The Development of Methods to Improve *In Vitro* Embryo Production in Pigs and Cattle

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

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Abbreviations

aCGH – Array comparative genomic hybridisation

Acr – Acrosome

ADO – Allele drop out

AI – Artificial insemination

AO – Acridine orange

ART – Assisted reproductive technology

ANOVA – Analysis of variance

AWERB – Animal welfare and ethical review body

BAF – B allele frequency

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

CASA – Computer assisted sperm analysis

CCs – Cumulus cells

CD – Cytoplasmic drop

CGs – Cortical granules

CI – Confidence interval

CNV – Copy number variant

COC – Cumulus-oocyte complex

COVID-19 – Coronavirus Disease 19

db-cAMP – Dibutyryl cyclic-adenosine monophosphate

ddH_2O – Double distilled H_2O

DF – Degree of freedom

DFI – DNA Fragmentation Index

DFR – Dominant follicle removal

DGS – Density gradient selection

DMSO – Dimethyl sulphoxide

DNA – Deoxyribose nucleic acid

E2 – Oestradiol

EDTA – Ethylenediaminetetraacetic acid

EGA – Embryonic genome activation

EGF – Epidermal growth factor

EGTA – Ethylene glycol tetraacetic acid

ET – Embryo transfer

EX – Extender

FBS – Foetal bovine serum

FCS – Foetal calf serum

FGF2 – Fibroblast growth factor 2

FISH – Fluorescence *in situ* hybridisation

FITC – Fluorescein isothiocyanate

FLI – FGF2, LIF, and IGF1

FSH – Follicle stimulating hormone

FT – Frozen-thawed

GG – Gabriel-Griffin

GH – Growth hormone

GEBV – Genomic estimated breeding values

GLM – Generalised linear model

GnRH – Gonadotropin-releasing hormone

GSH – Glutathione

GV – Germinal vesicle

GVBD – Germinal vesicle breakdown

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HDS – High DNA stainability

ICM – Inner cell mass

ICSI – Intracytoplasmic sperm injection

IETS – International Embryo Transfer Society

IGF1 – Insulin like growth factor 1

IM – Intramuscular

IUI – Intrauterine insemination

IVC – *in vitro* culture

IVD – *in vitro derived*

IVF – *in vitro* fertilisation

IVM – *in vitro* maturation

IVP – *in vitro* embryo production

LDL – Low density lipoprotein

LEY – Lactose egg yolk media

LH – Luteinising hormone

LIF – Leukaemia inhibitory factor

LIN – Linearity

LN_2 – Liquid nitrogen

LRR – Log R ratio

MAPK3/1 – Phosphorylated mitogen-activated protein kinase3/1

Mb – Mega base pairs

MCS – Microfluidic chip-based sperm

MMP – Mitochondrial membrane potential

mSOF – Modified SOF based sequential culture media

NCSU – North Carolina State University

NGS – Next generation sequencing

OEP – Orvus ES Paste

OPU – Ovum pick-up

OR – Odds Ratios

P4 – Progesterone

PB – Polar body

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PFA – Paraformaldehyde

PGF_2a - Prostaglandin

PGM – Porcine gamete media

PGT– Preimplantation genetic testing

PGT-A – Preimplantation genetic testing for aneuploidies

PGT-M – Preimplantation genetic testing for monogenic diseases

PGT-P – Preimplantation genetic testing for polygenic disorders

PGT-SR – Preimplantation genetic testing for structural rearrangements

PKA – Protein kinase A

PNA – Peanut agglutinin

PN – Pronucleus

POM – Porcine oocyte medium

PPV – Positive predictive value

PT – Pre-test

PVP – Polyvinylpyrrolidone

PXM – Porcine X medium

PZM – Porcine zygote medium

PZM5 – Porcine zygote medium 5

qPCR – Qualitative polymerase chain reaction

QTL – Quantitative trait locus

ROS – Reactive oxygen species

RT – Room temperature

sAC – Soluble adenylate cyclase

SEM – Standard error of the mean

SF – Slow freezing

sFF – Sow follicular fluid

SNP – Single nucleotide polymorphisms

SOFaai – Synthetic oviductal fluid with amino acids and myo-inositol

STR – Righteousness movement

T1/2 – Type 1/2

TCM-199 – Tissue culture medium 199

TE – Trophectoderm

TL – Tyrodes lactate

TLP – Tyrodes lactate medium with lactate and pyruvate

UPD – Uniparental disomy

VAP – Average path velocity

VCL – Curvilinear velocity

VMS – Vitrification with mini-straws

VP – Vitrification with pearls

VPD – Vitrification with pearls and prepared using DGS

VSL – Straight line velocity

WBC – White blood cell

WGA – Whole genome amplification

ZP – Zona pellucida

ZP3 – Zona pellucida sperm binding protein 3

Abstract

The global livestock industry is continually tasked with developing innovative solutions to meet rising food demand. In both economically developed and developing countries, sustainable supplies are essential for the continuous advances in productivity through genetic selection to improve feed conversion efficiency, disease resistance, and fertility. The interval between conception and birth, however, limits the rate at which these enhancements can be implemented. Furthermore, companies often export breeding animals to developing countries to boost genetic quality, but this comes with production, environmental, and logistical costs, as well as ethical issues.

In vitro embryo production (IVP) is an emergent technology that is progressively being applied to livestock breeding. IVP could bring incredible economic and environmental benefits, serving to increase selection intensity and facilitate the transport of genetically favourable livestock in a highly assistive, inexpensive, and bio-secure manner. Therefore, the main purpose of this thesis was to improve the efficiency of IVP procedures. IVP offers attractive benefits to breeders, such as increasing the offspring numbers derived from high genetic value animals, in less time, and at a cheaper cost than those produced *in vivo.* Moreover, it facilitates the study of the genetic constitution of the embryos to transfer only those carrying commercially desirable traits to improve genetic selection. IVP is key to reducing the transportation of live animals as the transport of embryos decreases the costs and reduces the risk of pathogen or disease transmission, favouring biosecurity. With this in mind, this thesis had five specific aims:

The first was to improve embryo quality with the addition of cytokines to porcine IVM media. This was successfully achieved as improvements were observed in oocyte maturation and developmental competence to produce higher quality embryos than those produced without cytokine supplementation. The second aim was to assess the effect of different sperm selection methods on basic boar sperm parameters and *in vitro* fertilisation (IVF) outcomes. This aim was partly successful in that it identified a microfluidic chip-based system as a selection method that produces similar parameters and IVF outcomes to density gradient selection, but with less morphological abnormalities. The third was to compare the slow freezing of boar sperm against modified vitrification protocols. The development of a suitable vitrification protocol was successful in preserving basic sperm parameters, but further work is needed to improve the efficiency compared to slow freezing, the “gold standard” in the breeding industry. The fourth aim was to use preimplantation genetic testing for aneuploidies (PGT-A) and SNP chip data from genomic estimated breeding values to screen *in vitro* produced bovine embryos. This allowed for the identification of chromosomal abnormalities and their origin, which when applied to embryo selection can yield improved pregnancy and live birth rates. The final aim was to use PGT-A to screen the inner-cell mass and trophectoderm of *in vivo* and IVP bovine embryos to identify and analyse chromosomal abnormalities. A comparison of the data between the inner-cell mass and trophectoderm revealed that trophectoderm biopsies reflect the true ploidy status of the embryo and demonstrate a reliable mean for screening embryos. Taken together, these results have improved the efficiency of porcine and cattle IVP procedures, furthering the development of techniques used for livestock animals.

1 Introduction

1.1 History of IVP

Since Louise Brown's birth in 1978, assistive reproductive technologies (ARTs) such as artificial insemination (AI), *in vitro* fertilisation (IVF), the cryopreservation of gametes, intracytoplasmic sperm injection (ICSI), and embryo transfer (ET) have been used to treat infertility and achieve pregnancy in humans (de Geyter *et al.,* 2020) resulting in more than 9 million IVF babies born (ESHRE, 2020). In domestic animals, assisted ART development has been less successful but has still played a key role in the development of biotechnology projects related to transgenic animals, used in cell therapies or xenotransplants, and in the development of new techniques for animal breeding (Cooper *et al.,* 2013, Choudhary *et al.,* 2016).

In cattle, the first calf born derived from *in vivo* mature oocytes was reported in 1982 (Brackett *et al.,* 1982). The obtention of immature oocytes from the ovaries of dead animals with high genetic value, or pre- or peripubertal slaughtered donors, to be matured *in vitro* also produced high quality embryos (Galli and Lazari, 1996). Nevertheless, compared to *in vitro* matured abattoir oocytes, blastocyst rates in cattle are greater when using *in vivo* matured ovum pick up (OPU) obtained oocytes (Rizos *et al.,* 2002). Due to this, since the early 90s, cattle IVP has been performed by inducing superovulation in live donors to release multiple mature oocytes that are collected via ultrasound-guided ovum pick up. These oocytes are then fertilised and cultured *in vitro* (IVC) before being transferred to donors (Van Wagtendonk-De Leeuw, 2006). This method was so successful that in 2018, the amount of IVP embryos transferred was greater than the amount of transferred *in vivo* derived (IVD) embryos, with 1,029,400 IVP and

469,967 IVD transferred embryos worldwide (28th annual global report of Embryo Transfer, IETS, 2019 - Viana, 2019). Nowadays, embryo technology is also being utilised commercially to choose and raise animals that carry highly desirable genetic traits (Lonergan, 2007; Sanches *et al.,* 2019), and when combined with genomic selection, the rate of genetic gain in cattle breeding programs has increased dramatically (Ponsart *et al.,* 2014).

Established during the 1990s, early porcine IVP techniques relied on the collection of ovulated oocytes which were fertilised with fresh boar sperm (Cheng *et al.,* 1986). This was the first report of the successful production of piglets using gametes (Cheng, 1986). Prior to this, porcine IVP attempts remained largely unsuccessful. Since then, IVP live offspring have been generated on multiple occasions, though the most significant difference was the use of abattoir material as this was readily available (Mattioli *et al.,* 1989; Yoshida *et al.,* 1990, Rath *et al.,* 1999; Abeydeera and Day, 1997). Mattioli et al. (1989) demonstrated that embryo development, normal pregnancy, and the birth of live piglets was possible with abattoir-sourced oocytes. Nevertheless, the efficiency of porcine IVP remained low, particularly in comparison with other livestock species (Grupen, 2014).

Through early studies, it became apparent that porcine IVP deficiencies stemmed from suboptimal culture conditions. In terms of *in vitro* maturation (IVM), initial experiments replicated conditions used to mature sheep and cattle oocytes. The addition of sow follicular fluid (sFF) and hormones in the medium led to considerable developments in the field and resulted in improved nuclear and cytoplasmic maturation (Yoshida *et al.*,1992b; Funahasi *et al.*,1994). Funahasi *et al.* (1994) demonstrated that the addition of different media

additives throughout the stages of oocyte maturation increased the developmental potential of the oocytes, further facilitating the development of porcine IVP systems. However, the strategy of stepwise IVM systems has not been greatly improved and the efficiency remains quite low.

Initial attempts in culturing *in vitro* derived porcine embryos throughout the pre-implantation stage failed as embryos stopped developing at the four-cell stage. To overcome the limitations and low efficiency of porcine IVP, the collection and transfer of pig embryos has been performed surgically (Hazeleger and Kemp, 2001), yet this method is impractical and undesirable since it has various detrimental effects on the animal. Furthermore, whilst non-surgical embryo collection or OPU is widely established in cattle breeding, the complex anatomy of the sow reproductive tract makes the collection very difficult (Geisert *et al.*, 2020). The development of new strategies for non-surgical embryo transfer have increased the efficiency of porcine IVP (Martinez *et al.*, 2016), with live-born piglets successfully achieved using IVP blastocysts derived from slaughterhouse oocytes, and surgical (París-Oller *et al.,* 2021; Suzuki *et al.,* 2006; Yoshioka *et al.,* 2003) and non-surgical embryo transfer methods (Suzuki *et al.,* 2004). Nevertheless, the developmental competence of IVP embryos is very low compared to their *in vivo* counterparts (Kikuchi *et al.,* 1999). The application of ET requires the improvement of other procedures, such as embryo preservation and embryo production, since large numbers of embryos are needed.

1.2 The Oestrous Cycle

The oestrous cycle, also known as reproductive cycle, is defined as the behavioural and physiological changes that occur in female mammals after

puberty to make them sexually receptive and increase the chances of successful mating and reproduction. Both cows and sows are non-seasonal polyoestrous animals, and each oestrous cycle is between 18-24 days or 21 days on average (Forde *et al.*, 2011; Geisert, 2020).

The oestrus cycle consists of a follicular phase, 4-6 days in cows and 5-7 days in sows, followed by a luteal phase that lasts around 14-18 days in cows and 13-15 days in sows. The follicular phase is the period when ovarian follicles grow and mature and the follicular cells secrete oestradiol (E2). The follicular phase is followed by ovulation (known also as pro-oestrus and oestrus, respectively), in which the oocyte-cumulus complex is released from the mature follicles. This is followed by a luteal phase and the formation of the corpus luteum, which secretes progesterone (P4). The pattern of hormone secretion (E2 and P4) is what determines the changes in behaviour, the probability of gestation, acceptance or not of the male, and of artificial insemination (Forde *et al.*, 2011; Geisert, 2020).

1.3 Regulation of Preovulatory Follicle Maturation

Preovulatory follicle maturation regulation is a multi-step process involving numerous cellular and physiological systems. This procedure ends with the release of a developed oocyte during ovulation. The inside of the follicle is lined with mural granulosa cells whilst the outer part is lined with theca cells (as seen in Figure 1.1), both of which are required to produce oestrogens (Mcgee and Hsueh, 2000). Theca cells convert cholesterol into androgens, such as testosterone and androstenedione, in response to LH. Whereas in the granulosa cells, follicle stimulating hormone (FSH) induces aromatase (CYP19) to convert androgens into oestrogens (LaVoie, 2017).

Each follicle contains an oocyte that is surrounded by the zona pellucida (ZP). Mammalian oocytes are surrounded by a small mass of somatic cells called cumulus cells (CCs), forming concentric layers that remain attached to the oocyte once outside the follicle. This forms the so-called cumulus-oocyte complexes (COCs; Russel *et al*., 2006). Within the follicle, oocytes mature via intricate routes and communication between granulosa cells, theca cells, cumulus cells, and the oocyte itself. These intracellular connections occur via gap junctions between the cells and the oocyte which facilitates the bidirectional transport of ions and small molecules, helping to balance the cellular metabolism by supplying nutrients and peptides (Feng *et al*., 2013).

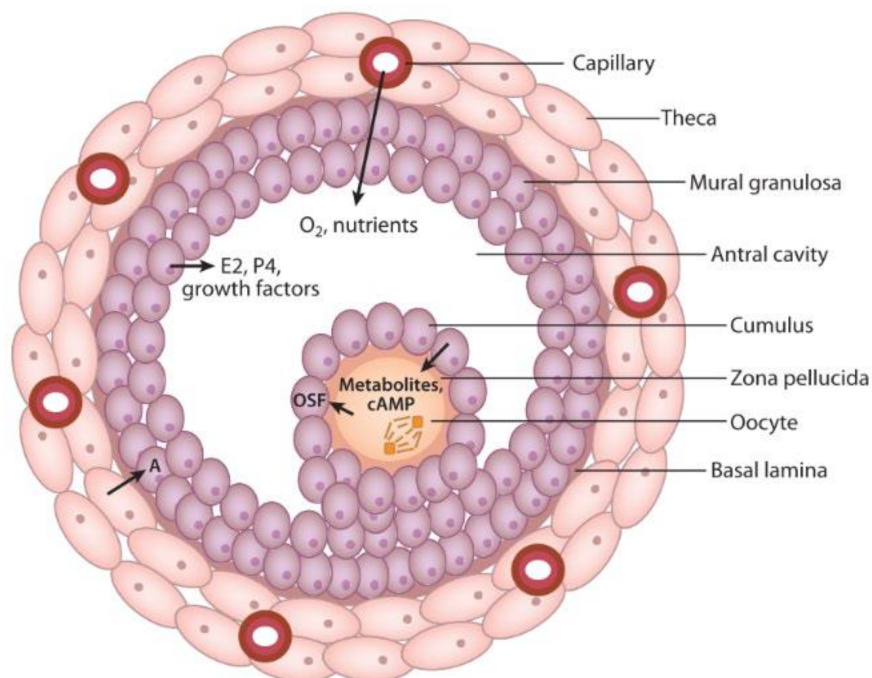

Figure 1.1: Ovarian preovulatory follicle diagram. The image shows the several cell types involved in follicular environment maintenance and oocyte competence. A: androgen; Camp: cyclic AMP; E2: oestradiol; OSF: oocyte-secreted factors; P4. progesterone (Krisher, 2013).

During the follicular phase, the hypothalamus releases gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to release FSH. FSH recruits many small- to medium-sized antral follicles and promotes their development with the help growth factors (Noakes *et al.,* 2018). The increase of

E2, secreted from the granulosa cells of the growing follicles, triggers a surge of luteinising hormone (LH) from the pituitary gland.

The exposure to the LH surge induces ovulation and a prompt reduction in the E2 production, in addition to a brief decrease in the mRNAs for the genes that code the steroidogenic apparatus. This is then followed by a marked increase in the expression of the genes that control the biosynthesis of P4 by the luteal cells (LaVoie, 2017). P4 controls gonadotropin secretion, limiting the growth of small- to medium-size follicles. When fertilisation does not occur, the functional regression of the corpus luteum, approximately in Day 16 of the oestrus cycle, leads to a decrease in P4 levels. This decline allows the continuation of growth for many of the small- to medium-size antral follicles that would develop into the next batch of preovulatory follicles.

1.3.1 Role of Growth Factors and Cytokines in Oocyte Maturation, Fertilisation, and Embryo Development

In addition to hormones, growth factors and cytokines play important roles in regulating the growth and development of follicles and oocytes, subsequently improving embryo development at early stages and implantation.

Insulin-like growth factor-1 (IGF-1) is essential for the regulation of follicular growth and corpus luteum function, which in turn enhances oocyte maturation. Additionally, IGF-1 increases the proliferation of granulosa and theca cells, enhancing steroid production (Furnus *et al.*,1998). Other growth factors with a key role in follicle development and oocyte maturation is growth hormone (GH); however, its effect is follicle-size dependant, being more noticeable on the cells of large follicles (Gregoraszczuk *et al.*, 2000).

The epidermal growth factor (EGF) is found in both the ovary and follicular fluid, affecting the ovarian cell function, and promoting the maturation of the pig oocyte and CC expansion (Li *et al.*, 2002; Uhm *et al.*, 2010). Furthermore, EGF receptors have been identified on mammalian sperm, improving sperm motility (Oliva-Hernandez and Perez-Gutierrez, 2008; Tan and Thomas, 2015).

Cytokines such as leukaemia inhibitory factor (LIF) and fibroblast growth factor 2 (FGF2) also promote the quality of oocyte maturation, and subsequently the ability to achieve fertilisation (Dang-Nguyen *et al.*, 2014; Barros *et al.*, 2019). After fertilisation, these cytokines also improve blastocyst development in both porcine (Dang-Nguyen *et al.,* 2014), bovine (Mo *et al.,* 2014), and ovine (Zheng *et al.,* 2008).

1.4 Porcine *In Vitro* Production

Embryo production by the non-surgical extraction of mature oocytes from the ovaries via OPU is mostly unavailable in pigs, though 2020 reported the first piglets born after OPU-IVP (Yoshioka *et al.*, 2020). IVM is still postulated as the most suitable method to produce porcine IVP embryos due to the large number of oocytes that can be matured simultaneously (Gil *et al.*, 2010). Despite all these factors, pig IVP remains less efficient and prone to more variability compared to other livestock species.

The success of IVP depends on a source of oocytes for manipulation. In species such as cattle, horse, sheep or goat, the collection is routinely performed by inducing superovulation in live donors to release multiple mature oocytes from their ovaries (Mullart and Wagtendonk-de Leeuw, 2000). These mature oocytes are retrieved using an OPU technique based on the puncture and aspiration of

ovarian follicles and guided by an ultrasound scanner equipped with a transvaginal probe, with the scanning performed by rectal exploration (Van Wagtendonk-de Leeuw, 2006; Boni, 2018). Nevertheless, although this technique is widely used, it has been demonstrated that repeated superovulation has various detrimental effects on female reproduction.

It is not always possible to use OPU to obtain mature oocytes, as is the case with animals of high genetic value that cannot be subjected to superovulation, are sub-fertile or have died. This also applies to the complex morphology of the sow reproductive tract morphology. Nonetheless, the application of OPU for oocyte collection in porcine IVP has been published on a few occasions, though it is not a technique widely recommended (Yoshioka *et al*., 2020). In domestic species such as pigs, ovaries tend to be collected from sows or gilts from abattoirs. Prepubertal gilt ovaries with unknown background are commonly used in IVP investigations because they are slaughtered in large numbers at commercial abattoirs, are readily available, and inexpensive.

The oocytes from slaughtered animals can be collected using different techniques, such as aspiration of the follicular fluid with a needle connected to a vacuum pump or a syringe, or by the “slicing” method, where the whole ovary gets dissected. Using oocytes from primiparous and multiparous sows rather than prepubertal gilts, on the other hand, results in improved IVP outcomes (Grupen *et al*., 2003; Bagg *et al*., 2007; Pawlak *et al*., 2015). Moreover, outcomes in cattle differ amongst breeds with varied reproductive performance (Fischer *et al*., 2000; Abraham *et al*., 2012).

1.4.1 *In Vitro* Maturation of Oocytes

During the oocyte maturation process, CCs participate indirectly in the maintenance of oocyte competence and promote meiotic resumption by regulating cyclic adenosine 3',5' monophosphate (cAMP) and hormone levels (Dumesic *et al*., 2015; Ferré *et al*., 2016). Furthermore, CCs undergo their own maturation process, exhibiting a morphological transformation that consists of mucification and expansion by the secretion of hyaluronic acid (Motlik *et al.,* 1986). A greater cumulus expansion after maturation has been reported to be an indicator of high oocyte developmental potential, referring to the capability of the COCs to complete meiosis (Figure 1.2), undergo fertilisation, and develop into a viable embryo (Han *et al.,* 2006; Qian *et al.,* 2003; Conti and Franciosi, 2018).

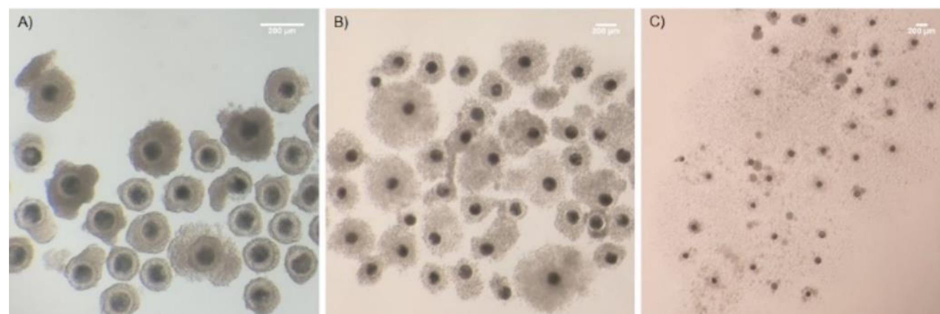

Figure 1.2: Porcine Cumulus-oocyte complexes (COCs) at different stages during IVM. A) Immature COCs with no cumulus cell expansion. B) COCs after 24h in culture where there is a degree of cumulus expansion. C) COCs after 44h of culture with a great cumulus cells expansion (M. Serrano Albal).

As previously mentioned in section 1.3, *in vivo* maturation of the COCs is regulated by the gonadotropins FSH and LH, which is transported to the ovaries via the blood (Senger, 2012). COC maturation starts inside the follicle and requires granulosa cell proliferation, under the influence of FSH, that activates the aromatase system and induces LH receptors, permitting the granulosa cells to later respond to LH. The LH peak induces the oocyte to resume meiosis,

reducing the levels of cAMP and completing nuclear maturation to the metaphase II (MII) stage of meiosis in readiness for fertilisation, via the granulosa and cumulus cells. The main objective of porcine IVM is to obtain mature oocytes outside the follicular environment, mimicking *in vivo* conditions that are suitable for embryo development. To achieve this, COCs from antral follicles are collected before being stimulated by the LH surge. Generally, slaughtered prepubertal gilts that have reached the adequate weight (typically about 120-160 kilograms when they are around 6 months old) are the source of unstimulated ovaries. These ovaries usually have a large number of follicles (Figure 1.3) with COCs in the immature germinal vesicle (GV) stage that can be retrieved and matured *in vitro* (Wang *et al*., 1997).

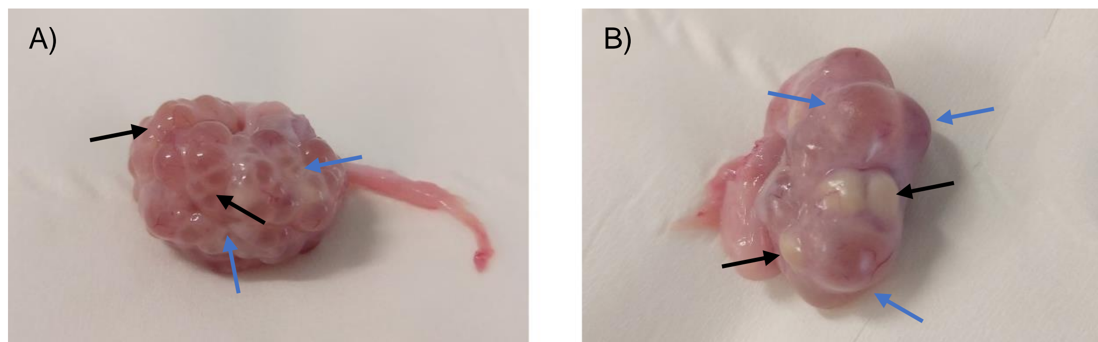

Figure 1.3: Ovaries obtained from slaughtered guilts. A) Ovary from a prepubertal guilts with a high number of medium (black arrow) and small follicles (blue arrow). B) Pubertal guilt ovary from with a few big follicles (not viable to be used in IVM; pointed with blue arrows) and three visible corpus albicans (black arrows), which are the scar that forms when the oocyte is released and the corpus luteum degenerates (M. Serrano Albal).

In both cattle and pig, the oocyte quality and hence its developmental competence depends on the size of the follicles (Bagg *et al.,* 2007; Carolan *et al.,* 1996; Marchal *et al.,* 2002; Qian *et al.,* 2001). This is due to differences in the quantity of organelles within the cytoplasm, such as mitochondria, and the abundance of gene transcripts associated with the regulation of oocyte

maturation, which is lower in those COCs obtained from small follicles (Kohata *et al.,* 2013). Follicles with less than 3 mm in diameter are commonly classified as small, medium follicles with a diameter between 3 and 6 mm, and large follicles with a diameter greater than 6 mm (Knox *et al.*, 2005). Medium follicles have a better developmental competence, so normally those are selected for IVM.

Prior to IVM, every trace of follicular fluid, endogenous gonadotropic, and growth factor activity needs to be removed. During IVM culture, COCs need to undergo two essential procedures, nuclear and cytoplasmic maturation, required for oocyte developmental competence (Channing *et al.*, 1980; Eppig, 1996).

1.4.1.1 **Nuclear Maturation**

Nuclear maturation refers to the processes that reverses meiotic arrest at prophase I and resumes meiosis to MII. In porcine IVM, roughly 70-85% of oocytes complete nuclear maturation and reach MII (Somfai *et al.,* 2005; Gil *et al.,* 2017). Before the LH surge, oocytes remain in prophase I where the chromatin is fully decondensed (GV stage). After the LH surge, the chromatin starts condensing and the nuclear membrane begins to degrade (germinal vesicle breakdown, GVBD). Following that, chromosomes are fully condensed in diakineses (prometaphase) and begin to align with the mitotic spindle, followed by the metaphase I stage in which chromosomes are plainly visible in the metaphase plate. In Anaphase I, chromosomes migrate towards the spindle poles, followed by chromosomal separation at the poles in telophase I. Finally, the oocytes will enter metaphase II, displaying chromosomes in the metaphase

plate as well as the first polar body that is extruded. This process is summarised in Figure 1.4.

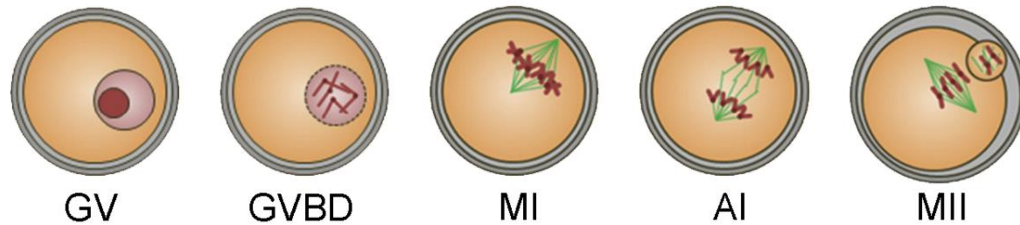

Figure 1.4: Schematic representation of the nuclear maturation within a mammalian oocyte. GV: Germinal vesicle. GVBD: Germinal vesicle breakdown. MI: Metaphase I. AI: Anaphase I. MII: Metaphase II. (Modified from Fan and Sun, 2019).

Porcine COCs are cultured for 40-44 hours, which is longer than the time required in other species, for example 22-24 hours in cattle. The GV stage has been observed until 18-24 hours after the start of maturation, during which the oocytes are cultured with hormones and a meiotic inhibitor, such as dibutyryl cyclic-cAMP (db-cAMP), to control the resumption of the meiosis (Mattioli *et al.*, 1991; Funahashi *et al.,*1997; Laforest *et al.*, 2005; Appeltant *et al.*, 2015). After this point, GVBD is initiated and metaphase I is typically observed 30-36 hours later; the anaphase and telophase stages are only visible for a short period of time around 36 hours. Finally, *in vitro* matured oocytes reach MII with polar body extrusion around 36-42 hours.

Porcine IVM has been carried out using a variety of fundamental culture media types. The most commonly used is Tissue Culture Medium 199 (TCM-199; Mattioli *et al.,* 1989; Yoshida *et al.,* 1990; Coy *et al.,* 1999), but there are others such as the modified Tyrode's medium containing lactate and pyruvate (TLP; Yoshida *et al.,* 1992a; Yoshida 1993), Waymouth medium (Yoshida *et al.,*

1992a; *Coy et al.,* 1999), Whitten's medium (Funahashi *et al.,* 1994), and North Carolina State University (NCSU; Petters and Wells 1971). These medium frequently include foetal calf serum (FCS; Yoshioka *et al*., 2005) or sow follicular fluid (sFF), as well as supplements such growth factors, that are added to the IVM medium to aid oocyte maturation by limiting asynchrony between nuclear and cytoplasmic maturation (Serrano Albal *et al*., 2022).

1.4.1.2 **Cytoplasmic Maturation**

A complete nuclear maturation and extruded first polar body may not ensure the competence of the oocyte to be fertilised. Alongside nuclear maturation, several cytoplasmic modifications are required to prepare the oocyte for activation and preimplantation development; this process is known as cytoplasmic maturation (Hirao *et al.,* 1994; Abeydeera, 2002; Coy *et al.,* 2002).

There are many morphological and ultrastructural changes which occur in the cytoplasm, such as the reorganisation and migration of different organelles, (including cortical granules and mitochondria (Liu *et al.,* 2010), which have been correlated with cytoplasmic maturation. In immature oocytes, cortical granules and mitochondria are scattered throughout the cytoplasm, but by the end of the maturation process they migrate to the peripheral area near the oocyte membrane (Cran and Cheng, 1985; Pawlak *et al*., 2012; Wang *et al.,* 1997). Cortical granules are required for the cortical reaction that occurs after sperm cell penetration to prevent polyspermy (Coy and Avilés, 2010), whereas mitochondria are required for energy production and frequently migrate to areas of high energy consumption, such as polar body extrusion (Babayev and Seli, 2015).

Cytoplasmic maturation is improved by the reduction of oxidative stress caused by reactive oxygen species. Glutathione (GSH) is an antioxidant synthetised by cumulus cells throughout oocyte maturation to protect oocytes against oxidative stress. In oocytes, the concentration of GSH increases when they reach the MII stage. Higher levels of GSH benefit sperm nuclear decondensation and male pronucleus formation, as well as good blastocyst development and a higher cell number in blastocysts (Yoshida *et al.*, 1993; Abeydeera *et al.*, 1998; Maedomari *et al.*, 2007). Previous studies have shown that the addition of cysteine, β-mercaptoethanol, and sFF to maturation media positively affected cytoplasmic maturation, resulting in an increase in GSH levels and blastulation rates in both pig and cattle (Abeydeera *et al.*, 1998; Furnus *et al.*, 1998; Kobayashi *et al.*, 2006; Yoshida *et al.*, 1992). Supplementing IVM medium with growth factors like EGF is also associated with the acquisition of porcine oocyte developmental competence by increasing mitochondrial activity (Mao *et al.*, 2012; Ritter *et al.*, 2015).

1.4.2 *In Vitro* Fertilisation

After natural mating or AI, sperm are deposited in the female reproductive tract where they start their ascent to reach and fertilise the oocyte. During this ascent, sperm undergo several biochemical and physical modifications which allow sperm to acquire the ability to fertilise, known as 'capacitation' (Austin, 1952; Rodriguez-Martinez, 2007). Capacitation induces changes in the fluidity of the membrane, modifies the sperm surface proteins, and increases intracellular ion levels. These changes prepare the sperm for the acrosome reaction necessary for fertilisation. Some of these changes are quick and occur during ejaculation, such as the activation of sperm motility due to bicarbonate exposure since the

sperm are immobile when in the epididymis. Others require more time in the female genital canal (*in vivo*) or in a medium that can sustain this process (*in vitro*), and these slow capacitation changes only occur once the sperm acquires the ability to fertilise. When in the genital canal or media, the presence of high weight molecular proteins and high-density lipoproteins facilitates the sperm acrosomal reaction due to cholesterol efflux, allowing the binding of the sperm to the ZP (Fraser, 1998) and changing the pattern of motility to a more vigorous non-linear movement, known as hyperactivation (Ho and Suarez, 2001). Both quick and slow changes are regulated by Protein kinase A (PKA), bicarbonate, soluble adenylate cyclase (sAC), and cAMP. Bicarbonate induces the hyperpolarisation of the membrane potential, activating sAC by increasing the pH. This leads to a reorganisation of the lipids in the membrane and changes the position of cholesterol in the apical part of the sperm head. The subsequent decrease in the cholesterol/phospholipid ratio increases membrane fluidity and facilitates the entrance of calcium. Calcium enhances sAC activity which in turn increases the protein tyrosine phosphorylation via the cAMP/PKA pathway (Visconti, 2009). Furthermore, in this journey through the reproductive tract, only a small proportion of the sperm can reach the oviduct as the sperm cells are subjected to a rigorous selection process (Rodriguez- Martinez *et al.*, 2005; Holt, 2010). High quality sperm are naturally selected from the remaining ejaculate by active migration through the cervical mucus (Sakkas *et al.*, 2015; Alvarez-Rodriguez *et al.*, 2020), promoting a highly efficient fertilisation process (Fitzpatrick and Lüpold, 2014).

1.4.2.1 In Vitro Sperm Selection and Capacitation of Boar Sperm

The main goal of processing sperm prior to IVF is to capacitate the sperm artificially to control the time of the acrosome reaction, since only capacitated sperm can be identified by the oocyte and are able to respond appropriately to oocyte signals (Töpfer-Petersen *et al.*, 2000). In order to achieve capacitation, any trace of seminal plasma or extender (media to dilute sperm) must be removed as these contain decapacitation components. There have been different approaches for *in vitro* sperm selection that have been designed to mimic the process *in vivo*.

