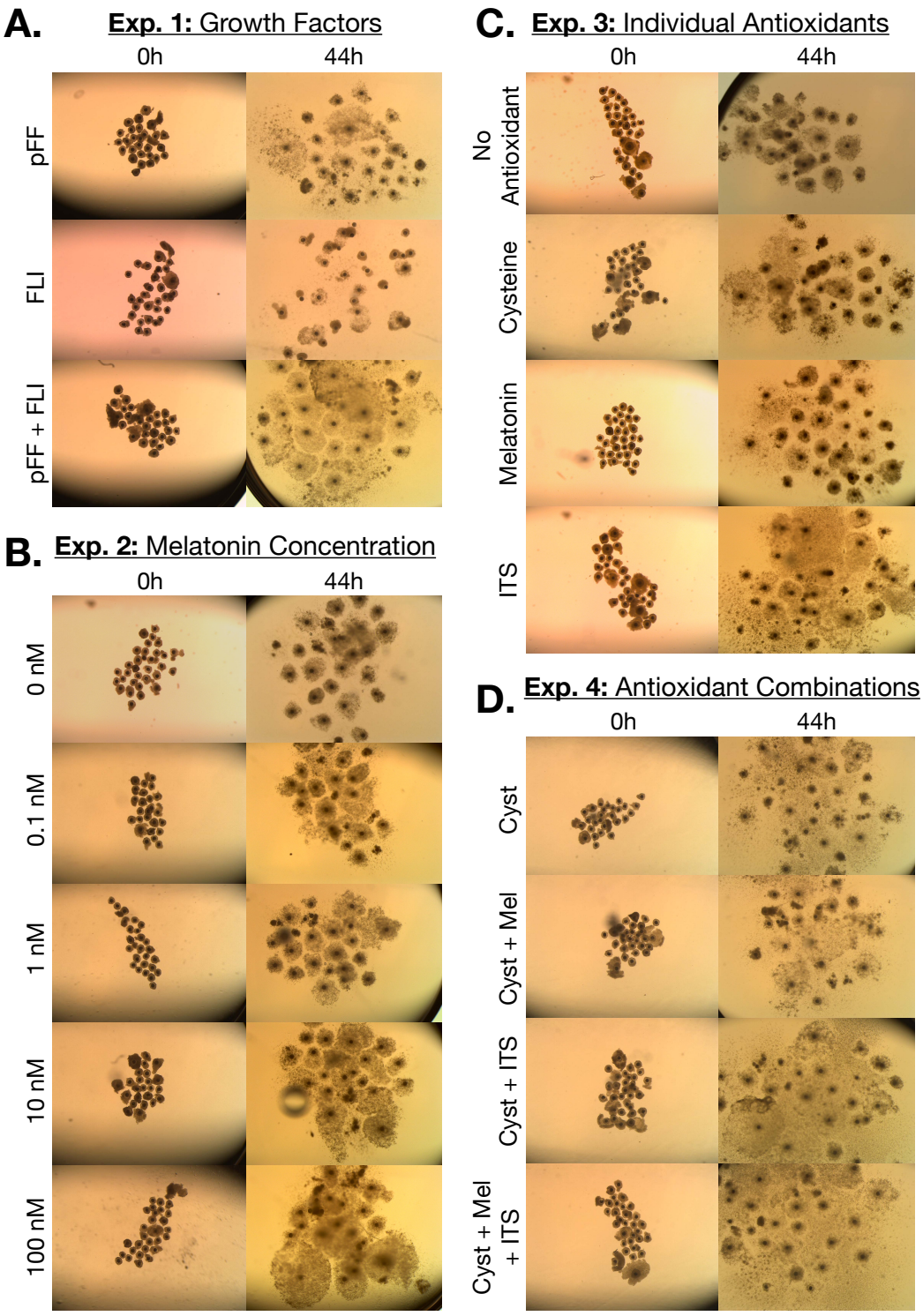
Antioxidant supplementation during IVM has an important impact for the production of embryos in vitro [29, 79, 80]. Commonly used antioxidants during porcine IVM include cysteine [81, 82], melatonin [33, 72, 73] and ITS [49, 50]. However, antioxidant demands are influenced by many factors, such as culture medium composition, culture atmosphere and oocyte source [83]. Recently, it has been shown that the addition of 1 nM melatonin to IVC medium improved embryo development and quality of in vitro-derived zygotes, but not their in vivo-derived counterparts [83]. This suggests that melatonin plays a specific role in embryos that were matured in vitro. Unfortunately, there is no agreement from the multiple studies that attempt to determine the optimal concentrations of melatonin for IVM in different species, especially in pigs [29, 33, 72, 73, 83, 84]. Thus, by testing different concentration of melatonin in an IVM medium containing pFF and FLI, we observed that both cumulus cell expansion and normal fertilization rates were improved using 100 nM melatonin. There is evidence from studies in bovine IVM suggesting that melatonin supplementation may improve fertilization by regulating the distribution of mitochondria and ER in oocytes, the peripheral migration of cortical granules, as well as the expression of Juno and distribution of IP_3R1 [42]. However, confirmation of these findings in porcine oocytes require further investigations. Melatonin has been shown to play a role in ER stress management in porcine oocytes however, with melatonin able to rescue meiotic maturation rates and expression of UPR signalling pathway markers in ER-stressed oocytes [74].

In this study, we observed that IVM in the presence of cysteine more than quadrupled the blastocyst rate of porcine oocytes matured in IVM medium supplemented with pFF and FLI in absence of antioxidants. Such an effect was not seen when IVM was supplemented with melatonin or ITS, which had only a partial impact on blastocyst rates. Part of cysteine's impact on blastocyst development can be explained by the increased nuclear maturation and cumulus cell expansion compared to the absence of antioxidants. However, there was no statistical differences in cumulus expansion, oocyte nuclear maturation and normal fertilization rates between oocytes that were

matured in presence of cysteine, melatonin and ITS. This suggests that even in the presence of direct ROS-scavengers like melatonin, oxidative stress management through glutathione plays a preponderant role in porcine oocytes. This could potentially be explained by GSH's auxiliary roles in addition to its antioxidant properties, with GSH known to promote multiple cellular processes including sperm head decondensation and subsequent male pronucleus formation during fertilization in pigs [28, 85], further emphasizing the importance of cysteine availability in vitro. These findings also suggest that different antioxidants could have an additive impact during porcine IVM. Yet, we observed that by pairing cysteine and melatonin in IVM media containing both follicular fluid and FLI yielded the best blastocyst rates and embryo cell numbers, albeit not significantly over cysteine alone. Thus, it is possible that porcine oocytes may benefit from both the direct effect of ROS-scavenging of melatonin, and the GSH-promoting effect of cysteine. However, results of combining ITS with cysteine or all three antioxidants were not improved over cysteine alone. It is worth highlighting however that oxidative stress management is a delicate balancing act, with some ROS beneficial and even necessary for proper fertilization to occur [86]. For example, ROS have been shown to play an important role in sperm capacitation in multiple species [87-89]. Hence, it is possible that an antioxidant combo requires further adjustment in the concentration of each antioxidant, as optimal concentrations may differ when used in combination with each other.

4.6. Conclusions

In conclusion, porcine in vitro embryo production can be improved by IVM in presence of pFF and FLI. Porcine IVM with pFF and FLI requires antioxidant supplementation, especially cysteine. Although melatonin and ITS supplementation enhanced porcine cumulus cell expansion, they did not significantly impact embryo development. Combining antioxidants during IVM may further impact porcine in vitro embryo production but additional studies are necessary to find the ideal concentrations of each antioxidant.



Supplemental Figure 4.5. Representative images of COC cumulus expansion before and after IVM. A: cumulus expansion for Experiment 1: Growth Factors. B: Cumulus expansion for Experiment 2: Melatonin Concentration. C: Cumulus expansion for Experiment 3: Individual Antioxidants. D: Cumulus expansion for Experiment 4: Antioxidant Combinations.

4.7. Declaration of competing interest

The authors declare that there are no conflicts of interest

CRediT authorship contribution statement

Luke Currin: investigation, formal analysis, writing – original draft preparation and review. **Werner Giehl Glanzner:** investigation. **Karina Gutierrez:** investigation. **Mariana Priotto de Macedo:** investigation. **Vanessa Guay:** investigation. **Hernan Baldassarre:** conceptualization, methodology, writing – review and editing. **Vilceu Bordignon:** conceptualization, methodology, writing – review and editing, supervision, funding acquisition.

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CONNECTING STATEMENT 2

As previously mentioned, to promote oocyte competence, we believe oocytes from prepubertal donors require specialized priming prior to fertilization both in vivo, inside the follicle prior to aspiration, and in vitro prior to fertilization. In Chapter 4, we established that porcine oocytes benefitted from in vitro maturation medium supplemented with FLI, follicular fluid and cysteine. Further supplementation of additional antioxidants, namely melatonin and/or ITS although beneficial did not yield a significant advantage over cysteine alone.

After establishing an optimal protocol in vitro (in pigs, which we believe to be a strong model for prepubertal buffalo), we turned our attention to developing a procedure to promote oocyte competence in vivo. The follicular micro-environment inside the follicle is critical for the future competence of the oocyte, since this is where the oocyte synthesizes and imports many of the proteins, nutrients and mRNA transcripts it will need for maturation, activation and early embryonic development. As discussed in the literature review, the HPO axis in prepubertal animals is immature and not fully functional. Consequently, ovarian stimulation with exogenous gonadotropins is necessary prior to LOPU to create a follicular environment required for the acquisition of an oocyte's developmental competence. Therefore, the objective of Chapter 5, and second objective of this thesis, was to develop the optimal gonadotropin stimulation protocol in water buffalo calves to promote acquisition of oocyte competence in vivo.

In Chapter 5, we investigated the optimal gonadotropin stimulation over three experiments: the effect of combining FSH with and without eCG, the effect of substituting regular FSH with a long-acting formulation of FSH, and finally the length of gonadotropin stimulation over 3, 4 and 5 days. Following LOPU, we used some of the lessons learned from Chapter 3 and matured COCs in an IVM medium we tailored for buffalo oocytes by addition of FLI growth factors, cysteine and ITS.

CHAPTER 5

Original Research Article 2

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Optimization of Gonadotropin Stimulation Protocols for In Vitro Embryo Production in Prepubertal Mediterranean Water Buffalo

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5.1. Abstract

Embryos can be produced from prepubertal donor animals using laparoscopic ovum pickup and in vitro embryo production technologies (LOPU-IVEP). Together, these tools can shorten the interval between generations, rapidly accelerating the rate of genetic gain. Here, we assessed the impact of different gonadotropin stimulation protocols in Mediterranean water buffalo heifer calves aged between 2 and 6 months old. Following gonadotropin stimulation, LOPU was performed at two-week intervals, with animals receiving different protocols on subsequent LOPUs. After collection, the cumulus-oocyte complexes (COCs) were matured and fertilized in vitro, and embryos were cultured to the blastocyst stage followed by transfer into synchronized adult recipients. The number and size of follicles aspirated during LOPU, the number and quality of COCs recovered, as well as cleavage, embryo development and pregnancy rates were assessed. First, we evaluated the impact of using FSH with and without eCG (administered 24-hours prior to LOPU) and found that a combination of FSH and eCG was able to significantly improve embryo development rates (20.6 ± 2.0% vs. 9.0 ± 3.6%; $P < 0.05$). Second, we compared this protocol to a slow-release formulation of FSH reconstituted in hyaluronan. In addition to requiring less work to prepare the animals for LOPU, this slow-release formulation yielded numerically higher, but not statistically different, average number of recovered COCs (14.4 ± 2.1 vs. 10.3 ± 2.0; $P > 0.05$) and embryo development rates (22.9 ± 4.7% vs. 14.1 ± 5.2%; $P > 0.05$) compared to FSH given every 12 hours. Next, we compared the length of gonadotropin treatment over 3-, 4- and 5-days prior to LOPU and found that as the length of gonadotropin treatment increased, although the number of COCs recovered steadily decreased (14.1 ± 2.4 vs. 8.7 ± 1.0 vs. 6.9 ± 0.7; $P < 0.05$), the embryo development rates steadily increased (14.4 ± 3.9 vs. 27.3 ± 4.4 vs. 35.9 ± 7.0; $P < 0.05$), presumably due to an increase in the proportion of large follicles at the time of LOPU. Numerically, the 4-day treatment yielded more transferrable embryos per donor per LOPU (2.70 ± 0.5) than 3-day (1.94 ± 0.6) and 5-day (2.25 ± 0.5) treatments. Finally, following embryo transfer, 26 of 90 recipient females became pregnant (28.9%). Pregnancies were established from all treatments, which suggests that post-implantation development was not affected among the gonadotropin treatments assessed.

5.2. Introduction

Domestic water buffalo (*Bubalus bubalis*) are an important livestock species globally, providing milk, meat, leather, and draught power. Despite being well-established in some regions of the world, water buffalo popularity has expanded in recent years, especially in the West [1, 2]. However, compared to dairy cattle, rearing Mediterranean water buffalo presents a unique set of challenges due to their late sexual maturity and long generational intervals, making rates of genetic gain slow [3]. Hence, water buffalo are an ideal candidate species for prepubertal reproductive technologies, also called 'juvenile in vitro embryo transfer' (JIVET). Using this approach, elite female calves can be selected through genomics shortly after birth, followed by collection of cumulus-oocyte complexes (COCs) by laparoscopic ovum pickup (LOPU), far before animals are sexually mature or large enough for ultrasound-guided OPU [4, 5]. Following in vitro embryo production (IVEP), the resulting embryos are then transferred into adult recipient animals to bring the pregnancies to term. LOPU-IVEP programs facilitate the production of multiple progenies from animals at a very young age, accelerating the rate of genetic gain by decreasing the generation interval [6]. Thereby, LOPU-IVEP has been used in buffalo heifer calves, as young as two months old, to produce multiple offspring in a rapid timeframe [4, 7, 8].

Multiple authors have shown that embryo development rates of oocytes from prepubertal donors remains lower compared to oocytes from their adult counter parts in multiple species including buffalo [9], cattle [10, 11], goat [12], sheep [13, 14] and pigs [15, 16]. Although the exact reasons and mechanisms underlying these differences are not fully understood, various discrepancies in oocyte size, organelle distribution, as well as gene and protein expression have been noted between prepubertal and adult oocytes [11, 17-19]. What is known however, is that before puberty, the hypothalamus-pituitary-ovarian (HPO) axis is immature, and not yet capable of fully supporting folliculogenesis, steroidogenesis and ovulation [20, 21]. However, follicular waves do occur in the ovary, with follicles continuously developing from primordial to antral stages when their growth stalls because they become gonadotropin-dependant and the immature axis does not provide such support. Prepubertal animals are capable of responding to exogenous gonadotropin stimulation however, yielding many follicles apt for aspiration [22, 23], making the employment of suitable ovarian stimulation protocols a key aspect affecting the success of LOPU-IVEP in prepubertal animals [24, 25]. Moreover, given the immaturity of the HPO axis, exogenous gonadotropin stimulation is required to support follicle growth and create a suitable follicular

micro-environment in addition to promoting oocyte growth and acquisition of developmental capacity prior to oocyte collection, thereby maximizing embryo development rates [5].

In the 1990s, gonadotropin stimulation protocols used in prepubertal LOPU-IVEP programs were originally adapted from superovulation protocols developed for adult animals [26]. As such, most early protocols used multiple injections of follicle-stimulating hormone (FSH), which is typically administered at 12 hours intervals, rather than one large singular dose due to its short-metabolic half-life [27, 28], which in cattle is around 5 hours [29, 30]. To simplify this, several protocols have combined a single large injection of FSH followed with one of equine chorionic gonadotropin (eCG), which has a longer metabolic half-life [26, 28]. In cattle, eCG has a half-life of around 40 hours, and persists in circulation for around 10 days [31]. These protocols, combining a single bolus of FSH with eCG, have resulted in similar ovarian responses to multiple FSH treatments, suggesting that the FSH is able to recruit a cohort of follicles, with eCG able to sustain their continued development until LOPU [28]. However, despite many variations of these original protocols being used over the years, with animals consistently responding well and producing many follicles, embryo development rates have remained low [24, 28, 32, 33].

Given the very short half-life of FSH and the immaturity of the HPO axis, prepubertal animals may benefit from gonadotropin stimulation in a more consistent manner by providing developing follicles a homogenous supply of FSH. In this regard, compared to FSH given at 12-hour intervals, administration of FSH at 8-hour intervals followed by eCG yielded significantly better embryo development rates in Holstein calves [34]. However, because administering FSH three times daily is stressful for the animals and labour-intensive, some studies have focused on developing protocols to allow sustained hormone delivery over a prolonged period, by slowing the release of FSH into circulation, thereby extending the half-life [35]. In adult animals, using a single injection of FSH diluted in hyaluronan (MAP5), a biodegradable polysaccharide found throughout the body, rather than multiple injections of regular FSH, resulted in similar numbers of follicles suitable for OPU, as well as cleavage, blastocyst development rates and pregnancy rates following embryo transfer [36-39]. To date, we are not aware of any studies attempting to use this approach in prepubertal buffalo.

Another important element for consideration is the length of gonadotropin stimulation prior to oocyte collection, due to its effect on follicle size, as there is a well-established positive correlation between follicle size and developmental competence of oocytes in multiple species

including buffalo [40, 41], sheep [42], goat [43], cattle [18, 44], and pigs [45, 46]. For example, previous studies in our laboratory working with prepubertal Holstein calves showed that FSH stimulation for 72 vs. 36 hours prior to LOPU increased the average size of follicles available for aspiration, yielding significantly more usable COCs and doubled the blastocyst rate following IVEP [25]. Similar trends have been noted in adult buffalo, with oocytes sourced from large follicles being significantly more competent than those originating from small follicles [41, 47, 48].