First, with the aim of destabilising the sperm membrane and achieving an acrosome reaction, highly concentrated sperm samples are centrifugated several times and pre-incubated in a medium with high calcium content (Pavlok, 1981). The sperm selection methods use density gradient solutions which have previously been used in human IVF and have resulted in live born piglets (Mattioli *et al.*, 1989). These selection solutions contain colloidal silica beads coated with polyvinylpyrrolidone that, via centrifugation, are able to capacitate the sperm, isolate only the motile sperm, and remove any traces of other seminal content. Its efficiency was such that despite being removing from human assisted reproduction protocols (Ng *et al.*, 1992), it is still the golden standard method for animal IVP in many livestock species such bull (Suzuki *et al.*, 2003; Oliveira *et al.*, 2011) and boar (Suzuki and Nagai, 2003; Coy *et al.*, 2009; Matás *et al.*, 2011).

There are other simpler and less invasive procedures that allow the sperm to swim and be selected based on their motility, and also result in the successful penetration of the oocytes (Nagai *et al.*, 1984; Yoshida *et al.*, 1993). Such

procedures include swim-up or microfluidics devices; swim-up (Park *et al.*, 2009) has been used successfully used to isolate a highly motile sperm population (Holt *et al.*, 2010), reducing polyspermy during IVF when using a pore-sized cell strainer (Park *et al.*, 2009). Although the efficacy of this method was lower compared to density gradients, recent updates on the protocol double the efficiency of swim up compared to density gradients (Canovas *et al.*, 2017; Navarro-Serna *et a*l., 2018).

Sperm selection using microfluidic devices result in a relatively high penetration rate (Sano *et al.,* 2010). The purpose of microfluidics is to study and control the movement of fluids through micrometre-sized channels (Sackman *et al.,* 2014). Due to this, they are a good option for sperm selection because the devices can simulate the micro-confined locations within the female reproductive system. Although there is not much published research using microfluidics, there has recently been publications showing promising developments in the field and provide a potential alternative to the centrifugation methods. It was first reported in the development of a device designed for porcine sperm (Matsuura *et al.*, 2012). After that, selection protocols incorporated sperm chemo-attractants (Vieira *et al*, 2020), thermotaxis (Pérez-Cerezales *et al.*, 2018), and oviductal epithelial cells (Ferraz *et al.*, 2018) to increase the efficiency.

1.4.2.2 **Mammalian Fertilisation**

At fertilisation, the sperm binds to zona pellucida sperm binding protein 3 (ZP3) of the oocytes, which induces the released of the enzymatic acrosomal contents that lyses the ZP (Senger, 2012). It has been proven that IZUMO1 protein, an acrosomal protein of the immunoglobulin family, is essential in gamete fusion (Inoue *et al.*, 2005). IZUMO1, after the acrosomal reaction, moves from the

acrosomal zone to the equatorial segment of the spermatozoon. From here, it interacts with the oolemma protein (JUNO). JUNO is a GPI-anchored protein expressed on the egg surface that is required to establish the biochemical connection that occurs between the oocyte and sperm during fertilisation (Bianchi *et al.*, 2014). Following penetration, the increase of intracellular Ca^{2+} prompts the cortical reaction in the oocyte (Figure 1.5). The exocytosis of cortical granules hardens the ZP, preventing penetration by other sperm cells.

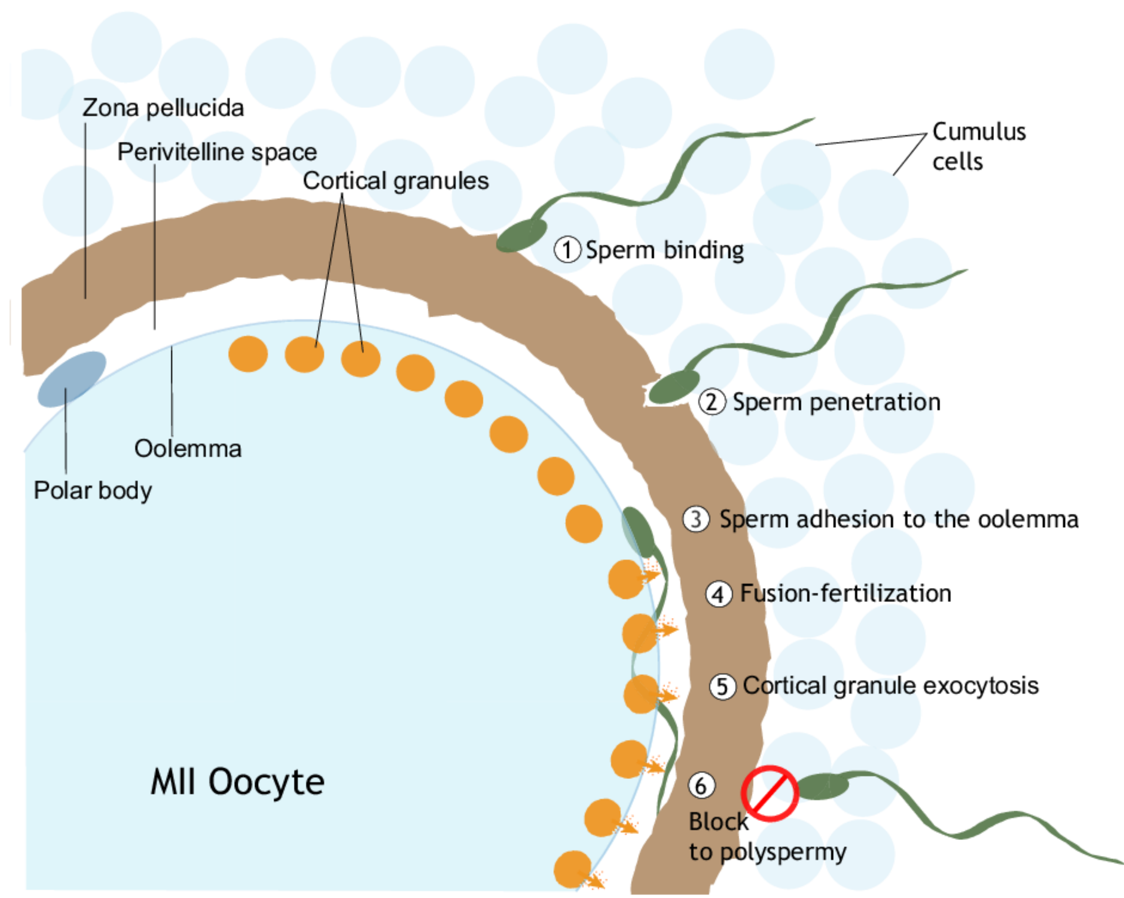

Figure 1.5: Mammalian oocyte fertilisation. Once sperm bond to the zona pellucida of the mature oocyte (Metaphase II, MII) (1), enter the zona (2), and attach to the oolemma of the oocyte (3). Sperm- oocyte fusion (4), induces the exocytosis of the cortical granules (5). The exocytosis of cortical granule content hardens the zona blocking the penetration of other sperm cells (6), ensuring monospermic fertilisation (Hanisha *et al.*, 2019).

Sperm penetration also induces the resumption of meiosis II and thus the extrusion of the second polar body (Sun and Nagai, 2003). Next, the sperm

nucleus and the oocyte chromatin undergo decondensation to form the female and male pronucleus that will fuse to form the zygote (Kaser *et al.*, 2019). Fresh and frozen-thawed sperm from ejaculated or epididymal samples have been used successfully in the *in vitro* fertilisation of IVM oocytes (Wang *et al.,* 1991; Rath and Niemann, 1997; Romar *et al.,* 2005; Suzuki *et al.,* 2005). Although, sperm survival and motility after freeze-thawing are frequently lower than in fresh sperm (Yeste, 2017).

Fertilisation media must contain supplements that induce sperm capacitation, such as caffeine or theophylline, to facilitate sperm penetration (Romar *et al.,* 2016). Caffeine is still often used in IVF protocols; however, it is associated with promoting greater rates of polyspermy (Funahashi and Nagai, 2001). On the other hand, theophylline has been shown to enhance sperm penetration into IVM oocytes without causing polyspermy (Yoshioka *et al.,* 2003). One of the limiting variables in porcine IVF has been identified as a high prevalence of polyspermy, which occurs when an egg cell is fertilised by more than one sperm cell (Funahashi, 2003; Suzuki *et al.,* 2003). Polyspermic zygotes can develop into blastocysts with good morphology, but only a few polyspermic blastocysts have been reported to develop into foetuses and piglets after transfer (Han *et al.,* 1999a; Han *et al.,* 1999b). The majority do not develop into viable piglets due to chromosomal abnormalities which reduces the efficiency of porcine IVP (Yoshioka *et al.,* 2008; Gil *et al.,* 2010; Martinez *et al.,* 2017). Various studies have investigated reducing porcine polyspermy by reducing the number of sperm cells present per oocyte (Xu *et al.,* 1996; Gil *et al.,* 2007), reducing co-incubation time (Almiñana *et al*., 2005; Gil *et al*., 2004), or using different IVF

methods to increase sperm selection and reduce the number of sperm cells (Park *et al.*, 2009; Li *et al.*, 2003).

1.4.3 *In Vitro* Culture of Embryos

After amphimyxis (the union of the two pronuclei) the zygote already has its own genome, however it is not yet active. Thus, the zygote cytoplasm has the necessary components to start the first cell division, in which maternal mitochondrial RNA will be involved. The first division, known as cleavage, takes about 24h, then the following divisions take about 12h. However, the first division can take up to 48h *in vitro*. Divisions continue to occur, increasing the number of cells until the morula stage (16 cells) is reached. During these divisions the cells become smaller and smaller, but the size of the embryo does not change, due to the ZP (Cross and Rossant, 2001).

After morula stage, embryos initiate a compaction process. During this process, cells bind to each other by releasing a series of proteins that allow the adhesion of the cell membranes and begin to differentiate, losing totipotency and changing their morphology. The surface cells elongate and flatten, acquiring the appearance of epithelial cells, while those in the center are larger and rounder in shape. The surface cells are joined by desmosomes, while the inner ones by interstitial junctions GAP-like interstitial junctions to maximise cellular communication. The surface cells form the trophectoderm (TE) and those in the center form the inner cell mass (ICM). The cells of the TE secrete fluid into the morula and begin to form a cavity called a blastocele, at which point the morula is called a blastocyst (Cross and Rossant, 2001).

Embryonic genome activation (EGA) is a gradual process regulated by cell cycle-dependent mechanisms and is subjected to environmental influences (Latham and Schultz, 2001). EGA requires a sharp shift in embryonic transcriptional patterns that involves significant epigenetic changes, such as chromatin architecture, chromatin accessibility, DNA methylation, and histone modifications (Eckersley-Maslin *et al.*, 2018). This critical event is a species-specific time point that normally occurs from 4 to 16 cells and affects embryo quality and developmental competence, which can compromise foetal, prenatal, and postnatal viability (Niemann and Wrenzycki, 2000). The switch from using the mRNA derived from the maternal genome to the embryonic genome occurs at the stage of 4 cells in porcine embryos (Tomanek *et al.*, 1989), and at the 8-cell stage in bovine embryos (Memili and First, 2000).

The culture of embryos, from zygote until blastocyst stage, is the longest step in IVP. Cell cleavage is often assessed at 2-3 days post-fertilisation (Figure 1.6) and porcine embryos are normally cultured until day 5 or 6 post-IVF, when they reach blastocyst stage. Not all mature oocytes are fertilised, and not all fertilised zygotes grow to the blastocyst stage. Approximately 15-30% of pig oocytes that are cultured develop into blastocysts (Grupen *et al.,* 2003; Kidson *et al.,* 2004; Martinez *et al.,* 2017), with developmental block often occurring at the 4-cell stage during IVC (Cao *et al.,* 2014). This blockage has also been observed in other species and is correlated with EGA (Meirelles *et al.,* 2004).

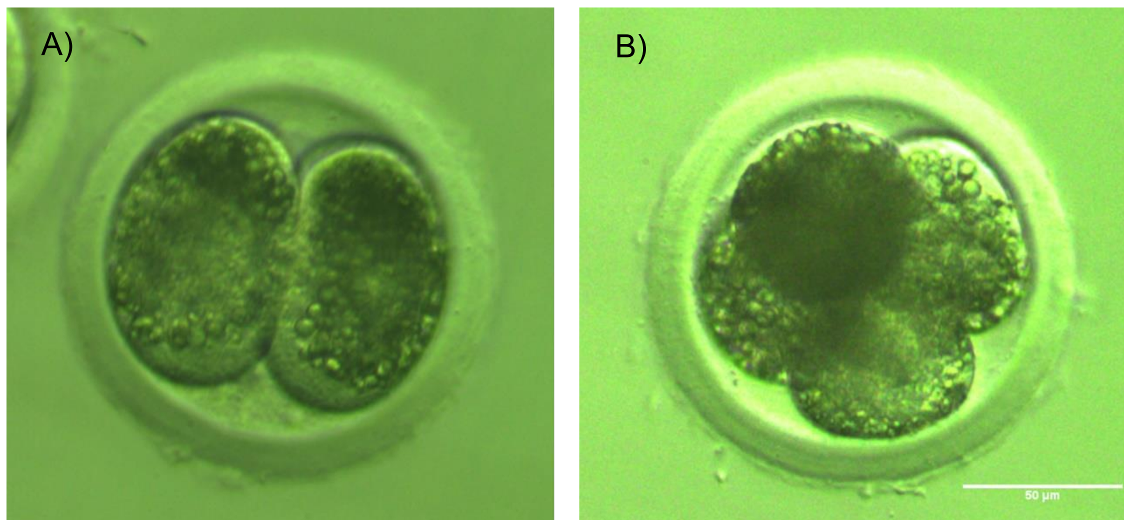

Figure 1.6: Porcine embryos at 2-cell (A) and 4-cell (B) stages (M. Serrano Albal).

The media most commonly used is NCSU- 23 because, whilst less effective than physiological systems, it was associated with the highest blastocyst rate (Macháty *et al.,* 1998). NCSU-23 media uses glutamine and glucose as the main energy sources, and taurine and hypotaurine to mediate osmolarity. However, NCSU- 23 does not contain pyruvate, lactate, or other amino acids. On the other hand, the composition of Porcine zygote media (PZM) variants are based on the oviductal fluid content, therefore they contain amino acids and pyruvate, and lactate instead of glucose, but it does not include taurine (Chen *et al.*, 2021). Many research groups have promoted the addition of serums to increase the efficiency of IVC, such as FCS as a source of proteins (Dobrinsky *et al.*, 1996; Koo *et al.*, 1997) or the use of oviductal epithelial cells as supplementation for porcine (Nagai and Moor, 1990; Romar *et al.,* 2001) and bovine IVC systems (Katska *et al.,* 1995; Katska *et al.,* 1998). Nonetheless, others have still defended the use of chemically defined media to standardise IVC conditions (Iwasaki *et al.,* 1999).

1.5 Embryo Quality assessment

The quality of porcine IVP blastocysts (Figure 1.7) is known to be lower than IVD blastocysts. IVP embryos show a decrease in the number of cells that form the ICM and the TE (Bauer *et al.,* 2010). This could be caused by a deficient metabolism and differences in lipid content that have been shown in IVP blastocysts when compared to IVD blastocysts (Swain *et al.,* 2002; Romek *et al.,* 2009). Consequently, prior to embryo transfer, IVP embryos need to be assessed on their quality.

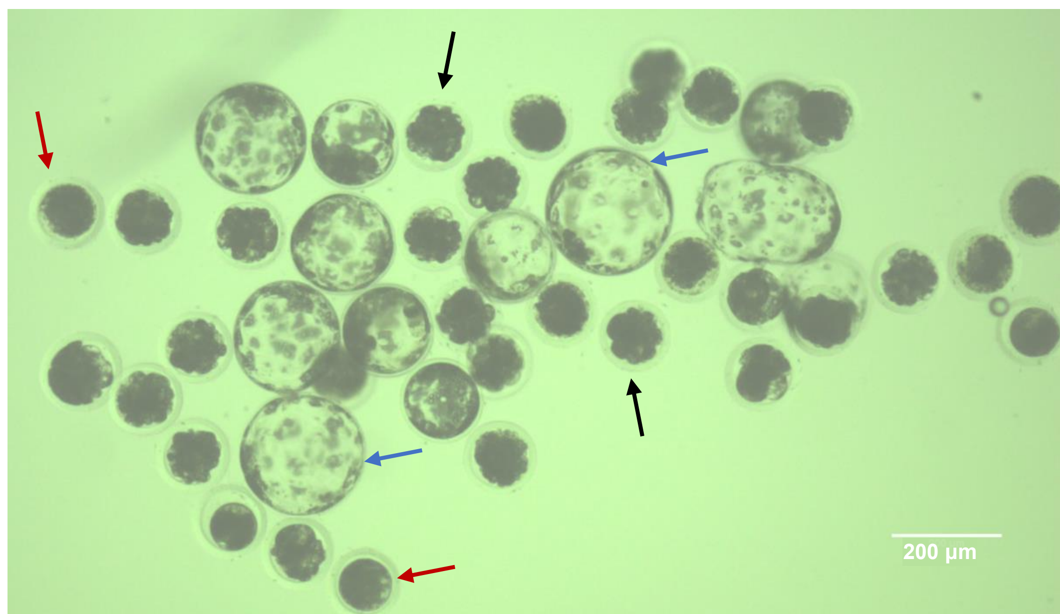

Figure 1.7: Porcine embryos cultured *in vitro* for 6 days. Embryos at blastocyst stage are pointed with blue arrows. Red arrows mark arrested embryos (with no cleavage). Morula stage embryos, that are embryos with more than 16 cells are shown with black arrows (M. Serrano Albal).

Blastocyst quality can be assessed by various non-invasive and invasive parameters such as scoring the morphology, kinetics of development by timelapse, counting total blastocyst cell number or TE cells and the ICM, or studying chromosomal abnormalities, blastocyst metabolism, and apoptosis (Soom and Boerjan, 2002).

1.5.1 Chromosomal Abnormalities in Blastocysts

Polyspermic blastocysts have been reported to develop into liveborn animals after transfer (Han *et al.,* 1999a; Han *et al.,* 1999b), but a high proportion of them do not develop into viable piglets due to chromosomal abnormalities. As previously mentioned, this reduces the efficiency of pig IVP (Gil *et al.,* 2010; Martinez *et al.,* 2017; Yoshioka *et al.,* 2008). Roughly one third of all human blastocysts that have been tested for hereditary disorders are aneuploid (Toft *et al.,* 2020), similar to previously published data reporting a 31% aneuploidy incidence in TE biopsies from cultured bovine blastocysts (Turner *et al.,* 2019). Furthermore, it has been reported that the evaluation of IVP bovine blastocysts only by their morphology might lead to the transfer of embryos with chromosomal abnormalities that are able to establish pregnancy but conclude in miscarriages, increasing economic losses for cattle breeding companies (Silvestri *et al.*, 2021).

Research has indicated that the vast majority of the abnormalities in the embryos are of a maternal origin (Obradors *et al.,* 2010). The ovarian reserve in mammals is created at birth, so oocytes remain in arrest for years waiting to be matured and released. During the maturation process, the oocytes are subjected to several start and stop signals, their divisions are not controlled by centrosomes (as with other somatic cells), and they are highly asymmetric in size when comparing the size of the oocyte with the size of the extruded polar bodies. These factors make the process extremely prone to errors (Hunt and Hassold, 2002). However, meiosis during spermatogenesis is less complicated, is a continuous process once it begins at puberty, and the entire process does not take more than a week. Furthermore, the checkpoint mechanisms appear to be more stringent in males than females (reviewed by Hunt and Hassold, 2002).

1.5.1.1 Types of Chromosomal Abnormalities

Chromosomal abnormalities can be divided into two categories: structural or numerical, based on the mechanism that causes the aberration. Numerical chromosome abnormalities, also called aneuploidies, are caused by errors in the segregation of homologous chromosomes during meiosis. This results in the gain or loss of one or more chromosomes (Theisen and Shaffer, 2010) and is summarised in Table 1.1. When the gain affects the whole chromosome set, due to genomic doubling, polyspermy or gametic nonreduction (Otto and Whitton, 2000), it is classified as polyploidy.

Table 1.1: Types of aneuploidies and polyploidies (adapted from Pierse, 2015).

	Type	Meaning	Representation
Aneuploidy	Nullisomy	Loss of homologous pair of chromosomes	$2n - 2$
	Monosomy	Loss of one chromosome	$2n - 1$
	Trisomy	Gain of one chromosome	$2n + 1$
	Tetrasomy	Gain of two homologous chromosomes	$2n + 2$
	Double trisomy	Gain of two chromosomes (nonhomologous)	$2n + 1 + 1$
	Double monosomy	Loss of two chromosomes (nonhomologous)	$2n - 1 - 1$
Polyploidy	Triploidy	Individual with three sets of chromosomes	$3n$
	Tetraploidy	Individual with four sets of chromosomes	$4n$
	Pentaploidy	Individual with five sets of chromosomes	$5n$

On the other hand, structural chromosome abnormalities are the consequence of alterations to the normal structure of the chromosome (Korf and Sathienkijkanchai, 2009; Figure 1.8), resulting in missing segments of the chromosome (deletion), additional segments (insertion or duplication), flipped segments (inversions) or the relocation of segments to another chromosome (translocation) (Theisen and Shaffer, 2010; Kalkan, 2017).

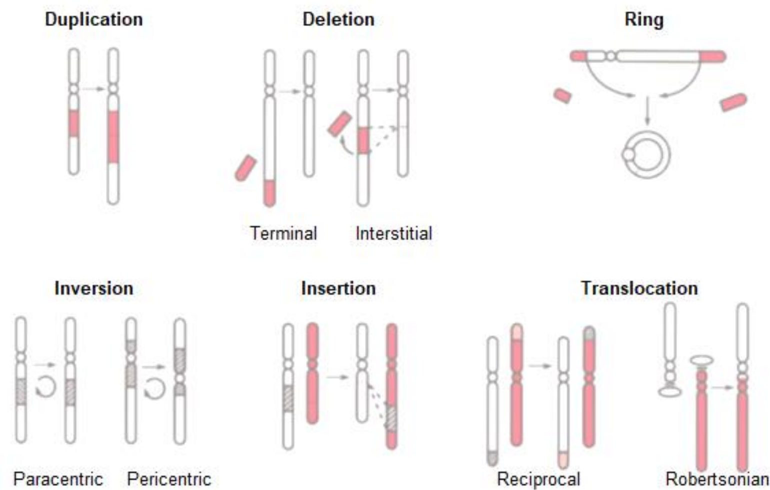

Figure 1.8: Type of abnormalities that affect the normal structure of the chromosomes (Arsham, Barch, and Lawce, 2017).

1.5.1.1.1 Uniparental Disomy

Uniparental disomy (UPD) occurs in disomic cells when homologous chromosomal segments or whole homologous pairs are inherited exclusively from one parent (Engel, 1980). UPD is associated with imprinting disorders, carcinogenesis, or other diseases triggered by the expression of recessive gene mutation or incomplete trisomic rescue (Robinson, 2000; Tuna *et al.*, 2009).

UPD has three different causes: the first is a meiotic trisomy which results in chromosomal loss to revert to a disomic pair during the zygote formation (trisomic rescue). The second is a monosomy that undergoes monosomy rescue by duplicating the chromosome, and the third is by gamete complementation, the fusion of both gametes to produce two chromosomes of one gamete and none from the other. When both chromosomes are identical, it is defined as uniparental isodisomy, which occurs when the same chromosome is duplicated, indicating an initial meiosis II or very early mitotic mistake. On the other hand, when both homologous chromosomes are different, it is referred to as

uniparental heterodisomy; this occurs due to an error during meiosis I (Gardner *et al*., 2011).

1.6 Preimplantation Genetic Testing for Aneuploidies

The most reliable source of information for chromosome abnormalities in early embryos is preimplantation genetic testing (PGT). PGT is a medical procedure routinely used in human IVF, with its main application being to improve the success rates of IVF and reduce the risk of miscarriage, whilst also decreasing the possibility of transferring genetically defective embryos in an ART setting. Its main use is to increase the success of conceiving a karyotypically normal child, typically under circumstances where the parents have had many failed IVF treatments or risk passing genetic diseases to their offspring, or both.

PGT is usually coupled with IVP in livestock species (Parikh *et al.*, 2018), given that combining IVP and genomic selection could potentially lead to improved rates of genetic gain through selective transfer of embryos that are proven carriers of desirable traits (Ponsart *et al.,* 2014). There are four approaches of PGT, PGT for monogenic diseases (PGT-M), PGT for structural rearrangements (PGT-SR), PGT for polygenic disorders (PGT-P), and PGT for aneuploidy (PGT-A), the last one being perhaps the most controversial. The use of PGT-A has been interrogated for a variety of reasons, including its debatable value in improving live birth rates per treatment cycle and the unpredictability of transferring identified and chromosomally mosaic embryos (Griffin and Ogur, 2018).

PGT-A is a branch of PGT that encompasses all the techniques used to select euploid IVP embryos for transfer. Early versions of PGT-A used fluorescence *in situ* hybridisation (FISH, Figure 1.9). Initially, FISH was used to clinically sex embryos using probes specific to the X and Y chromosomes (Griffin *et al.,* 1993; Delhanty *et al.,* 1993). Subsequently, it was used to assess the copy numbers of the most prevalent trisomies linked to live birth abnormalities, including chromosomes X, Y, 13, 18, and 21 (Schrurs *et al.,* 1993; Munné *et al.,* 1993). The main problem is that FISH can only provide information for a few chromosomes at a time since it is limited by the number of fluorophores that can be used in a single experiment. Advances in technology and the development of protocols based on microarray techniques are able to bypass this limitation and test the ploidy status of all chromosomes simultaneously.

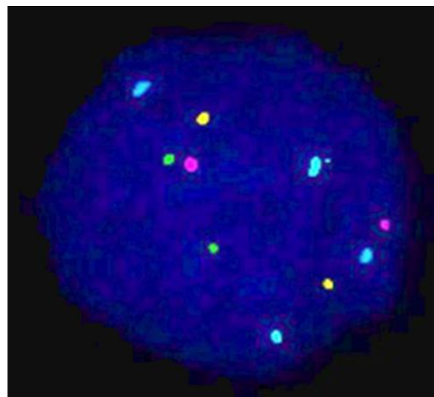

Figure 1.9: FISH using 5 different probes to assess the chromosomes 13, 16, 18, 21 and 22 on a human embryo blastomere (Image adapted from Griffin and Ogur, 2018).

1.6.1 Improved Methods for PGT-A

In human ART, there has been a transition towards aneuploidy screening of TE biopsies to produce at least one blastocyst that is karyotypically normal (Griffin and Ogur, 2018). Rapid advancements in diagnostic techniques, such as array comparative genomic hybridisation (aCGH) and next generation sequencing

(NGS) have helped to facilitate this transition. Both evaluate copy number abnormalities by comparing the relative signal intensities of the sample against a reference on a deoxyribonucleic acid (DNA) microarray chip. However, NGS is a quicker and more accurate method, having 100% specificity and sensitivity and allows the processing of several samples simultaneously (Kung *et al.,* 2015). Additionally, NGS has the ability to detect tiny copy number variants (CNVs), which can negatively impact embryo development and cause serious birth abnormalities (Fan *et al.,* 2015).

NGS and aCGH techniques allow for comprehensive chromosomal analysis, and the use of single nucleotide polymorphism (SNP) arrays has been utilised in human PGT to detect both chromosomal and monogenic disorders (Handyside *et al.,* 2010). SNPs are bi-allelic genome positions where both variations appear in a significant portion of the population, comprising a major part of these DNA variants. The two alleles of a SNP are often arbitrarily labelled as A and B. Therefore, since each individual usually inherits one copy of each SNP position from each progenitor, the individual's genotype at a SNP site is typically either AA, AB or BB.

The availability of a complete genome sequence for *Bos taurus* (Elsik *et al.,* 2009) allowed the discovery of thousands of SNPs associated with quantitative trait locus (QTL) which could be used as high-density markers for the production of genomic estimated breeding values (GEBVs) and for the selection of livestock based on genomic data, a process termed “genomic selection”. In the U.K., GEBVs are routinely established for cattle using data from the GCP 50K SNP array (Illumina, Cambridge, UK), making the SNP-array technique applied by Turner *et al.* an appropriate tool to adopt for PGT-A. This approach is appealing

as, from TE biopsies, it could provide breeders the opportunity to base their selections on the genetic merit (breeding value) of the embryo (Mullaart and Wells, 2018) and its likelihood to produce a viable pregnancy after ET following PGT-A. In an embryo, those SNPs whose parental origin is easy to identify, since one parents is homozygous while the other is heterozygous, are named “Informative” or “key” SNPs. This key SNP data can be applied to produce Karyomaps, Gabriel-Griffin Plots or calculate B Allele Frequency (BAF) graphs and the Log R Ratio (LRR), which are different techniques to identify aneuploidies in embryos.

1.6.1.1 Karyomapping

Karyomapping was originally created in 2010 and is based on genetic recombination and the inheritance of chromosomal haploblocks, which are segments of DNA that are inherited together (Handyside *et al.,* 2010). In order to map the origin of each chromosome inherited (and any crossovers between grandparental chromosomes), the mother, father, and another family member such a sibling (born or in embryo stage) are used as references to screen the embryos being evaluated. In human IVF, not only can Karyomapping be used to diagnose genetic disorders, but it can also deliver information on chromosomal imbalances (monosomy, uniparental disomy or trisomy). The plots provide a simple visualisation of chromosomes from both normal and abnormal cases, with the regions of chromosomal crossover clearly visible. The euploid chromosomes are characterised by the alternating blocks of red/blue (paternal) and yellow/green (maternal), representing alternating haploblocks (Figure 1.10A). Trisomy appears as frequent and short blocks of alternating

colours (Figure 1.10B), whilst monosomy appears as complete or almost complete lack of information for a full chromosome (Figure 1.10C).

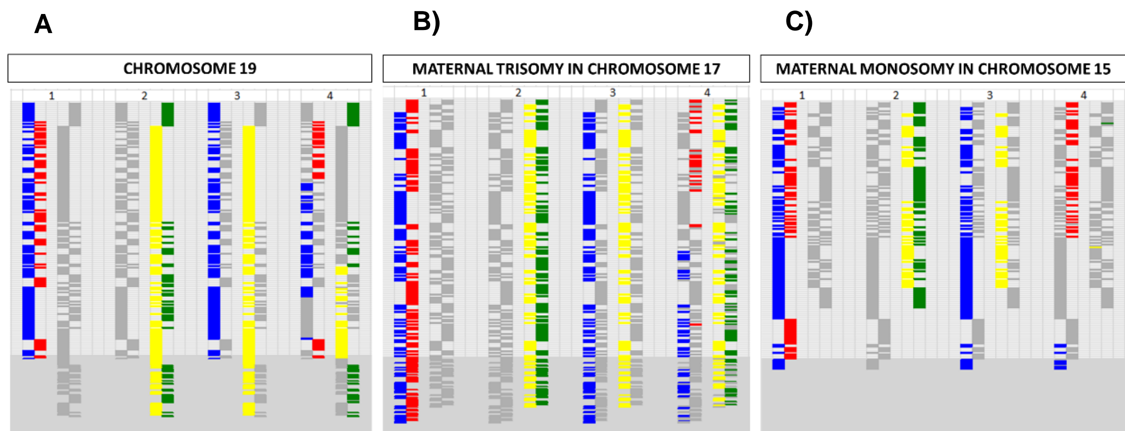

Figure 1.10: Chromosome ideograms in the Karyomaps of cattle embryos. Each chromosome is represented by two columns; 1 is the paternal sample, 2 is maternal sample, 3 is the reference sibling, and 4 is the embryo being tested. Blue blocks represent the paternal informative SNPs shared between the embryo and the reference, while red blocks are paternal SNPs which differ from the reference. Yellow blocks represent maternal informative SNPs shared between the embryo and the reference, while green blocks are maternal SNPs which differ from the reference. A) The tested embryo is euploid for Chromosome 19. B) Shows a trisomy of maternal origin in chromosome 17. C) Shows a monosomy of maternal origin in chromosome 15. Ideograms generated by M. Serrano Albal.

1.6.1.2 B Allele Frequency and Log R Ratio Graphs

BAF graphs and LRR can determine how much the tested embryo differs from normalised data. Once these are plotted, the information can be used to infer chromosome copy number (Esteki *et al.*, 2015). In euploid samples, all the points of a LRR graph will accumulate around the expected mean (which is set to zero, as seen in Figure 1.11A). When the sample is aneuploid, the deviation from the mean can be easily spotted, showing monosomies or partial deletions when it measures below zero (Figure 1.11B) or trisomies and duplications when above zero (Figure 1.11C).

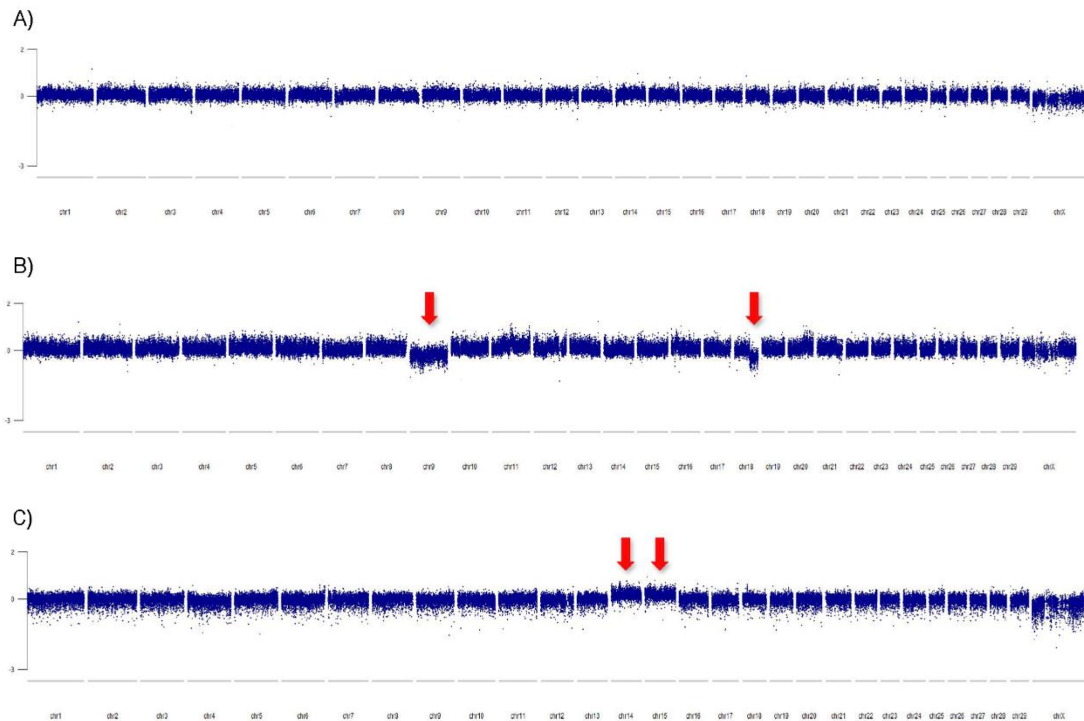

Figure 1.11: Log R Ratio Graphs. A) Euploid embryo. B) Monosomic embryo for chromosome 9, and a deletion in chromosome 18, shown by the decrease in the signal intensity and denoted with the arrows. C) Trisomy of chromosome 14 and 15, shown by an increase in the signal intensity on these chromosomes (indicated by the arrows). M. Serrano Albal.