In this study, we assessed multiple gonadotropin stimulation protocols on prepubertal Mediterranean water buffalo heifer calves between 2 and 6 months of age based on LOPU and IVEP results. First, we compared whether using eCG in combination with multiple injections of FSH was advantageous over FSH alone. Second, we compared FSH reconstituted in a slow-release hyaluronan formulation to regular FSH given at 12-hour intervals. Finally, we tested the effect of length of gonadotropin stimulation (3 vs. 4 vs. 5 days). Stimulation protocols were evaluated based on the number and size of follicles suitable for aspiration, the number and quality of COCs recovered by LOPU, embryo development rates following IVEP, the average number of embryos produced per donor per LOPU, and the pregnancy rates after embryo transfer to adult females.

5.3. Materials and Methods

5.3.1. Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were purchased from the Sigma Chemical Company (Millipore Sigma; Oakville, Ontario, Canada).

5.3.2. Animals

All experimental procedures were reviewed and approved by the Facility Animal Care and Use Committee of McGill University (AUP # MCGL-7552) in accordance with Canadian Council of Animal Care regulations. A total of 133 LOPUs on 42 Mediterranean water buffalo heifer calves between 2 and 6 months of age were performed. LOPU was performed every two weeks up to 5 times per animal. Donor animals were housed in an indoor barn at Macdonald Campus of McGill University, located in Sainte-Anne-de-Bellevue, Quebec, Canada (45.4252° N, -73.9654° W). Animals were weaned and fed good quality second-cut hay and water offered ad-libitum, as well as a grain concentrate (Optivia®, Shur-Gain, Brossard, Quebec, Canada) twice daily according to

their body weight. Due to the large individual variations in prepubertal animals, whenever possible, different protocols were tested in the same group of animals on an alternating basis over subsequent LOPUs, i.e., within experiments, each animal received each treatment. Recipient adult animals were housed at the Ontario Water Buffalo Company Farm in Stirling, Ontario, Canada (44.3377° N, -77.5798° W).

5.3.3. Ovarian Stimulation

Animals were treated intramuscularly (IM) with FSH (Folltropin-V®, Vétoquinol, Lavaltrie, Quebec, Canada) and eCG (Novormon® 5000, Partnar Animal Health Inc., Ilderton, Ontario, Canada), according to the experiment described below. In the first experiment, we assessed the impact of combining FSH with eCG (multi-FSH + eCG), compared to FSH alone (multi-FSH). In the second experiment, we assessed the impact of FSH reconstituted in a slow-release formulation (FSH-MAP5 + eCG) compared to conventional FSH (multi-FSH + eCG). In the final experiment, we assessed the impact of gonadotropin stimulation length over 3, 4 and 5 days. To prepare regular 'multi-FSH', the 400 mg lyophilized FSH vial was reconstituted in 20 mL of 0.9% saline solution. In the slow-release formulation (FSH-MAP5), the 400 mg lyophilized vial of FSH was reconstituted in 10 mL of 0.5% hyaluronan (MAP-5®, Vétoquinol). Regardless of treatment, all animals received two vaginal progesterone implants (330 mg each) in the form of a small ruminant CIDR (EAZI-Breed™ CIDR® 330, Zoetis Canada Inc., Kirkland, Quebec, Canada) inserted five days prior to LOPU.

5.3.3.1. Effect of FSH with and without eCG

In this experiment, LOPU was performed on a group of eight animals a total of five times (n=40). Of these 40 replicates, 20 used the 'multi-FSH' protocol, while the other 20 used 'multi-FSH + eCG'. In both treatments, regular FSH was given at 12-hour intervals starting 72 hours prior to LOPU. The 'multi-FSH' treatment contained 6 injections (total 140 mg FSH), while the 'multi-FSH + eCG' treatment consisted of 4 injections of FSH (total 100 mg FSH) along with 400 IU eCG given 24 hours prior to LOPU.

5.3.3.2. Effect of FSH + eCG with and without MAP5

In this experiment, LOPU was performed on a group of 15 animals a total of between 2 and 3 times (n = 37). Of these 37 replicates, 15 received the 'multi-FSH + eCG' treatment, while 22 received the 'FSH-MAP5 + eCG' treatments. The 'multi-FSH + eCG' treatment was identical to the one described above, with FSH administered every 12 hours. The 'FSH-MAP5 + eCG' treatment consisted of FSH diluted in hyaluronan given in a single injection 72 hours prior to LOPU (100 mg FSH total) and 300 IU eCG given 24 hours before LOPU.

5.3.3.3.Effect of Treatment Length

In this experiment, LOPU was performed on a group of 19 animals between 2 and 4 times each (n = 56). Of these 56 replicates, 17 received the 3-day treatment, 23 received the 4-day treatment and 16 received the 5-day treatment. Regardless of treatment length, all animals received 300 IU eCG 24 hours prior to LOPU. In the 3-day treatment, FSH-MAP5 was given in a single injection 72 hours prior to LOPU (100 mg FSH total). In the 4-day treatment, FSH-MAP5 was given in two doses, 48 hours apart (100 mg FSH total), starting 96 hours prior to LOPU. The 5-day treatment was identical to the 4-day treatment, with the addition of 200 IU eCG 120 hours before LOPU (eCG + FSH-MAP5 + eCG).

5.3.4. Laparoscopic Ovum Pickup (LOPU)

Animals were fasted of hay for 36 hours, concentrate for 24 hours, and water for 18 hours before LOPU. Anesthesia was induced using intravenous administration of 0.05 mg/KBW xylazine (Xylamax, Bimeda Canada, Cambridge, Ontario, Canada), 2 mg/KBW ketamine (Ketalean, Bimeda Canada) and 0.1 mg/KBW diazepam (Sandoz, Boucherville, Quebec, Canada). Animals were then intubated, attached to a mechanical ventilator and maintained under general anesthesia using 2% isoflurane USP (Fresenius Kabi Canada, Toronto, Ontario, Canada).

The laparoscopic ovum pickup procedure has been described previously [49]. Briefly, once fully anesthetized, calves were restrained in the Trendelenburg position on a cradle. Under laparoscopic observation, follicles were aspirated using a 20G needle mounted to the end of an acrylic pipette, which was then connected to a collection tube and vacuum pump. The laparoscopic equipment (Richard Wolf, Knittlingen, Baden-Württemberg, Germany) consisted of a 5mm 0° laparoscope, 3 trocar/cannula ports, atraumatic grasping forceps and cabled light source. The vacuum pump was adjusted to a pressure of 50 mmHg at the pump and 60 drops of media reaching

the collection tube per minute using a flow-valve on the vacuum line tubing. The aspiration medium was HEPES-buffered TCM-199 (Life Technologies, Burlington, Ontario, Canada) supplemented with 2 mg/mL BSA, 2 µL/mL gentamicin and 0.05 mg/mL heparin. Prior to oocyte collection, the aspiration medium was pre-warmed, with 2 mL aspirated into the collection tube which was maintained at 38°C during the LOPU procedure using a tube warmer. Following the aspiration of follicles, the ovarian surface was rinsed with a warmed 0.9% saline solution to remove any blood on the ovarian surface. Finally, the incisions were closed using surgical glue, each animal had their CIDR removed, and received 1mL/10KBW long-acting oxytetracycline (Oxymycine® LA, Zoetis, Kirkland, Quebec, Canada) as well as 1.5mL/50 KBW ketoprofen (Anafen® 100 mg/mL, Merial Canada Inc., Baie d'Urfé, Quebec, Canada). Animals were monitored until they had fully recovered and were eating normally.

5.3.5. Washing and Grading of COCs

Following LOPU, the collection tube containing the aspirate was transferred to the laboratory and its contents were poured into a 100 mm petri dish and observed under a stereoscope to search for COCs. The COCs were then washed in manipulation medium (HEPES-buffered TCM-199 (Life Technologies) supplemented with 2 mg/mL BSA and 2 µL/mL gentamicin). Based on their morphology, COCs were graded as either grade 1 (>3 layers of compact cumulus cells and evenly granulated ooplasm), grade 2 (1-3 layers of compact cumulus cells and evenly granulated ooplasm), grade 3 (absent cumulus oophorus), or grade 4 (expanded cumulus oophorus, heterogeneous ooplasm or degenerated). Grade 1 and 2 COCs were selected as usable and transferred into IVM.

5.3.6. In Vitro Embryo Production (IVEP)

Unless otherwise indicated, IVEP was conducted in commercial media (L'Alliance Boviteq, St. Hyacinthe, Quebec, Canada).

5.3.6.1. In Vitro Maturation

After grading and selection, COCs were washed twice and placed into 50 µL drops of maturation media under mineral oil (Fisher Scientific, Ottawa, Ontario, Canada) in groups of around 10. Maturation media consisted of TCM-199, 100 µg/mL cysteine, 5 IU/mL hCG

(Chorulon®; Merck Animal Health, Kirkland, Quebec, Canada), 10 µg/mL FSH (Folltropin-V®), 1 µg/mL 17 β Estradiol, 0.2 mM pyruvate, 2 mM L-carnitine, 5 µL/mL insulin-transferrin-selenium (ITS), 3 µL/mL stock FLI, 10 ng/mL epidermal growth factor (EGF; Life Technologies), 50 µg/mL gentamicin, 10% v/v FBS. COCs were incubated for 24 hours at 38.5°C in 5% CO_2 and 95% air. Stock FLI contained 20 ng/mL recombinant human leukemia inhibitory factor (LIF; Peprotech, Cranbury, NJ, USA), 20 ng/mL recombinant human insulin-like growth factor 1 (IGF1; Peprotech) and 40 ng/mL recombinant human fibroblast growth factor 2 (FGF2; Gold Biotechnology, St. Louis, MO, USA).

5.3.6.2. In Vitro Fertilization

Following IVM, COCs were washed in FERT medium and placed in groups of around 5 COCs into 45 µL droplets of FERT medium containing 1 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine and 10 µg/mL heparin plated under mineral oil. A straw of frozen water buffalo semen of known fertility was thawed in a water bath at 37°C for 1 minute and filtered through a discontinuous gradient (45% over 90%) of Bovi-Pure® (Nidacon Laboraties AB, Göthenborg, Sweden) by centrifugation at 600 X G for 10 minutes. Following centrifugation, the supernatant was discarded, and the pellet resuspended in gradient medium. The sperm was then spun down for another 5 minutes at 600 x G before the pellet was resuspended in 1 mL FERT medium. Sperm motility was assessed, and spermatozoa counted in a haemocytometer. The concentration was then adjusted to 3 x 10^7 motile sperm per mL, and 5 µL was added to each 45 µL droplet containing COCs for a final concentration of 3 x 10^6 motile sperm per mL in the 50 µL droplet. COCs and sperm were co-incubated overnight for around 18 hours at 38.5°C in 5% CO_2 and 95% air.

5.3.6.3. In Vitro Culture

Following IVF, presumptive zygotes were gently pipetted in manipulation medium to remove any remnant cumulus cells and sperm stuck to the zona pellucida. Following denudation, zygotes were then washed in culture medium before being placed in 30 µL droplets of the same media in groups of around 10 zygotes plated under mineral oil and incubated at 38.5°C with 100% humidity in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After 4 and 6 days of culture, embryos were transferred into new droplets of culture medium. Cleavage was assessed 96 hours after IVF

and embryo development rates were assessed after 7 days. Excellent grade, compact morulae and blastocysts were determined to be of transferrable/cryopreservation quality based on morphology.

5.3.7. Staining and Fluorescent Microscopy

Embryos not deemed suitable for transfer or cryopreservation on day 7 were left in culture until day 8 when they were fixed and stained to count the number of cells present. Any resulting morula or blastocysts were fixed in 4% paraformaldehyde for 15 minutes and then permeabilized in a PBS solution containing 0.3% BSA and 0.1% Triton X-100. Following fixation, embryos were stained by exposure to 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) for 20 minutes. Samples were then washed in the same PBS-BSA-Triton solution, until being mounted onto microscope slides using mowiol 40-88 mounting medium and assessed under a fluorescent microscope. The number of cells per embryo was then determined by counting the number of nuclei.

5.3.8. Embryo Transfer and Pregnancy Detection

Embryos were loaded into embryo transfer straws in the laboratory and transported to the farm in a portable incubator at 38.5°C. Adult recipient animals were synchronized to be in heat on the same day as LOPU, for embryo transfer to occur 8 days later. Twenty-one days before ET, a bovine progesterone implant was inserted (EAZI-BREED™ CIDR® 1380; Zoetis Canada), which was removed 10 days later together with the administration of 400 IU eCG (Folligon, Intervet Canada, Kirkland, Quebec, Canada) and 375 µg Cloprostenol (Estrumate®, Merck Animal Health, Kirkland, Quebec, Canada). Animals received 50 µg gonadorelin GnRH (Cystorelin®, Merial Canada) 48 hours later. Recipients were assessed for presence of corpora lutea the day before ET. One to two embryos were transferred non-surgically into the uterine horn ipsilateral to the corpus luteum. Pregnancy was checked 30-40 days after ET and confirmed a few weeks later using trans-rectal ultrasonography.

5.3.9. Statistical Analysis

All data was analyzed using the JMP software (SAS Institute Inc., Cary, North Carolina, USA). In each experiment, normality of data was tested using the Shapiro-Wilk W test and normalized when necessary. A two-tailed t-test was used to assess significance for single

comparisons, while a one-way ANOVA followed by a Tukey-Kramer HSD test was performed for multiple comparisons. Embryo Transfer and pregnancy data was compared using a chi-squared test in a contingency table. Results are presented as average ± standard error of the mean. Differences were considered statistically significant at the 95% confidence interval ($P < 0.05$).

5.4. Results

5.4.1. Effect of FSH with and without eCG

Compared to multi-FSH alone, FSH + eCG resulted in a numerical, but non-statistically significant, increase in the mean number of follicles and COCs (18.0 ± 1.6 vs. 20.3 ± 2.4 and 15.0 ± 1.5 vs. 17.4 ± 2.4, respectively, $P > 0.05$; Fig 1A), the percentage of follicles that were over 5 mm in diameter (83.4 ± 4.2% vs. 88.0 ± 3.9%) and the percentage of COCs that were graded as G1 (32.6 ± 5.9% vs. 33.9 ± 5.6%; $p > 0.05$). There was no difference in the percentage of COCs collected that were deemed usable (G1 + G2; 81.4 ± 2.9% vs. 81.6 ± 4.1%) among treatments (Fig 1B).

Embryo cleavage rates were numerically higher, but not statistically significant ($P > 0.05$), for FSH + eCG (49.7 ± 5.9%) compared to FSH alone (36.8 ± 8.1%; Fig 1C). However, embryo development rates were significantly higher ($P < 0.05$) for the FSH + eCG treatment, when calculated over either oocyte (9.0 ± 3.6% vs. 20.6 ± 2.0%) or cleaved embryos (19.9 ± 6.7% vs. 49.0 ± 8.7%), compared to the multi-FSH treatment (Fig 1C). Collectively, this resulted in a significant increase ($P < 0.05$) in the mean number of transferrable embryos per donor per LOPU for the FSH + eCG treatment (2.70 ± 0.3) compared to the multi-FSH treatment (1.11 ± 0.4; Fig 1D).

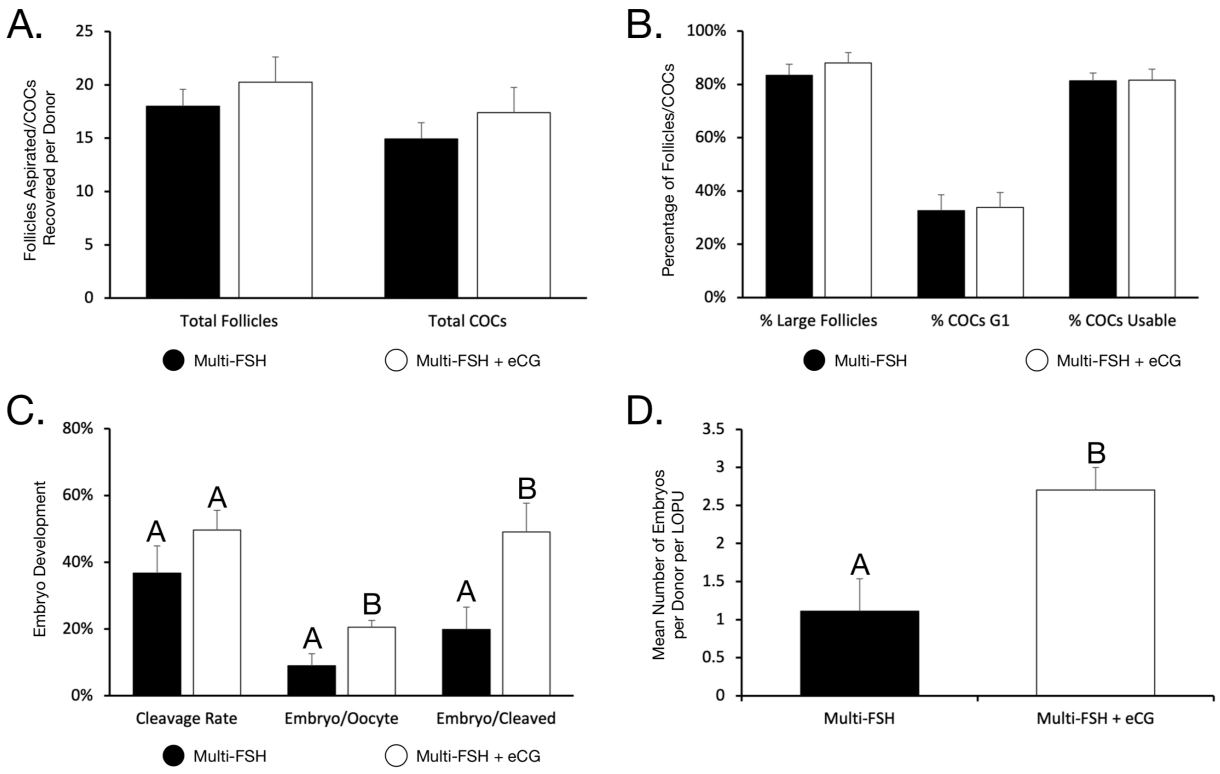

Figure 5.1. The effect of FSH ovarian stimulation, administered at 12-hour intervals, with and without eCG, 24 hours prior to LOPU in prepubertal buffalo calves. A. Average number of follicles and COCs per donor upon LOPU by gonadotropin treatment. B. Follicle size and COC quality by gonadotropin treatment. C. Embryo development rates by gonadotropin treatment. D. The mean number of transferrable embryos per donor per LOPU. Different scripts indicate statistical differences between treatments ($P < 0.05$).