On the other hand, BAF graphs compare the signal intensities of the A and B allele channels, so that euploid samples display three intensity clusters (Figure 1.12), one for each possible genotype (AA, AB, BB). Monosomies and deletions result in the total or partially loss of a cluster, respectively (Figure 1.12B). For trisomies, the graph shows a gain of a cluster (Figure 1.12C), resulting in a combination of three alleles (BBB, ABB, AAB, AAA).

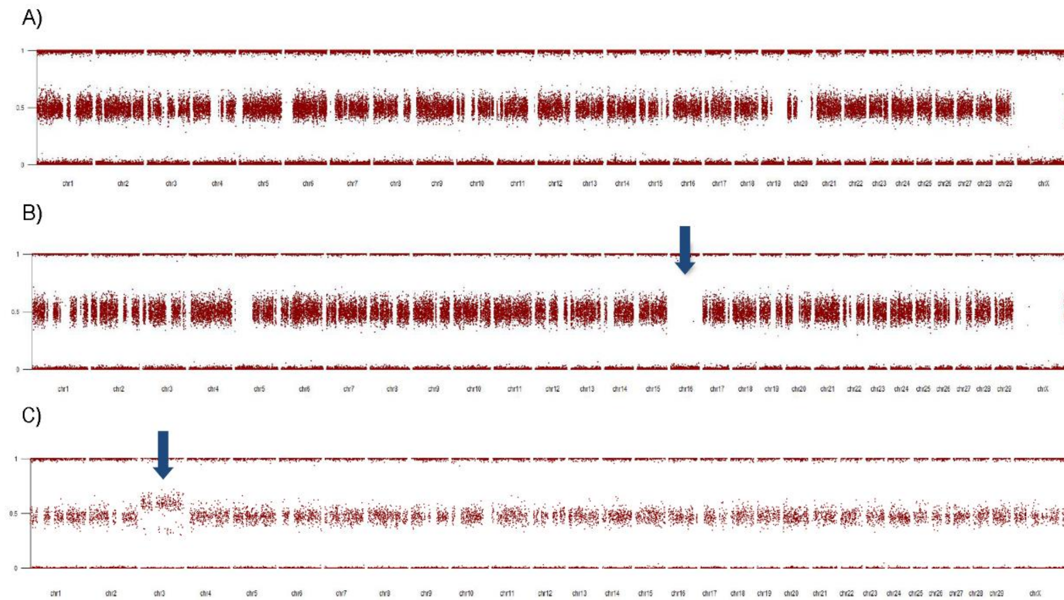

Figure 1.12: B Allele Frequency (BAF) Graphs. A) Euploid embryo, displaying 3 signal clusters according to each genotype (BB = 1, AB = 0.5, AA = 0). B) Monosomic embryo for chromosome 16(loss of the cluster noted with the arrow).C) Trisomy in chromosome 3, pointed with the arrow (allele combination ABB). M. Serrano Albal.

1.6.1.3 Gabriel-Griffin Plots

The algorithm created by Gabriel *et al.* (2011), referred to as Gabriel-Griffin (GG) plots, allows for the determination of the origin of trisomies. GG plots can identify errors at the stages of meiosis I, meiosis II, mitotic errors, and its parental origin, either during gamete maturation or embryo development. Although this method also uses informative SNP data, it does not require a reference in contrast to the Karyomapping requirements. Key SNPs are labelled as “type 1” (T1) if the SNPs have a paternal origin or “type 2” (T2) if its origin is maternal. Once the SNP data from the tested embryo is compared to their parental samples (T1 and T2), heterozygous SNPs (AB) will appear in red, while homozygous SNPs (AA or BB) are plotted in yellow (Gabriel *et al.*, 2011).

In the diagrams (an example shown in Figure 1.13), meiosis I errors are characterised by 100% heterozygous SNPs around the centromere

(Figure 1.13C), while meiosis II errors are characterised by ~50% homozygous SNPs around the centromere in red/yellow stripped pattern with the rest of chromosome with heterozygosis in red (Figure 1.13B). Mitotic errors are identified when the whole chromosome has a red/yellow stripped pattern (Figure 1.13A). If the error is seen on either T1 or T2, the mistake was inherited from either the paternal or maternal side, respectively (Gabriel *et al.*, 2011).

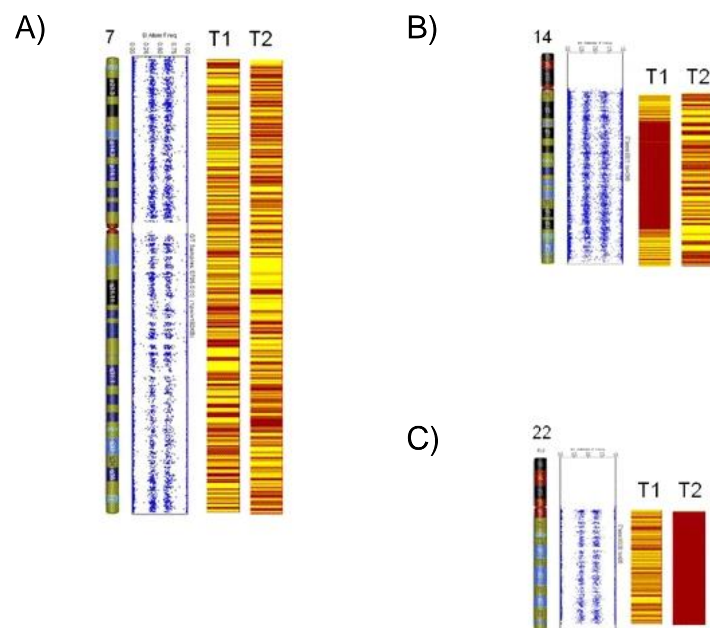

Figure 1.13: Ideograms of trisomic chromosomes with Gabriel-Griffin Plots (adapted from Gabriel *et al.*, 2011). The ideograms on the right are B Allele Frequency (BAF) graphs, created using Illumina genome viewer v1.6. to confirm the presence of trisomy. In the Gabriel-Griffin Plots (on the right) the red represents heterozygous genotypes while the yellow represents homozygous genotypes. A) Mitotic error. B) Meiosis II error of paternal origin with two cross overs. C) Meiosis I error of maternal origin with no crossovers.

1.7 Sperm Cryopreservation

The cryopreservation of gametes is crucial for the preservation of genetic resources, not only for the breeding industry but also to develop new strategies to ensure the survival of endangered animals at risk of extinction. Furthermore, is a useful tool to maintain the genetic diversity of the species.

Since the commercialisation of porcine blastocysts is not quite established yet, there are other areas that can enhance the efficiency of the porcine breeding industry, such as the sperm market. The sperm market involves the distribution and commercialisation of AI samples to facilitate the dissemination of high genetic resources worldwide. Transport of boar sperm, stored at 17°C in liquid form, is very popular in porcine AI practises. However, even though the samples are shipped within a few hours of collection, packages can take hours or even days to get to the breeder, affecting the sperm quality and hence its fertilisation capacity (reviewed in Paschoal *et al.*, 2021). The shipping of cryopreserved sperm, stored in liquid nitrogen vapours, increases the distance and time that the samples can be shipped without further detriment to the fertilisation capacity.

Cryopreservation is the artificial pausing of cell physiology through a freezing process, with the aim of preserving cell tissues for prolonged periods and conserving their functionality. Nevertheless, there are various factors affecting cell viability during the freeze-thawing technique which needs be considered (Baust *et al.*, 2017). The first publication about modern cryobiology was in 1949 where Polge *et al.* published the recovery of bull sperm frozen in glycerol. Four years later, the first human birth from frozen sperm was reported (Bunge *et al.,* 1954). In the porcine breeding industry, the first descendants from AI frozen-thawed (FT) sperm were reported in 1957 (Hess *et al.*, 1957). Despite the biotechnological applications which have been applied since then (Rath *et al.*, 2009), only ~1% of all inseminations conducted worldwide use FT semen (Knox *et al.*, 2015; Yeste, 2015).

In human IVF clinics, the cryopreservation of sperm is very reliable and well established using both fast and slow freezing techniques, being a key method

to preserve male or couple fertility by facilitating the sperm donation and increasing the efficiency of the sperm epidydimal and testicular extraction. The use of cryopreserved sperm would benefit the porcine meat production companies in terms of biosecurity, flexibility of use, and international trade, making distribution of this genetic resource possible between countries. Furthermore, the use of cryopreserved sperm in research allows for an increase in reproducibility as sperm from the same ejaculate can be used over a long period of time and throughout many experiments.

Nevertheless, these potential benefits have been somehow disregarded since the economic gain is not as prominent as with frozen bull sperm commercialisation. This is due to the lower cryotolerance of boar sperm compared to other species. Boar sperm membranes are more susceptible to thermal shock (Parks and Lynch, 1992). This could be explained by the different composition of phospholipids in the boar sperm membrane when compared to bull sperm. Boar sperm has a lower percentage of phosphatidylcholine and a higher percentage of phosphatidylethanolamine and sphingomyelin, which impacts membrane stability. Another factor is the low proportion of cholesterol and its asymmetrical distribution, resulting in greater proportions in the outer layer of the plasma membrane, disrupting the inner monolayer and lowering resilience to lower temperatures (Johnson *et al.,* 2000). In turn, this leads to a decrease in the functionality of the FT sperm, reducing their fertilising capacity, and in turn birth rates and litter size (Eriksson *et al*., 2002). The main damaging effects of slow freezing are alteration of plasma membrane (increasing its permeability), acrosome and DNA integrity (Thurston *et al*., 2003; Yeste *et al*., 2013; Gadani *et al*., 2017), decrease in total and progressive motility and other

kinetic parameters (Eriksson *et al.,* 2002), increase in the production of reactive oxygen species (ROS) (Juarez *et al*., 2011), and the reduction in mitochondrial activity (Hu *et al*., 2009) which also affects intracellular flow of Ca^{2+} (Kumaresan *et al*., 2014).

Despite all of the above and the fact that not all boars are “excellent sperm freezers”, improvements made in cryopreservation during the last 20 years, such as the development of new freezing and thawing extenders, have led to cryopreserved AI doses with good sperm quality that function properly when thawed (Yeste *et al*., 2015).

1.7.1 Sperm Cryopreservation Methods

Currently, there are two successful methods available for cryopreserving sperm: slow freezing and vitrification.

1.7.1.1 Slow Freezing

Slow freezing is the method that is better established for sperm cryopreservation. In slow freezing, sperm cells are diluted in a suitable freezing extender, which is exposed to a gradual decrease of temperature. This can be done manually or using a freezing device that can be adjusted to the species-specific sperm requirements (Medrano *et at*., 2009). The progressive cooling stimulates the production of ice crystals and increases the concentration of solutes in the extracellular medium, causing water to flow from the intracellular to the extracellular media. The range with the maximum level of ice crystal generation is between -5°C and -60°C (Yeste, 2016). When temperatures drop below -80°C, the sperm solution transforms from a viscous consistency into a metastable glassy stage and can then be stored in liquid nitrogen (LN_2) at a

temperature of -196°C. LN_2 has become the standard storage liquid in cryopreservation since it is not toxic, is chemically stable, affordable, and readily available.

1.7.1.2 Vitrification of Sperm Samples

Vitrification techniques have recently been applied to cryopreserve sperm in smaller volumes, simplifying and improving sperm cryopreservation (Isachencko *et al.*, 2008; Sánchez *et al.*, 2011; Díaz-Jiménez *et al.*, 2018). This is because vitrification is a super-fast process that reduces the mechanical damage of the samples. During vitrification, the direct plunging into LN_2 provides an ultra-fast cooling process where the intracellular and extracellular water acquires a vitreous state, preventing the formation of ice crystals (Isachenko *et al.*, 2003, Isachenko *et al.*, 2005; Tavukcuoglu *et al.*, 2012). Vitrification has been successfully performed in humans (Isachencko *et al.*, 2008), donkeys (Díaz-Jiménez *et al.*, 2018), ram (Jiménez-Rabadán *et al.*, 2015), rabbit (Rosato *et al.*, 2013), and fish, specifically in rainbow trout (*Oncorhynchus mykiss*; Merino *et al.*, 2011). Still, porcine sperm vitrification has not been successfully performed (Arraztoa *et al.*, 2017). Generally, vitrification extenders have a high content of penetrating and non-penetrating cryoprotectant agents (Fahy and Wowk, 2015).

1.7.2 Cryoprotectant Agents

In any type of cell, freezing causes damage by two distinct mechanisms. The first deleterious effect is caused by the chemical and osmotic effects of concentrated solutes in the residual unfrozen water, located between the ice crystals, producing an electrolytic imbalance and can lead to cell lysis during the thawing process (Benson *et al.*, 2012). The second is mechanical damage as the shape of cells is distorted by ice crystals formed inside and outside of the

cell (Woods *et al.*, 2004; Yeste, 2016). Consequently, the addition of chemicals to the extenders is necessary to reduce the freezing point of water (increasing the viscosity of intracellular and extracellular medium) and reduce cell dehydration and osmotic pressure. These compounds are called cryoprotectants, in which there are two types: penetrating and non-penetrating, and their concentration in the extender varies depending on the method used.

1.7.2.1 **Penetrating Cryoprotectant Agents**

Penetrating cryoprotectant agents are typically small molecules with low toxicity, including glycerol, dimethyl sulphoxide (DMSO), ethylene glycol, and propylene glycol, all of which are capable of penetrating the cell membranes and preventing the excessive dehydration of the cell (Yeste, 2016). These compounds successfully preserve sperm function after thaw, and for boar sperm cryopreservation, glycerol is one of the most commonly used penetrating cryoprotectants. Furthermore, the combination of glycerol with lactose, trehalose, and coconut water enhances sperm viability post-thaw (Zeng *et al.,* 2014).

1.7.2.2 **Non-penetrating Cryoprotectant Agents**

Non-penetrating cryoprotectant agents are high molecular weight compounds that act on an extracellular level since they do not penetrate the sperm plasma membrane. Despite being less toxic, their use is optional and is typically utilised to reduce the amount of penetrating cryoprotectants. This group includes proteins present in milk and egg yolks, disaccharides, polyethylene glycol, or polymers such as polyvinylpyrrolidone (PVP) and hydroxyethyl (Yeste, 2016). Once the temperature starts to decrease during the freezing process, these

molecules promote cell dehydration and form hydrogen bridges with the water, reducing ice crystal formation (Ávila-Portillo *et al.,* 2006).

Egg yolk is the cryoprotectant most widely used in the sperm freezing extenders of several species and its positive effects are linked to the amount of low-density lipoprotein (LDL) in the egg yolk (Bergeron and Manjunath, 2006). Moreover, the addition of detergent Orvus ES Paste (OEP) within extenders improves the interaction of the egg yolk proteins and boar sperm membrane; when egg yolk and OEP is combined with glycerol, it improves the seminal properties such as mitochondrial function, overall motility, plasma membrane integrity, and acrosomal integrity (Fraser *et al.,* 2014).

Disaccharides such as lactose are often preferred in freezing extenders, though trehalose has been shown to increase the viability and *in vitro* fertilisation of boar ejaculates (Malo *et al.*, 2010). The addition of trehalose or lactose to the extender reduces the final concentration of glycerol, enhancing motility and cell viability (Gutiérrez-Pérez *et al.*, 2009). In the vitrification of human sperm, the use of sucrose has resulted in high motile sperm post-vitrification (O'Neil *et al.*, 2019). Additionally, sucrose has been reported as a suitable cryoprotectant for boar sperm vitrification, resulting in a 21% increase of total motility post-thaw (Serrano Albal *et al.*, 2022 - unpublished data).

1.7.3 Thawing of Sperm Samples

Frozen boar sperm is typically thawed at 37°C for 20 seconds (Casas *et al.,* 2009; Didion *et al.*, 2013). However, there are other protocols that increase thawing temperatures and reduces the time of sperm exposure (for example 70°C for 8 seconds) that have shown an improvement in motility and other

kinetic parameters, such as velocity, when compared to 37°C for 20 seconds (Tomás *et al.*, 2014). Supplements in the thawing media can increase the viability of sperm, for instance seminal plasma, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA) and Ca^{2+}-chelating agents improves acrosomal integrity and fertilisation capacity, enhancing pregnancy rates in AI with frozen sperm (Gómez-Fernández *et al.*, 2012; Yeste *et al.*, 2015; Okazaki *et al.*, 2011).

1.8 Limitations in the Use of IVP in Livestock Animals

The main problem faced in animal IVP is the developmental failure of embryos from peri-pubertal pigs and heifers, due to deficiencies in oocyte maturation caused by the suboptimal developmental competence of the oocytes. These oocytes often have a reduced number and an unfavourable distribution of cytoplasmic organelles, both of which have deleterious effects on the cell metabolism and is related with an increased incidence of aneuploidy (Pawlak *et al.*, 2012; Leoni *et al.*, 2015; Reader *et al.*, 2015).

Furthermore, the IVP systems in place for mature animals, in both pigs and cattle, are suboptimal. The development of new chemically-defined IVM protocols that could mimic biochemical and cellular events occurring during *in vivo* maturation are necessary to eliminate the addition of non-defined fluids of animal origin to IVM media, such as serum or follicular fluid (Albuz *et al.*, 2010). However, limitations in IVP should not focus solely on oocytes; improvements are also required for the selection and freezing of sperm to ensure the IVP steps following fertilisation are successful, so establishing methods to enable this is key. All the above is reflected in poor fertilisation and subsequent development during IVP (May-Panloup *et al.*, 2005; Pawlak *et al.*, 2016).

With regards to cattle IVP, which is more successful than porcine IVP and has very well-established protocols, the focus should be on enhancing pregnancy outcomes following ET. The development of different approaches for the preimplantation evaluation of the embryos are required to improve aneuploidy screening of biopsied embryos.

1.9 Specific Aims of this Thesis

There are unexplored areas and unanswered questions within the field of livestock IVP due to various limitations, including unoptimised experimental procedures, human error, and technological limitations. With the above factors taken into consideration, the overarching hypothesis of this thesis was to demonstrate that the outcomes of porcine and cattle IVP can be improved through the use of new/improved methodology. Thus, the aim of this thesis was to address the gaps in our knowledge through the pursuit of the following specific aims:

Specific Aim 1: To test the hypothesis that the addition of cytokines within porcine *in vitro* maturation media enhances cytoplasmic maturation in prepubertal gilts oocytes and improves embryo quality

Specific Aim 2: To compare the effect of two different sperm selection methods by assessing basic boar sperm parameters and *in vitro* fertilisation outcomes

Specific Aim 3: To modify vitrification protocols for use on boar sperm and compare them against slow freezing, the standard cryopreservation method used in the boar industry

Specific Aim 4: To test the hypothesis that the use of PGT-A to screen *in vitro* produced bovine embryos using key SNP chip data from GEBVs improves pregnancy and live birth rates

Specific Aim 5: To test the hypothesis that PGT-A is a suitable method to identify and analyse chromosomal abnormalities within the inner-cell mass and trophectoderm of *in vivo* and *in vitro* produced bovine embryos

2 Materials and Methods

All chemicals and reagents used were acquired from Sigma-Aldrich (Gillingham, United Kingdom) unless otherwise detailed. The description of the stock solutions used along the experiments that are not described below are described in supplementary tables S1-8.

2.1 Ethical Approval

All pig embryos produced at the University of Kent during the course of this project were from abattoir-derived material and destroyed by day 6 post-IVF. Due to this, this part of project did not fall within the Animals (Scientific Procedures) Act 1986 and did not require review by an Animal Welfare and Ethical Review Body of the University of Kent.

The production and handling of bovine embryos did not occur at the University of Kent. As it did not require a bespoke intervention on live animals, ethical approval was not necessary. However, all protocols used by the University of Nottingham adhered to the Animals (Scientific Procedures) Act, 1986 and complied with the ARRIVE guidelines. These were approved by the University of Nottingham Animal Welfare and Ethical Review Body (AWERB).

2.2 Porcine Oocyte Collection and IVM

Prepubertal gilt ovaries were collected from either Cranswick Country Foods (Hull, UK) or C&K Meats Limited (Suffolk, UK) and transported to the laboratory within 5 hours in a sealed bag submerged in water at 30-35°C. At slaughter, the animals weighed approximately 160 kg and their prepubertal status was confirmed by the absence of developed ovarian corpora lutea. Before aspiration, the ovaries were washed 2-3 times in 1x phosphate buffered saline (PBS) and

kept at 28°C in a water bath. The retrieval of COCs was performed by manual aspiration from non-atretic follicles (3-6 mm) using a non-pyrogenic/non-toxic syringe (Henke-Sass Wolf GmbH, Tuttlingen, Germany) fitted with an 18-gauge needle. The follicular fluids were washed three times in a modified HEPES-buffered Porcine X Medium (PXM (Yoshioka *et al.*, 2008); 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.40 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 25 mM HEPES, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 4 mg/mL bovine serum albumin (BSA)); warmed at 38°C.

The chosen COCs were washed 3 times in Porcine Oocyte Medium (POM (Yoshioka *et al.*, 2008); 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.0 mM glucose, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 2.0 mM glutamine, 5.0 mM hypotaurine, 0.1 mM cysteine, 20 µL/mL BME amino acids 50x,10 µL/mL MEM non-essential amino acids 100x,10 ng/mL EGF, 50 µM β-mercaptoethanol, 10 µg/mL gentamycin, 4 mg/mL BSA) previously equilibrated overnight at 38.5°C and 5.5% CO_2 in humidified air. COCs were assigned to groups of 50. During the first 20 h of culture in POM, COCs were supplemented with FSH (0.5 IU/mL), LH (0.5 IU/mL) and db-cAMP (0.1 mM). In the subsequent 24 h, oocytes were cultured in POM in absence of hormones and db-cAMP, at 38.5°C and 5.5% CO_2 in humidified air.

2.3 Evaluation of Nuclear Stage and Cortical Granule Distribution in Pig Oocytes After IVM

The nuclear stage evaluation and cortical granule distribution was assessed using a modified protocol based on the protocol published in Zhang *et al.* (2010). In total, 763 denuded oocytes were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at 4°C, and then washed three times in PBS containing 0.3%

BSA and 100 mM glycine for 5 minutes each time. After a 5-minute wash with 0.1% Triton X-100 in PBS, oocytes were washed two additional times in standard PBS (5 minutes each). After that, oocytes were cultured in 100 mg/mL fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Invitrogen™, Inchinnan, United Kingdom) in PBS for 30 mins in a dark box to stain the cortical granules (CGs). Next, oocytes were washed three times in PBS with 0.3% BSA and 0.01% Triton X-100 in PBS.

To assess the nuclear status of the oocytes, they were then stained with 10 mg/mL of Hoechst H3570 (Invitrogen™) for 10 mins, mounted on non-fluorescent glass slides and visualised using an Olympus BX61 epifluorescence microscope equipped with a cooled CCD camera. Images were taken using DAPI and FITC filters at x200 total magnification using the software SmartCapture3 (Digital Scientific UK, Cambridge, United Kingdom). By implementing a set exposure time of 2 seconds in SmartCapture 3, CG distribution was quantified visually based on an increase or decrease in fluorescence.

COCs were graded as mature when they displayed a dye-stained metaphase plate and a polar body; otherwise, they were classified as immature. The distribution of CGs was classified in three ways as: central, peripheral or complete where: central distribution indicated a homogenous distribution of CGs throughout the cytoplasm; peripheral distribution, indicated that CGs had begun to localise subjacent to the oolemma, or complete distribution, where CGs were concentrated subjacent to the oolemma and around the polar body (an indicator of complete cytoplasmic maturation).

2.4 Boar Sperm Preparation and Capacitation

Extended boar semen (for commercial AI) was supplied by JSR Genetics Ltd. (Southburn, United Kingdom). The AI samples were shipped at room temperature (RT) in the post, then preserved at 17°C for up to two days before use.

2.4.1 Sperm Density Gradient Selection

Sperm preparation, using 17 boar semen samples, was accomplished using a 35%/70% (v/v) discontinuous density gradient system using BoviPure solutions (Nidacon, Göthenborg, Sweden) following the manufacturer's instructions. To avoid mixing the solutions, 1 mL 70% BoviPure solution was carefully layered on top of 1 mL 35% BoviPure in a 15 mL conic centrifuge tube, with 1 mL extended boar sperm carefully pipetted on top of the BoviPure solutions. The samples were then centrifuged for 15 mins at 400 G, the supernatant discarded with care to not disturb the sperm pellet, and 1 mL BoviWash (Nidacon, Göthenborg, Sweden) added prior centrifugation at 400 G for 5 mins. The supernatant was then removed, and the pellet resuspended in 2 mL of the Porcine Gamete Medium (PGM (Yoshioka *et al.*, 2008)).

2.5 Evaluation of Boar Sperm

10 extended boar semen samples were divided into four equal aliquots for each treatment group. The following tests for semen assessment were performed on the samples both before and after vitrification: total and progressive motility, sperm morphology and sample concentration, sperm viability and acrosome integrity, mitochondrial membrane potential, and sperm chromatin structure assay to measure DNA fragmentation and immature spermatozoa.

2.5.1 Total Motility

Total motility was evaluated using iSperm Swine Semen Analysis System (GenePro Inc., Fitchburg, Wisconsin) following the manufacturer's instructions. The assessed parameters, with an example presented in Figure 2.1, were the following: total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, expressed as percentage) and straightness (STR, ratio of VSL/VAP, expressed as percentage).

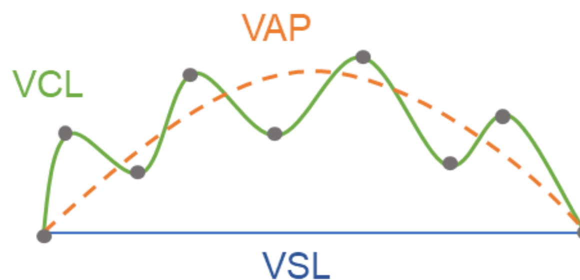

Figure 2.1: Motion Parameters obtain from computer-assisted sperm analysis (CASA) assays. Curvilinear velocity (VCL, in green), straight-line velocity (VSL, in blue), and the average path velocity (VAP, in orange). The grey dots show the position of the sperm each time its direction changes.

Samples with a low concentration that were not able to be analysed by our using iSperm system were evaluated using a warm stage and phase-contrast microscopy at x200 total magnification. Spermatozoa that showed stationary flagellation, curved motion, and twitching were considered as motile but non-progressive, while those that swam in a straight line were classified as motile and progressive.

2.5.2 Sperm Morphology

Sperm samples were fixed in 4% PFA for 30 mins at 4°C. Samples were then visualised using phase-contrast microscopy on an Olympus BX61 microscope equipped with a cooled CCD camera at x200 total magnification, and using SmartCapture3 imaging software (Digital Scientific UK, Cambridge, United Kingdom). The sperm was classified as normal (Figure 2.2A), with tail abnormalities (Figure 2.2B), with head abnormalities (Figure 2.2C) or with cytoplasmic drops (Figure 2.2D).

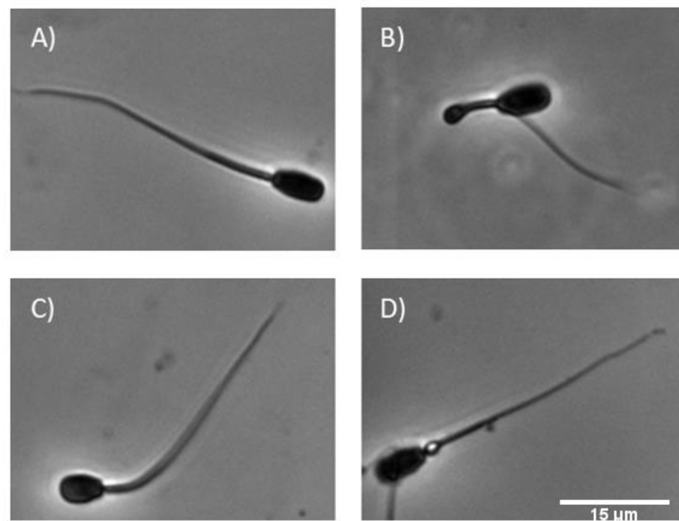

Figure 2.2: Boar sperm different morphologies (M. Serrano Albal). A) Sperm cell with normal morphology. B) Sperm with abnormal tail. C) Sperm cell with abnormal head. D) Sperm with a cytoplasmic drop at the base of the tail.

2.5.3 Sperm Concentration

The sperm count of each sample was performed on a Makler chamber via phase-contrast microscopy at x200 total magnification, using an Olympus BX61 epifluorescence microscope, equipped with a cooled CCD camera. Data is presented as million/mL.

2.6 Porcine *in vitro* Fertilisation and Embryo Culture

After IVM, matured oocytes were washed twice in PGM (Yoshioka *et al*., 2008]; 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.0 mM glucose, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 2.5 mM theophylline, 1 µM adenosine, 0.25 µM L-cysteine, 10 µg/mL gentamycin, 4 mg/mL BSA). The oocytes were then incubated with the prepared sperm for 2 hours. To minimise the risk of polyspermy, oocytes were moved to a clean well with PGM for another 2 hours (Figure 2.3).

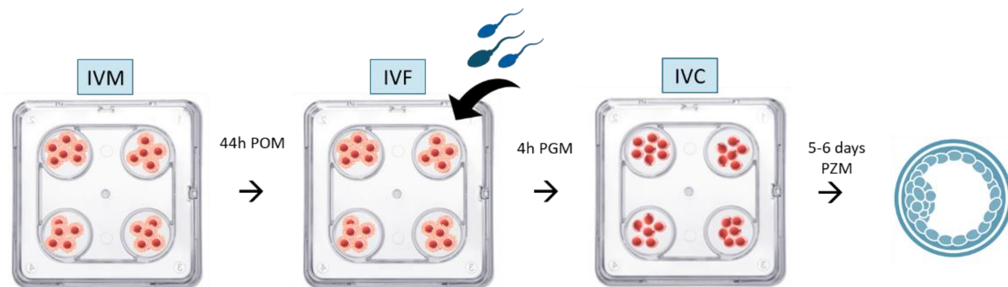

Figure 2.3: Schematic boar in vitro embryo production protocol. IVM) Group of COCs with at least two layers of cumulus cells incubate in maturation medium for 44h to reach metaphase II nuclear stage. IVF) Matured oocytes are in contact with sperm for 4h to be fertilised. IVC) Presumptive zygotes are denuded and cultured for 5-6 days to reach the blastocyst stage.

Hereafter, presumptive zygotes were denuded by pipetting up and down for 30 s in the well, and then washed twice in Porcine Zygote Medium 5 (PZM5 [Yoshioka *et al*., 2008]; 108 mM NaCl, 1 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 2.0 mM glutamine, 5.0 mM hypotaurine, 20 µL/mL BME amino acids 50x,10 µL/mL MEM non-essential amino acids 100x, 10 µg/mL gentamycin, 4 mg/mL BSA). The culture was performed in 500 µL wells of PZM5 overlaid with mineral oil and plates were incubated for 5-6 days at 38.5°C, in 5.5% CO_2, and 6% O_2 in humidified air.

2.7 Evaluation of Pig Blastocysts

The morphological appearance of blastocysts was assessed and scored using three grades (Figure 2.4): (1) excellent: fully expanded blastocyst, spherical, regular border, symmetrical with uniform size cells, obvious ICM and densely populated TE: (2) good, expanded blastocyst with few small blastomeres, fewer cells forming the ICM/TE; (3) poor: expanded or less developed blastocyst with numerous extruded blastomeres, loosely populated TE and possibly ICM. After grading, blastocysts from each treatment group were fixed in 4% PFA for 30 min at 4°C and stained with Hoechst H3570 (Invitrogen™) for cell counts. Blastocysts were visualised under an Olympus BX61 epifluorescence microscope equipped with a cooled CCD camera. Images were taken using DAPI at x200 total magnification using the software SmartCapture3 (Digital Scientific UK, Cambridge, United Kingdom).

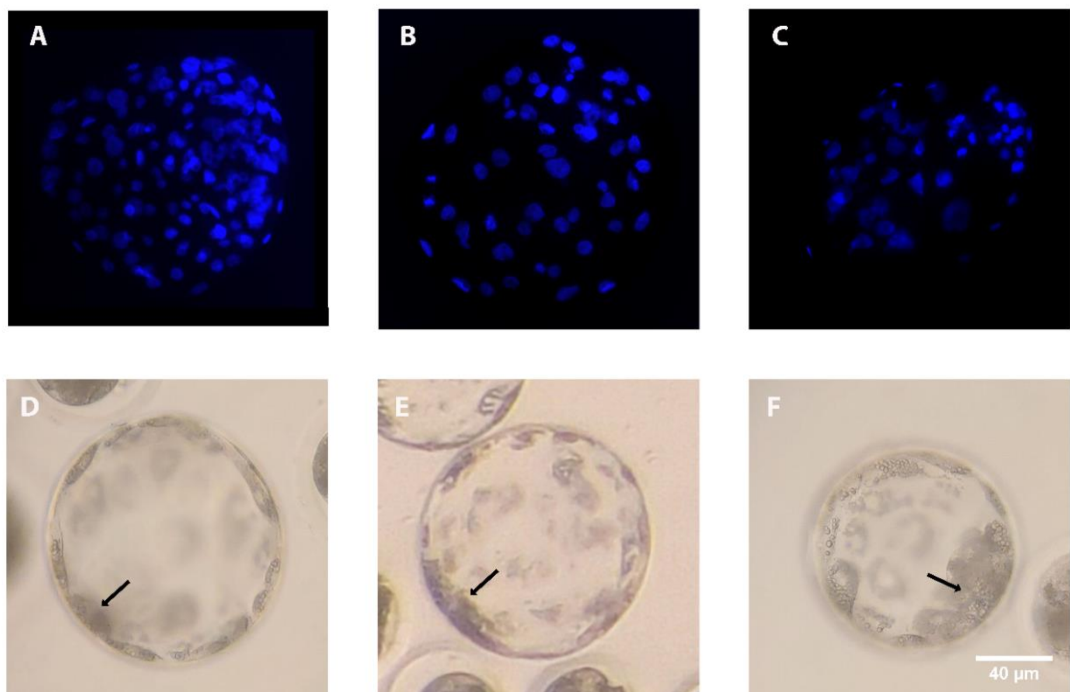

Figure 2.4: Pig blastocyst morphology. A, D) blastocysts of excellent morphology (grade 1). B, E) blastocysts of good morphology (grade 2). C, F) blastocysts of poor morphology (grade 3). Images A-C are taken using epifluorescence microscopy, with nuclei in blue (Hoechst). Images D-F are taken using phase-contrast microscopy. All images have a total magnification of x200. Arrows denote the inner cell mass of the blastocysts.

2.8 Production of Bovine Embryos

Bovine embryos were produced and provided by L'Alliance Boviteq Inc. (Québec, Canada) and the University of Nottingham (Nottingham, UK) . Further information on the methodology can be found in Sections 6.3 and 7.3, respectively.

2.9 PGT-A in Bovine Embryos

Whole genome amplified (WGA) DNA from the bovine embryos and parents were genotyped using different SNP arrays, which will be described further in section 7.3.5. The SNPs were then transferred to the University of Kent for blinded PGT-A analysis. Basic data manipulation on the database (cases extraction, file preparation for downstream analysis, filtering for shared SNPs) were performed using custom AWK and BASH scripts and resulted in the creation of output files for PGT-A analysis.

To perform a full chromosomal analysis, characterising the type of aneuploidy (mitotic/meiotic) and the origin of mutation (maternal/paternal), the SNP database was filtered in order to use only informative SNPs (Table 2.1). The tests used to assess the ploidy of the samples were Karyomapping, GG-plots, LRR graphs, and BAF graphs, all of which are further described below.

Table 2.1: Possible allele combinations that result in informative and non-informative SNPs. In red Informative SNPs (single nucleotide polymorphism)

Informative Parent	Sire	Dam	Embryo
Sire Informative SNPs	AB	AA	AB
			BB
			AA
	AB	BB	AB
			AA
			BB
Dam Informative SNPs	AA	AB	AB
			BB
			AA
	BB	AB	AB
			AA
			BB

2.9.1 Karyomapping

Karyomapping (as previously mentioned in 1.4.1.1) was used as a tool to detect many forms of aneuploidy and to identify their parental origin. The extraction of the SNP sample information and manipulation of the database was performed on MobaXterm (version 11.1, build 3860, Mobatek). Individual samples were clustered by parental origin, and sibling embryos were analysed together in the same output file. In each set, a randomly chosen embryo was selected to serve as a reference individual to permit Karyomapping haploblock tracing, as previously described (Turner *et al.*, 2019). After the analysis, the reference was swapped to a different sample to allow for the analysis of all samples. The analysis was completed via an upgraded version of the Microsoft Excel macro BoVision (version 3.1, University of Kent), previously described in Turner *et al.* (2019), which allowed for the simultaneous processing of multiple files. The

origin of chromosome errors was determined, with the exception of the Y and paternal X chromosome as sires only present a single copy of these chromosomes and cannot be detected by Karyomapping.

2.9.2 Analysis of LRR and BAF Graphs

LRR and BAF graphs (section 1.4.1.2) were used to detect the presence or absence of chromosome imbalances and to validate results obtained using Karyomapping. These graphs are able to discriminate between the loss of heterozygosity and monosomy and can detect some trisomies of mitotic origin that were not identify by Karyomapping. The analysis pertaining to LRR and BAF graphs were performed using R (R Core Team, 2014) and figures were produced using the package karyoploteR (Gel *et al.*, 2017). Euploid samples, as determined by Karyomapping, were used to calculate Theta (ThetaAA, ThetaAB, ThetaBB) and standard R (RAA, RAB, RBB) values for the combination of each SNP (defined in Sun *et al.*, 2009) from signal intensity data of X and Y available in SNP database. The data points plotted in the graphs were calculated using the formulas described in Sun *et al.* (2009), only when GC > 0.60 (Figure 2.5).

A) $\mathrm{R} = X + Y$ $\mathrm{LRR} = \log 2\left(\frac{R}{R\ of\ each\ genotype}\right)$

B) $\theta = \frac{\arctan(Y, X)}{(\pi/2)}$ $\mathrm{BAF} = \begin{cases} 0, if\ \theta < \theta_{AA} \\ 0{,}5\left(\frac{(\theta - \theta_{AA})}{(\theta_{AB} - \theta_{AA})}\right), if\ \theta_{AA} \leq \theta < \theta_{AB} \\ 0{,}5 + 0{,}5\left(\frac{(\theta - \theta_{AB})}{(\theta_{BB} < \theta_{AB})}\right), if\ \theta_{AB} \leq \theta < \theta_{AB} \\ 1, if\ \theta \geq \theta_{BB} \end{cases}$

Figure 2.5: Log R ratio (LRR) and B allele frequency (BAF) equations. (A) Formulas used to calculate LRR. (B) Equations to obtained BAF data, as referred on Sun *et al.*, 2009.

2.9.3 GG Plots

GG plots were used to identify the origin of the trisomies detected by Karyomapping or LRR/BAF graphs. GG plots identified if the aneuploidy originated during meiosis I, meiosis II or mitosis (Table 2.2) by processing the informative SNP data via the VBA macro BoVisionGG (version 1.0, University of Kent). BoVisionGG was also employed to automatically obtain the plots using the algorithm described in Gabriel *et al.* (2011).

Table 2.2: Chromosomal errors that can be detected by the different PGT-A test used. In this study. These tests are Karyomapping, GG and BAF/LRR ratio plots.

Aneuploidy class	Detected by Karyomapping	Detected by GG Plots	Detected by BAF and LRR	Detected by FISH	Detected by NGS
Trisomy:					
Meiotic	Yes	Yes	Yes‡	Yes‡	Yes‡
Mitotic	No	No	Yes‡	Yes‡	Yes‡
Monosomy:					
Meiotic	Yes	Yes	Yes‡	Yes‡	Yes‡
Mitotic	Yes*	Yes*	Yes‡	Yes‡	Yes‡
Segmental error (Loss or gain (*de novo*))	No	No	Yes	No	Yes
UPD	Yes	Yes	No	No	No
Haploidy	Yes	Yes	Yes	Yes	Yes
Triploidy	Yes	Yes	Yes	Yes	Yes
Hypotriploidy	Yes	Yes	Yes	No	Yes
Mosaicism	No	No	Yes†	Yes	Yes

*This only works if the monosomy is widespread within the cell population. A mitotic error will most likely cause mosaicism, but a mosaic monosomy will be masked in Karyomapping by the presence of other cells with a correct diploid number. The threshold (i.e., percentage of euploid cells required to mask a mosaic mitotic monosomy) is not known.
‡It will detect both but will not distinguish between them
†Rough ratios only

2.10 Statistical Analyses

Each data set was analysed on SPSS (Version 25 and 26, IBM) using the appropriate statistical analysis test. The data is presented as arithmetic mean ± standard error of the mean (SEM) or showing the 95% of Confidence Interval (CI). Results were considered to be statistically significant when $p < 0.05$. 95% CI were obtained using VassarStats (Lowry R., version 2021, http://vassarstats.net).

3 Supplementing Porcine *in vitro* Maturation Media with Cytokines to Enhance Cytoplasmic Maturation and Improve Embryo Quality

3.1 Background

In order to maximise the benefits of porcine IVP within the pig breeding industry, the protocols in place for the maturation of porcine oocytes need to be modified. Previous publications have hypothesised that incomplete nuclear and cytoplasmic maturation of the oocytes results in the poor developmental potential of the subsequent embryos. Improving cultures conditions for prepubertal porcine oocytes has been widely researched in recent years, with the goal to optimise the completion of cytoplasmic and nuclear maturation (Teplitz *et al.*, 2020; Uhm *et al.*, 2010; Appeltant *et al.*, 2016; Yuan, *et al.*, 2017).

Several intrinsic and extrinsic factors are known to affect the developmental potential of porcine oocytes and embryos (see section 1.4), typically through compromising the efficiency of IVP systems (Hunter, 2000; Weaver *et al.*, 2013; Teplitz *et al.*, 2020). One key extrinsic factor is the source of the oocytes which can be collected from either prepubertal gilts or adult donors, with oocytes derived from sows displaying enhanced developmental potential compared to oocytes derived from gilts (Marchal *et al.*, 2001). However, from a commercial standpoint, it is highly attractive to use ovaries from prepubertal gilts as this not only reduces generation times, but it also allows for the quick introduction of new genetics (Sherrer *et al.*, 2004). Despite these advantages, this approach is heavily defined by the ability in which the oocytes can successfully undergo cytoplasmic and nuclear maturation during IVM (Marchal *et al.*, 2001; Pawlak *et al.*, 2012).

To address the poor developmental competence of porcine oocytes, culture media for porcine oocytes has previously been supplemented with growth factors such as cytokines, being tested in combination or alone, in an attempt to design a defined IVM culture medium. Shown to aid meiotic progression during maturation, LIF and FGF2 promote oocyte quality and the ability to achieve fertilisation. LIF is known to phosphorylate signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein kinase (MAPK) 3/1 in oocytes, which is a particularly important pathway during IVM (Dang-Nguyen *et al*., 2014; Mo *et al*., 2014). Acting as a cofactor, FGF2 promotes high extracellular matrix quality and cumulus cell survival during IVM (Barros *et al*., 2019; Du *et al*., 2021). IGF1 is also found to improve increased cell survival in response to stress and IVM outcomes (Oberlender *et al*., 2013). In response to FSH, IGF1 aids in the expansion of porcine cumulus cells, enhancing the synthesis and retention of hyaluronic acid in porcine COCs, a process which is also activated by the MAPK3/1 pathway (Singh *et al*., 1997; Němcová *et al*., 2007). During IVM, the use of FGF2, LIF, and IGF1 (together known as “FLI”), improves IVF outcomes by improving the quality of oocytes, embryonic development and transfer outcomes, and embryo cryosurvival in cattle (Yuan, *et al*., 2017; Stoecklein *et al*., 2021).

Another commonly used supplement in porcine IVM media is sFF, as follicular fluid has been shown to play a key role throughout oocyte maturation in sows (Tatemoto *et al*., 2004; Pawlak *et al*., 2018). sFF provides the culture environment with several important growth factors that supports the *in vitro* development of oocytes (Lédée *et al*., 2008). These growth factors are also produced in the late stages of follicular development by ovarian somatic cells,

regulating oocyte maturation and developmental competence by acting as autocrine and paracrine mediators of ovarian function (Song *et al*., 2011). However, as the complete constitution of sFF is currently unknown, its use in IVM medium produces a non-defined culture system which could be considered as a potential biosecurity concern. Defining the complete constitution of sFF has proven to be difficult due to batch variation, with the composition known to change with the stage of the follicles and the age of the donor from which it is recovered (Sun *et al*., 2011). Varying concentrations of any sFF component has an impact on IVM outcomes and consequently the developmental competence of oocytes (Pawlak *et al*., 2018; De Oliveira *et al*., 2006).

In determining whether the use of cytokine supplements enhances porcine oocyte development, the localisation patterns of CGs throughout the cytoplasmic maturation process (as mentioned in section 1.2.1.2) is used to assess the degree of maturation. Present only in female germ cells and distributed randomly throughout the cytoplasm of immature oocytes, CGs are specialised secretory vesicles that migrate during meiotic maturation towards the cortical cytoplasm. Upon fertilisation, the hardening of the zona pellucida is achieved through the release of the CG contents via exocytosis (Burkart *et al*., 2012). Additionally, further research has demonstrated that the role of CGs is not limited to fertilisation processes, but also helps to regulate embryonic cleavage and preimplantation development (Liu, 2011; Kulus *et al*., 2020).

3.2 Specific Aim 1

The specific aim of this chapter was to test the hypothesis that the addition of cytokines within porcine IVM media enhances cytoplasmic maturation in prepubertal gilts oocytes, improving the developmental competence of the

oocytes and thus improving embryo quality. This was tested through the following aims:

- Specific aim 1A: To enhance oocyte nuclear maturation through the supplementation of IVM medium with FLI or sFF
- Specific aim 1B: To observe differences in CG distribution and thus cytoplasmic maturation through the supplementation of IVM medium with FLI or sFF
- Specific aim 1C: To compare differences between sFF and FLI on post-fertilisation outcomes

3.3 Materials and Methods Specific to this Chapter

In this chapter, 4 different POM supplementation were tested for IVM: 1) Control, POM was left unsupplemented; 2) FLI, media was supplemented with a combination of FGF2, LIF, and IGF1; 3) sFF, POM was supplemented with sFF; 4) sFF+FLI, media was supplemented with both FLI and sFF as can be seen in Figure 3.1.

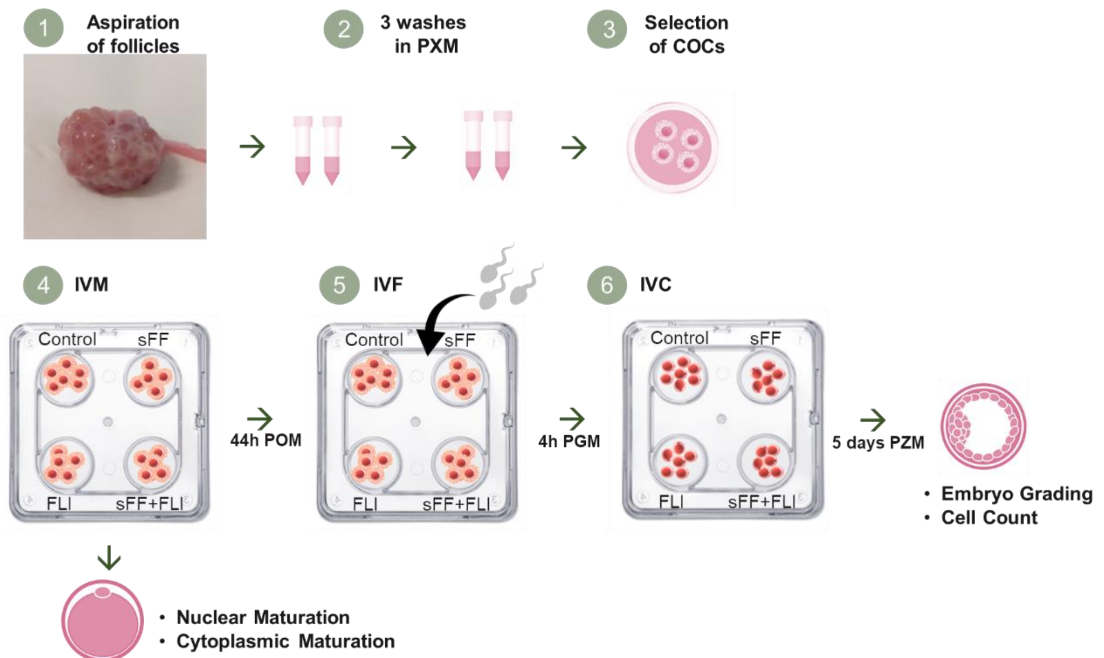

Figure 3.1: Schematic of the experimental flow conducted for testing the effect of FLI in IVM supplementation. 1) Follicles between 3-6 mm in size were aspirated and deposited in 15 mL falcon tubes. 2) The follicular fluid was left to precipitate and was washed three times with PXM. 3) Selection of COCs. 4) Distribution of the COCs in the four test groups, and *in vitro* matured in POM for 44 h. 5) IVF starts with the coincubation of oocytes with the sperm sample for 4 h in PGM. 6) IVC is initiated, and the presumptive zygotes are cultured for 5 days in PZM. FLI= combination of FGF2, LIF, and IGF1; sFF= sow follicular fluid.

In total, 1,896 oocytes were matured *in vitro* as described in section 2.2. From those, 763 (distributed among the 4 groups) were used assessed nuclear and cytoplasmic maturation. The remaining 1,133 mature oocytes, also distributed among the groups, continued the IVP process until IVC to evaluate the quality of the resulting blastocysts by their morphological appearance and the number of cells that each of them contained.

3.3.1 Protocol for Porcine sFF Collection and Filtration

The sFF was obtained from ovaries from slaughtered sows. After their collection, ovaries were transported in a thermos flask to the lab at 30-35°C. Follicles between 3-8 mm in size were aspirated with an 18-gauge needle attached to a 10 mL syringe. The aspirate was deposited in 15 mL falcon tubes and

centrifuged at 1500 G for 30 min. After that, the supernatant was filtered sequentially through 0.8, 0.45, and 0.2 μm corning filters. The sFF was then aliquoted and stored at -20 °C.

3.3.2 Supplementation of Culture Medium During IVM

In order to observe the effect of FLI supplementation on the meiotic competence of COCs throughout the IVM culture process, four groups of ~50 immature COCs were cultured using different cytokines. The IVM medium for the first group was supplemented with "FLI" as described by Yuan *et al.* (2017): 40ng/mL FGF2, 20ng/mL LIF, and 20ng/mL IGF. The second supplemented with 10% sFF, the third supplemented with both FLI and 10% sFF (to study a possible synergistic effect), and the fourth was not supplemented to serve as control. Each treatment group was repeated four times with a total of 40-50 oocytes per group per replicate (N= 763).

3.3.3 Assessment of CG Distribution

As noted in section 1.4.1.2, the level of cytoplasmic maturation in COCs can be assessed through differences in CG distribution. The treatment groups were as described in section 3.3.2. and each experiment was repeated three times, with 40-50 oocytes per group per replicate (N= 465). The most favourable outcome defined for this experiment is for the majority of oocytes to demonstrate "complete" CG localisation (*i.e.*, neither predominantly central nor peripheral).

3.3.4 Assessing Developmental Competence During IVF

The use of different supplements during the IVM process has been shown to affect the developmental competence of an oocyte following IVF. The treatment groups were as described in section 3.3.2 and each experiment was repeated

seven times, with 40-50 presumptive zygotes per group per replicate (N= 1133). In total, 149 blastocytes were assessed in order to evaluate the embryo quality of each treatment group.

This was evaluated using blastulation rate (blastocyst formation per inseminated oocyte), blastocyst cell counts, and blastocyst morphology. Total cell counts were estimated by staining nuclei with Hoechst H3570. By sequentially dividing the live fluorescence image of the embryo into smaller sections, the accuracy of the cell count was improved by bringing each section into focus.

3.3.5 Statistical Analysis

The data obtained from this chapter were analysed using SPSS (Version 26, IBM), and a binomial generalised linear model (GLM) with logit link functions was fitted to the statistical analysis of oocyte nuclear maturation and blastulation rates. Additionally, the number of blastomeres per blastocyst were log transformed and assessed using analysis of variance (ANOVA) tests. The Krustal-Wallis test was used to analyse the cytoplasmic maturation and morphology evaluation of the embryos; in the event that an interaction was detected amongst the variables, a Bonferroni correction was applied.

Data presented within tables in section 3.4 are presented as the mean ± SEM, whereas data presented within the graphs appear as the mean with error bars representing the 95% confidence interval of each variable. Results were considered to be statistically significant when $p < 0.05$.

3.4 Results

3.4.1 Specific Aim 1A: To Enhance Oocyte Nuclear Maturation Through the Supplementation of IVM Medium with FLI or SFF

In assessing the nuclear stage of 763 oocytes (Figure 3.2), the supplementation of IVM medium with FLI yielded the highest proportion of mature oocytes (N= 114/183; 70.9%). Oocytes treated with sFF yielded a result slightly lower than the control (no supplementation), with 61.5% (N= 115/187) and 62.3% (N= 114/183) respectively. The oocytes with the lowest nuclear maturation rate were those treated with a combination of sFF and FLI (N= 117/194; 60.3%). However, these differences were not considered to be statistically significant due to the intergroup variation (GLM; χ_1= 1.059; p >0.05).

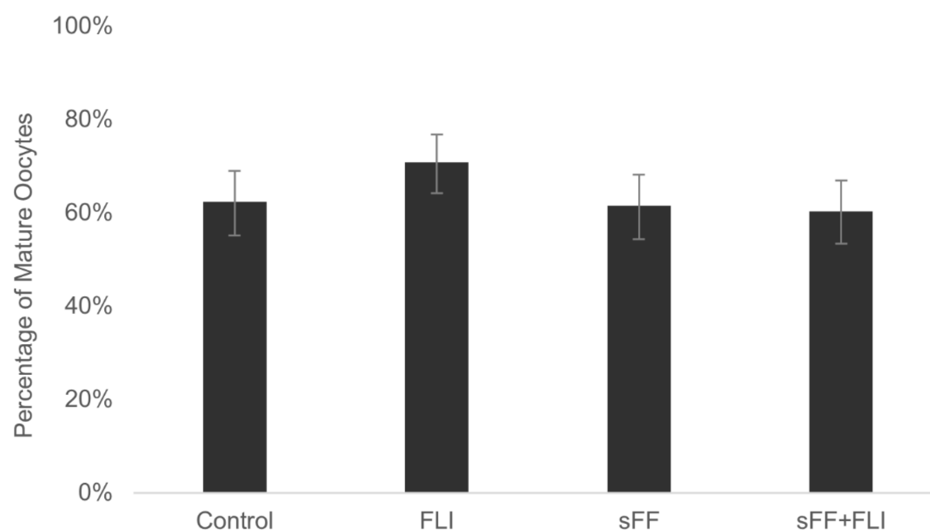

Figure 3.2: The effect of IVM medium supplements on the proportion of mature oocytes. Mean of the effects on nuclear maturation of prepubertal gilt oocytes after 44 hours of culture. Error bars represent the 95% confidence interval for N= 763 oocytes and groups did not differ statistically (p >0.5). FLI: combination of FGF2, LIF, and IGF1. sFF: sow follicular fluid.

3.4.2 Specific Aim 1B: To Observe Difference in CG Distribution and Thus Cytoplasmic Maturation Through the Supplementation of IVM Medium with FLI or sFF

The distribution of CGs after IVM culture (Table 3.1) demonstrated a higher prevalence of “complete” distribution (Figure 3.3, MII) in the groups treated with FLI (N= 60/123; 48.8%), followed by those treated with a combination of sFF and FLI (N= 49/114; 43.0%) and the control (N= 34/113; 32.7%). Oocytes treated solely with sFF had the lowest prevalence of “complete” distribution, with 28.7% (N= 43/115); these differences were considered to be statistically significant in an intergroup comparison (Krustal-Wallis; H_3= 10.995; p= 0.012). All treatments groups displayed a similar prevalence of peripheral CG distribution.

Table 3.1: Cortical granule distribution (cytoplasmic maturation) in oocytes after 44 hours of *in vitro* maturation.

Group	N	CG distribution		
		Central N (mean % ± SEM)	Peripheral N (mean % ± SEM)	Complete N (mean % ± SEM)
Control	113	37 (32.7 ± 6.7) [a]	42 (37.2 ± 9.1) [a]	34 (30.1 ± 3.4) [a]
FLI	123	22 (17.9 ± 1.4) [b]	41 (33.3 ± 10.6) [b]	60 (48.8 ± 11.7) [b]
sFF	115	23 (28.7 ± 2.0) [a]	39 (33.9 ± 3.5) [a]	43 (37.4 ± 4.5) [a]
sFF+FLI	114	30 (26.3 ± 2.4) [a, b]	35 (30.7 ± 3.2) [a, b]	49 (43.0 ± 3.4) [a, b]
p value	0.012*			

Data are shown as mean ± SEM (N= 3 replicates for each group).
[a,b] Different superscript letters indicate significant differences amongst the groups.
* Krustal-Wallis Test (H_3= 10.995).

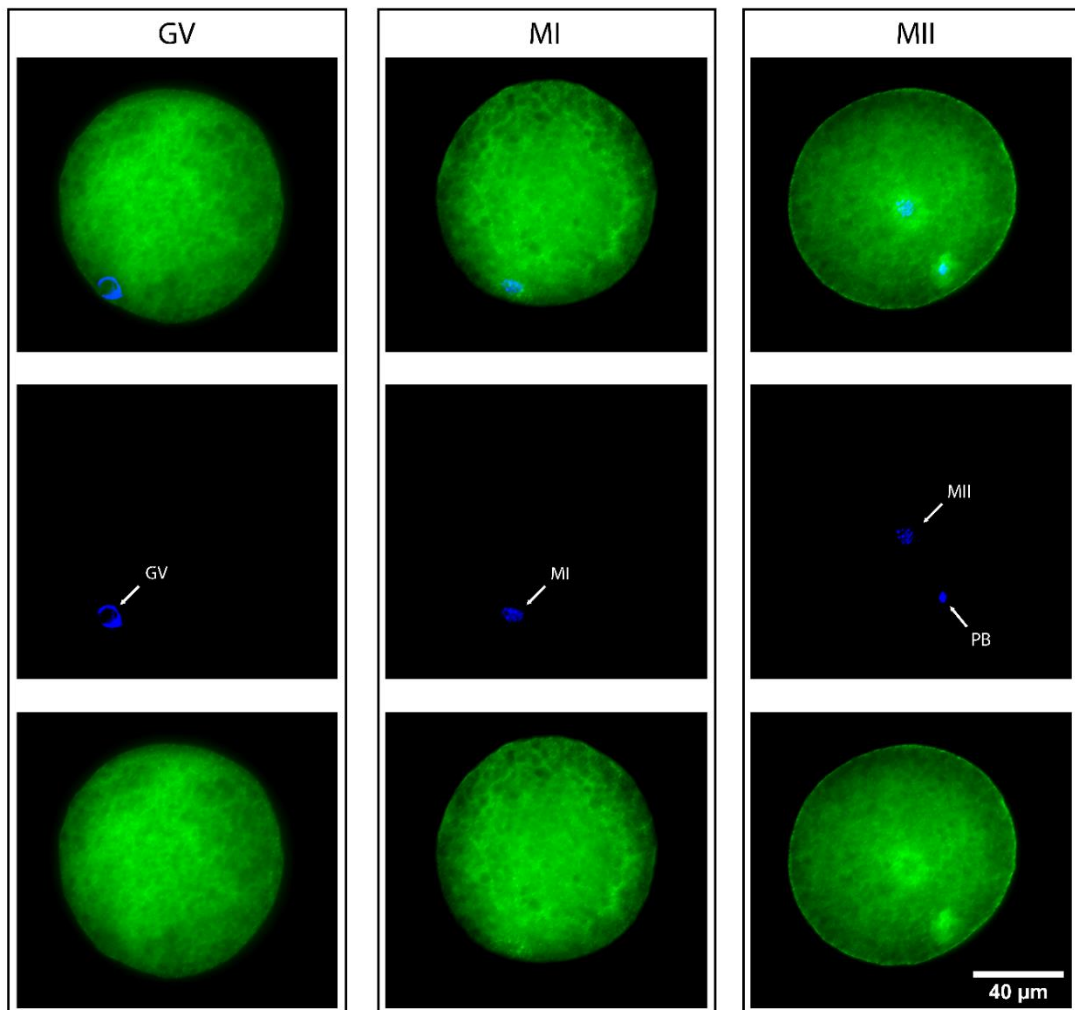

Figure 3.3: Distribution of CGs in oocytes after IVM. GV) Germinal vesicle stage oocyte demonstrating homogenous distribution of CGs throughout the cytoplasm. MI) CG localisation subjacent to the oolemma in an MI stage oocyte. MII) Concentration of CGs at the periphery of the cytoplasm and around the polar body (PB) in an MII mature oocyte. CGs stained in FITC-PNA (green), DNA stained in blue (Hoechst), x200 total magnification. Each column displays an individual oocyte; the top row displays a combined image, with subsequent images showing individual fluorophores (FITC or Hoechst).

3.4.3 Specific Aim 1C: To Compare Differences Between sFF and FLI on Post-fertilisation Outcomes

After fertilisation, the group treated with FLI was shown to have the highest rate of blastocyst formation per inseminated oocyte (N= 46/292; 15.7%), followed by the sFF treatment group (N= 40/279; 14.3%). The group treated with a combination of sFF and FLI (N= 34/284) had a similar proportion of blastocysts to the control group (N= 30/278), 12.0% and 10.8%, respectively. The

differences in blastulation rate between the groups (Figure 3.4) did not show any significant difference after statistical analysis (GLM; $\chi_1 = 0.044$; $p > 0.05$).

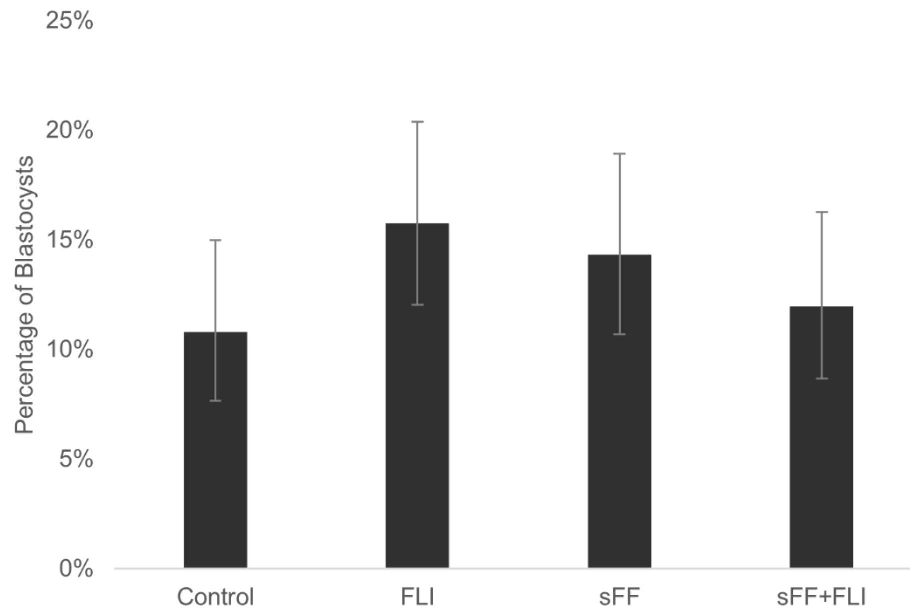

Figure 3.4: Percentage of blastocysts formed after IVM supplementation. Mean of the effects on blastulation rates after matured prepubertal gilt oocytes were fertilised. Error bars represent the 95% confidence interval for N= 149 blastocysts and groups did not differ statistically ($p > 0.5$). FLI: combination of FGF2, LIF, and IGF1. sFF: sow follicular fluid.

The morphological assessment of each treatment group revealed that the treatment of oocytes with FLI during IVM led to the development of higher quality blastocysts (Table 3.2). The FLI treatment group had the highest proportion of blastocysts (37.0% ± 12.1%) with excellent morphology, which was significantly higher than the groups treated with sFF or a combination of FLI and sFF (Krustal-Wallis; $H_3 = 8.162$; $p = 0.043$). The FLI treatment group also had a lower proportion of poor-quality blastocysts (19.6% ± 13.4%), in contrast to the control which had a higher percentage (34.5% ± 13.6%) of poor-quality blastocysts. Interestingly, the combination of FLI and sFF had the second highest proportion

of excellent quality blastocysts (29.4% ± 4.8%) as well as the second highest proportion of poor-quality blastocysts (29.4% ± 6.6%).

Table 3.2: Blastocyst quality after the different treatments on *in vitro* maturation.

Group	N	Grade		
		Excellent	Good	Poor
		N (mean % ± SEM)	N (mean % ± SEM)	N (mean % ± SEM)
Control	29	6 (20.7 ± 6.7) [a]	13 (44.8 ± 9.3) [a]	10 (34.5 ± 13.6) [a]
FLI	46	17 (37.0 ± 12.1) [b]	20 (43.5 ± 10.5) [b]	9 (19.6 ± 13.4) [b]
sFF	40	10 (25.0 ± 9.6) [a]	24 (60.0 ± 13.2) [a]	6 (15.0 ± 4.6) [a]
sFF+FLI	34	10 (29.4 ± 4.8) [a, b]	14 (41.2 ± 5.8) [a, b]	10 (29.4 ± 6.6) [a, b]
p value	0.043*			

Data are shown as mean ± SEM (N= 7 replicates for each group).
[a,b] Different superscript letters indicate significant differences amongst the groups.
* Krustal-Wallis Test (H_3= 8.162).

The number of cells were counted for each blastocyst to further assess the developmental competence. The average number of cells per blastocyst (Figure 3.4) was highest, with 62.1 cells per blastocyst, in the group treated with FLI (N= 23). This was followed by the control group with 57.1 cells per blastocyst, with the sFF (N= 17) treatment and the combination of FLI and sFF (N= 19) having the lowest number of cells (52.7 and 52.2 cells per blastocyst, respectively). A comparison of treatment groups indicated that they did not differ (ANOVA; F_3= 1.039; $p > 0.5$).

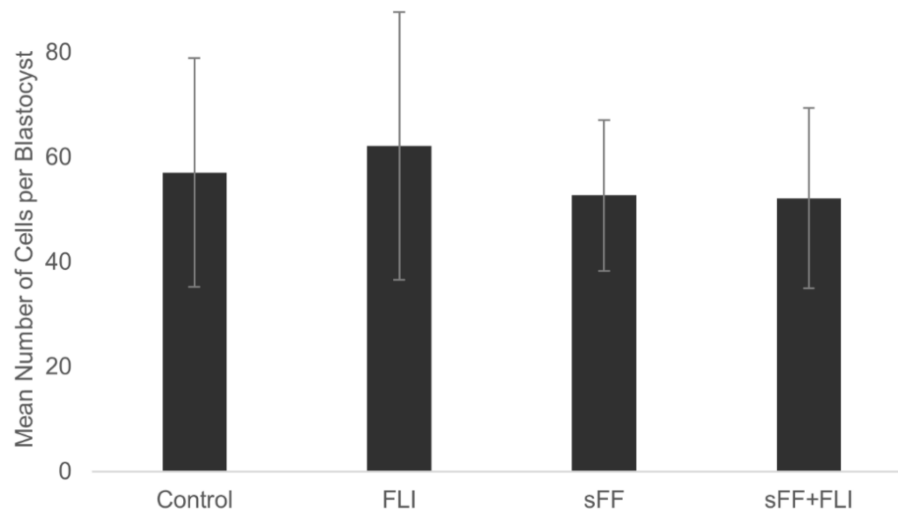

Figure 3.5: The average number of cells per blastocyst. Mean of the effects on the number of cells per blastocyst after matured prepubertal gilt oocytes were fertilised. Error bars show the 95% confidence interval for N= 73 blastocysts. Groups did not differ (p >0.5).

3.5 Discussion

3.5.1 Effect of FLI Supplementation on IVC and IVM

Despite the fact that not all findings were statistically significant, FLI supplementation during IVM consistently yielded better results and led to improved outcomes compared to the control and often to the other treatment groups. By contrast, sFF supplementation alone had a minimal impact on oocyte maturation and yielded results similar to that of the control group, with only marginally improved outcomes when combined with FLI. Statistically significant differences were observed in the distribution of CGs (cytoplasmic maturation) and blastocyst quality, suggesting that IVC also benefits from the positive effect of FLI supplementation. However, nuclear maturation, blastulation rate or the number of cells per blastocyst did not improve significantly with FLI supplementation, conflicting with data previously published by Yuan *et al.* (2017). This lack of significance could be influenced by the statistical power due

to differences in the number of blastocysts analysed compared to the total number of blastocysts obtained; a consequence of fixing of fragile blastocysts.

3.5.2 Improved Oocyte and Embryo Quality

It is well known that the very low survival rate of porcine IVP blastocysts is a result of their poor quality (Zijlstra *et al*., 2008). The data in this chapter shows that supplementing IVM medium with FLI improves the synchrony between cytoplasmic and nuclear maturation, leading to the overall improvement of oocyte quality (Marchal *et al*., 2001; Pawlak *et al*., 2012). It is possible that this synchrony positively influences the ability to undertake normal cell division and the developmental competence of the zygote, allowing for symmetrical blastomere division and lower fragmentation rates, as demonstrated in the results. Not only does the use of FLI improve the quality of embryos which develop to the blastocyst stage, but it has previously been shown to enhance embryo quality and the efficiency of sheep and cattle IVP (Stoecklein *et al*., 2021; Tian *et al*., 2021). Due to the limitations of what is possible within our lab at the University of Kent, the birth rates and viability to implant of the cultured embryos has not been tested. Nevertheless, previous research has demonstrated an increase in the post-implantation viability of embryos treated with growth factors (Biswas *et al.*,2018; Zheng *et al*., 2018).

The addition of supplements in culture medium is known to act additively to improve *in vitro* embryo production, with the treatment of cytokines (separately as well as in combination) shown to increase blastulation rates (Yuan, *et al*., 2017; Stoecklein *et al*., 2021; Valleh *et al*., 2017). Previous research by Liu *et al.* (2020) also demonstrated that the use of cytokines has a positive effect on promoting porcine oocyte maturation, and Redel *et al*. (2021) showed that the

improvement of cumulus cell expansion and oocyte development was enhanced by the combination of FLI with gonadotropins. Furthermore, Procházka *et al.* (2021) demonstrated that MAPK3/1 phosphorylation is increased in media supplemented with FLI during the first hour of culture, suggesting that the supplementation of FLI enhances MAPK3/1 stimulation in COCs and leads to the development of highly competent oocytes.