5.4.2. The effect of FSH + eCG with and without MAP5

Compared with multi-FSH, the FSH-MAP5 protocol resulted in a numerical but not statistically significant increase ($P > 0.05$) in the number of follicles available for aspiration (13.1 ± 1.9 vs. 17.3 ± 2.3) and COCs recovered (10.3 ± 2.0 vs. 14.4 ± 2.1, Fig 2A). Between the two treatments there were no statistically significant ($P > 0.05$) differences in the proportion of follicles that were greater than 5mm in diameter (72.0 ± 6.3% vs. 60.0 ± 7.4%), the percentage of COCs that were graded as G1 (50.1 ± 7.7% vs. 53.2 ± 6.5%), as well as the percentage of COCs recovered that were deemed usable (G1 + G2; 85.1 ± 4.2% vs. 88.2 ± 3.3%, Fig 2B).

In terms of embryo development, although no metrics were statistically significant ($P > 0.05$), there was a numerical increase in the cleavage rate (42.4 ± 5.9% vs. 54.4 ± 5.3%) and the

number of transferrable embryos assessed over either oocyte (14.1 ± 5.2% vs. 22.9 ± 4.7%) or cleaved embryos (35.1 ± 10.8% vs. 41.9 ± 7.5%, Fig 2C). This resulted in a numerical, albeit not significant (P > 0.05), increase in the mean number of transferrable embryos produced per donor per LOPU (1.93 ± 0.8 vs. 2.82 ± 0.7, Fig 2D).

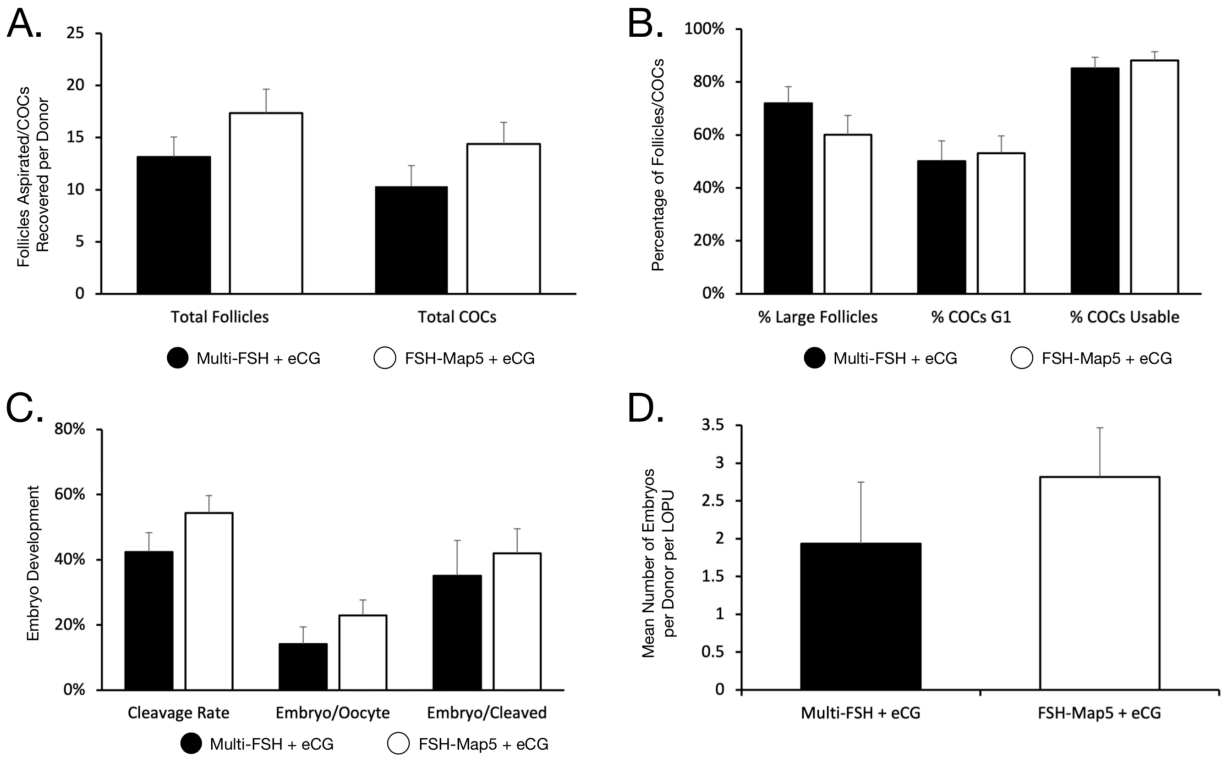

Figure 5.2. The effect of ovarian stimulation using FSH reconstituted with and without hyaluronan + eCG in prepubertal buffalo calves. A. Average number of follicles and COCs per donor upon LOPU by gonadotropin treatment. B. Follicle size and COC quality by gonadotropin treatment. C. Embryo development rates by gonadotropin treatment. D. The mean number of transferrable embryos per donor per LOPU.

5.4.3. The Effect of Treatment Length

The length of gonadotropin treatment over three, four or five days was assessed using FSH-MAP5 and eCG. As the length of gonadotropin stimulation increased, the mean number of follicles available for aspiration significantly diminished (17.3 ± 2.4 vs. 12.6 ± 1.5 vs. 9.2 ± 0.9; P < 0.05). This resulted in significantly less COCs recovered as the length of stimulation increased (14.1 ± 2.4 vs. 8.7 ± 1.0 vs. 6.9 ± 0.7; P < 0.05, Fig 3A). Interestingly, although the proportion of follicles that were larger than 5 mm in diameter significantly increased as the protocol lengthened

(44.3 ± 8.6% vs. 73.8 ± 5.5% vs. 76.3 ± 7.5%; $P < 0.05$), the proportion of COCs that were graded as G1 diminished (57.3 ± 5.8% vs. 41.8 ± 6.2% vs. 29.5 ± 6.1%; $P < 0.05$). This was partially due to a slight increase in the proportion of COCs with expanded cumulus upon recovery (1.0 ± 0.6% vs. 4.0 ± 1.5% vs. 8.2 ± 4.5%; $P > 0.05$). However, this did not affect the overall proportion of COCs that were usable (G1 + G2), with all three treatment lengths yielding similar results (89.3 ± 3.2% vs. 85.1 ± 2.6% vs. 79.8 ± 5.4%; $P > 0.05$, Fig 3B).

Embryo development rates significantly increased as the length of gonadotropin stimulation increased. Both cleavage rate (39.4 ± 5.9% vs. 57.0 ± 4.6% vs. 71.0 ± 5.4%) and transferrable embryo/oocyte rate (14.4 ± 3.9% vs. 27.3 ± 4.4% vs. 35.9 ± 7.0%) significantly increased ($P < 0.05$) as treatment lengths extended. The transferrable embryo/cleaved rate also increased (35.8 ± 9.1% vs. 45.8 ± 7.1% vs. 47.8 ± 8.2%, Fig 3C), although this increase was not statistically significant ($P > 0.05$). Collectively, with COC numbers decreasing, and embryo development rates increasing, the 4-day gonadotropin stimulation period yielded the highest number of transferrable embryos per donor per LOPU (2.70 ± 0.5), however this was not statically different compared to the short (1.94 ± 0.6) and long (2.25 ± 0.5) protocols (Fig 3D).

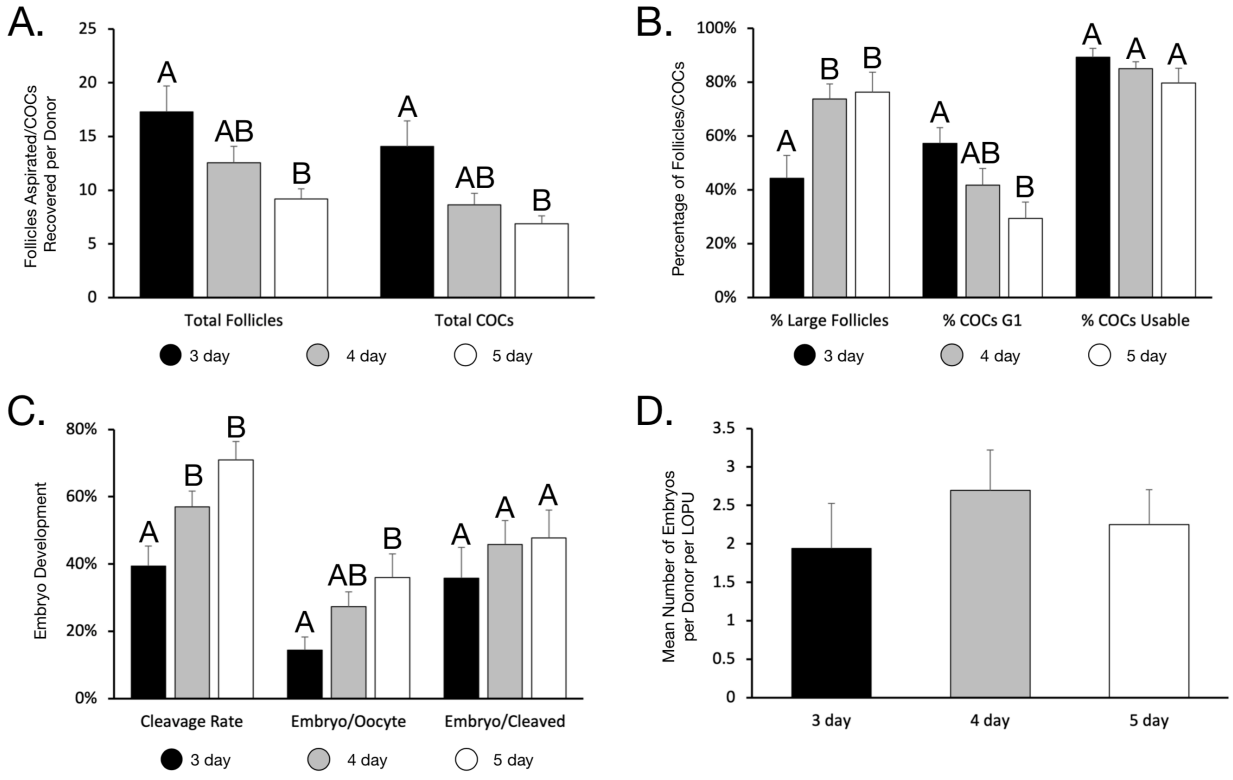

Figure 5.3. The effect of gonadotropin stimulation length using FSH-MAP5 and eCG over 3, 4, and 5 days prior to LOPU in prepubertal buffalo calves. A. Average number of follicles and COCs per donor upon LOPU by gonadotropin treatment. B. Follicle size and COC quality by gonadotropin treatment. C. Embryo development rates by gonadotropin treatment. D. The mean number of transferrable embryos per donor per LOPU. Different scripts indicate statistical differences between treatments ($P < 0.05$).

5.4.4. *Embryo Viability and Pregnancy Establishment*

Due to the limited availability of adult recipient animals (n=90), only a portion of the produced embryos were transferred on day 7 of development and the remaining were vitrified and stored for future studies. The overall pregnancy rate around 4-6 weeks following embryo transfer was 28.9% (26 of 90 recipients; Table 1). All treatments resulted in at least one pregnancy, ranging between 20.0% and 40.0% efficiency, however, there was no statistical differences among treatments due to the small sample size within experiments. Although most recipient animals were still pregnant at time of manuscript submission, ETs performed at the start of this study resulted in healthy, live calves, without signs of physical disability or distress.

Table 5.1. Embryo Transfer and Pregnancy Establishment using Embryos from Prepubertal Buffalo Calves by Gonadotropin Stimulation Treatment

Experiment	Treatment	Number of recipients	Pregnancies
Effect of FSH with and without eCG	Multi-FSH	3	1 (33.3%)
	Multi-FSH + eCG	7	2 (28.6%)
Effect of FSH + eCG with and without MAP5	Multi-FSH + eCG	9	2 (22.2%)
	FSH-MAP5 + eCG	5	2 (40.0%)
Effect of Treatment Length	3-Day	6	2 (33.3%)
	4-Day	50	15 (30.0%)
	5-Day	10	2 (20.0%)

Embryos which were not deemed to be suitable for embryo transfer or cryopreservation on day 7 of development were left in culture until day 8, when they were fixed and stained. Day-8 morula (n = 18) had an average of 38.4 ± 3.4 cells, while day-8 blastocysts (n = 21) had an average of 99.3 ± 5.7 cells (Fig 4).

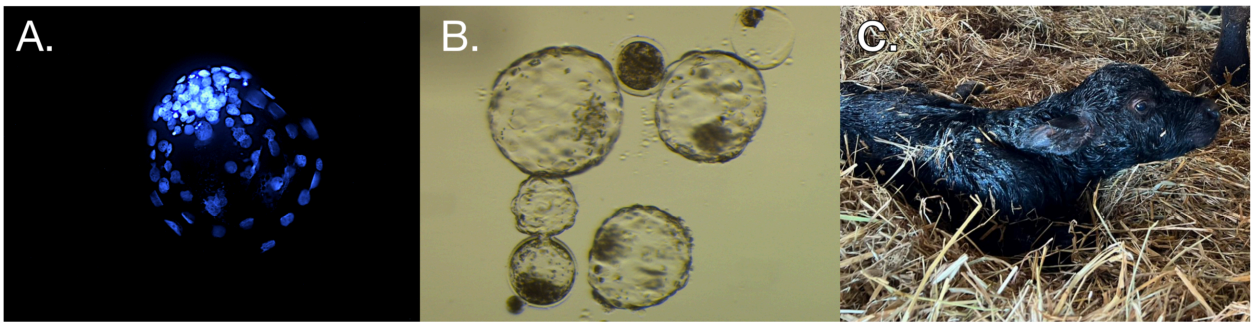

Figure 4. Embryo viability and birth of progeny following embryo transfer of embryos produced from prepubertal water buffalo. A. A representative image of a day-8 blastocyst stained to count the number of cells. B. A group of embryos viewed on day-7 from a single donor animal. C. A heifer calf born following embryo transfer.

5.5. Discussion

In this study, we assessed the effect of different gonadotropin stimulation protocols in prepubertal Mediterranean water buffalo including their impact on LOPU-IVEP results. Although there have been limited trials demonstrating this technology in the past [7, 9, 50], we believe this is the first study of its kind assessing the effect of different gonadotropin stimulation protocols on LOPU, IVEP and ET in buffalo heifer calves less than 6 months of age. The schedule and doses of our gonadotropin stimulation protocols were designed and adapted for buffalo calves based on previous results working with prepubertal Holsteins [25, 34]. We found that combining FSH-MAP5 stimulation with eCG, over a prolonged period of 4 to 5 days yielded the greatest overall results. In fact, the 5-day treatment, which consisted of an eCG + FSH-MAP5 + eCG regime, resulted in an embryo/oocyte rate approaching 36%, which is higher than blastocyst rates often achieved using oocytes from stimulated adult animals [9, 48, 51]. Following transfer, these embryos were capable to supporting development to term at rates similar to those produced in adults, resulting in live, healthy calves being born [9, 52, 53]. Collectively, these results highlight the potential of this technology going forward.

In our first experiment, we assessed the impact of combining multiple injections of FSH with or without eCG and found that using eCG at the end of the stimulation protocol significantly improved embryo development rates and the mean number of embryos per donor per LOPU. This is consistent with previous work in our laboratory that found combining FSH with eCG to be beneficial in prepubertal Holsteins [34]. This suggests that eCG provides additional support to the developing follicles over FSH alone. As eCG is known to possess both FSH- and LH-like activity [54, 55], it is possible that the LH-like activity towards the end of the follicle development protocol was the reason for the beneficial effect of eCG. To this effect, follicular fluid in dairy calves has been shown to contain around half the LH concentration compared to adult cows [56]. Yet, LH has major roles during folliculogenesis by regulating multiple pathways, including the CNP/NPR2 and EGF/EGF receptor pathways responsible for regulating meiotic maturation [57].

In our second experiment, we obtained similar results by using FSH in a slow-release formulation with hyaluronan compared to the traditional multi-FSH protocol, which is consistent with previous results in adult animals [35-39]. In addition to reducing stress to the animals, its ease of use and less labour required to prepare animals for LOPU, hyaluronan may provide a steadier, more sustained, even delivery of FSH without repetitive peaks and valleys in serum concentrations as FSH is repeatedly administered and metabolized by the body [36]. As such, the ovarian stimulation it provides may be physiologically similar to the one in adults, potentially improving COC quality. In support of this hypothesis, reducing the interval between regular FSH administration from 12 hours to 8 hours, tended to improve, albeit insignificant, embryo development in Holstein calves [34]. This FSH slow-release formulation could prove especially useful in prepubertal animals, as the HPO axis is not yet fully active with limited endogenous gonadotropin production [58]. To this effect, FSH stimulation in prepubertal heifers was able to mimic a functional HPO axis by upregulating expression of genes involved in follicular growth and preventing apoptosis [19]. Therefore, emulating a normal physiological state as closely as possible should be strived for, further emphasizing that the time spent by the COC inside the follicle is critical in gaining full developmental competence [59]. This is of particular importance for oocytes from prepubertal donors, as polyspermy and abnormal fertilization have been shown to be prevalent in these animals. For example, our previous work revealed polyspermy rates ranging from 10 to 45% following IVF of oocytes from prepubertal buffalo and Holsteins [7, 34]. This suggests that oocytes from prepubertal donors are not able to fully mobilize the resources to

effectively block polyspermy and oocyte activation upon fertilization. It has been found in bovine oocytes that calves have differences in both the duration and amplitude of calcium oscillations compared to cow oocytes [6], with calf oocytes displaying delayed and incomplete cortical granule migration following IVM [60]. Although this still needs to be assessed in water buffalo, it does underline the importance proper gonadotropin stimulation prior to LOPU, in order to provide COCs with the optimal conditions to promote full oocyte competence and ability to regulate fertilization and activation.

In our third experiment, we compared length of gonadotropin stimulation, starting three-, four-, or five-days prior to LOPU. Early attempts at stimulating prepubertal animals were mostly centred around short protocols starting 36- to 48-hours before LOPU [33, 61]. These protocols were popular because of their simplicity and ability to generate large follicular responses. In fact, many prepubertal donors often produce more follicles and COCs than average responses in adult animals [6]. For example, in this study we consistently recovered more than 10-15 COCs per donor per LOPU, whereas studies using slaughterhouse-derived ovaries often recover fewer than 3 COCs per ovary when working with adult buffalo [47, 62-64]. More recent work in cattle has suggested that prolonging gonadotropin treatments significantly improves embryo development rates [25]. Previously, we showed that stimulating Holstein calves for 72 hours prior to LOPU, instead of 36, significantly improved embryo development rates, in part due to an increased number of large follicles [25]. This is significant, since embryo development rates more than doubled (6.8% vs. 13.8%) when comparing prepubertal Holstein oocytes sourced from small (< 5mm) vs. large (> 5 mm) follicles [34]. Similar trends have also been seen in adult buffalo oocytes [41]. This is consistent with the notion that oocytes, cumulus and theca cells mediate bi-directional transport of various molecules to and from the oocyte during maturation to confer full competence during folliculogenesis [65]. In this manuscript, we showed that by pushing the length of gonadotropin stimulation beyond 72 hours, it is possible to improve embryo development rates. However, longer treatments reduced the number of follicles available for aspiration, possibly due to an increase in the percentage of large follicles being present for aspiration. With this interesting inverse relationship between number of follicles available for aspiration and embryo development rates, we found the 4-day treatment to yield the greatest number of transferrable embryos per donor per LOPU (2.7 ± 0.5). Our 5-day treatment followed an eCG + FSH-MAP5 + eCG regime, compared to the shorter 3- and 4-day treatments which only included eCG at the end of the protocol. Because

eCG has affinity for both FSH (higher) and LH receptors, further research will be needed to understand if the higher rates of embryo development obtained with this protocol are the result of the extra day of FSH-like stimulation or the impact of early LH-like stimulation.

Following embryo transfer, an average of 28.9% (26 of 90) recipient animals became pregnant. This is comparable with the average achieved using embryos derived from adult buffalo [9, 52, 53], and consistent with previous studies using embryos from prepubertal donor buffalo [8, 50]. These findings confirm that embryos produced from prepubertal animals are fully viable and have similar competence for post-implantation development compared to embryos from adult buffalo. This is consistent with the findings that regardless of gonadotropin stimulation treatment tested, all protocols were able to establish at least one pregnancy resulting in similar pregnancy rates across all treatments. Hence, the bottleneck for producing more embryos from prepubertal donors appears to rely more on increasing the developmental competence of oocytes rather than embryos. Embryos which were not deemed to be of sufficient quality for transfer (i.e. delayed development or morphologically questionable) were left in culture until day 8. Interestingly, a small percentage of these embryos did develop into blastocysts and had an average cell number of 99.3 ± 5.7 upon staining. Whether these embryos were viable if transferred into recipients remains to be seen, however, it does underline the difficulty in assessing the quality of bubaline embryos based on morphology and speed of development alone.

Future research should explore various parameters in vitro that affect embryo development rates in prepubertal buffalo. This is especially important since most research has focused on cattle, and hence, many protocols and media preparations are tailored for bovine embryos rather than buffalo. Although, in this study, we were able to successfully produce buffalo blastocysts using commercial media designed for cattle. Buffalo embryos are known to have a different molecular makeup compared to bovine embryos, most notably, a higher concentration of lipids, making them more vulnerable to lipid peroxidation and oxidative stress [2]. Hence, the availability of antioxidants and growth factors for buffalo embryos is crucial [66]. For example, studies have found adding L-carnitine to culture medium, which promotes lipid metabolism, improved cryotolerance in buffalo embryos [67, 68]. It is worth highlighting that oocytes from prepubertal donors are likely to be highly susceptible to oxidative stress, with high intracytoplasmic concentrations of reactive oxygen species, possibly due to an impaired ability to synthesize glutathione [69-71]. Hence, the use of antioxidants, for prepubertal buffalo IVEP is essential to

ensure success [69, 72-74]. In this study, we have also supplemented the oocyte maturation medium with a cocktail of the growth factors FGF2, LIF and IGF1 (known as FLI). In pigs, FLI supplementation was shown to significantly improve embryo development and viability rates [75, 76]. Although this may be the first report using FLI growth factors in buffalo IVM, further research would be required to establish if they confer any benefits to prepubertal oocytes since in the present study, the FLI growth factors were supplemented in the maturation media of all oocytes, without a control group.

Finally, it is worth mentioning the safety and efficiency of LOPU-IVEP programs in young animals. We have repeated LOPU more than 5 times at two-week intervals without animals displaying any adverse lesions or sequels on the reproductive organs or impaired fertility later in life. In fact, some of the donor animals at the start of our study became recipient animals used in the later stages of this study and successfully became pregnant following embryo transfer. This corresponds to other species, most notably small ruminants, where LOPU is more widely used, with animals showing no adverse fertility effects after repeated LOPU sessions [77, 78]. Hence, prepubertal LOPU-IVEP programs hold great promise in accelerating the rate of genetic gain, as well as the early propagation of valuable, transgenic or endangered animals, with minimal risk to the future reproductive health of those animals. For example, using the average of 2.7 transferrable embryos per donor per LOPU obtained in this study, with LOPU being repeated at 2-week intervals for 16 weeks, between 2 and 6 months of age, it would result in an average of 21.6 embryos being produced per calf. This is astonishing considering that buffalo are notorious for their poor reproductive response [79]. For example, despite being a popular technology in dairy cattle, multiple-ovulation embryo transfer (MOET) is not commercially viable in water buffalo, possibly due to the inability of the oviductal fimbria to effectively catch ovulations and transport multiple oocytes down the oviduct [9, 80, 81], consequently, attempts at MOET in water buffalo typically result in between 0 and 2 transferrable embryos per flush [8]. Hence, attention has been focused on OPU-IVEP as an alternative, however, OPU in Mediterranean water buffalo has also yielded poor responses, resulting in between 2 and 5 COCs per buffalo per OPU [48, 82-85]. Following IVEP, however, this typically only results in fewer than 2 transferrable embryo per animal per OPU as well [48, 83, 85, 86], further emphasizing the potential of prepubertal LOPU-IVEP programs in this species.

5.6. Conclusion

The findings presented here showed that combining eCG with FSH during gonadotropin stimulation significantly increased the embryo development rate of oocytes from prepubertal water buffalo. Using FSH reconstituted in hyaluronan, as a slow-release formulation, enabled similar results to regular FSH, despite requiring significantly less time, stress for animals and effort to stimulate animals prior to LOPU. Extending the gonadotropin stimulation period from 3 to 5 days increased embryo development rates but decreased the number or COCs recovered following LOPU. A 4-day treatment using FSH-MAP5 + eCG resulted in the highest number of transferrable embryos per donor per LOPU.

5.7. Declaration of Competing Interest

The authors declare that there are no conflicts of interest

CRediT Authorship Contribution Statement

Luke Currin: investigation, formal analysis, writing – original draft preparation and review. **Hernan Baldassarre:** conceptualization, methodology, investigation, writing – review and editing, supervision. **Mariana Priotto de Macedo:** investigation. **Werner Giehl Glanzner:** investigation. **Karina Gutierrez:** investigation. **Katerina Lazaris:** investigation. **Zigomar da Silva:** investigation. **Vanessa Guay:** investigation. **Maria Elena Carrillo Herrera:** investigation. **Caitlin Brown:** investigation. **Erin Joron:** investigation. **Ron Herron:** investigation. **Vilceu Bordignon:** conceptualization, methodology, writing – review and editing, supervision, funding acquisition.

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CONNECTING STATEMENT 3

In Chapter 5, we assessed the impact of gonadotropin stimulation in combination with an optimized in vitro maturation protocol we established in Chapter 4. Together, these procedures were able to yield very good LOPU and IVEP results, comparable to, if not slightly better than, those obtained in adult buffalo. Furthermore, we showed that the embryos that were produced were fully viable and resulted in healthy, live calves born after embryo transfer. Essentially, we showed that the technology does work. However, in order to fully understand and optimize the procedures required for future commercial implementation, it is important to understand what exogenous factors can affect LOPU-IVEP results, beyond the effect of gonadotropin stimulation and media composition. Moreover, for the technology to be implemented commercially, it is critical to produce embryos that can be cryopreserved, thereby separating (temporospatial) embryo production and embryo transfer.

Based on literature in both adult buffalo and prepubertal animals, we identified four factors which may have a significant impact on LOPU-IVEP results that had not been previously described in prepubertal water buffalo oocytes. The first factor was seasonality, since adult water buffalo are reproductively seasonal, which distinguishes them from cattle. The second and third factors were the effect of age and individual variation because we previously reported these had a significant impact on prepubertal Holstein calves. The fourth factor was the choice of sire used for IVF because previous literature had cited this to be a major limiting factor of IVF in adult buffalo oocytes. Finally, we selected vitrification as a preferred method of cryopreservation for in vitro produced embryos, especially embryos with high lipid content such as buffalo embryos.

Therefore, the goal of our third manuscript, and final objective of this thesis, was using the same parameters that were established in Chapters 4 and 5, to assess the impact of season, the age of the donor animal at the time of LOPU, individual variation among donor animals and the sire used for IVF in prepubertal water buffalo LOPU-IVEP. We also assessed the cryotolerance of embryos after transferring vitrified embryos into synchronized recipient adult females.

CHAPTER 6

Original Research Article 3

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Factors Affecting the Efficiency of In Vitro Embryo Production in Prepubertal Mediterranean Water Buffalo

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6.1. Simple Summary: Embryos can be produced from prepubertal animals using laparoscopic ovum pickup combined with in vitro embryo production technologies. However, due to their young age and unique reproductive physiology, there is currently very limited knowledge about what factors may affect the success of these programs in Mediterranean water buffalo. Here, we assessed how results are affected by season, age, individual variation and choice of sire used for fertilization. Specifically, we found that season and age only had limited impacts, but there were large variations between individual donors and sires. In addition, we assessed what factors can affect pregnancy rates after transferring the resulting embryos into recipient animals and found that regardless of number of embryos transferred, fresh or vitrified, all variables assessed were compatible with the establishment of pregnancies.

6.2. Abstract: Embryos from prepubertal water buffalo can be produced using laparoscopic ovum pickup (LOPU) and in vitro embryo production (IVEP). However, to date, it is unclear what factors and environmental conditions can affect LOPU-IVEP efficiency in prepubertal animals, especially buffalo. In this study, we explored the impact of season, age and individual variation among female donor animals, as well as the effect of the sire used for in vitro fertilization. Donor animals between 2 and 6 months of age were stimulated using gonadotropins prior to LOPU, which was performed at two-week intervals. Following in vitro maturation and fertilization, the resulting embryos were then cultured to the blastocyst stage until they were either vitrified or transferred into recipient animals. The number of follicles available for aspiration and embryo development rates was stable throughout the year. As animals became older, there was a slight trend for fewer COCs recovered from LOPU and better embryo development. There was a large individual variation in both ovarian response and the developmental competence of oocytes among donors. The bull used for fertilization also had a significant impact on embryo development. Upon embryo transfer, pregnancy rates were not affected by the number of embryos transferred per recipient. The best pregnancy rates were achieved when transferring blastocysts, compared to compact morula or hatched blastocysts. Finally, vitrification had no effect on pregnancy rate compared to fresh embryos.

Keywords: Seasonality, Age, Individual Variation, Bull Effect, Vitrification, LOPU-IVEP, Oocyte, Embryo Transfer.

6.3. Introduction

Famous for their mozzarella cheese, Mediterranean water buffalo (*Bubalus bubalis*) are an important livestock species, for both meat and dairy production worldwide. In the developed world, water buffalo milk is prized for its high protein and fat content [1], while, in developing countries, their meat offers an alternative and affordable healthy protein source [2,3]. With a sharp rise in demand for buffalo products in recent years, a key constraint for sustainable and efficient buffalo production is their unique and sometimes challenging reproductive biology [4]. Therefore, adept implementation and use of assisted reproductive technologies (ARTs) in buffalo is crucial for the long-term viability and profitability of raising this species [5].

Despite a wide array of ARTs used commercially in dairy cattle, implementation in water buffalo has lagged. Compared to dairy cattle, adult water buffalo respond poorly to multiple-ovulation and embryo transfer (MOET) programs with very few viable embryos being recovered upon flushing [5-7]. Hence, attention instead turned to ultrasound-guided transvaginal ovum pickup (OPU) followed by in vitro embryo production (IVEP). However, this has also yielded poor results compared to dairy cattle, with an average yield of < 2 transferable embryos per OPU in adult donors [8-10]. To further compound this problem, the onset of puberty and age of sexual maturity in water buffalo is typically delayed, making rates of genetic gain very slow [4]. Although the age of first calving is affected by various factors including nutrition [11], the interval between generations is a major factor impeding rates of genetic gain. Hence, a viable alternative is recovering cumulus-oocyte complexes (COCs) from prepubertal animals using laparoscopic ovum pickup (LOPU) combined with IVEP to produce embryos from elite donor animals at a very young age [12-14]. As LOPU can safely be repeated at regular intervals on animals as young as two months of age [14], prepubertal LOPU-IVEP programs represent an exciting biotechnology that dramatically decreases the generation interval and has been used in multiple ruminant species including buffalo [5,15], cattle [16-18], goat [19,20] and sheep [21,22]. For example, although embryo development rates from prepubertal animals have historically remained low compared adult animals [12], recent studies in our laboratory produced an average of 2 to 3 transferrable embryos per donor per LOPU [23], which is comparable or even slightly better than averages for OPU-IVEP/MOET programs in adult buffalo [10,24]. Following embryo transfer into adult recipient animals, these embryos produced healthy, live calves proving the in vitro embryos produced from prepubertal animals are fully viable [5,13]. However, to date, it is unclear what factors affect the efficiency of these programs.

One distinct aspect of reproductive physiology in water buffalo compared with dairy cattle is their seasonality. Adult water buffalo are known to be photoperiod-sensitive, with season being an important factor affecting the success of breeding programs [25,26]. Seasonal animals can be broadly divided into short-day breeders, which are most fertile in the autumn and winter, and long-day breeders, which are most fertile in the spring and summer [27]. Like sheep, goat, and deer, water buffalo are short-day breeders with fertility increasing as the daylength draws shorter in the autumn and winter [25,28]. As such, heat detection, artificial insemination, embryonic survival and pregnancy rates all improve during the autumn months [29-32]. These effects also carry over

into embryo development rates of OPU-derived oocytes during IVEP as well, with cleavage and blastocyst rates significantly affected by season [8,25]. Photoperiod-sensitivity is driven by the hypothalamic-pituitary-ovarian (HPO) axis with melatonin, which is produced by the pineal gland in response to darkness, modulating GnRH production, and consequently, gonadotropin secretion in accordance to daylength [33,34]. However, studies investigating the impact that season may play on very young prepubertal buffalo are lacking and needed, since, unlike adult animals, prepubertal animals have an under-developed and immature HPO axis [35]. Therefore, it is plausible that prepubertal buffalo may not respond to season in the same way as adult animals, although to our knowledge no one has investigated this.

Another key aspect affecting the success of prepubertal LOPU-IVEP is individual variation among animals. Multiple studies have shown that some individuals consistently respond very well to gonadotropin stimulation and produce many COCs and embryos, while other animals produce very few – if any, despite being housed in the same conditions [12,14,18,24]. This has made choosing which donor animals to select for LOPU-IVEP programs challenging. In a similar manner to the large variation observed among donor females, the choice of male for use in IVF is a key consideration in buffalo IVEP, as different bulls may have different fertility, and consequently, embryo development rates [5,36]. For example, working with adult Murrah buffalo, a study comparing 8 different bulls for IVF resulted in embryo development rates varying between 6.4 and 37.7% [5]. In fact, it has been suggested that only 10% of bulls are suitable for buffalo IVF [5,24]. As oocytes sourced from prepubertal females are known to have multiple molecular and organelle differences compared to oocytes from adult animals [17,37-39], it is possible that the bull used for IVF with prepubertal oocytes may have an even larger influence. Although the polyspermy rate in prepubertal oocytes has been shown to be abnormally high [14,18], to date, we are unaware of any authors investigating the effect of bull on oocytes from prepubertal buffalo.