The results of this chapter indicates that the combination of sFF and FLI does not have a synergist effect on IVP outcomes, yet further research is required as this may be due to variations in sFF composition (Sun *et al.*, 2011; De Oliveira *et al.*, 2006). Likewise, other factors such as the stage of the ovarian follicles and the donor's origin and age may also attribute to the differences between the results shown in this chapter and those previously published on the effect of FLI on porcine maturation (Yuan, *et al.*, 2017; Redel *et al.*, 2021).

3.5.3 Enhancing IVP Processes

Porcine IVP has continued to lag behind its bovine counterpart and is considered to be more challenging at all stages, so any incremental improvement is always welcomed. In terms of cost, environmental benefits for the transport of genetics (nationally and internally), and biosecurity, routine IVP has a huge potential in the pig breeding industry. This data demonstrates that there is great potential in using of peri-pubertal gilts as a source of ovaries, shortening generation intervals to allow for the rapid introduction of new genetics. If barriers to the production of porcine embryos can be overcome, these embryos hold great potential as a resource for biobanking and gene editing. Therefore, the results of this chapter represent a significant advance and provides the scope for continued enhancement of IVP processes.

3.6 Conclusion

Previous IVP processes have relied on the use of sFF in IVM media as it contains important growth factors, however its use raises concerns of biosecurity and batch variability. In using FLI to create a defined IVM system, it was shown to be an efficient substitute during both IVM and embryo culture without compromising oocyte cytoplasmic or nuclear maturation. The addition of FLI in the culture medium enhanced oocyte meiotic maturation and increased the developmental competence and quality of embryos, yet the combination of FLI and sFF showed no synergistic effects despite suggestions that sFF may have growth factors thought to be absent in FLI.

Publications arising from this chapter:

Serrano Albal, M., Silvestri, G., Kiazim, L.G., Vining, L.M., Zak, L.J., Walling, G.A., Haigh, A.M., Harvey, S.C., Harvey, K.E. and Griffin, D.K., 2022. Supplementation of porcine in vitro maturation medium with FGF2, LIF, and IGF1 enhances cytoplasmic maturation in prepubertal gilts oocytes and improves embryo quality. *Zygote*, pp.1-8.

Personal contribution:

- Design of the study
- Data collection and interpretation of statistical analyses
- Manuscript preparation: writing of the first draft and following modifications
- Corresponding author in the publication

4 Comparing Sperms Selection Methods Through the Assessment of Basic Boar Sperm Parameters and IVF Outcomes

4.1 Background

The increase in global pork consumption has prompted the pig breeding industry as a whole to improve production in a more efficient and environmentally sustainable manner (Kumar *et al*., 2021). This is being achieved through the combination of genetics and ART, yet the use of ART in pigs is limited to AI alone. This is primarily due to technical difficulties in producing pig *in vitro* embryos of comparable quality to *in vivo* produced embryos, as well as an inability to achieve adequate pregnancy rates following transfer (Macháty *et al*., 1998; Bauer *et al*., 2010).

Efforts for the optimisation of porcine IVP have primarily focused on oocyte in vitro maturation, with sperm selection and quality overlooked due to the ease in obtaining semen from boars with good reproductive performance. However, as seen in humans and cattle, the quality of sperm is critical to the success of IVF and embryo development (Salumets *et al.,* 2002; Cesari *et al.,* 2006; Gadea and Matás, 2000; Gadea, 2005; Gil *et al.,* 2008), and is commonly indicated by motility, viability, morphology and concentration in the ejaculate. In order to optimise IVF, sperm selection methods have been proposed to isolate the sperm populations with the greatest potential for fertilisation (Rodriguez-Martinez *et al*., 1997; Morrell, 2006). With the potential to increase fertilisation rates, improved sperm selection methods could be of significant economic importance within the pig breeding industry due to the use of AI (as mentioned above).

Each boar ejaculate consists of a diverse sperm population with varying degrees of normality, morphological and functional differentiation (Thurston *et al.*, 2001; Quintero-Moreno *et al.*, 2004), all of which has the potential to influence embryo production and pregnancy outcomes. As mentioned before in section 1.4.2, boar sperm undergo a strict selection process *in vivo* as they travel through the female reproductive tract. The sperm undergo biological transformations, known as capacitation (Austin, 1952), which alter the morphology and structure (Gadella, 2008) of the sperm. By actively migrating across the cervical mucus, high quality sperm are filtered out from the remaining ejaculate (Sakkas *et al.,* 2015; Alvarez-Rodriguez *et al.,* 2020). This promotes very efficient fertilisation (Fitzpatrick and Lüpold, 2014), with approximately 37 billion sperm released into the female reproductive tract but only ~5000 reaching the fertilisation site (Avilés *et al.*, 2015). Methods of sperm selection for IVP have been created to mimic this process, though establishing the best sperm selection method prior to IVF presents a number of difficulties. This method should ideally be non-invasive, affordable, and capable of isolating a large number of motile sperm cells without causing any damage. It should also be able to identify sperm of high quality to increase the likelihood of pregnancy and live birth rates (Henkel and Schill, 2003; Rappa *et al.*, 2016).

There are different sperm separation techniques that traditionally use centrifugation, including the swim-up method, the simple wash method, and density gradient selection (DGS; Mortimer, 1991 and 1994; Vaughan and Sakkas, 2019). The selection of superior quality sperm is typically obtained using density gradients, consisting of solutions with colloidal silica particles, and is widely used in many livestock species, including bulls (Suzuki *et al.,* 2003;

Oliveira *et al.,* 2011) and pigs (Suzuki and Nagai, 2003; Matás *et al.,* 2011). The gold standard method for porcine IVP is DGS as it enriches the quality of boar semen samples with respect to the morphology and motility (Noguchi *et al.*, 2015), demonstrating improvements in sperm penetration (Matás *et al.,* 2011), cleavage (Jeong and Yang, 2001), and blastulation rates (Jeong and Yang, 2001; Ohlweiler *et al.,* 2020) after IVF and embryo culture. Nonetheless, the centrifugation and contact with the solutions used in DGS have potentially adverse effects on sperm. This method of selection may result in mechanical damage (Mortimer,1991; Alvarez *et al.,*1993, Oliveira *et al.,* 2011) and high levels of oxidative stress, reducing the motility of spermatozoa and the ability to bind to oocyte membranes. This could, in turn, weaken the process of fertilisation and impair embryo development (Aitken *et al.*,1988, Tavalaee *et al.*, 2012; Betarelli *et al.*, 2018).

The most promising of the new sperm selection techniques has recently been suggested to be microfluidic chip-based sperm (MCS) sorting devices (Smith and Takayama, 2017; Suarez and Wu, 2017; Samuel *et al.,* 2018, Vaughan and Sakkas, 2019). These devices are a simple and affordable alternative for traditional sperm selection methods that perform a less invasive separation, mimicking the cervical and uterine pathways that sperm must travel through in order to naturally fertilise the mature oocyte by relying on the behaviour and movement of sperm in the absence of any external stimuli (Samuel *et al.,* 2018, Ahmadkhani *et al.,* 2022). In humans, MCS appears to produce preparations with a lower percentage of spermatozoa with DNA fragmentation (Asghar *et al.,* 2014; Quinn *et al.,* 2018; Parrella *et al.,* 2019), making the use of this technique advantageous for ICSI and IVF. Nevertheless, there are few studies utilising

MCS devices in livestock species. In 2018, Nagata *et al.* reported the improvement in bull sperm selection utilising a diffuser-type microfluidic sperm sorter. However, neither the use of boar sperm nor a device like the ZyMōt™ Multi has been investigated with this approach; nonetheless, the use of the ZyMōt™ Multi in human IVF clinics has shown an increase in the number of euploid IVF embryos (Parrella *et al.,* 2019; Anderson *et al.,* 2020; Beyhan, 2020). Recently, the ZyMōt™ Multi has been tested in equine IVP, producing noticeably higher quality sperm than DGS (Orsolini *et al.,* 2022).

4.2 Specific Aim 2

The specific aim of this chapter was to compare the effect of two different sperm selection methods (DGS and MCS) by assessing basic boar sperm parameters and IVF outcomes of selected unselected (extender-diluted) boar semen samples. This was tested through the following aims:

- Specific Aim 2A: To test the hypothesis that the use of a commercial microfluidic device improves the basic semen parameters and thus selection of boar sperm
- Specific Aim 2B: To test the hypothesis that the use of a commercial microfluidic device improves pig IVF outcomes compared to standard methods

4.3 Materials and Methods Specific to This Chapter

4.3.1 Experimental Overview

To summarise the series of events in this experiment, Figure 4.1 shows the experimental workflow.

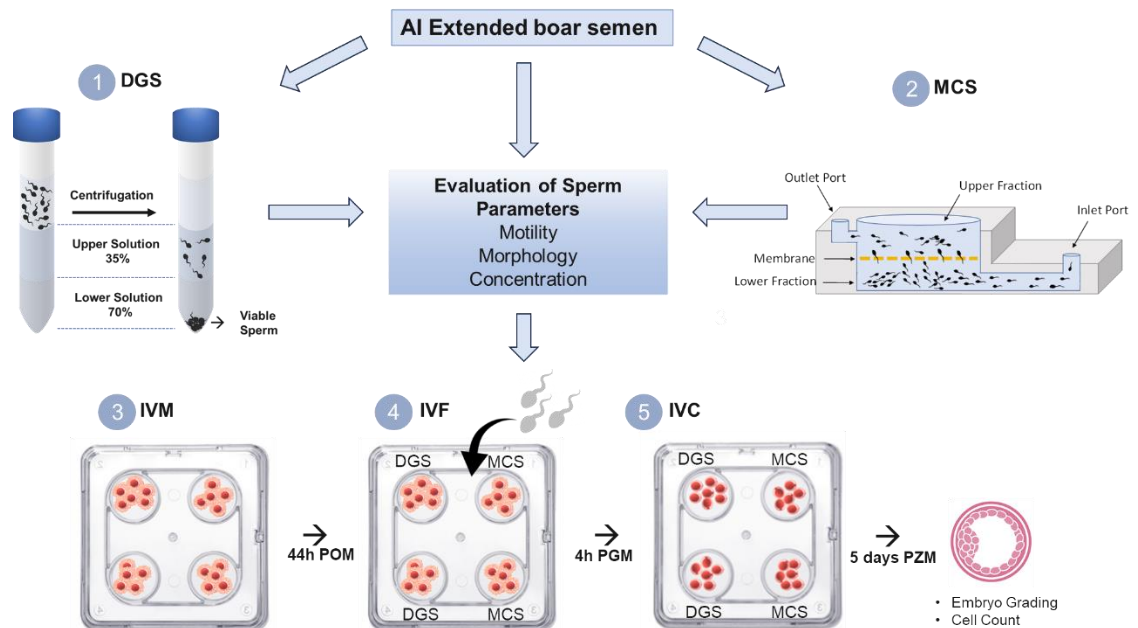

Figure 4.1: Schematic of the experimental flow conducted for testing the effect of FLI in IVM supplementation. Sperm parameters for AI extender boar semen were evaluated and prepared for IVF by two different methods: 1) DGS and 2) MCS. After preparation, sperm parameters of both samples were also evaluated. 3) COCs selected from follicles between 3-6 mm in size were distributed in groups and *in vitro* matured in POM for 44 h. 4) IVF starts with the coincubation of oocytes with the sperm sample (either prepared by DGS or MCS) for 4 h in PGM. 5) IVC is initiated, and the presumptive zygotes are cultured for 5 days in PZM. After that, blastocyst morphology and cell count were evaluated.

4.3.2 Boar Sperm Samples

Extended boar semen (for commercial AI) was supplied by JSR Genetics Ltd. (Southburn, United Kingdom). AI samples were shipped at RT in the post, then preserved at 17°C for up to two days before use. In total, there were 17 extended boar sperm samples, with each sample split into two equal aliquots for use in each experiment (MCS vs. DGS).

4.3.3 MCS Separation

Using the ZyMōt™ Multi (ZyMōt Fertility Inc., Maryland, USA), a MCS device, 3 mL extended boar semen (from 17 boar semen samples) was pipetted into the inlet port (Figure 4.2). 2 mL PGM was rapidly pipetted on top of the membrane prior to incubation at RT for 30 mins. After the incubation period, sperm located within the upper fraction of the device were gently aspirated by inserting a 1 mL syringe into the outlet port. This protocol was a modified based on the manufacturer's instructions.

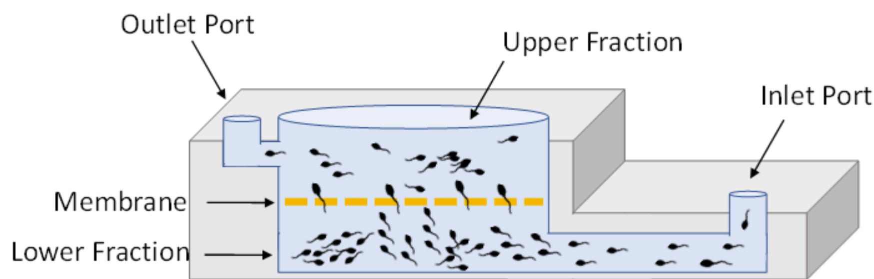

Figure 4.2: Diagram of the ZyMōt™ Multi. The device separates the sperm based on motility as the spermatozoa with the best mobility swim through the micropores within the membrane (image modified from ZyMot Fertility, 2019).

4.3.4 Statistical Analysis

SPSS was used to analyse the data (Version 25, IBM). The samples were analysed using one-way ANOVA in the parameters with a normal distribution as determined by the Kolmogorov-Smirnov Test. The Krustal-Wallis Test was used to test the data parameters that did not have a normal distribution. Results were considered to be statistically significant when $p < 0.05$. Violin graphs were plotted using https://www.bioinformatics.com.cn/en, a free online platform for data analysis and visualisation.

4.4 Results

4.4.1 Specific Aim 2A: To Test the Hypothesis that the Use of a Commercial Microfluidic Device Improves the Basic Semen Parameters and thus Selection of Boar Sperm

The sperm concentration was significantly reduced with the use of any selection method (Figure 4.3), with an average concentration of 33.4 ± 2.2 million sperm cells/mL obtained in the control group, 10.1 ± 1.2 million sperm cells/mL after using DGS, and 3.9 ± 0.6 million sperm cells/mL after using MCS. As expected, both test groups were lower than the control, however the concentration of sperm in the MCS group was significantly lower than DGS.

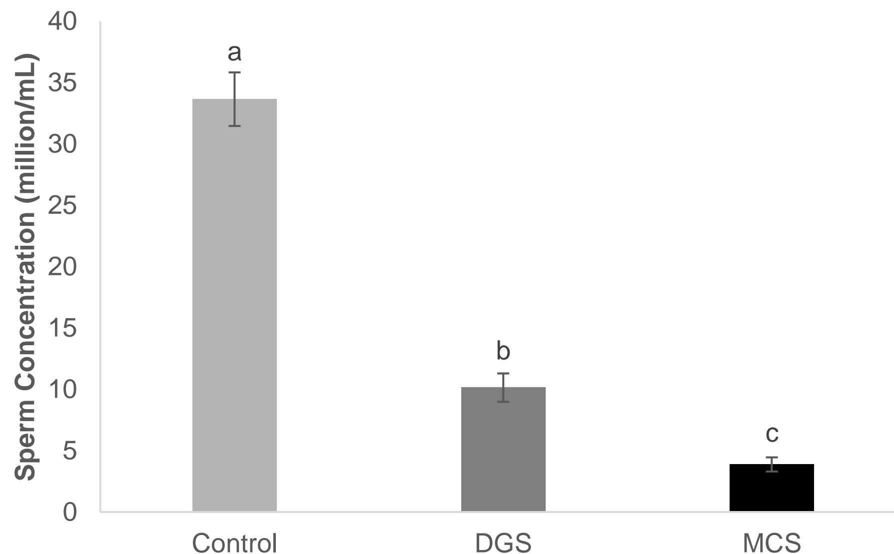

Figure 4.3: Effect of MCS and DGS selection methods on the final sperm concentration of the sample. Data are shown as mean % ± SEM for N= 17 replicates per group). As data were not normally distributed, a Krustal-Wallis Test was performed (gl= 2, p <0.001, H= 40.451). [a,b,c] = Different superscript letters indicate significant differences amongst each of the groups. MCS= Microfluidic chip-based sperm. DGS= density gradient system.

The percentage of total motility in the control and MCS samples did not differ significantly, with 58.8 ± 4.4% and 73.0 ± 3.8%, respectively (Figure 4.4A). The total motility of the DGS samples (75.9 ± 4.0%) did differ significantly from the

control group but was not statistically different from that of the MCS samples. The proportion of sperm with progressive motility was significantly lower in the control group (19.5 ± 1.4%) compared to that of the DGS and MCS group (Figure 4.4B), but differed significantly between the DGS and MCS groups, with DGS selected samples (65.2 ± 4.9%) showing greater progressive motility than MCS selected samples (45.7 ± 5.3%).

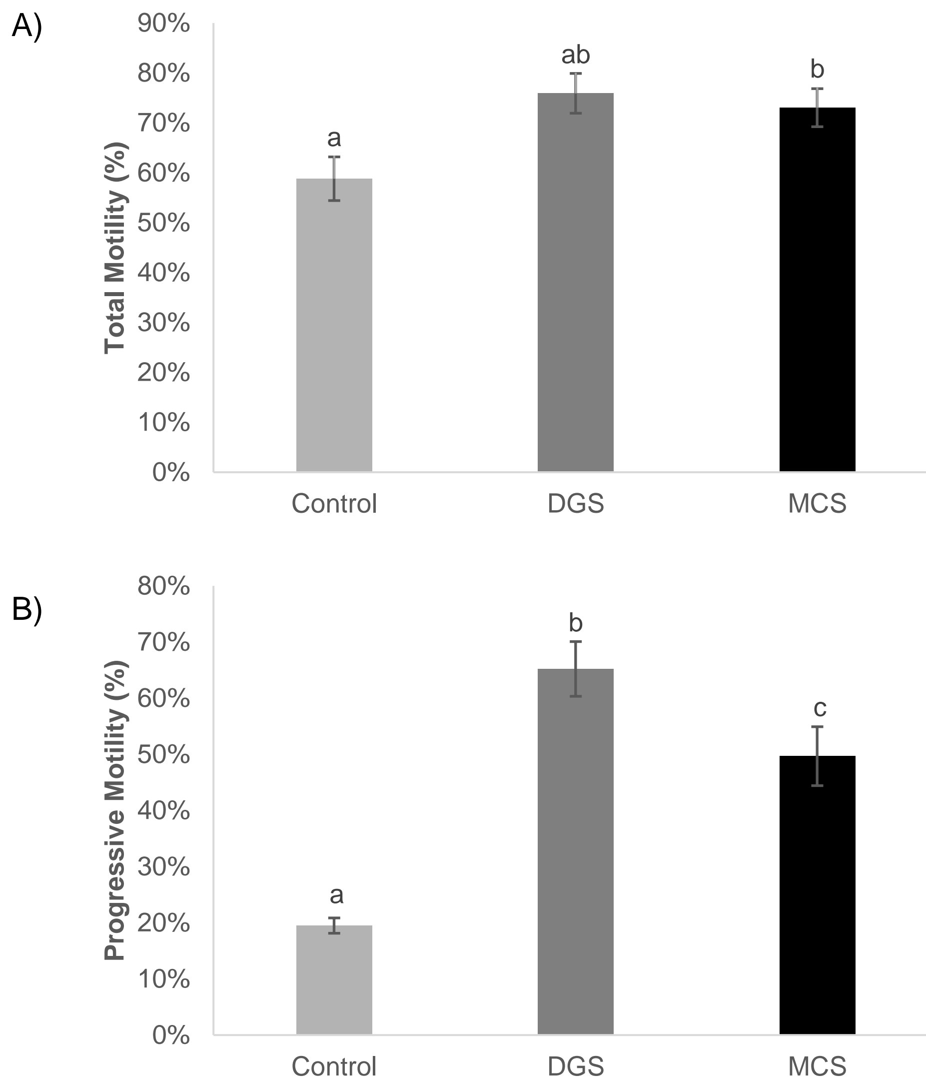

Figure 4.4: Effect of sperm selection method on the motility of the samples. A) Average percentage of total motility, ANOVA Test (gl= 2, p= 0.010, F= 5.075). B) Average percentage of progressive motility, ANOVA Test (gl= 2, p <0.001; F= 30.499). **a,b,c** Different superscript letters indicate significant differences between each of the groups, ab does not denote a significant difference but reflects similarities to both a and b. Data are shown as mean % ± SEM for N= 17 replicates per group. MCS= Microfluidic chip-based sperm. DGS= density gradient system.

4.4.1.1 Test Whether the Use of a Commercial Microfluidic Device Improves Pig IVF Outcomes Compared to Standard Methods

Analysis of the data obtained from the iSperm Swine Semen Analysis System, presented in Figure 4.5, showed that the VCL of the samples increased significantly in the DGS selected samples (92.5 ± 3.4 μm/s) compared to the control group (60.2 ± 2.7 μm/s) and MCS selected samples (71.1 ± 3.6 μm/s), though MCS samples did not differ significantly from control. The VAP and VSL of each selection method were significantly different to each other, with both variables being significantly increased in MCS samples (66.3 ± 3.3 μm/s and 55.1 ± 2.6 μm/s, respectively) compared to the control (48.3 ± 2.5 μm/s and 39.6 ± 2.4 μm/s, respectively). However, DGS selected samples did not display as great of an increase (83.1 ± 2.1 μm/s and 75.7 ± 2.9 μm/s, respectively).

The MCS and DGS selected samples demonstrated a statistically similar LIN, with 77.6 ± 2.1% and 79.9 ± 2.3% respectively, with both groups being significantly higher in comparison to the control (62.9 ± 2.1%). The STR was significantly different in DGS selected samples (89.2 ± 1.7%) compared to control (78.6 ± 1.9%) but did not differ significantly from MCS selected samples (83.4 ± 2.3%), and the MCS samples did not significantly differ from the control.

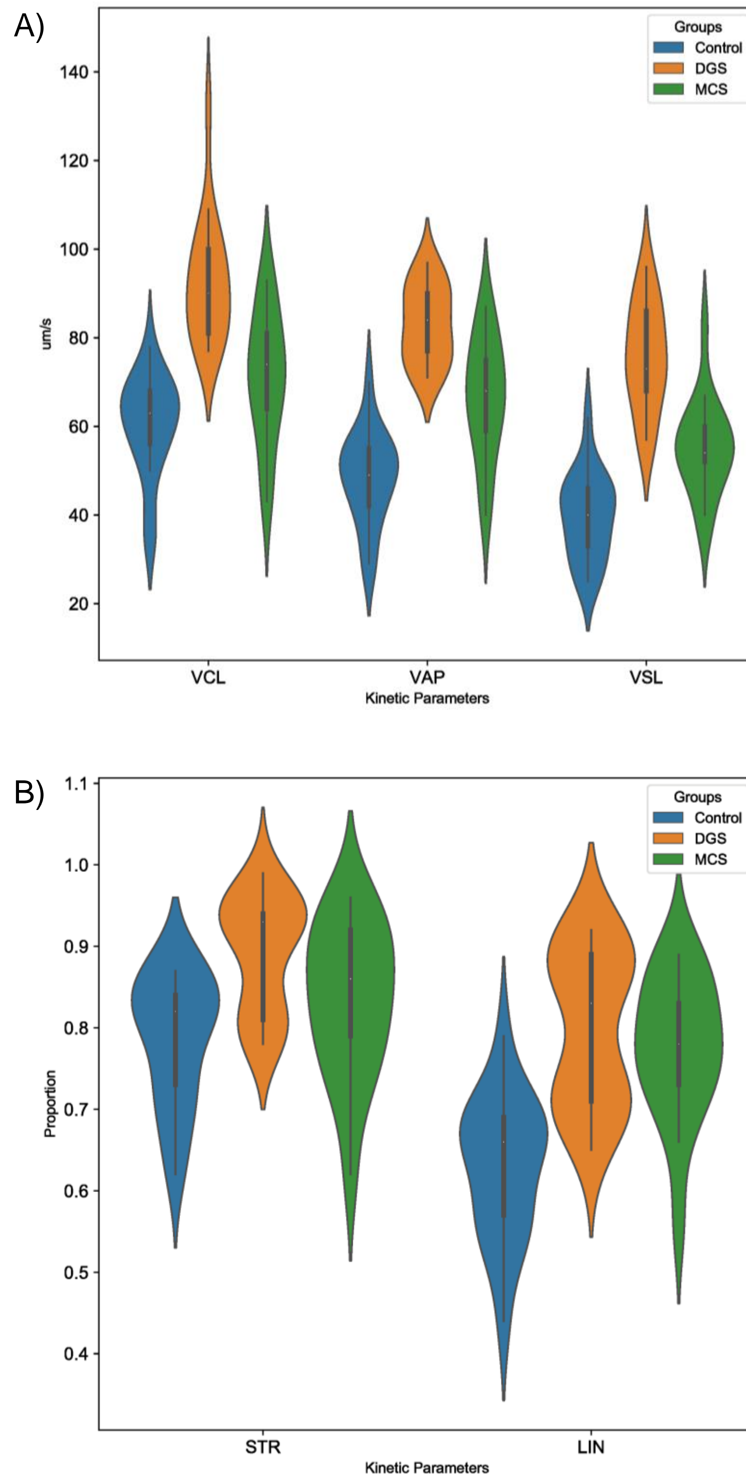

Figure 4.5: Effect of sperm selection method on sperm kinetic parameters (VCL, VSL, VAP, LIN and STR). A) From left to right: curvilinear velocity, average path velocity, and straight-line velocity. B) From left to right: Linearity and straightness of trajectory. N= 17 replicates for each group. ANOVA test (gl= 2, p value of all parameters <0.02). F_{VCL}= 25.557; F_{VAP}= 41.704; F_{VSL}= 46.637; F_{LIN} =18.235; F_{STR}= 7.600. F were calculated as variation between sample means/variation within the samples. MCS= Microfluidic chip-based sperm. DGS= density gradient system.

4.4.1.2 Evaluation of Sperm Morphology

The sperm morphology in both MCS and DGS selection methods showed a significantly lower number of abnormalities ($p < 0.01$) compared to the control group (29.6 ± 1.5%), as seen in Figure 4.6. However, the percentage of abnormalities in MCS selected sperm (15.2 ± 1.0%) was significantly lower than in the DGS selected sperm (24.7 ± 1.3%). No significant differences were observed in the percentage of head abnormality between all the studied groups, though the percentage of tail abnormalities found in the control group (10.5 ± 1.2%) was significantly higher than in MCS and DGS groups (Table 4.1). The lowest percentage of tail anomalies was seen in the MCS group (4.9 ± 0.6%), differing statistically from that of DGS (8.6 ± 1.0%). Significant differences were also observed between all three groups in the presence of cytoplasmic drop (CD) percentage between all the groups, with the highest found in the control group (11.5 ± 1.6%) followed by DGS (8.8 ± 1.2%) and then MCS (3.6 ± 0.6%).

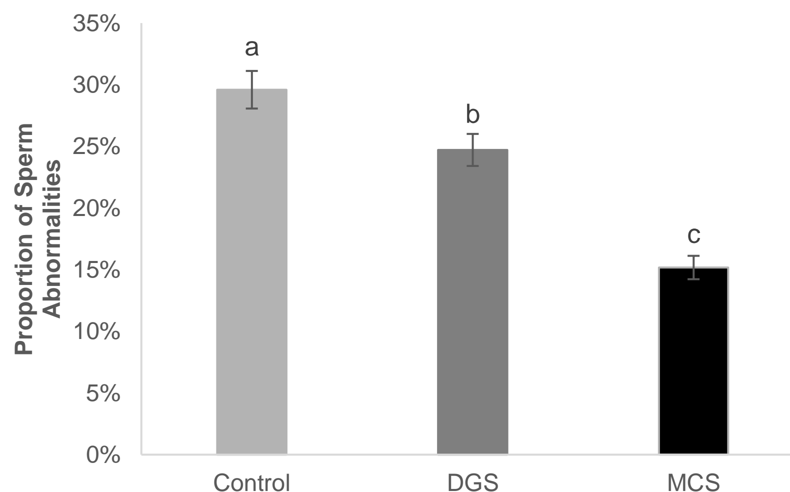

Figure 4.6: Proportion of sperm abnormalities from the evaluation of the sperm morphology. Data are shown as mean % ± SEM for N= 17 replicates per group. ANOVA Test (gl= 2, $p < 0.01$). [a,b,c] Different superscript letters indicate significant differences between each of the groups; groups that share the same letter are statistically indistinguishable from each other. F= 32.832 (calculated as variation between sample means/variation within the samples). MCS= Microfluidic chip-based sperm. DGS= density gradient system.

Table 4.1: Proportion of each morphological anomalies in sperm before and after sperm selection.

Group	Head Abnormality Mean % ± SEM	Tail Abnormality Mean % ± SEM	CD Mean % ± SEM
Control	7.6±0.7	10.5±1.2 [a]	11.5±1.6 [a]
MCS	6.7±0.5	4.9±0.6 [b]	3.6±0.6 [b]
DGS	7.4±0.5	8.6±1.0 [c]	8.8±1.2 [c]
p value*	0.402	<0.001	<0.001
H*	1.820	16.744	19.383

(N= 17 sperm samples in each group). MCS= Microfluidic chip-based sperm. DGS= density gradient system. CD= Cytoplasmic droplet. *Krustal-Wallis Test used for data not normally distributed (gl= 2). [a,b,c] Within each column, different superscript letters indicate significant differences amongst the groups; samples that share the same letter are statistically indistinguishable from each other. H was calculated as variation between sample

4.4.2 Specific Aim 2B: To Test the Hypothesis that the Use of a Commercial Microfluidic Device Improves Pig IVF Outcomes Compared to Standard Methods

There were no discernible changes when evaluating IVF outcomes (p >0.05) between the two sperm selection strategies, based on the proportion of embryos that underwent cleavage after 3 days of IVC (Figure 4.7A) and those that reached blastocyst stage after 5 days of IVC (Figure 4.7B). The cleavage rate of embryos fertilised using DGS selected sperm was 46.7% compared to 42.5% in embryos fertilised by MCS selected sperm. The blastulation rate of embryos fertilised using DGS selected sperm was 11.9 % compared to 10.4% in embryos fertilised by MCS selected sperm.

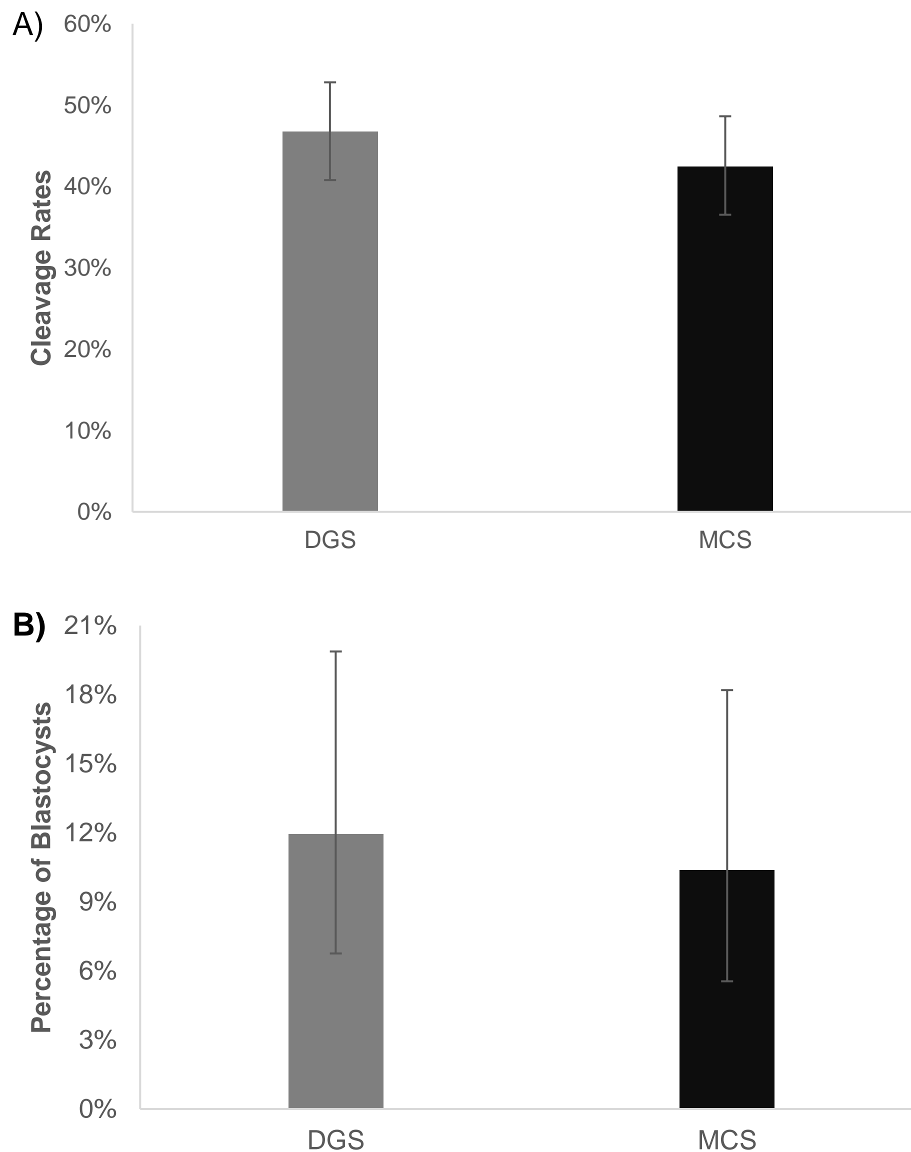

Figure 4.7: Effect of sperm selection on IVF outcomes. A) Proportion of cleaved embryos after the use of DGS and MCS. Error bars show the 95% confidence interval for N= 430 replicates. Groups did not differ (p >0.05). B) Proportion of blastocyst formation after the use of DGS and MCS. Error bars show the 95% confidence interval for N= 430 replicates. Groups did not differ (p >0.05). MCS= Microfluidic chip-based sperm. DGS= density gradient system.

4.4.2.1 Evaluation of Embryo Quality

An evaluation of the embryo morphology at the blastocyst stage (N= 48) showed that most of the blastocysts from the DGS group were classified as good quality (53.5%) or poor quality (38.5%), with only 7.8% of excellent quality. Conversely, embryos from the MCS group were of an overall higher quality, with 36.4%

classified as both good and excellent quality, and only 27.3% being classified as poor. However, no significant difference was observed in the quality of the blastocyst between the sperm selection groups (Figure 4.8).