In addition to season and individual variation, the age of the donor at the time of LOPU has been shown to impact results in dairy cattle. Compared to their adult counterparts, prepubertal animals have a large ovarian reserve and often respond very well to gonadotropin stimulation, yielding many follicles available for aspiration [40,41]. However, despite yielding many COCs, in vitro embryo production rates are low, with similar trends shown across multiple species including buffalo [5], cattle [17,42], goat [20], sheep [21,22] and pigs [43,44]. On this note, age has been shown to affect LOPU-IVEP success in other species [45,46]. For example, in Holstein cattle, we

showed that as animals became older, although the number of COCs recovered from LOPU gradually decreased, the competence of those oocytes grew, with embryo development rates improving [16]. Similar trends have been noted in goats [19]. Finally, it is important to understand the viability of embryos once they are transferred into recipient animals. Although multiple authors have investigated embryo development rates, fewer have assessed what factors may influence pregnancy rates following embryo transfer. Even though pregnancy rates established using embryos produced from prepubertal animals have generally been comparable with those using regular embryos in recent years [14-16,47], there is only limited information available about what factors can affect these rates.

In this retrospective study, working with buffalo heifer calves between 2 and 6 months of age, we assessed the impact of season, donor age, and individual variation in both the donors and the males on the efficiency of LOPU-IVEP. Specifically, we assessed the ovarian response based on the number of follicles aspirated, as well as the number of COCs recovered following LOPU. We also assessed cleavage and in vitro development to the blastocyst stage. Finally, we assessed factors that can affect the pregnancy rate following embryo transfer into recipient females, including season, the number of embryos transferred, as well as whether the prepubertal-derived embryos were transferred fresh or vitrified.

6.4. Materials and Methods

6.4.1. Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were purchased from the Sigma Chemical Company (Millipore Sigma; Oakville, Ontario, Canada).

6.4.2. Animals

A total of 256 LOPUs on 55 Mediterranean water buffalo heifer calves between the ages of 2 and 6 months old were performed in different seasons between the summer of 2017 and spring of 2022, every two weeks up to a maximum of seven times per animal. Donor animals were housed in an indoor barn at Macdonald Campus of McGill University, located in Sainte-Anne-de-Bellevue, Quebec, Canada (45.4252° N, -73.9654° W). The barn had large windows with lots of natural light; during the summer, the barn was kept as cool as possible with large fans, while during the winter it was heated to 15°C. Animals were weaned and fed good quality second-cut hay and

water offered ad-libitum, as well as a grain concentrate (Optivia®, Shur-Gain, Brossard, Quebec, Canada) twice daily according to their body weight. Recipient adult animals were housed in a free-stall barn with access to the outdoors at the Ontario Water Buffalo Company farm in Stirling, Ontario, Canada (44.3377° N, -77.5798° W).

6.4.3. Ovarian Stimulation

Animals were treated intramuscularly with a combination of 100-140 mg of FSH (Folltropin-V®, Vétoquinol, Lavaltrie, Quebec, Canada) with or without 300-600 IU eCG (Novormon® 5000, Partnar Animal Health Inc., Ilderton, Ontario, Canada) starting 72 to 120 hours prior to LOPU. FSH was diluted in either 0.9% saline solution, given at 12-hour intervals, or hyaluronan (MAP-5®, Vétoquinol), given in a single injection or two injections 48-hour apart. All animals received two vaginal progesterone implants (330 mg each) in the form of two small ruminant CIDRs (EAZI-Breed™ CIDR® 330, Zoetis Canada Inc., Kirkland, Quebec, Canada) inserted 120 hours prior to LOPU. Results regarding the effect of gonadotropin treatment were demonstrated in a previous publication from our group [23]. Since no significant differences among each parameter assessed was found, for the purposes of this retrospective study, data was pooled.

6.4.4. Laparoscopic Ovum Pickup (LOPU)

Prior to LOPU, animals were fasted of hay for 36 hours, concentrate for 24 hours, and water for 18 hours. Anesthesia was induced using 0.05 mg/KBW xylazine (Xylamax, Bimeda Canada, Cambridge, Ontario, Canada), 2 mg/KBW ketamine (Ketalean, Bimeda Canada) and 0.1 mg/KBW diazepam (Sandoz, Boucherville, Quebec, Canada) administered intravenously. Animals were then intubated, attached to a mechanical ventilator, and maintained under general anesthesia using 2% isoflurane USP (Fresenius Kabi, Toronto, Ontario, Canada). The laparoscopic ovum pickup technique has been described previously [48]. Briefly, once fully anesthetized, animals were moved into the Trendelenburg position in a cradle. Follicles were aspirated under laparoscopic observation using a 20G needle mounted on an acrylic pipette which was connected to a vacuum pump. The vacuum pump was adjusted to 50 mmHg of pressure at the pump and 60 drops of media reaching the collection tube per minute using a flow-valve on the vacuum-line tubing. The aspiration medium was composed of HEPES-buffered TCM-199 (Life Technologies,

Burlington, Ontario, Canada) supplemented with 2 mg/mL BSA, 2 µL/mL gentamicin and 0.05 mg/mL heparin. Following aspiration, the ovarian surface was rinsed with 0.9% saline and the incisions were closed using surgical glue. Finally, each animal had their CIDR removed and received 1mL/10 KBW long acting oxytetracycline 200 mg/mL (Oxymycine® LA, Zoetis, Kirkland, Quebec, Canada), 1.5mL/50 KBW ketoprofen (Anafen® 100 mg/mL, Merial Canada Inc., Baie d'Urfé, Quebec, Canada) and monitored until they had fully recovered.

6.4.5. Washing and Grading of COCs

Following transfer of the collection tube to the laboratory, COCs were recovered and washed in manipulation media composed of HEPES-buffered TCM-199 (Life Technologies) supplemented with 2 mg/mL BSA, 2 µL/mL gentamicin. Under a stereomicroscope, COCs were graded based on their morphology as either grade 1 (>3 layers of compact cumulus cells and evenly granulated ooplasm), grade 2 (1-3 layers of compact cumulus cells and evenly granulated ooplasm), grade 3 (absent cumulus oophorus), or grade 4 (expanded cumulus oophorus, heterogeneous ooplasm or degenerated). Grade 1 and 2 COCs were selected as usable and transferred into in vitro maturation.

6.4.6. In Vitro Embryo Production

Unless otherwise indicated, IVEP was conducted in commercial bovine media (L'Alliance Boviteq, St. Hyacinthe, Quebec, Canada).

6.4.6.1. In Vitro Maturation (IVM)

After grading and selection, usable COCs were placed into 50 µL drops of maturation media under mineral oil (Fisher Scientific, Ottawa, Ontario, Canada) in groups of around 10 after being washed. Maturation media consisted of TCM-199, 100 µg/mL cysteine, 5 UI/mL hCG (Chorulon®; Merck Animal Health, Kirkland, Quebec, Canada), 10 µg/mL FSH (Folltropin-V®), 1 µg/mL 17 β Estradiol, 0.2 mM pyruvate, 2 mM L-carnitine, 5 µL/mL insulin-transferrin-selenium (ITS), 3 µL/mL stock FLI, 10 ng/mL epidermal growth factor (EGF; Life Technologies), 50 µg/mL gentamicin, 10% v/v fetal bovine serum (FBS). Stock FLI contained 20 ng/mL recombinant human leukemia inhibitory factor (LIF; Peprotech, Cranbury, New Jersey, USA), 20 ng/mL recombinant human insulin-like growth factor 1 (IGF1; Peprotech) and 40 ng/mL recombinant human fibroblast

growth factor 2 (FGF2; Gold Biotechnology, St. Louis, Missouri, USA). COCs were incubated at 38.5°C in 5% CO_2 and 95% air for around 24 hours.

6.4.6.2. In Vitro Fertilization (IVF)

IVF was conducted in 45 µL droplets of FERT medium containing 1mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine and 10 µg/mL heparin plated under mineral oil. Following IVM, COCs were washed in FERT medium and placed into the droplets in groups of around 5. A straw of frozen water buffalo semen of known fertility was thawed in a 37°C water bath for 1 minute and filtered through a discontinuous gradient (45% over 90%) of Bovi-Pure® (Nidacon Laboraties AB, Göthenborg, Sweden) by centrifugation at 600 X G for 10 minutes. The supernatant was discarded, and the pellet resuspended in gradient medium. The sperm was then centrifuged a second time (5 minutes at 600 x G) before the pellet was finally resuspended in 1 mL FERT medium. Sperm motility was assessed and then counted in a haemocytometer. The concentration was then adjusted and 5 µL was added to each 45 µL droplet containing COCs for a final concentration of between 2-4 x 10^6 motile sperm per mL, as per the experiment described below. COCs and sperm were co-incubated overnight for around 18 hours at 38.5°C in 5% CO_2 and 95% air.

6.4.6.3. In Vitro Culture (IVC)

Presumptive zygotes were washed to remove cumulus cells and sperm stuck to the zona pellucida following IVF before being placed in 30 µL droplets of culture media supplemented with 2mM L-Carnitine in groups of around 10 zygotes, plated under mineral oil and incubated at 38.5°C with 100% humidity in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After 4 and 6 days of culture, embryos were transferred into new droplets. Cleavage was assessed 96 hours after IVF and embryo development rates were assessed after 7 days. Excellent grade, compact morulae and blastocysts were determined to be of transferrable/cryopreservation quality based on morphology.

6.4.7. Embryo Vitrification and Thawing

Embryos were vitrified using a protocol adapted from a previously described method [49]. Embryos were selected for vitrification in groups of 1 to 3. Vitrification was performed in a four-well NUNC plate on a warm plate with medium pre-warmed to 37°C. In wells 1 and 2, embryos

were washed in 800 µL holding medium composed of TCM-199 HEPES-buffered medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 20% fetal bovine serum (FBS). Embryos were then moved into well 3 containing 1 mL of VS1 medium composed of holding medium with 7.5% ethylene glycol and 7.5% dimethyl sulfoxide (DMSO) for three minutes. Embryos were then moved into the fourth well containing 1 mL VS2 medium composed of holding medium supplemented with 670 mM sucrose containing 16.5% ethylene glycol and 16.5% DMSO for 1 minute. Embryos were then loaded onto a properly labelled vitrification straw approximately 2 mm from the end in the smallest volume possible. Vitrification straws were made from 0.5cc semen straws with a 30° bevel cut into one end. The straw containing the embryos was then immediately plunged into liquid nitrogen and kept submerged for at least 30 seconds. Straws were then covered with a sheath and loaded into goblets before being arranged into canes for storage.

Embryos were thawed using a four-well NUNC plate on a warm plate with media pre-warmed to 37°C on the day of embryo transfer. Straws were removed from the liquid nitrogen and the tip containing the embryos was immediately plunged into the first well containing 1200 µL of holding medium supplemented with 333 mM sucrose. Embryos were recovered and moved into the second well containing the same medium and left to incubate for 5 minutes. Embryos were then moved into the third well containing 1200 µL of holding medium supplemented with 167 mM sucrose and incubated for another 5 minutes. The embryos were then moved into the final well containing 1200 µL of holding medium and washed thoroughly. Embryos were then washed in transfer medium and loaded into straws ready for transfer to the recipient females.

6.4.8. Embryo Transfer and Pregnancy Detection

Embryos were loaded into embryo transfer straws in the laboratory and transported in a portable incubator at 38.5°C. When transferring fresh embryos, adult recipient animals were synchronized to be in heat on the same day as LOPU, for embryo transfer to occur eight days later. Twenty-one days before ET, a bovine progesterone implant was inserted (EAZI-BREED™ CIDR® 1380; Zoetis Canada), and it was removed ten days later. Animals received 400 IU eCG (Folligon, Intervet Canada, Kirkland, Quebec, Canada) and 375 µg cloprostenol (Estrumate®, Merck Animal Health, Kirkland, Quebec, Canada) at the time of CIDR removal. Forty-eight hours later, animals received 50 µg gonadorelin GnRH (Cystorelin®, Merial Canada). The day before ET, recipients were assessed for the presence of corpora lutea. One to two embryos were transferred non-

surgically into the uterine horn ipsilateral to the corpus luteum. Pregnancy was assessed 30-40 days following ET using trans-rectal ultrasonography.

6.4.9. The Effect of Season

Over the period of five years, LOPU-IVEP was performed in a group of 53 animals up to a maximum of 7 times, for a total of 248 replicates. To assess the impact of season, the date of LOPU was divided into one of four seasons of equal length: spring (March-May; n = 88), summer (June-August; n = 55), autumn (September-November; n = 81) and winter (December-February; n = 28). The animal housing facility is located in the St. Lawrence Lowlands, at a 45° latitude, winters are cold and dark, while summers are hot, humid and lengthy in daylight. The average daily meteorological conditions for the study area recorded during the trial are shown in Table 1. For the effect of season on embryo transfer, the date of embryo transfer was considered rather than the date of LOPU. A total of 118 embryo transfers were performed across all four seasons: March-May (n = 31), June-August (n = 31), September-November (n = 23), December-February (n = 33).

Table 6.1. Average daily meteorological conditions according to season during the trial period

Season	Mean T (°C)	Min T (°C)	Max T (°C)	Humidity (%)	Precipitation (mm)	Daylength (h)
Mar-May	7.51 ± 7.2	2.41 ± 6.8	12.60 ± 8.1	63.8 ± 14.8	2.76 ± 6.9	13.55 ± 1.2
Jun-Aug	19.46 ± 3.4	14.51 ± 3.5	24.39 ± 3.7	68.4 ± 12.6	3.39 ± 7.8	15.30 ± 0.7
Sept-Nov	8.34 ± 6.4	3.82 ± 6.1	12.85 ± 7.3	73.3 ± 10.0	2.48 ± 5.0	10.69 ± 1.2
Dec-Feb	-6.07 ± 6.0	-10.92 ± 7.0	-1.22 ± 6.0	73.3 ± 10.5	4.18 ± 7.7	9.80 ± 0.8

Values are expressed as mean ± standard deviation. Data adapted from Environment and Climate Change Canada [50].

6.4.10. The Effect of Age

To evaluate the effect of age, LOPU results were assessed on a group of 55 animals, up to a maximum of 7 procedures, for a total of 256 replicates. Animals were pooled into one of three age categories according to their age at the time of LOPU: A) ≤ 120 days old (n = 78); B) between 120 and 150 days (n = 98), and C) ≥ 150 days old (n = 80).

6.4.11. The Effect of Individual Variation

The effect of individual variation was evaluated on a group of 6 animals on which we conducted LOPU a total of 4 times each, for a total of 24 replicates. The same frozen semen batch/bull was used for all replicates.

6.4.12. The Effect of Sire and Semen Concentration

The effect of sire on IVEP was assessed in 8 animals over 3-4 LOPUs, with a total of 5 different bulls tested. To reduce the error between subsequent replicates, for any given LOPU, oocytes from the same donor animal were split and fertilized with semen from two different bulls for donor animals who produced enough COCs. A total of 50 replicates were used in this study: bull A (n = 8), bull B (n = 8), bull C (n = 8), bull D (n = 7) and bull E (n = 19).

The effect of semen concentration was studied on a group of 8 donor females over 4 LOPUs. In all cases, IVF was performed using a single frozen semen source (bull D) using a concentration of 2, 3 or 4 million motile sperm/mL in the IVF drops. As described previously, for animals that produced enough COCs, multiple concentrations were tested in the same female and LOPU. A total of 35 replicates were used in this study: 2 million (n = 11), 3 million (n = 8), 4 million (n = 16).

6.4.13. Factors Impacting Embryo Transfer

We studied the impact of the number of embryos transferred (1 or 2), the stage of embryo development at the time of transfer (morula, blastocyst or hatched blastocyst) and the type of embryo transferred (vitrified or fresh).

6.4.14. Statistical Analysis

Data was analyzed using the JMP software (SAS Institute Inc., Cary, North Carolina, USA). The normality of data were tested using the Shapiro-Wilk W test and normalized when necessary. For the effect of season and age on LOPU and IVEP results, a one-way ANOVA followed by a Tukey-Kramer HSD test was performed. The effect of individual variation and sire used for IVF was compared using a student's t-test. Embryo transfer and pregnancy data was compared using a chi-square test in a contingency table. Results are expressed as the average ± the standard error of the mean. Differences were considered statistically significant at the 95% confidence interval ($P < 0.05$).

6.5. Results

6.5.1. The Effect of Season

The number of follicles available for aspiration was similar across all four seasons, varying between 16.0 ± 1.2 and 16.3 ± 1.0 follicles in the spring, autumn and winter, with a slight decrease during the summer months (14.3 ± 1.0; Fig 1A). This led to uniform number of COCs recovered throughout the year, varying between 12.3 ± 0.9 and 13.6 ± 1.0 (Fig 1A). The percentage of COCs that were deemed usable was numerically higher in the autumn and winter (82.1 ± 2.2 and 85.4 ± 3.3%) compared to the spring and summer (77.4 ± 2.1 and 81.3 ± 2.2%). Cleavage rates and embryo development rates, calculated over both oocyte and cleaved, were homogeneous regardless of season (Fig 1B). This led to the production of approximately two embryos per donor per LOPU throughout the year, varying between 1.84 ± 0.3 and 2.22 ± 0.4 (Fig 1C). Upon embryo transfer into adult recipient animals, there was a slight numerical increase in pregnancy rates in the autumn and winter (30.4 and 33.3%), however, because of the small sample size, this difference was not statistically significant compared to the spring and summer (25.8 and 22.6%; Table 2). When nulliparous heifer recipient animals were excluded from the data set, and only primiparous or multiparous cow recipients were considered, the pregnancy rate was numerically lower in the summer months, with only 1/16 transfers resulting in pregnancy (6.25%) compared to the other seasons which varied between 20.0 and 37.5%.

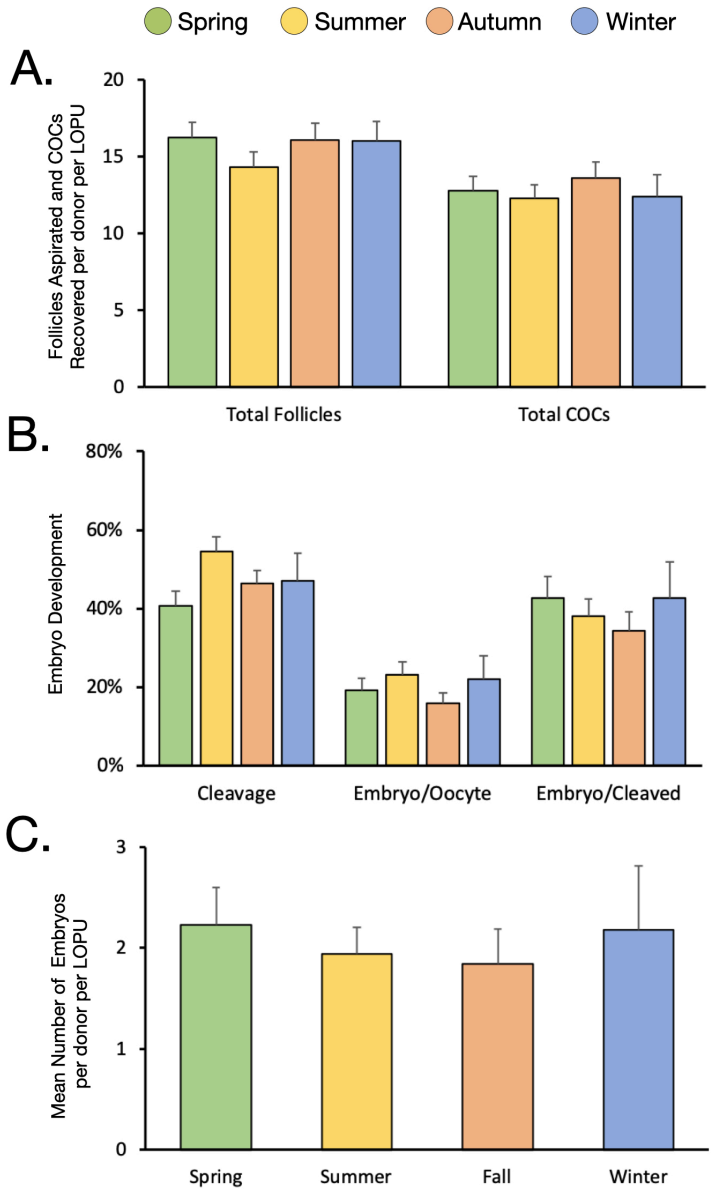

Figure 6.1. The effect of season on the number of follicles and COCs recovered during LOPU as well as in vitro embryo production rates. A: LOPU results by season. B: Embryo development rates by season. C: The mean number of embryos produced per donor per LOPU by season.

Table 6.2. Pregnancy rates after embryo transfer according to season.

Season	N	Pregnancies
Mar-May	31	8 (25.8%)
June-Aug	31	7 (22.6%)
Sept-Nov	23	7 (30.4%)
Dec-Feb	33	11 (33.3%)

6.5.2. The Effect of Age

As donor animals got older, there was a slight numerical, albeit statistically insignificant ($P > 0.05$), decrease in the number of follicles aspirated from 17.3 ± 1.2 to 15.5 ± 1.1. This led to a similar decline in the number of COCs recovered following LOPU, falling from 14.7 ± 1.1 to 12.9 ± 1.1 (Fig 2A). However, the proportion of COCs that were deemed usable slightly increased with age, increasing from 79.5 ± 1.8 to 81.7 ± 2.3% ($P > 0.05$). Following IVEP, the trends reversed, with cleavage (44.1 ± 4.4 to 52.1 ± 2.8%), embryo/oocyte (20.8 ± 3.6 to 23.5 ± 2.6%) and embryo/cleaved (41.3 ± 5.8 to 43.0 ± 3.9%), all slightly increasing with age, although this was not statistically significant (Fig 2B). Collectively, this resulted in a mean number of between 2 and 3 embryos per donor per LOPU (2.11 ± 0.4 vs. 2.44 ± 0.3 vs. 2.54 ± 0.3; $P > 0.05$; Fig 2C).

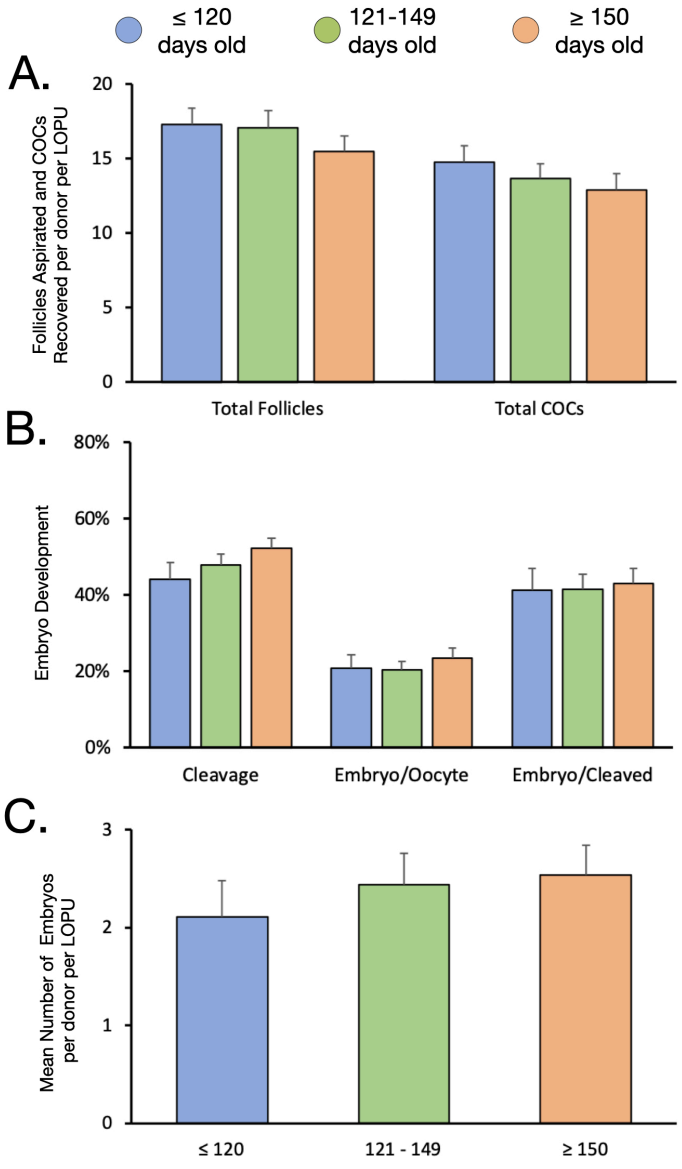

Figure 6.2. The effect of age on the number of follicles and COCs, embryo development rates and mean number of embryos per donor per LOPU. A: LOPU results according to age showing number of follicles aspirated and number of COCs recovered. B: Embryo development according to age showing cleavage and embryo development rates calculated over oocyte and cleaved embryos. C: The mean number of embryos produced per donor per LOPU according to age category.

6.5.3. The Effect of Individual Variation

There was a large individual variation among donor females, both in ovarian response, and embryo development rates during IVEP. For example, the number of follicles aspirated per female varied from a mean of 5.0 ± 1.6 to 24.5 ± 1.4 per LOPU. Over the course of 4 LOPUs, this resulted in a range from 20 to 98 follicles aspirated per animal. In terms of number of COCs recovered per animal, there was a similar large variation from 4.5 ± 1.0 to 18.0 ± 2.8 per LOPU or from 18 to 72 total COCs per animal over 4 LOPUs.

There was also a large variation in embryo development rates among females, despite using the same conditions (bull, semen concentration, capacitation factors, etc.) for all animals during IVEP. For example, cleavage rates varied from 34.9 ± 3.0% to 74.3 ± 15.2% and mean embryo development rates calculated over oocyte varied from 8.0 ± 5.9% to 52.0 ± 9.8%. This resulted in a mean number of embryos per donor ranging from 0.75 ± 0.3 to 5.5 ± 1.6, resulting in a total number of blastocysts per donor ranging from 3 to 22 for the four LOPUs. It is interesting to note that animals who had good ovarian responses did not necessarily result in superior IVEP responses and vice versa.

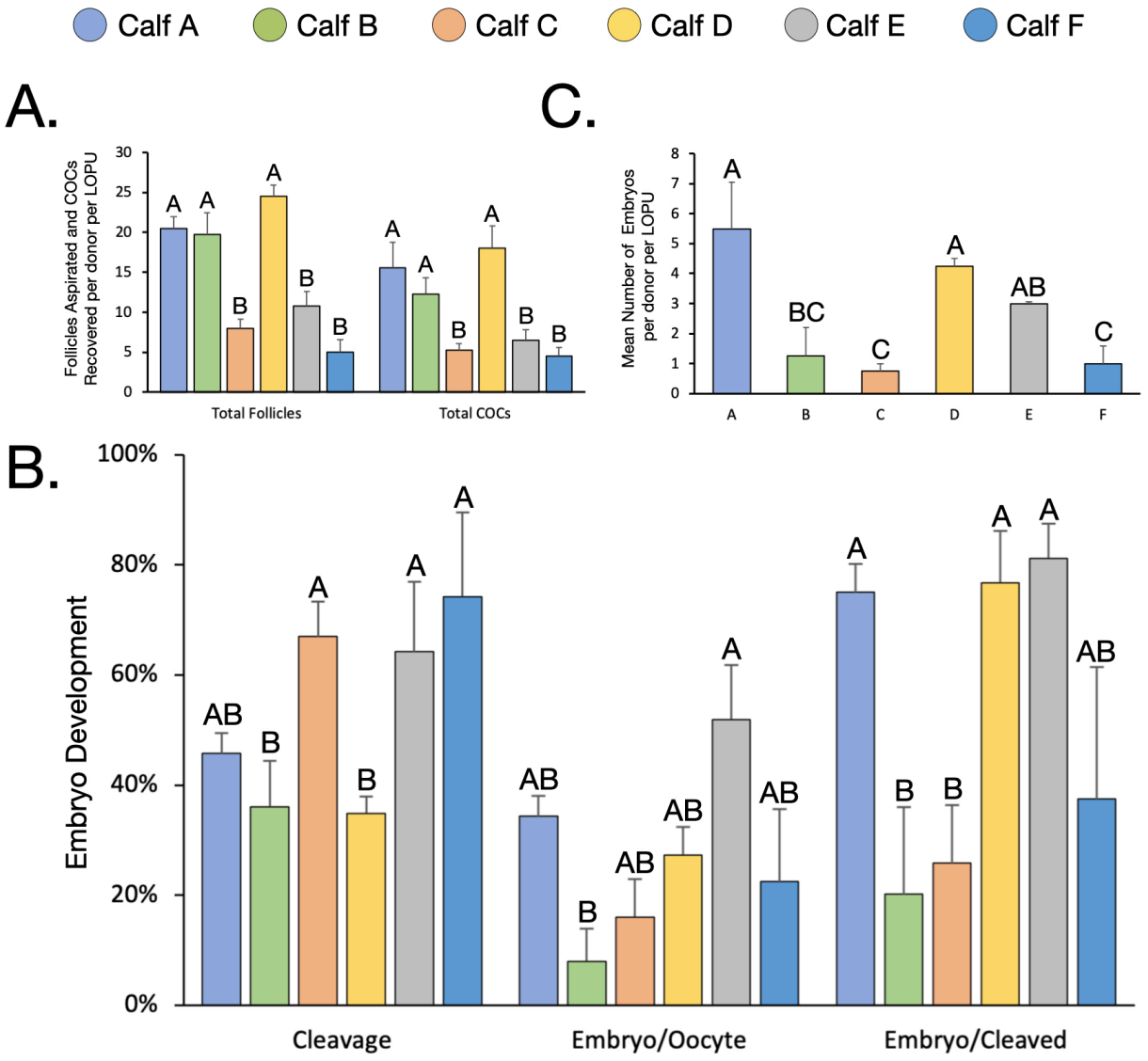

Figure 6.3. The effect of individual variation. A. The mean number of follicles aspirated and COCs recovered per animal. B. The embryo development rates per animal. C. The mean number of embryos produced per animals per LOPU. Values within the same chart with different script (A, B, C, D) differ significantly ($P < 0.05$).

6.5.4. The Effect of Sire and Semen Concentration

In a similar manner to the large individual variation among donor females, there was a large variation on IVEP results depending on which bull was used for IVF. In the same group of eight female donor calves, we tested five different bulls, labelled A through E (Fig 4A). We used the same concentration for each bull, 3 million motile sperm/mL. Among bulls, there was a large

variation in cleavage rate, ranging from 10.1 ± 6.5 to 73.6 ± 4.4%. Due to the small sample size, there were relatively large variations in embryo development rate calculated over cleaved, as evidenced by the error bars. However, the mean varied between 25.0 ± 16.4 and 66.3 ± 14.2%. It is interesting to note that although bull B resulted in the highest cleavage rate, it resulted in the second lowest embryo rate. Collectively, this resulted in an average embryo rate/oocyte varying between 3.9 ± 3.1 and 47.0 ± 6.0%.

Next, we assessed the effect of semen concentration using a single group of donor animals (n=8) and bull D (Fig 4B). Although not statistically significant (P > 0.05), compared to 2 and 4 million, inseminating COCs with 3 million motile sperm/mL led to the greatest cleavage (52.2 ± 9.7%) and embryo development rates calculated over both cleaved and oocyte (56.1 ± 13.1 and 29.8 ± 7.9, respectively).

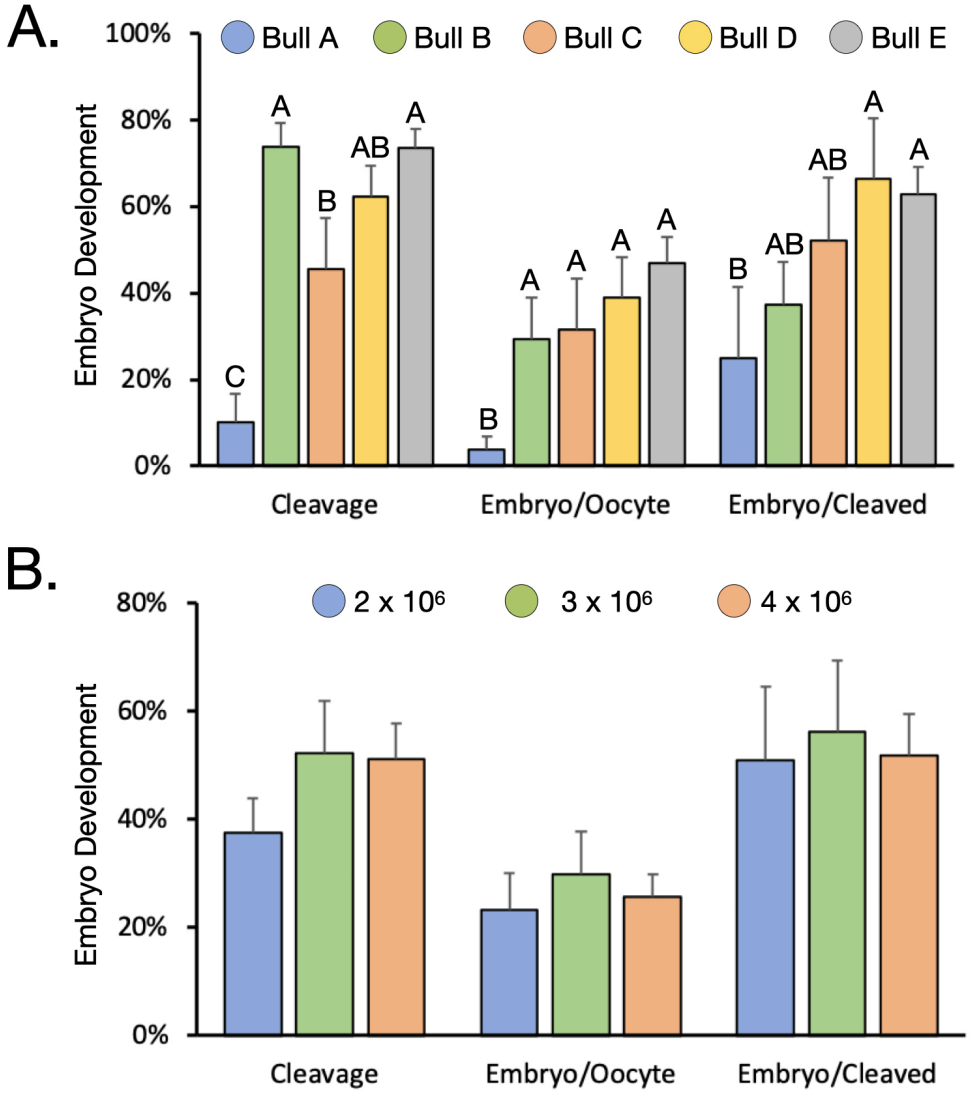

Figure 6.4. The effect of sire and semen concentration. A. Embryo development according to bull used for IVF. B. The effect of semen concentration for bull D. Values within the same chart with different script (A, B, C) differ significantly ($P < 0.05$).