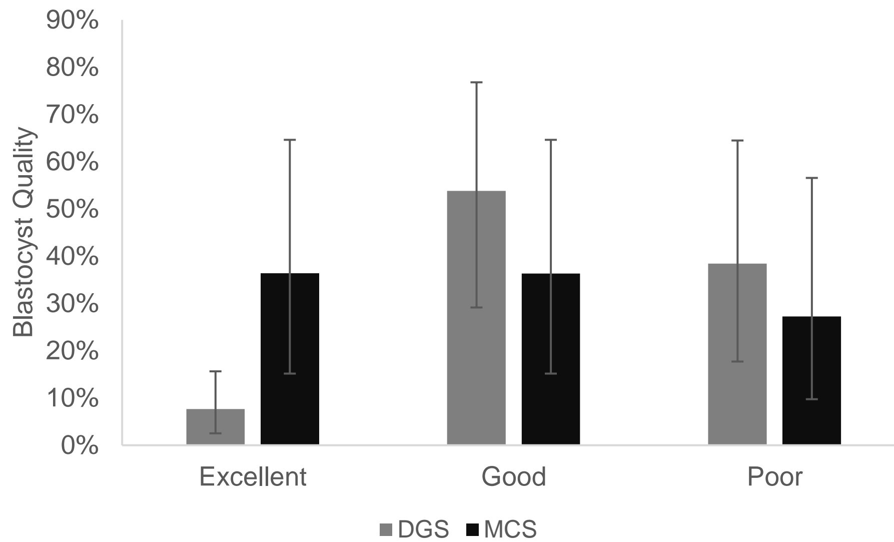

Figure 4.8: Effect of both sperm selection methods on blastocyst quality. Error bars show the 95% confidence interval for N= 48 replicates. Groups did not differ (p >0.05). MCS= Microfluidic chip-based sperm. DGS= density gradient system.

An analysis of the mean number of cells per blastocysts (N= 48) derived using MCS selected sperm revealed a significantly difference compared the embryos derived using DGS selected sperm (Figure 4.9). The average number of cells per blastocyst in the MCS group was 49.6 ± 3.0 cells compared to 38.7 ± 1.8 cells in the DGS group (ANOVA; F_1= 12.389; $p < 0.05$).

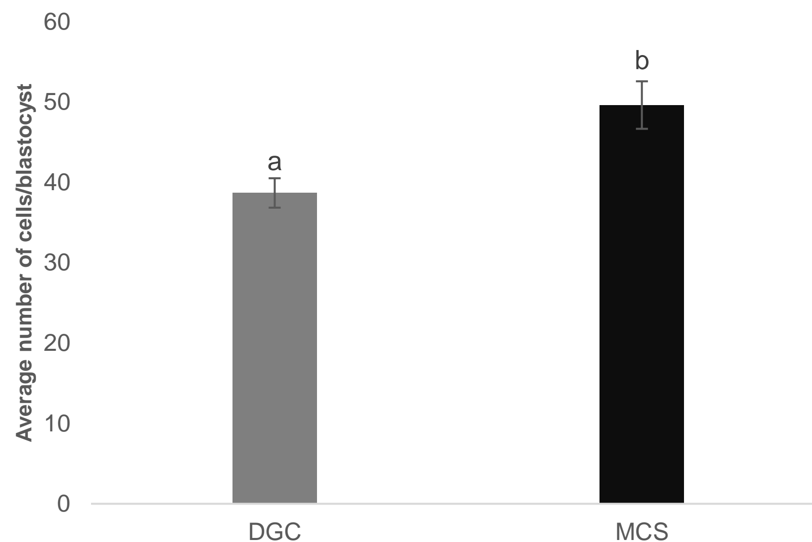

Figure 4.9: Effect of sperm selection method on average number of cells per blastocyst. ANOVA Test (gl= 1, p >0.05, F= 12.389. F is calculated as variation between sample means/variation within the samples. Error bars show the SEM for N= 48 replicates. [a,b] Different superscript letters indicate significant differences between the two groups. MCS= Microfluidic chip-based sperm. DGS= density gradient system.

4.5 Discussion

4.5.1 Concentration of Sperm Between Selection Methods

For boars with lower concentrations or lower quality sperm, the significant concentration reduction of the samples selected by MCS may present a challenge in IVF (Gil *et al*., 2008). Since centrifugation facilitates the aggregation of sperm when using DGS, the selection is less harsh, and it always results in a higher sample concentration compared to MCS. Nonetheless, using centrifugation increases sperm hyperactivation, which is known to cause sperm aggregates to form during sperm capacitation and could make the fertilisation process more challenging. The use of MCS devices like the ZyMōt™, which handle sperm more gently, decreases the formation of aggregates which in turn may improve the success rate of IVF. When it comes to other ARTs, like ICSI, MCS would be ideal as it enables the embryologist to select good sperm without significantly capacitating it (Garcia-Rosello *et al*., 2006). Anderson *et al.* (2020)

demonstrated that the use of MCS devices to sort sperm for ICSI increased the number of euploid human embryos produced.

4.5.2 Sperm Motion Parameters

Hyperactivation is one of the modifications sperm undergo during capacitation (Suarez *et al.,* 1992, Ho *et al*., 2001). This mechanism is described as the increased beating amplitude of the sperm flagellum to produce a more vigorous movement, which is necessary for penetrating COCs (Yanagimachi, 1994). Sperm chosen by DGS had much higher progressive motility. Sperm selected by DGS had significantly higher progressive motility. Additionally, the examination of velocity parameters VCL, VAP, and VSL, acquired from the CASA analysis, revealed improved performance in DGS samples. The linearity and straightness of the trajectory in the DGS and MCS samples, however, were similar between the two methods. Although the same IVF media were used for both selection methods in this chapter, the absence of centrifugation to promote capacitation when utilising MCS may require the addition of supplements during IVF that have been reported as capacitating agents, such as raising intracellular calcium concentration using a calcium ionophore (Suarez *et al.,* 1992; Suarez, 2008) or caffeine (Funahashi and Day, 1993, Funahashi and Nagai, 2001).

4.5.3 Evaluation of Sperm Morphology

In terms of sperm morphology, density gradients are known to reduce the number of abnormalities in semen samples by eliminating dead sperm cells, sperm with head or tail anomalies or cells with CDs and debris that are known to generate a high proportion of ROS (Noguchi *et al*., 2015). The data obtained in this chapter demonstrates that MCS performs a more stringent selection, further lowering the levels of abnormalities in the sample. According to

previously published studies, centrifugation damages the structural integrity of sperm, reducing both their lifespan and capacity to fertilise (Aitken and Clarkson, 1988). In bovine sperm, Avery and Greve (1995) hypothesised that the presence of unbound polyvinylpyrrolidone in the density gradient solutions would have a negative impact on the cleavage rates following *in vitro* insemination.

4.5.4 Effect of Sperm Selection Methods on IVF Outcomes and Embryo Quality

As the data revealed similar cleavage and blastulation rates, both DGS and MCS will serve as suitable sperm selection methods in pig IVF in terms of fertility and embryo development. However, the ongoing issue for pig IVF is the high incidence of polyspermy, which hinder embryo growth and cell count (Han *et al.*, 1999), and is something to be investigated further. Although there was no significant difference in the blastocyst quality between the two selection methods, the embryos derived from the MCS selected sperm had a significantly higher number of blastomeres.

4.6 Conclusion

The use DGS to prepare sperm samples results in the selection of sperm with higher motility which ultimately helps to achieve successful fertilisation. However, the potential damage caused by centrifugation and the likely toxicity of the solutions could have adversely affect the sample quality. MCS sorting is an alternative technique for the selection of high-quality sperm which does not require centrifugation, nor any toxic solution, to prepare the sample for fertilisation. This less invasive approach produces the same number of embryos with a higher number of cells, which may be indicative of better developmental potential and is likely to improve the reproductive outcomes in IVF.

Publication (in preparation) arising from this chapter:

Serrano Albal, M., Aquilina, M.C., Kiazim, L.G., Zak, L.J., Ellis, P.J., and Griffin, D.K. Effect of Two Different Sperm Selection Methods on Basic Boar Sperm Parameters and *in vitro* Fertilization Outcomes. *To be submitted to Theriogenology in January 2023*.

Personal contribution:

- Presented the results of the study in the form of oral presentation at ICAR 2020+2
- Design of the study
- Data collection and interpretation of statistical analyses
- Manuscript preparation: writing of the first draft and following modifications

5 Comparing New Vitrification Protocols for Boar Sperm Against the Slow Freezing

5.1 Background

Within the pig breeding industry, boar sperm is routinely frozen as it allows for the shipping and storage of high-merit genetic material for future use. As a bank of genetic resources that has been accessible for commercial use since 1975, cryopreservation is a vital tool for the preservation of important productive lines (Westendorf *et al.*,1975*;* Johnson *et al.*,1985). The most commonly used cryopreservation method for preserving boar sperm is slow freezing (SF) (Pezo *et al.*, 2019). Yet only 1% of artificial inseminations performed globally use frozen-thawed boar sperm since the cryopreservation process reduces sperm survivability (Yeste, 2016). Despite the fact that cryopreservation causes damage to spermatozoa, leading to changes in membrane lipid composition, acrosome status, sperm motility, and viability (Paoli *et al.,* 2014; Gangwar *et al.,* 2018), cryopreservation is still the best technology for storing boar sperm for an extended period of time and facilitating sample transport for commercial purposes (Rodríguez-Gil *et al.*, 2013; Yeste *et al.*, 2017).

The varying compositions of cryopreservation media, such as the addition of antioxidants to freezing and thawing extenders, directly correlates to the level of damage observed in sperm cells. Cryopreservation media, which includes substances such as glycerol or egg yolk that act as cryoprotectants, is used to perform slow freezing (Yeste, 2016). Additionally, non-penetrating substances in the media promote cell dehydration and form hydrogen bridges with the water molecules, which reduces the formation of ice crystals (Ávila-Portillo *et al.*, 2006). Sugars, such as sucrose, are extracellular cryoprotectants that are

commonly used in the cryopreservation of sperm cells across different species (Díaz-Jiménez *et al*., 2019). Despite this, the formation of intracellular crystals during the cooling process is still very noticeable. Sperm cells and their membranes must withstand significant damage from freezing (Medrano *et al*., 2002), which reduces sperm motility and mitochondrial activity (Johnson *et al.,* 2000; Vadnais and Althouse, 2011) whilst increasing the proportion of morphological abnormalities in the samples and/or compromising DNA integrity. In comparison to other species, boar sperm are more susceptible to thermal shock (Parks and Lynch, 1992) and have lower cryotolerance (Johnson *et al.,* 2000) due to the asymmetrical distribution of cholesterol between the layers of the membranes. The detrimental effects of freezing result in a decrease in the different functional sperm parameters, reducing the fertilising capacity of frozen-thawed sperm by roughly 20-30% in comparison to semen in extender stored at 17°C (Eriksson, 2002; Knox, 2016).

Vitrification has been widely used in recent years for the cryopreservation of oocytes and embryos of various species (Cervera and García-Ximénez, 2003; Kasai and Mukaida, 2004; Davidson *et al.,* 2014), and has been increasingly applied as a method for preserving sperm in smaller volumes quantities. By plunging sperm directly into LN_2, it prevents intracellular ice crystal formation as it provides an ultra-fast cooling process (Isachenko *et al*., 2003; 2005). Early advances in this field were made using human samples (Isachencko *et al*., 2008) but were quickly extended to include non-human species (Sánchez *et al*., 2011; Díaz-Jiménez *et al*., 2018).

In recent years, there have been some cases of successful recovery of sperm samples after vitrification in pearls, in which the post-warmed sperm viability was

significantly better in the vitrification groups (Aizpurua *et al.,* 2017; Pabón *et al.*, 2019). It is also considered to be more effective than alternative freezing techniques, such as the freeze-dry method, which was previously proposed to replace slow freezing (Men *et al.*, 2013; Olaciregui and Gil, 2017). Spermatozoa that have been freeze-dried lose their motility and viability and exhibit increased DNA damage (Kwon *et al.*, 2004), whereas vitrified samples exhibit less damage and maintain their motility (Isachencko *et al.*, 2008; Aizpurua *et al.*, 2017; O'Neill *et al.*, 2019). However, it has yet to be performed successfully on boar sperm (Arratzoa *et al.*, 2017).

Early sperm vitrification protocols simply required submerging a metal strainer in a polystyrene box filled with LN_2 for the collection of pearls. Aliquots of sperm were pipetted directly into the LN_2, producing spheres that floated on the surface for a few seconds before solidifying. Since having multiple pearls in the same strainer increased the possibility of pearls adhering together and generating larger drops of a greater volume, only one drop could be created at a time.

Furthermore, the sperm spheres were gathered with a spoon in order to deposit the pearls in cryovials, resulting in a tedious and time-consuming collection process. Recent papers described the use of a Kitazato BioPharma device that facilitates the formation of pearls (O'Neill *et al.*, 2019; Pabón *et al.*, 2019). This device is a metal funnel with a metal grate that enables the simultaneous formation of multiple pearls. The device also contains a cryovial fastened to the bottom of the funnel, making it easier to collect the spheres and reducing the handling time. However, the Kitazato device is an open system, which is a major limitation as the risk of cross-contamination between samples is increased by having the sample in direct contact with LN_2 (O'Neill *et al.*, 2019).

Other studies have developed sperm packaging methods for cryopreservation to perform vitrification techniques in a more aseptic manner (Erickson and Rodríguez-Martínez, 2000). Vitrification of small volumes of sperm (30-50 µL) using sterile mini-straws reduces cross-contamination between the samples (Díaz-Jiménez *et al.*, 2018). This technique uses straws of two different diameters; the inner, smaller straw contains the sperm while the outer, larger straw serves as a protective function. The samples remain isolated since the outer straws are sealed on both sides, decreasing the risk of cross-contamination.

5.2 Specific Aim 3

The specific aim of this chapter was to modify vitrification protocols for use on boar sperm, comparing the use of the Kitazato device against slow freezing in straws, the standard cryopreservation method used in the boar industry. This was tested through the following aim:

- Specific Aim 3A: To compare boar sperm parameters between three different cryopreservation techniques (slow freezing, vitrification using pearls, and vitrification using mini-straws)

5.3 Materials and Methods

5.3.1 Sperm Preparation and Cryopreservation

10 extended boar semen samples, kept at 17.5°C, were supplied by JSR Genetics Ltd®. (Southburn, United Kingdom). Each sample was split into 4 equal fractions and then prepared at RT using a 35%/70% (v/v) BoviPure discontinuous density gradient system (Nidacon, Göthenborg, Sweden) following manufacturer's instructions. After DGS, each sample split into four equal aliquots for use in each experiment: 1) PT; the sperm parameters of samples resuspended in PGM were evaluated without conducting any cryopreservation. 2) Slow freezing (SF). 3) Vitrification in pearls (VP) 0.4) Vitrification in mini-straws (VMS). An overview of the freezing groups is shown in Figure 5.1.

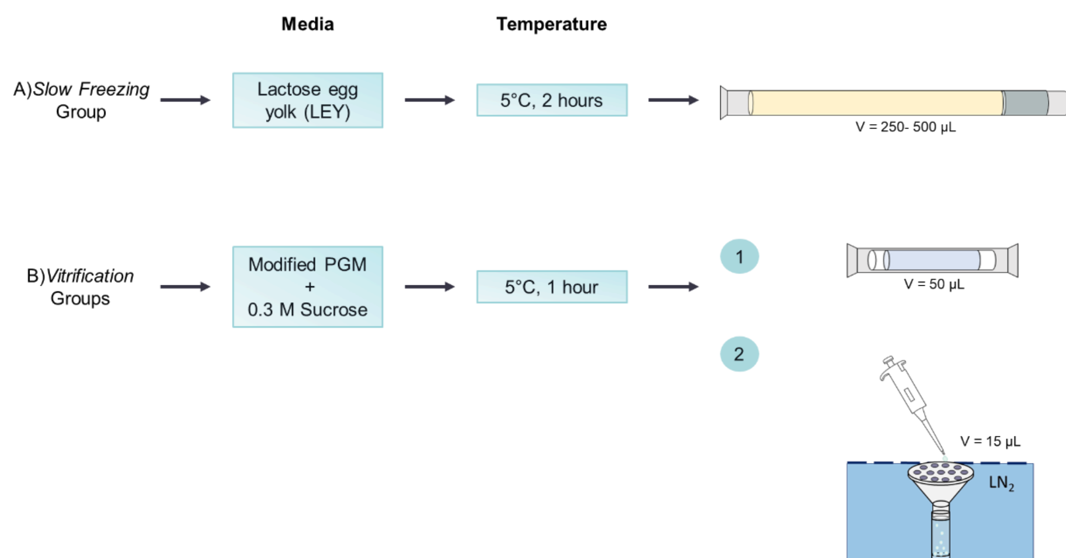

Figure 5.1: Schematic of freezing protocols. A) Slow Freezing protocol where prepared sperm samples were loaded in 0.5-0.25 mL straws and sealed. B) Vitrification protocol where prepared samples were frozen in two distinct methods: mini-straws vitrification method (B.1) and pearl vitrification method (B.2).

5.3.2 Slow Freezing Media

The first medium prepared for the slow freezing sperm method was a lactose and egg yolk-based extender (LEY), composed of 20% (v/v) egg yolk, 80% (v/v) lactose, and 500 µg/mL streptomycin. A second medium (referred as LEY-GE) was composed of LEY, 9% glycerol, and 1.5% Equex-paste (Minitube, Tiefenbach, Germany).

After sperm preparation, the samples were resuspended in LEY and cooled for 2 hours at 5°C. Then, two parts of the LEY solution and sperm were mixed with one part of LEY+ GE (also cooled 2 hours at 5°C) to provide a final concentration of 3% glycerol per sample. The sperm solution was then loaded into 0.25-0.5 mL using a 100-1000 µL manual single-channel pipette, and then heat-sealed. The sealed straws were cooled for 15 min by exposure to LN_2 vapours before being plunged into LN_2. Once the freezing process was complete, the samples were stored a LN_2 tank.

5.3.3 Vitrification Methods

Samples were resuspended in modified PGM containing HEPES buffer and then cooled to 5°C for 1 hour. A cooled sucrose solution (cooled at 5°C) was added to the sample, resulting in a final sucrose concentration of 0.3 M. Immediately after, samples were vitrified in the form of pearls or using mini-straws.

5.3.3.1 Pearl Vitrification

In order to create the frozen sperm pearls, a specialised device was designed by Kitazato Co. (Fujinomiya, Japan) for previous research (O'Neill *et al.*, 2019; Pabón *et al.,* 2019). 15 µL sperm diluted in vitrification media was pipetted into

the device, which was fully submerged in LN_2. The pearls were formed by direct contact with LN_2 and were funnelled into the attached cryovial (Figure 5.2).

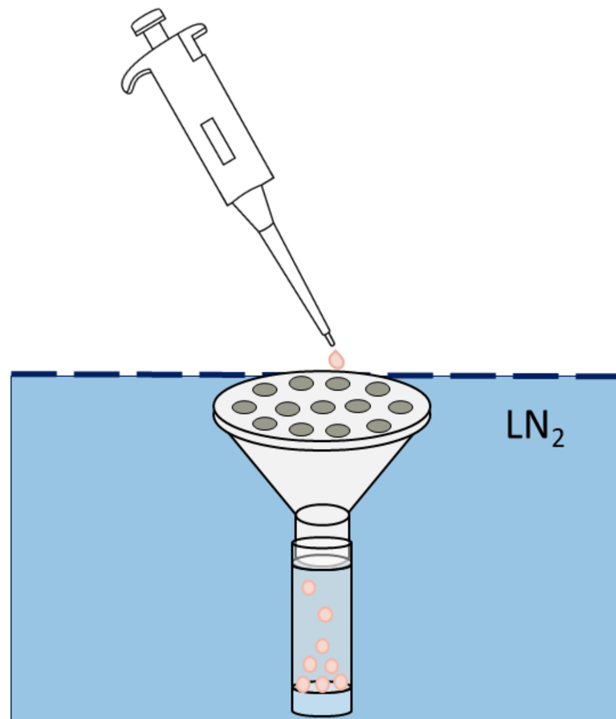

Figure 5.2: Diagram of the Kitazato vitrification device. Droplets of sperm were pipetted into the submerged device, and once vitrified, sink into the cryovial.

5.3.3.2 Mini-Straws Vitrification

50 µL sperm in vitrification medium were injected into a cut 0.25 mL straw. These straws were inserted into a cut 0.5 mL (with a bigger diameter) straw and then sealed before being plunged into LN_2 (Figure 5.3), as published in Isachenko *et al.*, 2005. All VMS samples were stored in the same LN_2 tank.

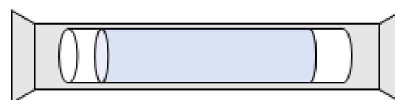

Figure 5.3: Diagram of the mini-straw used to vitrify sperm. 50 µL sperm, diluted in vitrification media, were pipetted into a cut 0.25 mL straw, that was introduced into a cut 0.5 mL straw, sealed before being submerged in liquid nitrogen.

5.3.4 Thawing/Warming and Post-thaw of Samples

Every cryopreservation method had a different thawing (for SF samples) or warming (for vitrification groups) process. These methods are described below.

5.3.4.1 Thawing and Post- thaw Preparation of SF Samples

SF samples were thawed by placing the straws in warm water (37°C for 30s) and resuspended in 500 μL of PGM (Figure 5.4). These samples were prepared using DGS as explained in section 2.4.1. After centrifugation, the supernatant was removed and the pellet was resuspended in 500 μL fresh PGM.

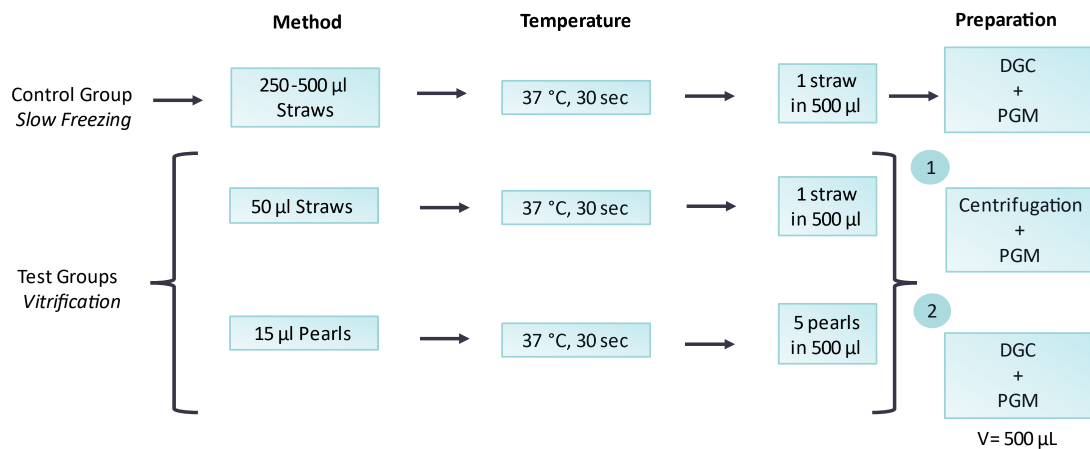

Figure 5.4: Schematic thawing and post-thaw preparation protocol of the different cryopreservation groups.

5.3.4.2 Warming and Post-warming Preparation of Vitrified Samples in Mini-straws

VMS samples were warmed by immersion of the straw in water at 37°C for 30 s. After that, the sealed straw was cut and the content was diluted in 500 μL warm PGM. Subsequently, each sample was centrifugated at 600 G for 7 mins. The supernatant was then discarded, and the pellet resuspended in 500 μL fresh PGM.

5.3.4.3 Warming and Post-warming Preparation of Vitrified Samples in Pearls

Pearls were warmed by submerging 5 pearls of the same sample in 500 µL warm PGM (37°C for 30 s). Prior to sperm evaluation, the samples were prepared in two ways: centrifugation and DGS.

5.3.4.3.1 Centrifugation

The warmed sample was centrifuged for 7 min at 600G. Once the supernatant was removed, the pellet was resuspended in 500 µL fresh PGM. This method was used in the samples that formed the group labelled as VP.

5.3.4.3.2 DGS

The samples that formed the VPD group were prepared using the DGS method. The warmed samples were prepared by DGS as explained in 2.4.1. After the supernatant was removed, pellets were resuspended in 500 µL fresh PGM.

5.3.5 Analysis of Seminal Parameters by Flow Cytometry

Flow cytometry analyses were performed simultaneously, with the help of Marie Claire Aquilina (PhD student at the School of Biosciences, University of Kent), on a BD Accuri C6 Plus (BD Biosciences, Berkshire, United Kingdom). This flow cytometer model has a 14.7 mW laser power at 640 nm wavelengths (Diode Red Laser) and 20 mW laser power at 488 nm wavelengths (Solid State Blue Laser).

In each test, the flow cytometer collected the data from 10,000 events per sample. Analysis of the data was conducted using the BD C6 Plus Software (BD Biosciences, Berkshire, United Kingdom) by gating the forward and side scatter to eliminate remaining debris or aggregates.

5.3.5.1 Sperm Viability and Acrosome Integrity

Sperm viability was assessed using propidium Iodide (PI) and acrosome status was determined using fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Invitrogen™, Inchinnan, United Kingdom) following the protocol explained in (Robles and Martinez-Pastor ,2013).

500 µL staining solution with 1.5 µM PI and 1 µg/mL of FITC-PNA (diluted in PBS) was pipetted into a flow cytometer tube. The sperm was then added to the staining solution at a concentration of 1-2 x 10^6 sperm cells/mL. Prior to analysis, samples were incubated in the dark for 15 minutes at RT. For this test, the positive control samples were placed on dry ice for 1 hour before staining. After testing, the positive control was expected to show that >90% of sperm was dead and acrosome reacted (if not, a new staining solution would be prepared).

5.3.5.1 Sperm Chromatin Structure Assay

The detection of DNA damage and protein alteration in sperm nuclei was assessed via flow cytometry of acridine orange-stained sperm following the protocol explained in Evenson (2014). All solutions used were prepared at the lab and stored at 4°C, unless specified otherwise.

Acid detergent solution was prepared by adding 20 mL 2 M HCl, 4.39 g 0.15 M NaCl, 0.5 mL 0.1% Triton X-100 (HFH10; ThermoFisher, Dartford, UK), and topped up with double distilled water (ddH_2O) to reach 500 mL. The pH was then adjusted to 1.2 using 5.0 M HCl. To prepare the staining buffer, 630 mL 0.2 M sodium phosphate (28.4 g Na_2PO_4 debased to 1.0 L ddH_2O), 370 mL 0.1 M citric acid (21.01 g/L citric acid monohydrate in 1.0 L ddH_2O), 372 mg 1 mM EDTA disodium. and 8.77 g 0.15 M NaCl was stirred for 6 hours, on a stirrer plate, to

ensure complete dissolution of EDTA. The pH was then adjusted to 6.0 using NaOH pellets (ThermoFisher, Dartford, UK).

Acridine orange (AO) staining solution was prepared by adding 600 µL AO stock solution (1.0 mg/mL) to 100 mL of staining buffer. This solution was used for a up to 2 weeks and was stored in amber glass vials. The AO equilibration buffer was prepared by adding 400 µL acid detergent solution to 1.2 mL AO staining solution. The working solution of 1x TNE buffer was prepared by adding 60 mL 10x TNE (Tris-HCl, 52.6 g NaCl, and 2.23 g disodium EDTA in 600 mL ddH$_2$O) and 540 mL ddH$_2$O. After that the pH was adjusted to 7.4.

Prior to testing, the sperm concentration of each sample was calculated as explained in section 2.5.3. Each sample was diluted to a concentration of 1-2 x 106 cells/mL with TNE buffer for a final volume of 200 µL in a conical test tube (12x75 mm). 400 µL acid-detergent was then added, and after 30 seconds, 1.2 mL AO staining solution was added, prior to the sample being inserted in the flow chamber to be measured for 3 replicates. At the end of testing, the flow cytometer was set to perform a sip rinse with ddH$_2$O between samples and a sip clean involving FACS clean solution (BD Biosciences, Berkshire, United Kingdom). Furthermore, during each testing cycle, three quality control checks were performed. First, CS&T RUO calibration beads (661414; BD Biosciences, Berkshire, United Kingdom) were used as the first quality control check to evaluate the performance of the flow cytometer lasers. To check the quality of the reagents, reference samples of known DNA fragmentation levels were analysed, being acceptable up to a 5% coefficient of variation between the samples. For the third quality control check, one of the samples to be assessed

during that day was incubated for 1 hour at 50ºC resuspended in of 5 µL 30% H_2O_2, with the aim of increasing sperm DNA fragmentation levels.

5.3.5.2 Mitochondrial Membrane Potential Assay

The assessment of the mitochondrial membrane potential (MMP) was conducted using Mitotracker Red CMXRos (Invitrogen™, Inchinnan, United Kingdom) following the protocol explained in (Peña *et al.*, 2018). Stock solution of MitoTracker Red (100 µM) was prepared in PBS. 2 µL MitoTracker Red stock was added to each sample (1-2 x 106 cells/mL), giving a final concentration of 200 nM. Samples were incubated in the dark at 37°C for 30 min. Prior to testing, samples were centrifuged (600 G for 5 mins) and then resuspended in prewarmed PBS. To ensure protocol efficiency, a sperm sample was assessed every day for a week to check how the levels of mitochondrial activity dropped with the days (labelled in Figure 5.5A and 5.5B in green as High MMP). As a positive control, an aliquot of the same sample was frozen in dry ice for 1 hour and then thawed at RT before staining (Figure 5.5C). A negative control the sample was assessed with no staining (Figure 5.5D)

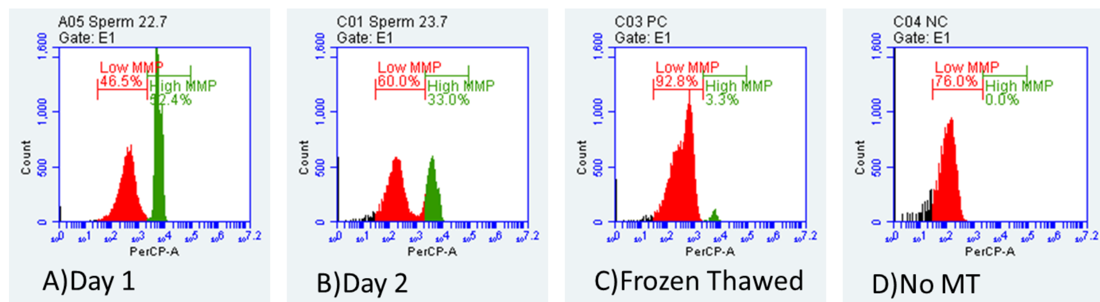

Figure 5.5: Positive and negative controls for Mitochondrial Membrane Potential evaluation (MMP). Figure A shows in read the low MMP and in green high MMP of the sperm sample the day that arrives to the lab. Figure B shows how the MMP decreases when the same sample is tested the following day from its arrival at the lab. Figure C shows how MMP drastically decreases when the sample is frozen in dry ice. Figure D shows how High MMP % is cero in absence of Mitotracker Red CMXRos.

5.3.6 Statistical analysis

Data were analysed on SPSS (Version 25, IBM). The statistical analysis of the samples, both before and after cryopreservation, and two different methods of post-thaw (VP and VPD) were assessed using one-way ANOVA in those parameters that had a normal distribution. The data parameters that did not have a normal distribution were tested using the Krustal-Wallis Test.

5.4 Results

5.4.1 Specific Aim 3A: To Compare Boar Sperm Parameters Between Three Different Cryopreservation Techniques (Slow Freezing, Vitrification Using Pearls, and Vitrification Using Mini-Straws)

5.4.1.1 Total and Progressive Motility Prior to Freezing and After Thawing

In comparison to the average motility (PT= 80.20 ± 3.8%) of samples prior to cryopreservation (N= 10), all cryopreservation techniques significantly reduced the progressive and total motility after thawing (Figure 5.6). When comparing only vitrification groups, the total motility of the vitrified samples using the pearls method (26.15 ± 2.8%) was significantly higher than that of the mini straws (9.39 ± 0.9%). Moreover, when the samples were prepared using DGS after warming, this parameter was significantly reduced for VP (26.15 ± 2.8%) and VPD (5.93 ± 1.5%). However, slow freezing had a significantly higher percentage (42.20 ± 4.3%) of total motility when compared to vitrified groups. Progressive motility was significantly higher in the SF group (25.28 ± 4.4%) but did not differ between VP (8.31 ± 1.4%), VPD (2.78 ± 1.1%), and VMS (1.44 ± 0.6%).

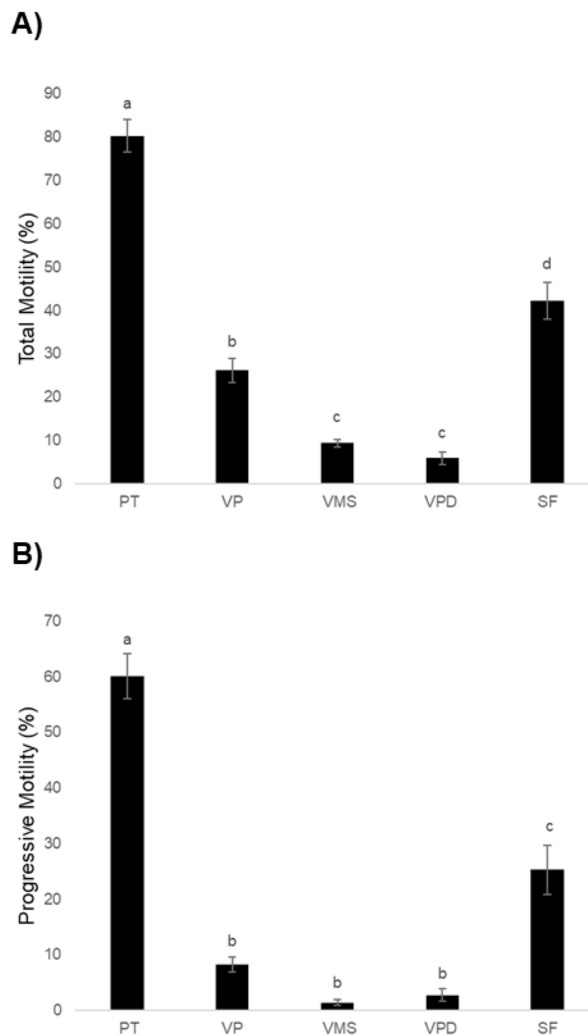

Figure 5.6: Proportion of total (A) and progressive motility (B) of each group prior and post cryopreservation. Data are shown as mean percentage ± SEM for N= 10 replicates per group. As data were not normally distributed, a Krustal-Wallis Test was performed (gl= 4, p <0.001; $H_{Total\ Motility}$= 42.119; $H_{Progressive\ Motility}$= 37.165). ^{a,b,c,d} Different superscript letters indicate significant differences between groups, samples that share the same letter are not statistically significant from each other. PT= Pre-test. VP= Vitrification pearls. VMS= Mini-straws. VPD= Vitrification pearls + DGS. SF= Slow freezing.

Cryopreservation also has a negative impact on mitochondrial activity (PT= 29.46 ± 7.13%). The results shown in Figure 5.7 indicate no differences in mitochondrial activity between the vitrification methods, but the mitochondrial membrane potential was significantly higher in the SF group (15.73 ± 3.4%) compared to VP (5.70 ± 1.1%), VPD (5.01 ± 1.2%), and VMS (2.10 ± 0.5%).

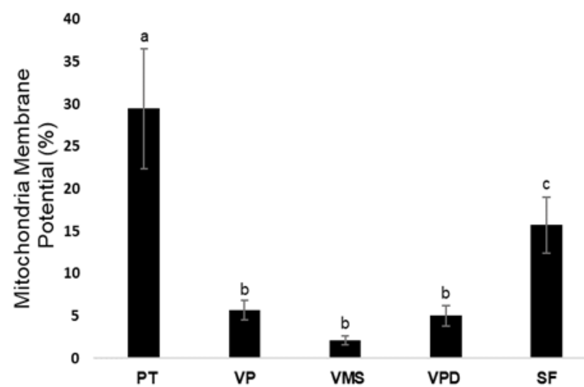

Figure 5.7: Mitochondria membrane potential of each freezing group pre- and post-cryopreservation. Data are shown as mean percentage ± SEM for N= 10 replicates per group. As data was not normally distributed, a Krustal-Wallis Test was performed (gl= 4, p= 0.002; H= 16.803). [a,b,c,] Different superscript letters indicate significant differences amongst the groups; samples that share the same letter are not statistically different from each other. PT= Pre-test. VP= Vitrification pearls. VMS= Mini-straws. VPD= Vitrification pearls + DGS.