6.5.5. The Effect of Embryo Transfer

There was a limited availability of adult recipient animals, so embryos produced from LOPU-IVEP were either vitrified or transferred directly into recipient animals on day 7 of development. Either one or two embryos were transferred into recipient animals at different stages of development: compact morula, blastocyst or hatched blastocyst (Table 3). Although most of the recipient animals were still pregnant at the time of manuscript submission, to date, the vitrified embryos have resulted in five healthy, live calves born (4 heifers and 1 bull). We believe these are the world's first live calves born from vitrified embryos produced from a prepubertal LOPU-IVEP program in buffalo.

Table 6.3. Embryo Transfer and Pregnancy Establishment.

Variable	Treatment	N	Pregnancies
Number of embryos transferred	1	18	4 (22.2%)
	2	99	29 (29.3%)
Stage of embryo development	Morula	20	4 (20.0%)
	Blastocyst	19	9 (47.4%)
	Hatched	15	4 (26.7%)
Vitrification	Fresh	87	25 (28.7%)
	Vitrified	28	8 (28.6%)

6.6. Discussion

In this first of its kind study, we showed that embryos from prepubertal buffalo heifers can be produced year-round, despite large individual variations among oocyte donors and sires used for IVF. Furthermore, we showed that embryos produced from prepubertal buffalo oocytes were fully viable after vitrification, producing healthy live young, which may substantially facilitate the technology's applicability going forward. The efficiency of LOPU-IVEP in prepubertal buffalo has been growing, with specially tailored gonadotropin stimulation and in vitro maturation procedures yielding promising results [23]. Further elucidating the impacts of exogenous factors

on LOPU-IVEP programs is crucial for adoption of this technology on a commercial basis in the near future.

In this study we found that the effect of season did not have a large impact on LOPU-IVEP results, obtaining similar follicular and embryo development results year-round. In adult Mediterranean water buffalo, although season was shown to only have a small impact on the number of follicles available for aspiration and COCs recovered during OPU, embryo development rates significantly improved during the autumn months compared to the spring and summer (30.9% vs. 13.3%) [8]. This contrasts with our results, where we obtained embryo development rates around 20% year-round. This could potentially be explained given the immaturity of the HPO axis in prepubertal animals, therefore, without being dependant on, or affected by endogenous gonadotropin production, the administration of exogenous gonadotropin stimulation prior to LOPU resulted in similar responses year-round. While many factors affect the seasonality of buffalo, with both exogenous (e.g., climate, nutrition) and endogenous (e.g., genomic, endocrine) elements playing a role, photoperiod is likely the principal regulator [28]. This is especially true at higher latitudes, where day length is more variable than sub-tropical areas closer to the equator [10,51]. Consequently, the recipient animal was probably the major driver behind the higher pregnancy rates achieved during the autumn and winter (18/56 = 32.1%), compared to the spring and summer (15/62 = 24.2%). This is consistent with previous studies in adult animals showing similar changes in pregnancy rate according to season [52,53]. Our findings further emphasize the role photoperiod has on seasonality, as our results are similar to other studies across the northern hemisphere [54], despite the fact that Canada's climate involves much milder temperatures during spring and summer compared to other regions of the world (southern Italy for example). It is interesting to note, however, that the physiological rise in melatonin plasma concentrations in response to darkness is lower in buffalo heifers compared to adult cows [55], with seasonal effects on fertility more pronounced in adult cows than in heifers [54,55]. The fact that only 1/16 transfers to adult cows resulted in pregnancy during the summer months seems to reflect this. It is also possible that cows were in a higher degree of metabolic and heat stress during summer due to lactation demands, explaining their higher degree of difficulty to become/remain pregnant in the summer compared with heifers. Although we did not directly measure melatonin production in this study, future research investigating melatonin synthesis across multiple age groups, in both

gonadotropin-stimulated, and unstimulated calves remains an interesting avenue for future studies that could shed light on the mechanisms affecting results in younger animals.

Although there was a slight trend in our results, we did not see a large variation in results according to age. Previous results in our lab using Holstein calves of similar ages to buffalo calves used in this study showed that as animals got older, although the number of COCs recovered from LOPU gradually decreased, embryo development rates improved [16]. Since buffalo mature at a far slower pace than Holsteins, reaching puberty at a much later age [4], it is possible the timeframe in this study was too narrow for us to detect a significant effect. However, our results are consistent with previous knowledge that prepubertal animals produce substantially more follicles and COCs compared to adults [40,41,56]. For example, although we saw a slight trend in lower numbers of COCs being recovered as animals became older (14.7 ± 1.1 to 12.9 ± 1.1), this is still substantially higher than an average of fewer than three COCs recovered per ovary from slaughterhouse-derived embryos [57-60]. This is consistent with other research which following LOPU in prepubertal, and OPU in adult buffalo, recovered an average of 10.9 ± 3.3 and 5.8 ± 1.3 COCs, respectively [47]. One drawback of our experimental design was that since LOPU was repeated at two-week intervals, we were unable to discriminate between the effect of age and repeated gonadotropin stimulation. It is possible that animals began to build an immunogenic response to gonadotropins after repeated gonadotropin stimulation, and consequently, produced fewer follicles. One way to avoid this potential source of error would be to only assess each animal once; however, a major source of error would then be introduced with individual variation among animals. Nonetheless, previous research in adult goats has shown that repeated LOPU does not have an impact on the number of follicles available for aspiration [61].

We showed that there was a large variation among donor animals, which is consistent with previous studies, in both prepubertal [14], and adult buffalo [24]. This is a potential challenge for choosing which animals are best suited to be enrolled in LOPU-IVEP programs. For example, in this multi-year study, considering the sum of 4 LOPUs per donor, our best animal produced 29 transferrable embryos, while our poorest animal produced zero. In adult animals, there is a correlation between antral follicle count (AFC) and the number of blastocysts produced in subsequent OPU-IVEP sessions [24]. However, because prepubertal animals are too small for rectal ultrasound imaging, this metric is less useful in young animals. Concentrations of anti-Müllerian hormone (AMH) have been proposed as a reliable marker for gonadotropin-stimulation

response in adult buffalo [47,62]. Although it is unclear whether this remains true in prepubertal buffalo, studies in *Bos taurus* and *indicus* calves have shown it to be a valuable and accurate tool for identifying animals with high AFC [63].

Despite the fact that all bulls tested in this study were proven to be fertile when used for artificial insemination, we found a large variation in IVF results (3.9 to 47.0% embryo/oocyte rate) among five bulls. This is consistent with previous research that found only around 10% of bulls were suitable for IVF in buffalo [5,24]. To further complicate this issue, it has also been shown that there is a large variation in fertilization kinetics among bulls in buffalo, with different males requiring different co-incubation lengths to successfully penetrate oocytes [64]. Although in cattle there is a correlation between AI and IVF fertility [65], the large variation among bulls in buffalo IVF suggests different bulls require different conditions in vitro. Although we did not test different parameters in this study, previous studies in buffalo found that different bulls expressed different abilities to capacitate in vitro regardless of treatment, and that different bulls responded differently to different capacitation factors [66] and heparin concentrations [67]. This further suggests that various bulls may require capacitation factors specifically tailored to their needs. Another possibility is that different bulls may require different sperm concentrations in order to perform optimally. Although we did not test this specifically, we found that 3 x 10^6 motile sperm/mL to be optimal for bull D. However, this is higher than the industry-standard concentration of 2 x 10^6 [67,68], further lending evidence to the theory that different bulls may require specific IVF conditions.

We observed that pregnancy rate was numerically higher but not statistically different when two embryos were transferred together compared to one embryo (29.3 vs. 22.2%). Since we had limited access to recipients and we predicted in vitro produced embryos from prepubertal buffalo would have a rather low survival rate, we thought transferring embryos in pairs would allow more calves on the ground with a rather low incidence of twins (3/29 pregnancies = 10.3%). Since this did not work the way we expected, the recommendation is to transfer embryos individually, as they resulted in a better pregnancy rate per embryo (22.2 vs. 16.2%). The results also reaffirm the importance of the recipients in the overall success of an embryo transfer program [53]. On the embryo side, one factor that does appear to have an impact on results is development stage at the time of transfer. Indeed, we observed that transferring blastocysts led to the highest pregnancy rates (47.4%) compared to morula (20.0%) or hatched blastocysts (26.7%).

One particularly noteworthy finding of our study was that vitrified embryos were able to establish pregnancies at the same rate compared to embryos transferred fresh (28.6 vs. 28.7%). Further opening the possibility of embryo sale and export when prepubertal LOPU-IVEP is used commercially. Embryo quality is known to be a major factor affecting cryotolerance [69,70], further emphasizing the quality of embryos that were obtained in our study. Indeed, although most recipients were still pregnant at the time of manuscript submission, vitrified embryos did yield healthy, live calves proving the embryos we produced were fully viable. To date, a total of five calves have been born from vitrified embryos, four heifers and one bull. One factor that may partially explain this is that oocytes from prepubertal donor animals appear to be less sensitive to cryoprotectants than adult oocytes [71]. However, following vitrification of caprine blastocysts, re-expansion rates were lower in those produced from prepubertal donors compared to adult goats [20]. In adult OPU-derived embryos, pregnancy rates between fresh and vitrified embryos were 43.4 and 37.1% after 30 days and 41.7 and 31.4% after 60 days, respectively [9]. However, similar to our results, other researchers found no significant differences in pregnancy rates between direct transfer and vitrified buffalo embryos [47]. One particular characteristic of buffalo embryos making vitrification difficult is their high lipid content [68]. To address this, we supplemented our IVEP medium with L-carnitine, which has been shown to promote lipid metabolism [72,73], provide antioxidative support and improve post-thaw survivability [74-77].

6.7. Conclusions

Findings from this study revealed that season does not appear to affect LOPU-IVEP efficiency in prepubertal buffalo, indicating that LOPU-IVEP can be performed year-round with negligible effects on efficiency. In addition, pregnancy rates were slightly better in the autumn and winter, presumably due to the seasonality of recipient animals, and were not affected by embryo vitrification compared to fresh embryo transfer. As such, it may be beneficial to vitrify embryos produced during the spring and summer from particularly valuable donor animals, for transfer in the autumn and winter to further maximize the number of calves born. Furthermore, we found a large individual variation in both donor females and the male used for IVF, but donor age only had a small impact on results, suggesting that prospective donor animals and the bull used for IVF should be carefully selected.

6.8. Author Contributions: Conceptualization: L.C., H.B., V.B., methodology: L.C., H.B., V.B., writing – original draft preparation: L.C., writing – review and editing: L.C., H.B., V.B., data curation: L.C., investigation: L.C., H.B., M.P.M., W.G.G., K.G., K.L., V.G., M.E.C.H., Z.S., C.B., E.J., R.H., V.B., visualization: L.C., supervision: H.B., V.B., project administration: H.B., V.B., funding acquisition: H.B., V.B.

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Institutional Review Board Statement: The study was approved by the Facility Animal Care Committee of McGill University, in accordance with Canadian Council of Animal Care regulations (AUP# MCGL-7552). The AUP was reviewed and approved annually over the course of this study with last approval on April 25, 2022.

Data Availability Statement: The data presented in this study is available upon reasonable request from the corresponding author

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CHAPTER 7
DISCUSSION AND CONCLUSION

7.1. Overall results and general discussion

Taken as a whole, this thesis explores and describes strategies to optimize LOPU-IVEP for oocytes from prepubertal livestock. The knowledge that using prepubertal donor animals would facilitate a significant increase in the rate of genetic gain in livestock has been well established for decades. Despite multiple attempts using different approaches since the first attempts in the early 1970s in cattle [1-3], embryo development rates using oocytes from prepubertal donors has remained stubbornly low, preventing the widespread adoption and implementation of the technology [4, 5]. As highlighted in the literature review, the reasons for this are most likely multi-faceted, including the immature HPO axis resulting in the presence of small follicles in the ovary giving rise to oocytes with an impaired developmental competence, liable to polyspermy and early embryonic developmental arrest. Therefore, this thesis aimed to improve the development of embryos derived from prepubertal animals through improved in vitro maturation (Chapter 4) and gonadotropin stimulation protocols (Chapter 5), as well as characterize exogenous factors that can affect results in order to guide eventual commercial implementation in the future (Chapter 6). Demand for efficient ARTs for prepubertal livestock has grown in recent years, due to the implementation of genomics for herd management, making prepubertal LOPU-IVEP-ET research more pertinent than ever [6-8]. We decided to focus our attention on water buffalo, a species which is extremely economically important worldwide, and would also benefit greatly due to their long generational intervals [9]. Furthermore, there has only been very limited research in water buffalo; in fact, the first calf born as a result of a prepubertal LOPU-IVEP-ET program in buffalo was only reported in 2017 [10].

One reason that water buffalo are an excellent candidate species for prepubertal LOPU-IVEP programs is their small preantral follicle count compared to other livestock species. The number of primordial follicles in buffalo has been shown to be between 10 and 12 thousand, a 10-fold deficiency compared to cattle [11]. As such, the total and usable number of COCs recovered from buffalo ovaries has been reported to be up to 25 times lower compared to cattle [11]. One of the major advantages of prepubertal LOPU-IVEP is that the large follicular reserve in young

animals can be harnessed. In contrast to males that continuously produce germ cells throughout adulthood, females are born with a lifetime's supply. In this manner, newborns have the highest number of primordial follicles that are not regenerated [12]. Thus, although a very small percentage of these follicles will be ovulated, the vast majority (more than 99%) of this reserve is lost to atresia [13]. Therefore, with a large ovarian reserve still present in young animals, the follicular response to LOPU is usually very good, which is especially useful in water buffalo due to their already low reserve to begin with. As such, although there are large variations among individuals (as highlighted in Chapter 6), when subjected to LOPU, prepubertal animals typically produce significantly more COCs than the average number recovered from adults undergoing OPU [14, 15]. This trend was evident in our results, which are presented in Table 7.1., compared with our previous results working with prepubertal Holstein calves (adapted from data presented in [16] and [17]) along with results in both prepubertal (Murrah) and adult (Mediterranean and Murrah) buffalo published by other groups [10, 14, 15, 18, 19].

Collectively, across all results, in a group of 55 water buffalo calves subjected to a total 256 LOPUs, we aspirated an average of 16.6 ± 0.6 follicles per calf per LOPU. Although slightly lower than our previous experience working with Holstein calves (19.2 ± 1.0), this is still substantially better than an average of between 4.2 and 7.2 in adult buffalo undergoing OPU [14]. From those 16.6 ± 0.6 follicles, we recovered an average 13.7 ± 0.6 COCs per donor per LOPU, which is roughly a 4-fold improvement compared to the average in adult Mediterranean buffalo [14, 15]. Furthermore, although 13.7 ± 0.6 is slightly lower than the 15.0 ± 0.9 COCs recovered from Holsteins, it is certainly not like the large discrepancy observed between adult buffalo and cattle. Compared to adult buffalo, we obtained similar cleavage (48.1 ± 1.8% vs. rates between 37.6 and 65.6%) and embryo development rates (21.3 ± 1.5% vs. rates between 11.5 and 30.9%). This is a very encouraging and exciting result, considering previous results between prepubertal and adult animals in different species. Finally, we produced an average of 2.38 ± 0.2 transferrable embryos per donor per LOPU. This is substantially better than rates achieved using Holstein calves and adult buffalo. However, it should be noted that OPU in adult buffalo can be performed on a more regular basis with intervals of twice weekly, compared to the two-week interval used in LOPU. Our pregnancy rate was comparable to rates reported in adult buffalo [18, 20, 21], but lower than the rates we achieved in Holsteins.

Table 7.1. Overall results in prepubertal Mediterranean water buffalo compared to Holstein and Murrah calves as well as adult buffalo subjected to LOPU/OPU and IVEP.

	Water Buffalo Calves		**Bovine Calves**	**Adult Water Buffalo Cows**			
	This Research Project (Chapters 5, 6)	Baruselli *et al.* 2020 [10, 18]	Currin *et al.* 2017 [16, 17]	Gasparrini *et al.* 2014 [14]	Di Francesco *et al.* 2012 [15]	Petrovas *et al.* 2020 [19]	Baruselli *et al.* 2020 [10, 18]
Animals	55	8	14	8	9	18	10
Breed	Mediterranean	Murrah	Holstein	Mediterranean	Mediterranean	Mediterranean	Murrah
LOPU/OPU Repeats	Up to 7	1	Up to 9	28	18	4	1
LOPU/OPU Interval	14 days	–	14 days	Twice weekly	Twice weekly	6 days	–
Ovarian Stimulation	Yes	Yes	Yes	No	No	Yes	No
Season	Year-round	–	Year-round	–	Autumn	Autumn	–
Follicles Aspirated	16.6 ± 0.6	–	19.2 ± 1.0	5.3 ± 0.2	4.2 ± 0.2	7.2 ± 0.5	–
COCs Recovered	13.7 ± 0.6	10.9 ± 3.3	15.0 ± 0.9	2.7 ± 0.2	2.4 ± 0.1	4.6 ± 0.5	5.8 ± 1.3
Cleavage Rate (%)	48.1 ± 1.8	30.3	71.1 ± 2.8	53.5 ± 2.0	65.6 ± 3.2	51.5 ± 2.3	37.6
Embryo/Oocyte (%)	21.3 ± 1.5	5.1	18.2 ± 2.5	11.5 ± 1.8*	30.9 ± 3.5	23.6 ± 2.4*	15.4
Embryo/Cleaved (%)	41.6 ± 2.5	–	23.8 ± 2.9	–	46.7 ± 4.5	–	–
Transferrable Embryo	2.38 ± 0.2	1.0 ± 0.6	1.28 ± 0.2	0.3 ± 0.1*	–	0.9 ± 0.1*	1.1 ± 0.4
Pregnancy Rate	33/118 (28.0%)	3/8 (38.0%)	13/21 (61.9%)	–	–	–	–

Results presented as the mean ± the standard error of the mean. Follicles aspirated, COCs recovered and Transferrable Embryos are presented as the mean per donor per LOPU/OPU. *Data denoted with an asterisk is presented as a blastocyst rate rather than embryo rate. Blank cells (–) denote data that was not reported in the original publication.