5.4.1.2 Sperm Morphology Prior to Freezing and After Thawing

The evaluation of the sperm morphology (N= 10) before and after freezing (Table 5.1) revealed a significantly higher percentage of anomalies in all cryopreservation groups relative to PT (17.8 ± 2.6%). The VPD and SF groups, where the samples were prepared using DGS after warming, had the highest number of abnormalities (39.6 ± 3.8% and 40.4 ± 1.9%, respectively). This was followed by the VMS group (35.2 ± 1.8%), but was not significantly different to VPD and SF. The VP group had a significantly lower percentage of all the cryopreservation groups (28.6 ± 2.1%).

When taking into account the different types of abnormalities, no significant differences were observed in the percentage of CDs present in any of the groups. The percentage of tails abnormalities observed in the control group (PT= 5.7 ± 1.5%) was significantly lower than that of the cryopreservation groups but there were no significant differences in tail abnormalities between VP

(18.3 ± 2.0%), VPD (23.2 ± 3.1%), VMS (18.2 ± 2.9%), and SF (26.7 ± 2.5%). The percentage of head abnormalities revealed more intergroup variation. After the control group (5.9 ± 1.1%), VP had the second lowest (8.8 ± 0.9%) levels of head abnormalities, albeit there was no statistically significant difference between the two groups. The SF group had a significantly higher (10.0 ± 1.2%) proportion of head abnormalities than PT but did not differ significantly from VP, whereas VPD (12.6 ±1.5 %) did significantly differ from PT and VP, but not from SF. Lastly, VMS (13.0 ± 1.6%) revealed a significantly higher proportion of head abnormalities compared to all the groups apart from VPD.

Table 5.1: Results obtained from the evaluation of the sperm morphology and sperm concentration before and after cryopreservation.

Group	Abnormal Mean % ± SEM	Head Abnormality Mean % ± SEM	Tail Abn. Mean % ± SEM	CD Mean % ± SEM	[] sperm Million/mL Mean ± SEM
PT	17.8 ± 2.6 [a]	5.9 ± 1.1 [a]	5.7 ± 1.5 [a]	6.0 ± 1.9	12.9 ± 2.6 [a]
VP	28.6 ± 2.1 [b]	8.8 ± 0.9 [ab]	18.3 ± 2.0 [b]	1.5 ± 0.6	0.8 ± 0.1 [b]
VMS	35.2 ± 1.8 [c]	13.0 ± 1.6 [d]	18.2 ± 2.9 [b]	4.0 ± 1.3	1.1 ± 0.1 [b]
VPD	39.6 ± 3.8 [c]	12.6 ± 1.5 [cd]	23.2 ± 3.1 [b]	3.9 ± 1.8	0.5 ± 0.1 [b]
SF	40.4 ± 1.9 [c]	10.0 ± 1.2 [bc]	26.7 ± 2.5 [b]	3.7 ± 1.0	2.6 ± 0.6 [b]
p value*	<0.001	0.002	<0.001	0.261	<0.001
F*	15.176	5.162	11.969	1.370	17.320

Data are shown as mean percentage ± SEM (N= 10 replicates for each group).
* ANOVA Test (gl= 4). **a,b,c,d** Within each column, different superscript letters indicate significant differences amongst the groups; samples that share the same letter are not statistically significant from each other. F calculated as variation between sample means/variation within the samples. PT= Pre-Test; VP= Vitrification Pearls; VMS= Mini-Straws; VPD= Vitrification Pearls + DGS; SF= Slow Freezing.

5.4.1.3 DNA Damage and Stainability Prior to Freezing and After Thawing

In using the flow cytometer to stain sperm cells with acridine orange (to assess DNA damage), there was no significant difference in the DNA fragmentation index (DFI) percentage between the control (1.6 ± 0.2%), VP (1.2 ± 0.2%), VPD (1.2 ± 0.4%), and SF (1.5 ± 0.3%) groups, as seen in Figure 5.8A. However, VMS had a significantly higher DFI (5.1 ± 0.1.7%) in comparison. The lowest percentage of immature spermatozoa, identified as spermatozoa having high DNA stainability (HDS), was the VPD group (0.8 ± 0.1%) which was significantly different to the VMS and VP groups. VMS has the highest (2.2 ± 0.2%) HDS levels, which was significantly higher than the VPD and SF groups.

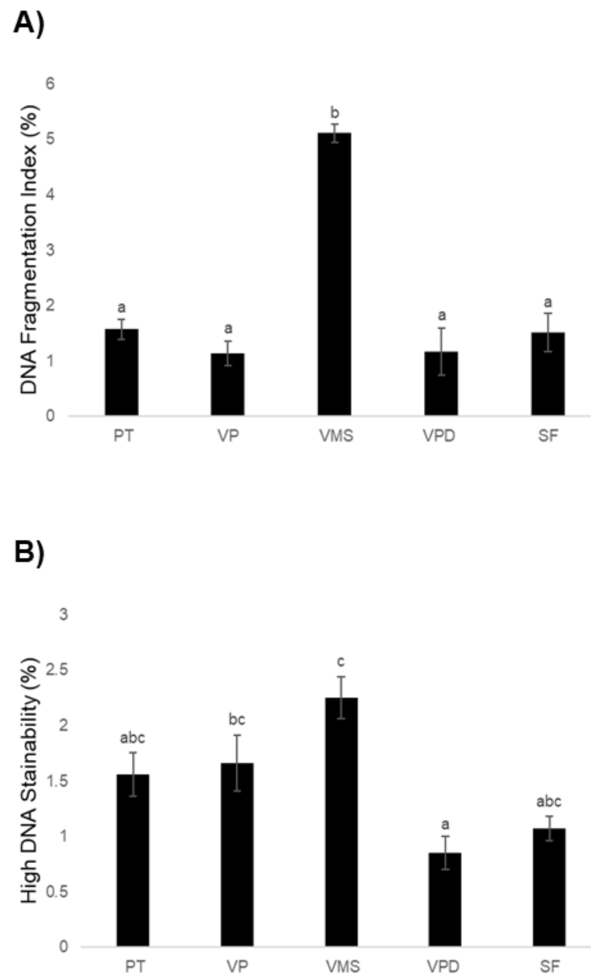

Figure 5.8: Results from the evaluation of DNA Fragmentation Index (A) and high DNA stainability (B) under the flow before and after cryopreservation. Data are shown as mean ± SEM for N= 10 replicates per group). As data was not normally distributed, a Krustal-Wallis Test was performed (gl= 4; p_{DFI}= 0.025, p_{HDS} <0.001; H_{DFI}= 11.131, H_{HDS}= 21.004.). [a,b,c,d] Within each column, different superscript letters indicate significant differences amongst the groups; samples that share the same letter are not statistically significant from each other. H was calculated as variation between sample means/variation within the samples. PT= Pre-Test. VP= Vitrification Pearls. VMS= Mini-Straws. VPD= Vitrification Pearls + DGS. SF= Slow Freezing.

5.4.1.4 Sperm Vitality and Acrosome Status Prior to Freezing and After Thawing

In the SF group, the total percentage of alive sperm cells (Table 5.2) was not significantly different to the control group (81.6 ± 5.4%). VP and VPD had similar percentages (32.0 ± 3.0% and 33.7 ± 8.3%, respectively), both of which were significantly lower the control and SF. The lowest percentage of alive sperm was

in the VMS group, which was significantly different to the other groups. The proportion of alive sperm with reacted acrosome (Alive-Acr) is not significantly different between the groups, with the highest percentages being found in the control and VP groups (15.8 ± 8.2% and 15.4 ± 4.3%, respectively).

Table 5.2: Results from the evaluation of vitality and acrosome status of the sample under the flow before and after cryopreservation.

Group	Alive Mean % ± SEM	Alive-Acr Mean % ± SEM	Total Alive Mean % ± SEM
PT	65.8 ± 9.0 [a]	15.8 ± 8.2	81.6 ± 5.4 [a]
VP	16.6 ±3.3 [b]	15.4 ± 4.3	32.0 ± 3.0 [b]
VMS	6.7 ± 1.5 [b]	3.8 ±1.2	10.6 ± 2.5 [c]
VPD	25.4 ± 6.8 [b]	8.3 ± 2.7	33.7 ± 8.3 [b]
SF	71.4 ± 2.3 [a]	6.1 ± 1.4	77.5 ± 2.8 [a]
p value*	<0.001	0.138	<0.001
H*	31.878	6.965	37.333

Data are shown as mean ± SEM (N= 10 replicates for each group).
*Krustal-Wallis Test performed for data not normally distributed (gl= 4). [a,b,c] Within each column, different superscript letters indicate significant differences amongst the groups; samples that share the same letter are not statistically different from each other. H calculated as variation between sample means/variation within the samples. PT= Pre-Test; VP= Pearls; VMS= Mini-Straws; VPD= Vitrification pearls + DGS; SF= Slow Freezing; Alive-Acr= alive sperm with reacted acrosome.

5.5 Discussion

5.5.1 Limitations of the Experiment

Initial experimental plans for this chapter included the use of frozen-thawed sperm samples for IVF experiments to highlight any potential impacts these methods may have on subsequent embryo quality and development. However, due to the impact of the Coronavirus Disease 2019 (COVID-19) pandemic in which porcine ovaries were unavailable (see section 8.1), these confirmatory experiments were unable to be performed.

5.5.2 Total and Progressive Motility Prior to Freezing and After Thawing

Slow freezing is still more efficient than vitrification as cold shock deteriorates mitochondrial activity in vitrification groups, resulting in a drastic decrease in the membrane potential when compared to slow freezing. The preservation of sperm mitochondrial activity is critical for preserving sample quality since it is directly related to a reduction in motility (Kasai *et al.*, 2002), so modifying the thawing method might help to mitigate the damage. The standard method to thaw in boar sperm is to warm the sample at 37°C for 20-30 seconds (Yeste, 2016; Carvajal *et al.*, 2004; Arraztoa *et al.,* 2017). However, vitrification protocols for oocytes and blastocysts showed a reduction in ice formation and osmotic stress post-thaw with a shorter and drastic change of temperature (Ding *et al.*, 2004; Parmegiani *et al.*, 2014). Moreover, previous publications have demonstrated that shorter periods of time at higher temperatures correlate with improvements in sperm parameters post-thaw (Tomás *et al.*, 2014; Casas *et al.*, 2009).

5.5.3 Sperm Morphology Prior to Freezing and After Thawing

As expected, the number of abnormalities in cryopreserved sperm samples were higher than pre-cryopreservation numbers. Nevertheless, the data in this chapter revealed that the structural damage caused by the slow freezing of boar sperm samples was significantly higher than when using the most suitable vitrification method, VP. The gradual decrease in temperature during the slow freezing process promotes the formation of intra- and extracellular ice crystals, which can cause morphological damage (Yeste, 2016). The ultra-fast drop in temperature during the vitrification process, caused by the direct contact of samples with LN_2, minimises the time in which samples are between -5°C

and -60°C to prevent ice crystal formation (Rodriguez-Martinez *et al.,* 2011). When comparing VP to VMS, the increase in abnormalities in VMS might be caused by the larger sperm volumes used and the insulation provided from the straws, reducing the speed in which the sample is vitrified. The differences found between VP and VPD can only be caused by the warming protocol as the vitrification method is the same; indicated by the significant decrease in viability, sperm which have been vitrified are more susceptible to damage and are less likely to tolerate the damage caused by the centrifugation or solutions from DGS.

5.5.4 DNA Damage and Stainability Prior to Freezing and After Thawing

It has been demonstrated that DFI over 30% in humans, as measured by the sperm chromatin structure assay, decreases fertility both *in vivo* and *in vitro* (Cissen *et al*., 2016; Kumaresan *et al*., 2017; Simon *et al*., 2017). However, DFI levels greater than 5% in boar sperm samples are considered poor quality (López-Fernández *et al*., 2008). In this chapter, vitrification using mini straws was the only method that resulted in DFI levels being greater than 5%. Additionally, the lowest number of viable sperm following thawing was from the use of vitrification using mini straws. Therefore, based on these findings, it can be confirmed that vitrification with mini straws is not ideal for use in boar sperm. This contradicts what was published by Díaz-Jiménez in 2018 for the vitrification of donkey sperm, where the use of straws demonstrated a better efficiency than pearls.

5.5.5 Sperm Vitality and Acrosome Status Prior to Freezing and After Thawing

Despite the decrease in morphological damage and the maintenance of DNA integrity, the vitality of the vitrified samples was drastically reduced. This is also associated with the decreased in the mitochondrial activity of the sperm which is better preserved by slow freezing. The results also showed a decrease in the percentage of sperm with reacted acrosome. This result was unexpected since the cryopreservation process and the centrifugation steps during the thawing/warming process need to have an effect on the acrosome status.

However, the media used in each protocol may have been crucial in preserving the vitality of the sample. Slow freezing has the advantage in that the media used for this method has been extensively studied and implemented for decades (Amidi *et al.,* 2016), whereas the vitrification media consisted of a standard IVF medium with the addition of sucrose, a cryoprotectant used in sperm vitrification protocols for other species (Isachencko *et al.,* 2008; O'Neill *et al.,* 2019; Díaz-Jiménez *et al.,* 2019). It is possible that increasing the complexity of the vitrification media through the addition of antioxidants or chelating agents might be useful to increase the efficiency of vitrification and prevent cooling damage (Silva *et al.,* 2015).

5.6 Conclusion

The data presented in this chapter corroborates the fact that vitrification decreases the viability of the samples after thawing, as with any other type of cryopreservation. However, compared to the current literature, the results demonstrate better outcomes when performing boar sperm vitrification, allowing for the successful recovery of samples with improved survivability, a reduction

in DNA damage, and the preservation of motility after thawing. The optimal vitrification-thaw protocol for boar sperm was determined, with the pearl method being the most suitable of the vitrification techniques for use with boar sperm. This demonstrates that vitrification is also a method available for boar sperm cryopreservation, but slow freezing is still more sustainable and efficient to cryopreserve boar sperm so further research is needed to improve the efficacy of vitrification.

Publication (in preparation) arising from this chapter:

Serrano Albal, M., Aquilina, M.C., Zak, L.J., Ellis, P.J., and Griffin, D.K. Successful Recovery of Motile and Viable Boar Sperm after Vitrification with Different Methods (Pearls and Mini straws) Using Sucrose as a Cryoprotectant. *To be submitted to Cryobiology in January 2023*.

Personal contribution:

- Presented the results of the study in the form of poster presentation at ICAR 2020+2
- Design of the study
- Data collection and Interpretation of statistical analyses
- Manuscript preparation: writing of the first draft and following modifications

6 Improving Pregnancy and Live Birth Rates by Using PGT-A to Screen IVP Bovine Embryos

6.1 Background

At the preimplantation stage of development, cattle are one of the most studied mammalian species (after humans and mice). With the increased demand for beef and dairy products, IVP approaches are extensively used for breeding to reduce generation intervals and enable the movement of genetic resources in a biosecure manner (Ferré *et al.,* 2020), and has prompted the application of novel IVP strategies that integrate the recent developments in embryology with bioinformatics and genomics (see section 1.6). Prior to the implementation of genetic analyses, embryos were primarily selected for transfer based on morphological assessments and scoring. However, the morphological assessment of embryo quality is not a guarantee of ploidy status.

Approximately one million IVP cattle embryos are transferred each year, with a growing number being checked for GEBVs prior to ET (Mullaart and Wells, 2018; Fujii *et al.,* 2019). As noted in section 1.6, using a genomic evaluation to determine an individual animal's breeding value is not a novel concept (Meuwissen *et al.,* 2001). This trend has been expedited by the in-depth characterisation of the bovine genome sequence, which has enabled the development of precise models that describe the relationship between certain genetic markers (typically SNPs) and desirable traits. GEBVs have typically been determined shortly after birth, but modern screening at the embryonic stage is advantageous by reducing generation intervals even further (enabling the rapid introduction of new genetics) and increasing the selection intensity (by increasing the pool of screened individuals). This method consists of four steps,

which are summarised as follows: biopsy of the trophectoderm, WGA, SNP array interrogation, and subsequent bioinformatics analysis (see section 1.6.1). The embryo transfer subsequently proceeds as it would during any other IVP process. Depending on the quality of the embryos and the production system used (for a comprehensive review, see Hansen, 2020), the proportion of live births per ET using current IVP methods is around 50%. Furthermore, no GEBVs have been identified to date that can predict the likelihood of embryo implantation.

The origin of aneuploidy can occur in either gamete (predominantly the oocyte) during meiosis or within the embryo during the mitotic divisions of preimplantation development. The latter can result in mosaicism, a mixture of euploid and abnormal cells, and there has been much debate surrounding the use of PGT-A to screen for chromosomal abnormalities prior to ET. Even though the higher viability of euploid embryos appears to be well established, the results of a recent randomised controlled experiment were conflicting, revealing that PGT-A had no overall benefits other than in older age groups (Munné *et al.,* 2019). A negative impact on embryo viability due to the biopsy procedures (a requirement of established PGT-A methods) or incorrect diagnoses resulting in embryo wastage could explain this discrepancy (Pagliardini *et al.,* 2020). However, it has been suggested by many cohort and retrospective studies that PGT-A is beneficial (Griffin and Ogur, 2018).

Chromosomal abnormalities have been observed in 25 to 40% of embryos produced by bovine IVP (Hornak *et al.,* 2016; Turner *et al.,* 2019; Tutt *et al.,* 2021), which can result in the failure of early embryo development, pregnancy loss or the birth of infertile offspring, as seen in humans. Nevertheless, PGT-A

has only recently been adapted to cattle screening, demonstrating promising results (Turner *et al.,* 2019) despite not being widely established in animal breeding. Breeders have the opportunity to improve embryo selection and increase the likelihood that each transferred embryo will result in a pregnancy by analysing SNP chip data to combine GEBV with PGT-A. In using genotyped embryo biopsy material, the chromosome copy number (Attiyeh *et al.,* 2009), the position of recombination events and parental origin of aneuploidy (Handyside *et al.,* 2010), and the mitotic or meiotic origin of trisomies (Gabriel *et al.,* 2011) can be determined.

6.2 Specific Aim 4

The specific aim of this chapter was to test the hypothesis that the use of PGT-A to screen IVP bovine embryos using key SNP chip data from GEBVs improves pregnancy and live birth rates. This was tested through the following aims:

- Specific aim 4A: To compare the live-birth rate of *in vitro* produced bovine embryos in euploid and aneuploid embryos thereby testing the hypothesis that aneuploid embryos have little chance of being viable, and that PGT-A is thus effective for cattle
- Specific aim 4B: To determine the nature and incidence of chromosomal abnormalities in bovine development

6.3 Materials and Methods Specific to this Chapter

6.3.1 General Considerations

The methodology described in this chapter (Sections 6.3.2 to 6.3.4) relating to ovarian stimulation, generation of embryos, embryo biopsies, WGA, SNP genotyping, and ETs were performed by L'Alliance Boviteq inc. between 2016 to 2018 in a controlled commercial environment The contribution from the University of Kent was the data collection and statistical analysis, including the creation and assessment of karyomaps, GG plots, LRR graphs, and BAF graph analysis.

No live animals or embryos were used to obtain the data for this chapter. All work undertaken was performed entirely *in silico* using archived information from L'Alliance Boviteq inc. (Saint-Hyacinthe, Canada) as part of their routine business operations.

6.3.2 *In Vitro* Production of Embryos by L'Alliance Boviteq Inc.

48 h after the dominant follicle was removed, dams underwent ovarian stimulation for 3 days (Landry *et al.*, 2016), including six injections of FSH (Folltropin-V, Bioniche Animal Health, Belleville, Canada) administrated at 12 h intervals. This was followed by a period of 44 h in absence of FSH. COCs were retrieved by OPU in warm HEPES-buffered Tyrode's medium with heparin (10 IU/mL). Recovered COCs were matured *in vitro* for 24h at 38.5°C in atmospheric CO_2, in HEPES-buffered media as, described in Turner *et al.* (2019).

For IVF, frozen-thawed spermatozoa were selected by discontinuous gradient centrifugation (BoviPure, Nidacon, Göthenborg, Sweden). The gametes were

co-incubated in modified Tyrode's lactate medium supplemented with 6 mg/mL BSA, 0.2 mM pyruvate, 2 μg/mL heparin, 50 μg/mL gentamycin, and PHE (Miller et *al.*, 1994) at 38.5°C for 18-22 h under 5.5% CO_2. The obtained embryos were cultured in droplets of modified SOF medium supplemented with nonessential amino acids, 3 μM EDTA, and 4 mg/mL BSA at 38.5°C under 6.5% CO_2 and 5% O_2.

6.3.3 Blind Retrospective Study Design (L'Alliance Boviteq Inc.)

The data collected in this period consisted of genotyped DNA from embryos (N= 1,737) and their parents (N= 168 Holstein dams and N=73 Holstein sires) using the Illumina SNP chips summarised in Table 6.1. A requirement of Karyomapping analysis is the need for two half siblings or one full sibling, meaning 24 embryos were excluded from the analysis.

Table 6.1: Variety of SNP chips employed used to generate data. All SNP chips used were manufactured as part of llumina's Bovine range.

SNP Chip Name	Number of SNPs	Embryos Tested (n)	Parents Tested (n)
GGP Bovine HD 150k v01	138,892	379	-
GGP Bovine HD 150k v03	139,376	1,241	-
GGP Bovine HD 150k v04	140,668	112	-
GGP Bovine LD v04	30,105	5	-
GGP BovineSNP50	45,187	-	241

Any information pertaining to embryo stage, quality, outcome following ET, and parental age were blinded from the dataset, and only disclosed by L'Alliance Boviteq inc. after PGT-A analysis.

6.3.4 Limitations of Karyomapping Analyses

Due to the limited number of SNPs available for chromosome Y, the Karyomapping haploblock tracing could not be implemented, meaning the chromosome could not be analysed reliably. Similarly, any errors present on paternal chromosome X could not be analysed as haploblock tracing is prevented due to the single copy number in the sire.

6.3.5 Statistical Analysis

The statistical analysis of binomial data was completed on SPSS (version 26, IBM) and compared by fitting a binomial or a multinomial GLM with logit link functions. Multiple comparisons were performed using the Bonferroni correction, and ANOVA or linear regression analyses were used to investigate continuous variables as appropriate. Percentages were reported with 95% CIs, and the significance was set at $p = 0.05$. All embryos transferred had pregnancy and live birth checks conducted in this study ($N = 1713$), with checks for birth weight performed on 656 out of 700 live born calves.

6.4 Results

6.4.1 Specific Aim 4A: To Compare the Live-Birth Rate of *in vitro* Produced Bovine Embryos in Euploid and Aneuploid Embryos Thereby Testing the Hypothesis that Aneuploid Embryos Have Little Chance of Being Viable, and that PGT-A is thus Effective for Cattle

In total, 1713 embryos were selected for SNP genotyping and subsequent analysis, resulting in 892 pregnancies and 700 live births. Of the 700 live births, 688 were derived euploid embryos and 12 from aneuploid embryos.

The combination of Karyomapping and LRR/BAF plots allowed for the determination of aneuploidy levels, with 14.1% (N= 241/1713) of embryos carrying a chromosomal abnormality. Analysis of ET outcomes found that euploid embryos resulted in a pregnancy rate of 59.6% (N= 878/1472) and a live birth rate of 46.7% (N=688/1472). In comparison, chromosomally abnormal embryos resulted in a pregnancy rate of 5.8% (N= 14/241) and a live birth rate of 4.9% (N= 12/241; detailed overview in Table 6.2), with the difference being statistically significant in both cases ($p < 0.0001$). In favour of euploid embryos, the associated Odds Ratios (OR) for pregnancy and live birth rates were 24.0 (95% CI: 13.8-41.5) and 16.7 (95% CI: 9.3-30.2). Therefore, the positive predictive value (PPV) associated with the PGT-A test performed for live birth rates was 98.3% (95% CI: 97.1% - 99.0%).

Table 6.2: Overview of live birth cases from aneuploid embryos. UPD= Uniparental disomy. Chr= chromosome.

Sample	Sex	Embryo Stage	Embryo Grade	Diagnosis	Chr
1536	M	6	1	Maternal Monosomy	4
816	F	5	2	Maternal Monosomy	22
1173	M	7	1	Maternal Trisomy	4
37	M	7	1	Maternal Trisomy	26
1437	M	6	1	Maternal Trisomy	26
1127	M	6	1	Maternal Trisomy	27
1524	M	5	2	Mitotic Trisomy	6
257	M	6	2	Segmental Deletion	17
482	F	6	2	UPD	6
1021	M	8	1	Hypotriploidy (polyspermy)	-
353	M	6	2	Hypotriploidy (polyspermy)	-
1407	M	6	2	Hypotriploidy (polyspermy)	-

According to the archived database, transferring embryos without performing PGT-A resulted in a pregnancy rate of 52.1% and a live-birth rate of 40.9%, implying that elective transfer of PGT-A selected euploid embryos would have increased pregnancy rates by 7.5% (p <0.0001, OR=1.36) and live-birth rates by 5.8% (p=0.001, OR=1.27). The exclusive use of euploid embryos would have reduced the number of ETs needed to obtain a livebirth from 2.45 to 2.14 ETs/livebirth (Figure 6.1).

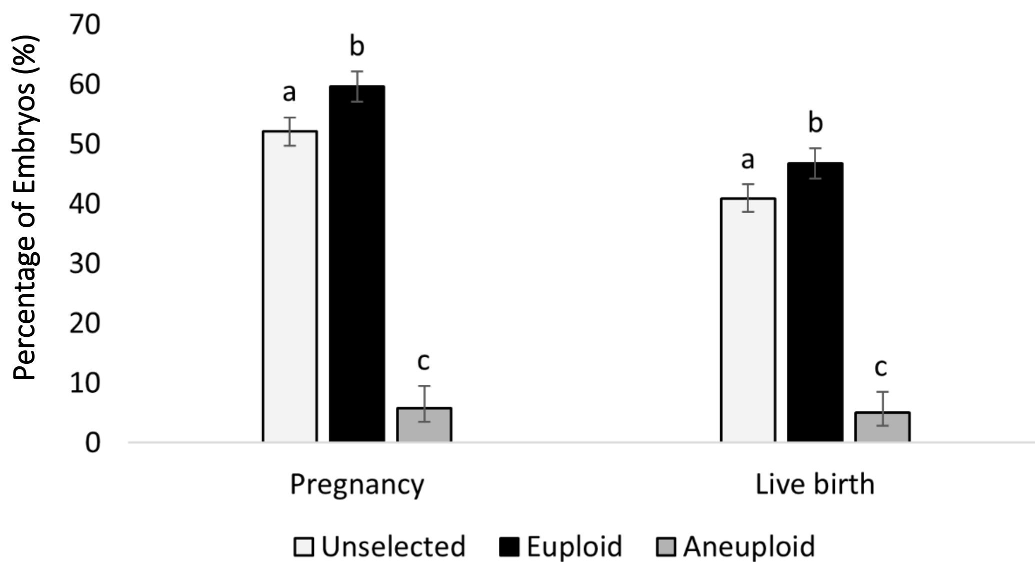

Figure 6.1: Effect of embryo ploidy on pregnancy and live birth outcomes. Embryos diagnosed as euploid (N= 1472) after PGT-A analysis had higher pregnancy and live birth rates than embryos that did not undergo PGT-A selection or chromosomally abnormal embryos. Data represented as percentage with 95% CI for N= 1713 replicates.

Of the 878 euploid embryos transferred, 688 (78.4%) were able to establish a clinical pregnancy at D60 post-transfer and yield a live birth. Conversely, the live birth rate was 85.7% (N= 12/14) among the few aneuploid embryos that also led to clinical pregnancies at Day 60. This difference was not statistically significant (p= 0.51); however, it was not possible to distinguish between early pregnancy loss (before Day 60) and embryo mortality since there was no information

available on miscarriage within the first few weeks. Despite the limitations presented by the low number of aneuploid embryos surviving in utero after Day 60, the data seems to indicate the presence of a bottleneck for aneuploid embryos before Day 60 (whether this be an embryo attachment failure or early miscarriage), with aneuploid and euploid embryos being indistinguishable in terms of their ability to proceed to term.

6.4.2 Specific Aim 4B: To Determine the Nature and Incidence gf Chromosomal Abnormalities in Bovine Development

6.4.2.1 Maternal Meiotic Errors are the Most Common Chromosomal Abnormality

A variety of chromosomal abnormalities were identified using a combination of PGT-A algorithms, including whole chromosome and segmental errors, and determined whether the paternal or maternal gamete was the origin of the error. Furthermore, the use of GG plots allowed for the origin of chromosomal errors (mitosis, meiosis I, meiosis II) to be identified. However, for monosomies, the same method was unable to specify whether the error originated during meiosis or during embryo development; monosomies that were only discovered in LRR/BAF plots and not by Karyomapping were considered to have originated *de novo* in the embryo (thus of mitotic origin).

Overall, with an incidence of 47.6% (N= 139/292), monosomy was the most common chromosomal abnormality detected. In comparison to monosomies, trisomies were less prevalent with 38.7% (N= 113/292). The remaining chromosomal abnormalities identified included UPD, triploidy/hypotriploidy, and segmental errors (Table 6.3).

Table 6.3: Types of chromosomal abnormalities and frequency identified via PGT-A. A) Overview of all detected chromosomal errors, including a breakdown by origin (paternal germline, maternal germline, embryonic) and origin. B) Summary of whole chromosome errors, broken down by origin and error class (trisomy, monosomy, uniparental disomy (UPD)). Further information is provided for trisomies to indicate whether the errors arose during meiosis I, meiosis II or embryonic development (mitotic errors). For UPDs, the origin is considered to be from the embryo in cases of a mosaic configuration or from the parent passing two copies of its own chromosomes.

A)		*Origin*		
Aneuploidy class	**N**	**Dam**	**Sire**	**Embryo**
Segmental errors	21	2	14	5
Triploidy and hypotriploidy	16	6	10	-
Whole chromosome	255	212	16	27
Total errors	292	220	40	32

B)		*Origin*		
Whole chromosome	**N**	**Dam**	**Sire**	**Embryo**
Trisomy	113	90	1	22
MI	84	83	1	-
MII	7	7	-	-
Mitotic	22	-	-	22
Monosomy	139	120	15	4
UPD	3	2	-	1

In the cases for which a parental origin could be identified, the majority of chromosomal errors originated in the oocyte (75.3%, N= 220/292). Specifically, the use of GG plots for trisomy analysis (complete breakdown in Supplementary Table S9) revealed that the errors which arose during maternal meiosis I accounted for 73.5% of all trisomy events (N= 83/113), suggesting that a similar percentage of monosomies may have had the same origin. The origins of segmental errors and triploidy/hypotriploidy (a potential consequence of polyspermy and/or meiotic errors) were the only abnormalities which could be more prevalently attributed to the paternal germline.

6.4.2.2 Parental Effects on Aneuploidy Incidence

The average age of the donors (N= 168) was 10.3 ± 0.7 months. Groups were used to record sire age (N= 63) and were defined as less than 10 months, 10 to 10.5 months, 10.5 to 11 months, 11 to 12 months, and greater than 12 months. Samples were collected from most males at either 10.5 to 11 months (N= 9) or greater than 12 months of age (N= 12).

Aneuploidy rate seemed to be influenced by the specific parent (sire or dam) used in the IVP cycle. There was no evidence of a sire-specific effects (p= 0.636), but certain dams were more likely to produce aneuploid embryos (p= 0.0002). Conversely, the age of the donor dam appeared to have no effect on the incidence of aneuploidy (p= 0.678), most likely a result of the young average age of the dams. Interestingly, a significant effect of sire age on the incidence of aneuploidy was discovered (p= 0.002); subsequent analyses suggest this result may be due to a significant decrease in the incidence of aneuploidy associated with sperm samples collected from sires between the ages of 11 and 12 months (Figure 6.2). Nevertheless, it should be noted that a comprehensive study on the effects of parental age on aneuploidy incidence is not truly applicable to the wider breeding population given the relatively young age of the animals employed.

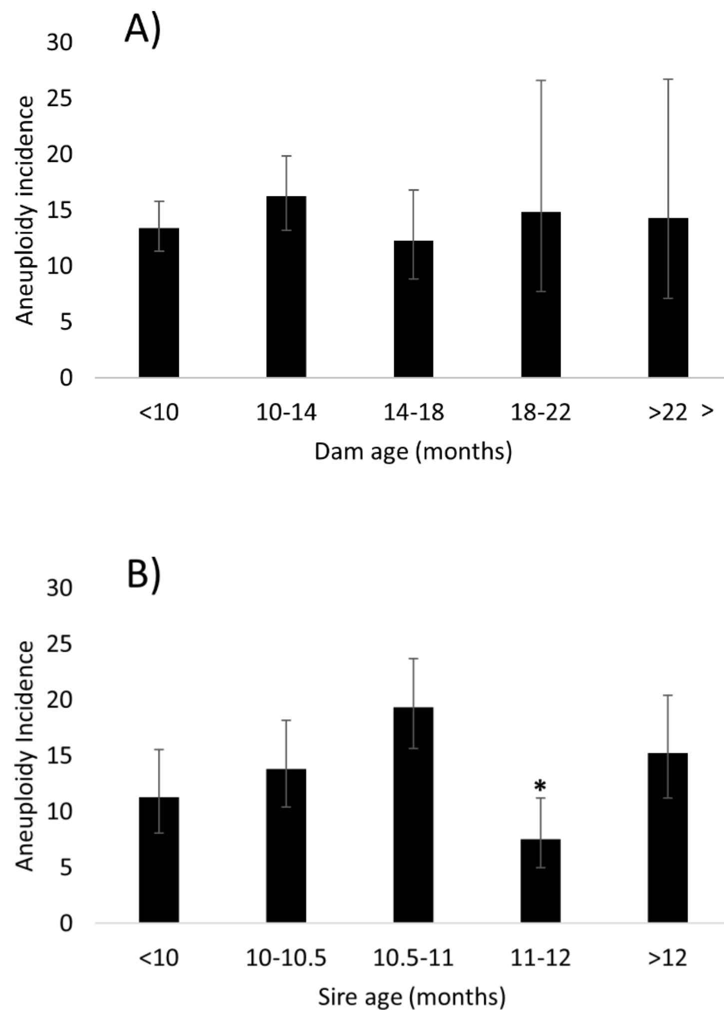

Figure 6.2: Incidence of embryo aneuploidy by parental age. A) Regression analysis indicating there is no significant interaction between dam age (N= 168) at oocyte collection and aneuploidy incidence in the resulting embryos (R^2= 0.0142, p >0.05). B) The use of older sires (N= 63) did not have a negative effect on embryo ploidy; the asterisk denotes a statistically significant decrease in the incidence of aneuploidy for that age group. Data presented as percentage (%) with 95% C.I.

6.4.2.3 Relationship Between Embryo Stage and Grade with Aneuploidy, Pregnancy and Live Birth Rates

The developmental stage of the embryo at the time of biopsy had a significant impact on the overall aneuploidy incidence (p <0.0001), with lower rates of chromosomal errors observed in more developmentally advanced embryos; pregnancy and live birth outcomes also reflected this trend, favouring embryos at later stages of development (Table 6.4). An in-depth analysis also revealed that the prevalence of specific aneuploidy types varied substantially across

embryo development stages, as reported in Figure 6.3A. Particularly, the incidence of whole chromosome errors exhibited a trend in which the prevalence of monosomies was significantly less in the later stages of embryo development, whereas trisomies and hypotriploidies followed the opposite trend. The incidence of segmental errors, on the other hand, remained consistent across all developmental stages studied.