7.2. The effect of antioxidants and growth factors and their impact on polyspermy

In Chapter 4, we assessed the impact of supplementing in vitro maturation medium with growth factors, follicular fluid, and antioxidants on porcine in vitro fertilization and early embryo development. Although previous studies had already shown that IVM supplementation with FLI is beneficial for porcine IVF and SCNT embryos in a defined medium [22] as these factors are naturally present in follicular fluid [23-25], it was unclear whether supplementing IVM medium with FLI already containing pFF would offer any benefit. One particularly intriguing aspect of this research article which we did not discuss in Chapter 4 was that supplementing pFF media with FLI appeared to have a more significant impact on IVF results compared to PA embryos, even though the same trends were observed in both models. This suggests that the supplementation of FLI growth factors may confer an additional ability for an oocyte to properly manage oocyte activation endogenously compared to when it is induced artificially. Evidence that IGF1 is involved calcium signalling including SERCA and IP_3R expression seems to support this hypothesis [26]. This could be especially helpful in prepubertal animals as we believe oocyte activation to be a major stumbling block in these oocytes [27]. We also showed that cysteine is essential to IVM maturation medium, and, although further supplementation with melatonin yielded the highest development rates, it was not statistically significant.

The original reason we chose to work with porcine oocytes in Chapter 4 was that IVF in porcine oocytes typically results in high polyspermy rates compared to other livestock species [28-30]. As oocytes from prepubertal donor animals have also exhibited abnormally high polyspermy rates in the past, we decided swine would be a good model to investigate what factors can be used to improve IVEP. For example, the overall efficiency of porcine IVF, i.e. the percentage of monospermic oocytes out of the total number inseminated has been reported to be as low as 30-40% [31]. Previous studies in our laboratory working with both Holstein and Buffalo calves showed similar results, with polyspermy rates of up to 45% [27]. Using porcine oocytes as a model allowed us to work with a large number of oocytes given the wide availability of porcine ovaries at local abattoirs and meant animals would not be subjected to LOPU unnecessarily, in line with the 3Rs (replacement, reduction, refinement) principle of animal use in research [32]. For example, across the four experiments conducted in Chapter 4, a total of 5858 oocytes were assessed, a number that simply would not have been feasible using prepubertal oocytes collected by LOPU.

This large oocyte availability was very useful for having large numbers of oocytes assigned to assessing meiotic maturation and polyspermy following fixation and staining.

Our collective results in Chapter 4 indicated that cysteine was required during porcine IVM and that the most blastocysts were obtained using IVM supplemented with FLI, pFF, cysteine and melatonin. Therefore, in Chapters 5 and 6, we supplemented our buffalo IVM media with FLI, cysteine and ITS. We elected to use FBS in our maturation media, which is widely used with adult buffalo, over pFF as a protein source, as it is bovine-derived and possibly more physiologically suitable for buffalo oocytes compared to porcine follicular fluid [33, 34]. We elected to use ITS over melatonin in our maturation media as we observed that ITS promoted cumulus expansion in Chapter 4, a characteristic we felt was important since buffalo IVF is conducted with the cumulus cells intact [34], in contrast to porcine IVF where denuded oocytes are used [35]. The expanded cumulus cells therefore act as the first barrier to polyspermy prevention, acting as a "sperm trap" [36], slowing the passage of sperm to the zona pellucida, and selecting a subpopulation to eventually reach the oolemma [34, 37]. The presence of cumulus cells during IVF have also been shown to be beneficial by increasing cleavage and blastocyst rates, presumably due to their ability to secrete glycosaminoglycans, to aid in inducing sperm capacitation and acrosome reaction [34, 38-40].

Previous experience has indicated that polyspermy is a significant problem in prepubertal animals [16, 27]. This observation is supported by microscopy studies that showed an impaired cortical granule distribution and migration in prepubertal cattle [41]. Accordingly, we found lower semen concentrations to yield preferable results in Holsteins. This contrasts with our results in this thesis which indicated that higher semen concentrations (1.5 to 2.0x the industry standard) seemed to yield better results with prepubertal buffalo oocytes. Another particularly curious finding was that, although our cleavage rate in buffalo was lower compared to Holstein calves (48.1 ± 1.8 vs. 71.1 ± 2.8), our embryo/cleaved rate was much higher (41.6 ± 2.5 vs. 23.8 ± 2.9), lending further evidence that polyspermy did not seem to be a problem in buffalo considering polyspermic embryos often appear to "cleave". Collectively, this seems to indicate the supplemental antioxidants and growth factors developed in Chapter 4 and the hormonal stimulation protocols developed in Chapter 5 did indeed improve oocyte competence, allowing the oocyte to better defend itself against polyspermy. However, future research would be needed to confirm this hypothesis, as we did not assess polyspermy in buffalo in this research.

7.3. Were pigs a suitable model? Comparison of porcine and bubaline embryos

In addition to their similarities to prepubertal ruminant oocytes, porcine embryos also have multiple similarities to bubaline embryos. Although bovine oocytes have been the de-facto model, with many bubaline protocols derived from those developed for cattle, multiple differences between the two species exist. The most obvious difference when looking at buffalo oocytes/embryos is their dark colour, due to their high lipid content. Lipids are important for a variety of cellular functions [42]; for example, fatty acids are an important energy source [43], while cholesterol is the main precursor for most steroid hormones [44]. However, lipids are also a major contributing factor to oxidative stress [45]. As such, intra-cytoplasmic lipid concentration impact oocyte maturation and embryo development rates as well as embryo quality and cryo-tolerance [42, 46-48]. Studies have shown different species to exhibit marked variations in lipid content [49]. For example, although mouse oocytes only contain around 3.8 ng of lipids, cattle oocytes contain 63 ± 6, while those from pigs contain an overwhelming 161 ± 18 ng [50]. Therefore, as lipid peroxidation is a major source of oxidative stress and a significant hinderance to the cryotolerance of embryos [51], we believe pigs to be a good model given their very high lipid content. The fact that we found cysteine, a major GSH-precursor molecule, increased embryo development rates from 5.3 ± 2.6% to 23.5 ± 7.7% compared to a control medium containing no antioxidants supports these findings.

Another key similarity between porcine and bubaline embryos is the timing of embryonic genome activation (EGA). EGA is the point at which embryos switch from using maternally-inherited mRNA and proteins to embryonic mRNA transcription, marking the point when the embryo itself gains exclusive genetic control [52]. EGA marks a major turning-point in embryonic development, which, in addition to oocyte activation, marks two of the major hurdles an embryo must overcome to successfully develop into a blastocyst. With the intense chromatin remodelling that occurs during this period, along with the recruitment of embryonic transcription machinery for the first time, many embryos that fail to reach to the blastocyst stage arrest during this period [53]. The developmental stage at which embryos undergo EGA is species-dependant and variable among species. Although in bovine, EGA occurs at the 8-16 cell-stage of development [54], in pigs and buffalo EGA occurs earlier at the 4-8 cell stage of development [54, 55]. This is significant since cellular function prior to EGA is dependent on maternally supplied proteins and

mRNA accumulated during oogenesis [53]. Therefore, in addition to their effect on activation and fertilization, the maturation period could also affect EGA. In other words, the IVM medium tested in Chapter 4 and the gonadotropin stimulation tested in Chapter 5 may also carry over and affect future embryo development and not only an oocyte's competence to undergo activation and fertilization. Previous experience working with embryos from prepubertal Holsteins indicated a high proportion of embryos do indeed arrest around the 8-16 cell stage (unpublished). In fact, of embryos which cleaved but failed to reach the blastocyst stage, around 38.4% arrested between the 8 and 16 cell stage (unpublished). Whether this was indeed due to the embryos failure to undergo EGA is unknown, however, it does suggest an interesting avenue for future research. Not only in water buffalo, but in prepubertal animals in general as very little is known.

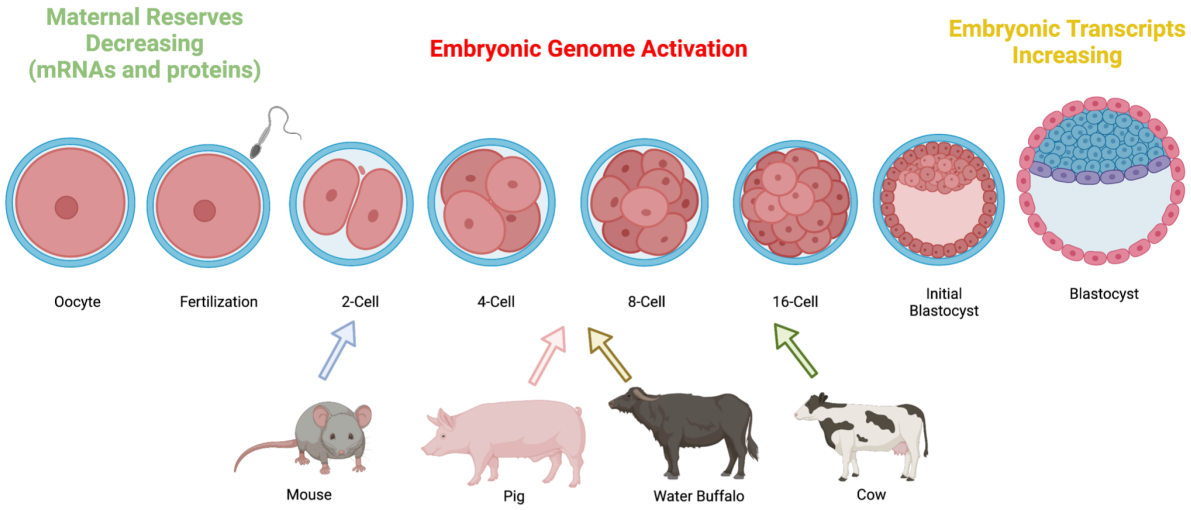

Figure 7.1. Embryo Genome Activation (EGA) Among Multiple Species. Until EGA, embryos are transcriptionally silent, using maternally inherited reserves of mRNA and proteins, when they begin transcribing their own mRNA. The timing of EGA is variable among species occurring at the 2-cell stage in mice, the 4-8 cell stage in pigs and buffalo, and the 8-16 cell stage in cattle. Figure created using BioRender.com

7.4. Gonadotropins and their importance in prepubertal animals

In Chapter 5, we focused on assessing the optimal gonadotropin stimulation protocol to promote acquisition of oocyte developmental competence in vivo. Due to the immaturity of their

HPO axis, prepubertal animals require gonadotropin stimulation prior to LOPU to produce competent oocytes [16]. In Mediterranean water buffalo calves between 5-9 months of age, compared to a control group which received no treatment, gonadotropin stimulation yielded more follicles available for aspiration 20 ± 9.3 vs. 29 ± 4.1, although this difference was not statistically significant [56]. However, in the control group that received no stimulation, 92.2 ± 5.3% of those follicles were small, while in the treated group 70.2 ± 10% were large in size [56]. Although in this study the authors did not assess embryo development rates, it was still a significant finding, considering the well-established link between follicle size and oocyte competence [57, 58]. In adult buffalo subjected to OPU, stimulation with six doses of FSH was able to significantly increase the number of follicles available for aspiration (7.2 ± 0.5 vs. 3.4 ± 0.2), and also significantly increased their size [19]. This resulted in the blastocyst rate more than doubling from 10.3 ± 7.0% to 23.6 ± 2.4% [19]. We also noted similar results working with prepubertal Holstein calves, finding that, although gonadotropin stimulation over a 3-day period did not yield more follicles available for aspiration compared to a control group (19.8 ± 9 vs. 16.9 ± 10), in the control group more than 90% of those follicles were less than 3mm in diameter. As such, once subjected to IVEP, blastocyst rates more than doubled from 16.7 ± 9% to 36.7 ± 26% in animals which received treatment [16].

Another noteworthy finding when working with Holstein calves was that combining FSH stimulation with eCG given 24 hours prior to LOPU resulted in moderately better embryo development rates despite having no effect on the number of follicles [17]. Therefore, the first objective of Chapter 5 was to confirm this was also the case in water buffalo. Indeed, we showed that combining FSH with eCG more than doubled the embryo/oocyte rate from 9.0 ± 3.6 to 20.6 ± 2.0, which resulted in an average of 2.7 ± 3.0 embryos per donor per LOPU, compared to 1.11 ± 0.4 for the FSH only group. The reason for the addition of eCG being more beneficial to buffalo compared to cattle remains unclear. One possibility is that the LH-like activity of eCG was able to promote steroidogenesis, stimulating theca cells to produce androgens (Testosterone and DHEA) as precursors for estradiol, necessary for creating the follicular micro-environment required for the acquisition of oocyte competence. On this accord, the estradiol concentration in follicular fluid has been shown to be substantially lower in prepubertal calves compared to adults [59]. Androgens have also been shown to be essential for fertility, with androgen receptors (AR) expressed throughout the follicle (including granulosa, theca and oocytes), and AR conditional knock-out

mice severely sub-fertile [60]. However, the possible role androgens play in prepubertal ruminants remains unclear, with more research necessary. Research into the biochemical signature of bubaline follicular fluid is ongoing, with multiple groups investigating extracellular vesicles, microRNAs (miRNAs), proteomics, hormonal and lipid profiles [61-64].

7.5. Seasonality, individual variation and cryotolerance

In Chapter 6, one particularly surprising finding was that season did not appear to affect LOPU-IVEP in prepubertal calves. This was rather unexpected, considering that the effects of seasonality are well described in adult animals [65, 66]. However, this could be explained considering the immaturity of HPO axis in prepubertal animals, resulting in little endogenous support, and the use of exogenous gonadotropin stimulation. On this note, a great deal of research has been conducted in adult animals in order to develop hormonal therapies to promote fertility out of the breeding season [67]. Often referred to as out of breeding mating strategies (OBMS) [66], these protocols are widely used in Italy to ensure the availability of Mozzarella cheese during the summer months when demand is at its highest, but milk production wanes [68]. Therefore, it would be interesting to assess seasonal differences in unstimulated animals in order to better understand whether prepubertal animals are indeed unaffected, or whether the gonadotropin stimulation was simply masking the effects. However, from a commercial perspective, this is encouraging news as it suggests that LOPU-IVEP can be performed year-round. However, season did have an effect on pregnancy rates following embryo transfer, meaning cryopreservation of embryos will likely play a significant role as a countermeasure.

Vitrified embryos were able to establish pregnancy at the same rate as fresh embryos. In fact, we believe our work resulted in the world's first healthy, live calves being born from a vitrified buffalo embryo produced using oocytes from prepubertal buffalo donors (Fig 7.2). To date, a total of five calves have been born, four heifers and one bull. One obvious advantage is the simplification of distribution, sale and transport of embryos in the future, as well as the separation, both geographically and temporally, between LOPU-IVEP and ET. This means that embryos can be transported long distances and ET can be performed based on convenience (or a recipient's natural heat) rather than tied to when LOPU was performed. It also means that should LOPU-IVEP fail for one reason or another, recipients synchronized for fresh transfer do not go to waste and can

be transferred with embryos from the cryo-bank. This marks a substantial step-forward in making the technology commercially viable and profitable in the future. Future research should assess differences between vitrified and direct-transfer slow-freeze embryos in prepubertal animals. Recent research in adult animals has shown slow-freeze direct-transfer to result in similar but slightly better conception rates following transfer compared to vitrified embryos (30.4 vs 41.4%) [69].

Figure 7.2: Mediterranean water buffalo calves born from vitrified embryos produced using LOPU-IVEP in prepubertal calves. A: Duchess, a heifer calf, the world's first calf born using a vitrified embryo produced from a prepubertal calf. B: The second calf born using vitrified embryos, a heifer.

We found that individual variation in both the donor female and sire used for IVF had substantial impact on the outcome of LOPU-IVEP. Although these findings were not unexpected, they do remain significant hurdles that must be accounted for in order to streamline LOPU-IVEP efficiency and ensure consistency. More research in the future is needed to address this uncertainty, as AFC count is an inappropriate metric in prepubertal animals as they are too small for transrectal ultrasound imaging and the reliability of AMH as a predictor of AFC remains unclear in prepubertal buffalo. We also found that blastocysts yielded the highest pregnancy rates compared to morula and hatched blastocysts. Although our sample size was too small to find any statistical significance, this is important information for future optimization, highlighting the need for more research going forward.

7.6. Conclusion

To summarize, this thesis examined strategies to optimize the production of viable embryos from juvenile animals. A great deal of progress has been made in recent years, and the research accomplished in this thesis represent another substantial and promising step forward. In Chapter 4, we showed that supplementation of maturation medium with the growth factors FGF2, LIF and IGF1, as well as the antioxidants cysteine and melatonin yielded the greatest embryo development rates in pigs. In Chapter 5, we showed that a 4-day stimulation period consisting of two doses FSH reconstituted in hyaluronan administered 48 hours apart, combined with a singular dose of eCG 24 hours prior to LOPU to yield the most transferable embryos per donor per LOPU in Mediterranean water buffalo. Finally, in Chapter 6, we showed that prepubertal buffalo oocytes produced under the above optimized conditions can tolerate cryopreservation and produce pregnancies following thawing and transfer at rates comparable to those of fresh embryo transfer and vitrified adult embryos. We also showed that season and age did not have a significant impact on LOPU-IVEP results, but individual variation among both donor females and sire used for IVF did impact the results. Although our results presented in this thesis represent a promising step forward, it is also important to recognize that significant gaps in our current knowledge still exist and future research is necessary. This is especially important in buffalo, as very little research has been done in prepubertal animals of this species.

7.7. References

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