Table 6.4: Incidence of aneuploidy, pregnancy, and live birth rates in embryos at various stages of development. The developmental stage of the embryo affected all three parameters (p <0.001). The only one stage 4 embryo in the database was excluded from the analysis. Data is presented as mean with 95% C.I.

Embryo Stage	N	Aneuploidy incidence (%)	Pregnancy rate D60 (%)	Live birth rate (%)
5	308	24.0 (19.6 – 29.1)	47.7 (42.2 – 53.3)	35.4 (30.3 – 40.9)
6	786	15.0 (12.7 – 17.7)	50.5 (47.0 – 54.0)	41.0 (37.6 – 44.4)
7	515	9.1 (6.9 – 11.9)	54.9 (50.6 – 59.2)	42.1 (37.9 – 46.5)
8 - 9	103	1.9 (0.5 – 6.8)	62.1 (52.5 – 70.9)	49.5 (40.0 – 59.0)

A link was observed between aneuploidy and embryo morphology, with the overall incidence of aneuploidy being 19.7% in embryos classed as having Grade 2 “good” morphology (N= 640). In comparison, the aneuploidy incidence in embryos classed as Grade 1 “excellent” (N= 1073) was only 10.7%, the results of which are significantly different (p <0.0001).

The different aneuploidies were also unequally represented between Grade 1 and Grade 2 embryos, implying that embryo morphology is not only associated with aneuploidy incidence but also the aneuploidy class. Interestingly, trisomies were more prevalent in Grade 1 embryos with 44.3% (N= 51/115) carrying the abnormality compared to 25.4% (N= 32/126) of Grade 2 embryos (p= 0.002). Conversely, the incidence of monosomies was 50% (N= 63/126) in Grade 2

embryos compared to 38.3% (N= 44/115) in Grade 1 embryos, however this difference was not statistically significant (p= 0.067). There was an equal distribution of the other aneuploidy classes across the different embryo grades (Figure 6.3).

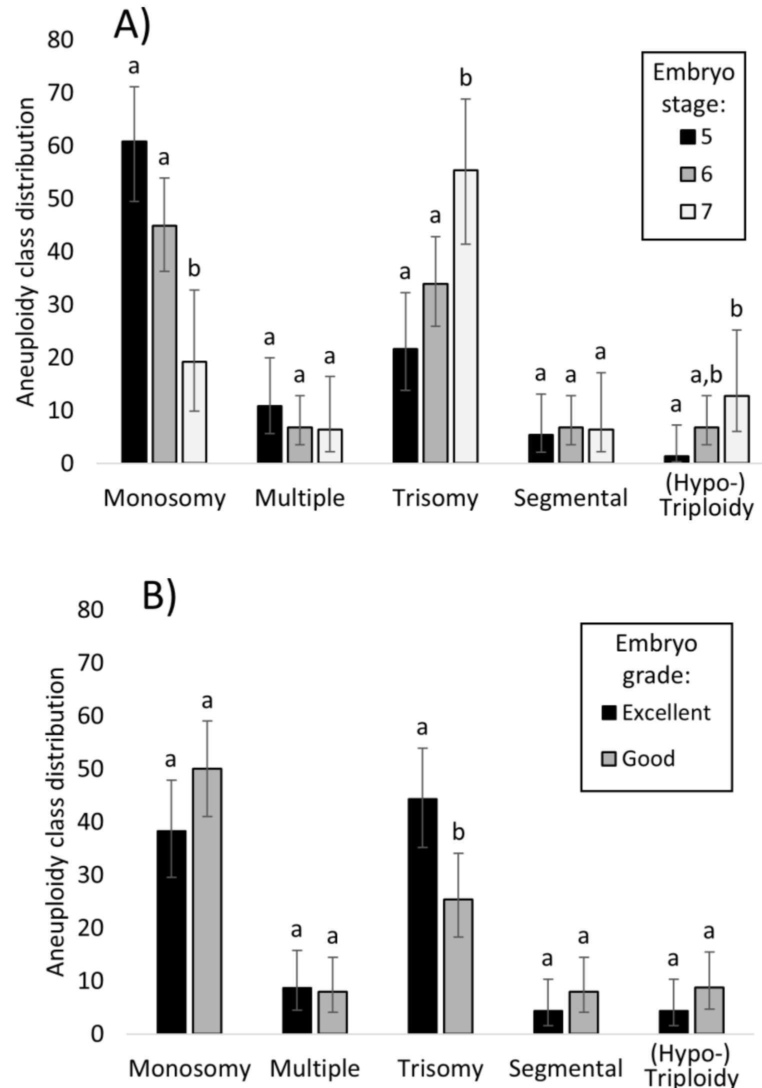

Figure 6.3: Embryo developmental stage and morphology grade influence the distribution of aneuploidy types. A) The prevalence of each aneuploidy class varies significantly across the developmental stages, with monosomy being the most prevalent in stage 5 embryos and trisomy in stage 7. Embryo stages 4, 8, and 9 were excluded from the graph due to the sample size being less than 3 per group. B) The distribution of overall aneuploidy class also varied between embryos with “Excellent” or “Good” morphology. Columns with different superscripts differ significantly. Data presented as percentage (%) with 95% CI for N=1713 replicates.

Notably, Grade 1 embryos resulted in higher pregnancy rates following ET than Grade 2 embryos, with 55.4% and 46.6% respectively (p= 0.0004). However, live births per transferred embryo were comparable across Grade 1 and Grade 2 embryos (41.8% versus 39.4%, p= 0.33), suggesting that the sole use of embryo morphology as an indicator of post-transfer developmental potential may not be reliable.

6.4.2.4 Male Embryos are Disproportionately Affected by Aneuploidy but Achieve Higher Morphology Scores

Due to the requirement of the breeder to transfer more male embryos, the database contained significantly more XY embryos compared to XX embryos in the database (N= 925 XY versus N= 788 XX, p= 0.0009). The morphology of XY embryos was generally better than that of XX embryos, with 67.1% (N= 621/925) of being classed as Grade 1 compared to 57.4% (N= 452/799) of XX embryos. Yet chromosomal errors were more common in XY embryos (N= 152/925) than XX embryos (N= 89/788), with 16.4% and 11.3% respectively (p= 0.002). On the other hand, there was equal representation of the different aneuploidy types between the sexes (Figure 6.4).

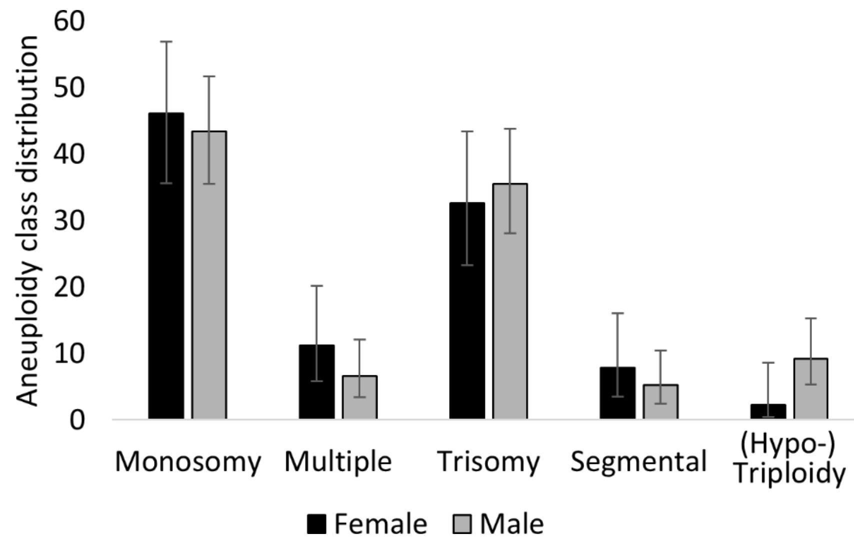

Figure 6.4: Relationship between aneuploidy and embryo sex. Despite the overall incidence of aneuploidy being higher in XY embryos than XX embryos, there was equal distribution of the aneuploidy classes. Data presented as percentage (%) with 95% CI for N= 1713 replicates.

6.4.2.5 Incidence of Aneuploidy by Chromosome

The chromosome-specific incidence of aneuploidy appeared to vary substantially when triploidy or hypotriploidy events were excluded from the analysis. The highest error frequencies were observed in chromosomes 14 and 26 (N= 31/255 and N= 18/255, respectively), with chromosome 26 having the greatest incidence of monosomes (N= 13/18) and chromosome 14 having the highest incidence of trisomies (N= 21/31). In contrast, no chromosomal errors were observed for chromosome 7 throughout the entire database, whilst chromosomes 21 and 22 only exhibited monosomies, and chromosomes 11, 13, 18, and 19 only exhibited trisomies (summary of all chromosomes in Figure 6.5).

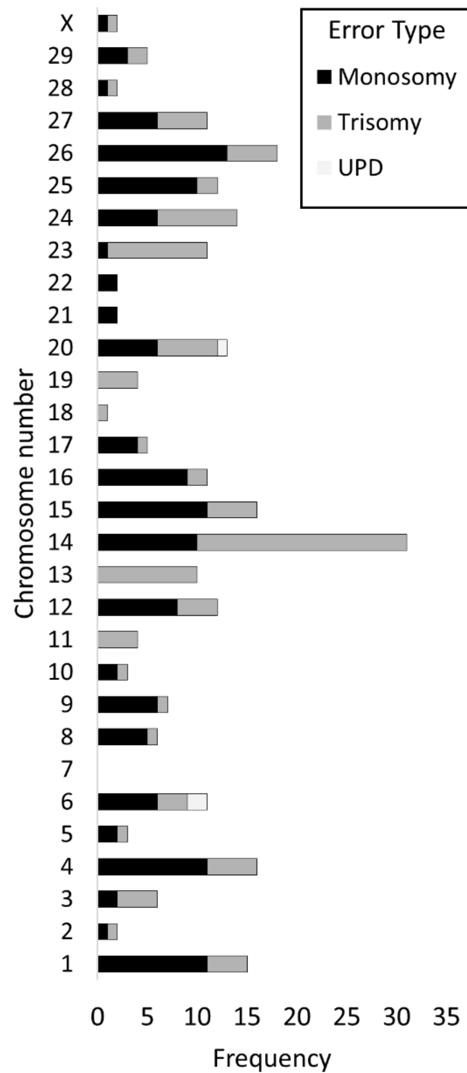

Figure 6.5: Frequency of whole chromosome errors by chromosome. Aneuploidy affected individual chromosomes at varying rates, with some chromosomes exhibiting a lower or higher incidence than anticipated. N= 255 whole chromosome errors.

6.4.2.6 Chromosomal Loss in Hypotriploidy

The analysis of 15 cases of hypotriploidy (embryos with a partial set of triploid chromosomes) showed that chromosome loss appeared at a higher frequency on chromosome 22 (N= 8/15) and chromosome 25 (N= 6/15), with no chromosome loss observed in chromosomes 6 and 17. A significant trend was apparent when arranging chromosomes by size (measured in Mb), indicating that chromosome loss in hypotriploidy more commonly affects smaller chromosomes (p= 0.016); this highlights the possibility of a mechanistic

connection between the two parameters, with Figure 6.6 proposing a visual representation of this analysis.

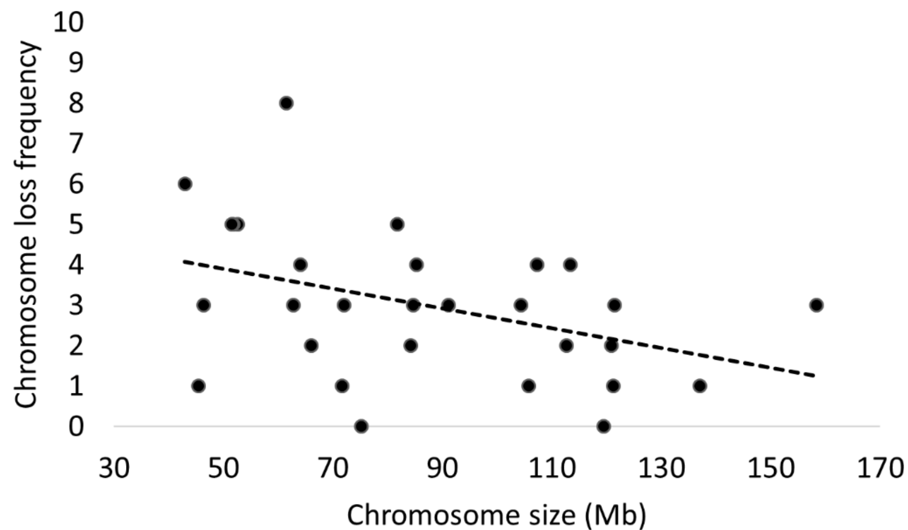

Figure 6.6: Incidence of chromosome loss in hypotriploidy. The trend observed suggests that smaller chromosomes are more frequently lost in hypotriploidy (p= 0.016), despite the goodness of fit being only modest (R^2=0.166) in this model.

6.5 Discussion

6.5.1 PGT-A Improves Pregnancy and Live Birth Outcomes

The results shown in this chapter suggest that PGT-A is an effective approach to improving selection strategies in cattle IVP, with the transfer of embryos diagnosed as euploid being more than 10 times more likely to implant in comparison to those diagnosed as aneuploid. Moreover, the pregnancy and live birth rates significantly improved with the sole use of embryos diagnosed as euploid whilst reducing the number of ETs needed to achieve a livebirth from 2.45 to 2.14 ETs/livebirth. The potential value of this would not only be the considerable financial savings, but also improved efficiencies with positive environmental benefits (as fewer dams would be required for ETs). The use of PGT-A has a high PPV of 98% and could be particularly effective for the cattle

industry, which is becoming progressively dependent on IVP as a breeding technology (Viana, 2019) and has begun to implement routine embryo biopsy and SNP genotyping to determine GEBVs. In gathering this data, breeders will have the necessary information to screen embryos for chromosomal abnormalities whilst also undertaking genomic selection.

6.5.2 Relationship Between Embryo Morphology and Chromosomal Abnormality

Previously published data reported the overall incidence of chromosomal abnormalities as 30% or higher (Hornak *et al.,* 2016; Turner *et al.,* 2019; Tutt *et al.,* 2021, whereas the incidence reported in this chapter is considerably lower (14.1%). However, the embryos within this database had already been pre-selected by breeders based on strict morphological criteria in order to increase embryo biopsy survival, cryosurvival, and live birth rates. As a result, the low incidence of chromosomal abnormalities is likely a considerable underestimation in bovine IVP.

The relationship between aneuploidy incidence and embryo morphology has already been observed (Alfarawati *et al.,* 2011; Tutt *et al.,* 2021), with the results in this chapter further strengthening the relationship, particularly for embryos that were biopsied at an earlier stage of development. Given that chromosomally abnormal embryos are capable of achieving the highest morphology scores, the data supports the additional benefit of PGT-A alongside morphology screening in IVP. Furthermore, there is substantial evidence that the incidence of aneuploidy varies significantly depending on the stage of the embryo, with embryonic stages 5 to 9 showing widely different results. This implies that any

comparison of the aneuploidy incidence between different studies should take into account the embryo developmental stage.

6.5.3 Aneuploidy Affects Certain Chromosomes More Than Others

With previous studies showing the breakdown of aneuploidy classes and chromosome errors affecting bovine embryos (Turner *et al.,* 2019; Tutt *et al.,* 2021), the considerably larger sample size used provides a more comprehensive analysis. The data highlights how some cattle chromosomes, such as chromosomes 2, 7, and 8, exhibit very few or no errors, whereas others such as chromosomes 14 and 16, are much more susceptible to aneuploidy. In cattle, the distribution of abnormalities among the chromosomes challenges the observation that the incidence of aneuploidy and chromosome size are inversely correlated (Baryshnikova *et al.,* 2013). Instead, it implies that the properties specific to each chromosome should be taken into account. However, since embryos were only assessed at the blastocyst stage, there may be a bias caused by the degree of lethality linked to the aneuploidy of specific chromosomes. For example, trisomies of chromosome 7 could cause a blastocyst to have a severely compromised morphology or result in early developmental failure, which could explain why trisomy of chromosome 7 was not detected. This latter hypothesis is further supported by the findings presented on chromosomal loss in hypotriploidy, which showed that smaller chromosomes were lost more frequently.

6.5.4 Trisomic Embryos Survive Longer Than Monosomic Embryos

In this database, monosomies were the most common abnormality detected, followed by trisomies. Trisomy rescue could result in euploid cells, which would explain why there are fewer trisomies than monosomies (McCoy, 2017;

Kahraman *et al.,* 2020). The relative incidence of specific aneuploidy types, however, has been observed to change considerably with embryonic stage, with advanced embryos being more frequently affected by trisomies. When considering the fact that trisomies are more tolerable in embryos than monosomies, this conclusion may not appear surprising. Similarly, trisomies and duplications were more common than monosomies and deletions at the later stages of development in the extended culture of human embryos (Popovic *et al.,* 2019).

6.5.5 The Origin of Aneuploidy in Cattle IVP

The maternal germline was the origin for the majority of whole chromosome errors, with GG plots indicating that 73.5% of trisomies arose during meiosis I in the oocyte. As GG plots cannot be used to reconstruct the meiotic origin of monosomies, it could be assumed that a similar percentage of these chromosome errors would also have occurred in meiosis I. These findings corroborate a trend in cattle which has previously been documented in human embryos (Hassold and Hunt, 2001; Kubicek *et al.,* 2019), and also supports a recent study (Tutt *et al.,* 2021) that suggested the considerable number of the aneuploidy errors found in IVP can be attributed to the *in vitro* maturation of cattle oocytes.

6.5.6 Differences between XX and XY embryos

The aneuploidy incidence varied dramatically between XY and XX embryos, with male (XY) embryos being affected more severely. Considering the morphology of XY embryos was generally better than that of XX embryos, an IVP system using embryo morphology as the sole selection criterion may result in an

unfavourable increase in the birth of male calves. In turn, this would unintentionally contribute to the overall incidence of aneuploidy in IVP.

6.5.7 Limitations of PGT-A

PGT-A allows for the diagnosis of euploid or aneuploid embryos, however these diagnoses may never be truly accurate due to the incidence of mosaicism (Taylor *et al*., 2014). The invasive nature of embryo biopsy means only 2-5 cells are aspirated from the blastocyst to ensure the survivability of the embryo and it is possible that the needle was inserted into an area of euploid cells of an aneuploid embryo (and vice versa; Griffin and Ogur, 2018). Unless another comprehensive screen was conducted to detect chromosomal abnormalities, the true success of the use of PGT-A can never be determined.

6.6 Conclusion

Overall, the data presented clearly supports the use of PGT-A to screen for chromosomal abnormalities in cattle IVP as the grading of the embryo morphology is not always indicative of its ability to develop to term. The selection of only euploid embryos would result in improved pregnancy and live birth rates per ET, resulting in a system that is more economical and environmentally sustainable and would ultimately reduce the number of ET surrogates. The current findings add a new approach to the research demonstrating the beneficial effect of PGT-A on ET outcomes. To truly test the reliability of this method, further research should be undertaken that implements a prospective design, whereby embryos are only transferred following PGT-A based recommendations.

Publications arising from this chapter, where I am first joint author:

Silvestri, G., Canedo-Ribeiro, **Serrano Albal, M.,** C., Labrecque, R., Blondin, P., Larmer, S.G., Marras, G., Tutt, D.A., Handyside, A.H., Farré, M., Sinclair, K.D. and Griffin, D.K, 2021. Preimplantation Genetic Testing for Aneuploidy Improves Live Birth Rates with In Vitro Produced Bovine Embryos: A Blind Retrospective Study. *Cells*, *10*(9), p.2284.

Personal contribution:

- Presented the results of the study in the form of poster and oral presentations at AETE 2019
- Creation of Karyomaps
- Assessed the ploidy of the embryos by Karyomapping, Gabriel-Griffin plots, log R ratio graphs, and B-allele frequency
- Data collection and statistical analysis
- Manuscript preparation: contribution to writing of the first draft and following modifications

7 Using PGT-A to Identify Chromosomal Abnormalities within the ICM and TE of *In Vivo* and *In Vitro* Produced Bovine Embryos

7.1 Background

In order for post-fertilisation development in mammals to be successful, maturational processes that occur in growing oocytes during antral-follicle development are essential. The main factor limiting the successful development of systems for the IVP of cattle embryos is a deficiency in these processes (Merton *et al.,* 2003; Abbara *et al.,* 2018; Luciano and Sirard, 2018). Although the development of IVM systems for GV stage oocytes from small to medium sized follicles from non-stimulated ovarian cycles has made considerable progress (Labrecque and Sirard, 2014; Gilchrist *et al.,* 2016), there are still deficiencies that prevent oocyte retrieval from such cycles from being widely used in commercial bovine IVP among *Bos taurus* breeds. Instead, the preferred methods are those that involve controlled ovarian stimulation in which oocytes experience some degree of *in vivo* maturation (Ferré *et al.,* 2019; Sakaguchi *et al.,* 2020).

In cattle, the development and refinement of protocols (Blondin *et al.,* 2002; Nivet *et al.,* 2012) for *in vivo* maturation is based on the concept that a brief period of gonadotrophin withdrawal (or "coasting"), within stimulated cycles and before follicular aspiration, can improve the oocytes' acquisition of developmental competency (Barnes and Sirard, 2000). High yields of transferrable-quality blastocysts per donor cycle are possible with these protocols. However, the molecular mechanisms in the follicular compartment that predict subsequent oocyte development from such cycles are just now beginning to be elucidated (Khan *et al.,* 2016), and the availability of published

data on the pregnancy outcomes after ET is limited (Durocher *et al.,* 2006). Genes involved in DNA replication, recombination, and repair, as well as those involved in chromosome stability and spindle integrity, are shown to be highly labile by array-based transcript analyses of oocytes from "coasting" protocols of varied duration (Labrecque *et al.,* 2013). Nevertheless, it is still unclear what the incidence of potentially lethal chromosomal abnormalities in pre-implantation embryos means.

The use of PGT-A to identify suitable embryos for transfer is of great interest as there is evidence of increased live-birth rates in single FET cycles following PGT-A in young women (Hou *et al.,* 2019), which closely parallels that of commercial bovine IVP-ET. Nonetheless, PGT-A remains a contentious topic in human reproduction, with opponents claiming that mosaicism and false positives render it inappropriate for routine use (Griffin and Ogur, 2018). In bovine, the birth of the world's first five karyomapped cows established the applicability of karyomapping to cattle embryos (Turner *et al*., 2019). Turner *et al*. were able to report the incidence of meiotic recombination and meiotic errors as well as the concordance between SNP information obtained from the blastocyst biopsies and cattle liveborn outcomes.

The purpose of this chapter was to employ PGT-A strategies involving SNP chip interrogation to identify chromosomal abnormalities within the ICM and TE of *in vivo* and *in vitro* produced bovine embryos. In so doing, the hypothesis was tested the diagnosis of the TE is an accurate reflection of the ICM.

7.2 Specific Aim 5

The specific aim of this chapter was to test the hypothesis that PGT-A is a suitable method to identify and analyse chromosomal abnormalities within the inner-cell mass and trophectoderm of *in vivo* and *in vitro* produced bovine embryos. This was tested through the following aims:

- Specific aim 5A: To determine the incidence of chromosomal abnormalities in oocytes from stimulated and non-stimulated ovarian cycles and thereby test the hypothesis that ovarian stimulation induces increased aneuploidy
- Specific aim 5B: To determine the precise nature of chromosomal abnormalities in aneuploid bovine embryos
- Specific aim 5C: To establish the concordance of chromosomal abnormalities between the ICM and TE lineages

7.3 Materials and Methods Specific to this Chapter

7.3.1 Generic Considerations

The methodology described in this chapter (Section 7.3.2 to 7.3.9) relating to the generation of embryos, immunodissection, DNA extraction, WGA, concordance, sexing, SNP genotyping, and statistical analysis was performed by the Sinclar Lab at the University of Nottingham. The contribution from the University of Kent was the creation and assessment of karyomaps, GG plots, LRR, and BAF graph analysis.

The University of Nottingham has its own farm in which it regularly uses to conduct research projects. All procedures used to generate embryos at the

University of Nottingham adhered to the Animals (Scientific Procedures) Act, 1986. Associated protocols complied with the ARRIVE guidelines and were approved by the University of Nottingham AWERB.

7.3.2 *In Vitro* Embryo Production

Nine sexually mature heifers of 16-20 months old underwent oestrous synchronisation using an intravaginal P4-releasing device (CIDR Vaginal Delivery System, 1.38 g, Zoetis UK Ltd, Leatherhead, UK) and intramuscularly (IM) administering 2 mL GnRH analogue (Acegon, Zoetis UK Ltd, Leatherhead, UK). Six and seven days later CIDR was removed and the 2 mL prostaglandin (PGF_2a) analogue Cloprostenol (Estrumate, MSD Animal Health, UK) was IM administered. To assist ovulation GnRH (2 mL IM) was administered again 48 h later.

Transvaginal ovarian follicular aspiration (OPU) commenced 4 days post oestrous onset and was repeated every 3-4 days for 3 weeks, leading to a total of 6 cycles of “non-stimulated” OPU. Following the “non-stimulated” cycles, the same donors underwent to three stimulated cycles of OPU, undertaken once every 14 days over a 5-week period. The aspiration of all 5 mm follicles (dominant follicle removal; DFR) was performed five days after the final non-stimulated session of OPU. Next, the heifers had a CIDR inserted and ovarian stimulation commenced 48 h later. Ovarian stimulation involved six IM injections of FSH (Folltropin, 70 IU dose per injection, Vetoquinol UK Ltd, Towcester, UK) given at 12 h intervals. 38-42 h after the las FSH injection started the first stimulated session of OPU, Following OPU, the CIDR was replaced and the subsequent cycle of DFR commenced 8 days later.

All OPU procedures were performed in a dedicated room kept in between 28-33°C. COCs were aspirated as described previously in Turner *et al.* (2019), using a Cook Medical vacuum pump with a 7.5 MHz ultrasound scanner (Exapad, IMV Imaging, Glasgow, UK) aspirating (70 mmHg aspiration pressure) with a 18G needle attached to a 1.4 m silicone tubing (of 1.4 mm internal diameter) into 5 mL of Tyrodes lactate (TL, Nivet *et al.*, 2012).

7.3.3 *In Vitro* Embryo Fertilisation and Culture

OPU fluids were passed through filter heated at approximately 37°C and transferred to 100 mm petri dishes on a heated stage (~38°C) for oocyte retrieval. COCs (N= 783) were graded 1-4 according to the references Sinclair *et al.* (2008) and Wrenzycki *et al.* (2018). All COCs with expanded, with few or absent cumulus cells and/or with fragmented or irregular cytoplasm were graded as class 4 and rejected. COCs graded 1-3 class were matured *in vitro* as described in Turner *et al.* (2019).

Fertilisation took place in 50 µL drops (5 oocytes/drop) under oil using frozen/thawed semen from a single sire. Media consisted of modified TL (Nivet *et al.*, 2012) supplemented with 0.6% (w/v) fatty acid free BSA, 1.5 µg/mL heparin, 0.2 mM sodium pyruvate, 0.08 mM penicillamine, 0.04 mM hypotaurine, 10 mM epinephrine, 50 µg/mL gentamicin. Sperm was prepared by centrifugation through a 45%/90% (v/v) BoviPure (Nidacon, Molndal, Sweden) density gradient. 2 µL sperm preparation was added to each drop with oocytes and co-incubated for 18-21 hours in a humidified environment at 38.5°C and 5% CO_2.

Embryos were incubated under oil in modified SOF based sequential culture media (mSOF) at 5% CO_2, 5% O_2, and 38.5°C in a humidified chamber (Nivet *et al.*,2012). On day 2, cleavage was evaluated and the embryos classified according to cell number (i.e. 1, 2-3, and >4 cells). Embryos were transferred on day 4 to 10 µL drops of the second culture media. Progression to morula was assessed 48 h later, when the embryos were transferred to 20 µL drops of the third medium. Stage and quality of the embryos (N= 459) were assessed again 48 h later (Day 8) following the International Embryo Transfer Society (IETS) guidelines for bovine embryo assessment (Stringfellow *et al.*, 2010).

7.3.4 *In Vivo* Embryo Production

Only 8 of the heifers were used to obtain *in vivo* embryos since one of them did not undergo oestrus synchronisation. Heifers underwent oestrous synchronisation using the drugs and doses described above and following this schedule: On day 10 the heifers had the first CIDR inserted, that was withdrawn on Day 2 with oestrus detected between Day 1 and 2. 48 hours later PGF2a was administered (Day 4). On day 7 of the cycle, a second CIDR was inserted followed by GnRH (IM) on Day 9, and initiation of ovarian stimulation began on Day 11. In this case, the treatment involved eight injections of FSH every 12 h, reducing the dose by 0.5 mL each day (from 2.5 mL to 1 mL). PGF_2a (IM) was administered on the morning of Day 13. AI started 48 h later and was repeated three times at 12 h intervals.

Day 7 post-AI embryos were flushed trans-cervically. This was performed following light sedation (0.25 mL/100 kg IM xylazine hydrochloride; Rompun 2% w/v, Bayer Animal Health, Reading, UK) and epidural (adrenaline, lignocaine hydrochloride; 3-5 mL 2% w/v, Lignol, Dechra Veterinary Products, Shrewsbury,

UK) anaesthesia, a Foley catheter was passed through the cervix and placed in each uterine horn. Each horn wash flushed 3 times using 50 mL syringes containing Vigro™ Flush Media (Vetoquinol UK Ltd, Towcester, UK). Flushes were passed over a 70 mM filter to recover embryos that were kept in 2 mL synthetic oviductal fluid with amino acids and myo-inositol medium (SOFaai) (Holm *et al.*,1999) treated with 25 mM HEPEs media at 38°C in a portable incubator until transported to the lab. The 20 embryos (in stages of blastocyst and morula) were cultured for 24 h in SOFaai culture media (Holm *et al.*,1999) in 20 µL drops (up to 11 per drop, within donor) under oil at 5% CO_2, 5% O_2, and 38.5°C, and then graded as described for *in vitro* produced embryos.

7.3.5 Immunodissection, DNA Extraction and Amplification

The TE and ICM from 389 Day 8 blastocysts were isolated via immunodissection, with 336 OPU-IVP derived (117 non stimulated, 219 stimulated), 33 abattoir-derived (limited to 4 ovary pairs), and 20 *in vivo* derived (same OPU donors). Immunodissection was performed as described by Solter and Knowles, 1975.

Removal of the ZP was achieved by exposure to 10 µg/mL pronase in TCM199 for 30-60 seconds. The ZP-free embryos were then incubated in 50% anti-bovine sera in mSOF for 60 mins and washed prior to incubation in 50% guineapig complement sera in mSOF for 2 mins. Each individual embryo was washed in PBS/PVP (Ca/Mg-free PBS + 0.1% PVP), deposited into 5 µL PBS/PVP under oil, and placed on a warm stage for 40 mins. A fine-bore glass pipette was used to induce disaggregation and the TE cells were transferred into a polymerase chain reaction (PCR) tube containing 4 µL PBS and before being

held on ice. Any adherent TE cells were removed by repeated pipetting the ICM, with the remaining ICM transferred into a new PCR tube.

Separation of the ICM and TE was confirmed by qualitative polymerase chain reaction (qPCR) using the expression of *GATA3* (Gilchrist *et al.,* 2016), a TE-specific marker (p = 0.003). The ICM and TE samples were then frozen at -80°C until needed.

7.3.6 Retrospective Sample Allocation

For chromosomal analysis, the ICM and TE from 9 blastocysts of IETS Stage 7 (grades 1 and 2), 8 and 9 (all grade 1) were selected per donor per treatment. When there were 9 or fewer embryos available, only stages 7, 8, and 9 were selected; if there were more than 9 embryos available, the stage of the blastocysts randomly selected were proportionate to the stages exhibited by that donor for non-stimulated and stimulated cycles. In total, 152 blastocysts were selected, with 82 from stimulated and 70 from non-stimulated OPU cycles. All the *in vivo* derived (N = 20) and immunodissected abattoir blastocysts (N= 27) of stages 7, 8, and 9 were selected.

7.3.7 DNA Extraction

Whole blood samples from the OPU donors were centrifuged at 2,500 RPM for 3 mins, followed by 14,000 RPM for 2 mins to form a pellet of white blood cells (WBCs). Semen samples from a single sire were washed in PBS prior to centrifugation at 14,000 RPM for 2 mins to form a pellet of sperm. Using samples from a single sire, the semen was washed in PBS and centrifuged at 14,000 RPM for 2 mins to form a sperm pellet.

A salting-out method was used to extract DNA from the sperm and WBCs as described by Montgomery and Sise, 1990. Briefly, sperm cells were lysed using 10 mM DTT and incubating overnight at 55°C; for WBCs, cells were lysed by incubating overnight in an 800 µL solution of 8 mM Tris/0.1 mM EDTA buffer with 0.01% (w/v) proteinase K and 0.5% (w/v) SDS. Proteinase K (37.43 µg/mL) was added to the lysed blood cells and incubated for a further 3 hours at 55°C to reduce clumping. Next, 360 µL 5M NaCl was added prior to centrifugation at 14,000 RPM for 5 mins to remove cell debris, and DNA from the supernatant extracted from by ethanol precipitation. Using a DNeasy kit (Qiagen, UK), DNA was extracted from using 20 mg ovarian tissue (for abattoir derived oocytes). The kit was used as per the manufacturer’s instructions, with the only exception being the elution of DNA in 10mM Tris-HCl (pH 8.5) instead of the supplied elution buffer.

7.3.8 Whole Genome Amplification

A REPLI-g single cell WGA kit (Qiagen, UK) was used to perform WGA as per the manufacturer's instructions. The amplified DNA was stored in the freezer at -20°C until needed.

7.3.9 Quality Assurance and Sexing

PCR was used to assess the DNA amplification, confirm concordance between ICM and TE lineages, and identify the sex of individual blastocysts. Two primer sets were utilised for multiplex PCR: one set of primers for *SRY* to identify male sec (GenBank accession no. EU58186.1), and the other for BSP as an autosomal marker (Kovar and Rickords, 1996). The 25 µL PCR mix consisted of 1.25 µL *SRY* primer set, 0.125 µL BSP primer set, 12.5 µL Immomix Red

mastermix (Bioline Reagents Ltd., UK), and 1 µL WGA DNA. The following cycle was run for the PCR (Table 7.1):

Table 7.1: Summary of the PCR cycling conditions for *SRY* and BSP.

Step	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	10 minutes	1
Denaturation	94	30 seconds	30
Annealing	55	30 seconds	
Extension	72	60 seconds	
Final Extension	72	10 minutes	1

7.3.10 SNP genotyping and chromosomal analysis

Genotyping was performed using GGP 50K SNP arrays (Illumina, Cambridge, UK) by Neogen Europe Ltd (Ayr, Scotland, UK) using parental DNA and whole genome amplified embryonic DNA (both the ICM and TE). The fraction of SNPs successfully genotyped (average "call rate") was 95.8% ± 0.18%, the average GC10 Score was 0.424 ± 0.005, and the average GC50 Score was 0.747 ± 0.001.

7.3.11 Statistical Analysis

GenStat statistical package (19th Edition, VSN International, 2018; https://www.vsni.co.uk/) was used to perform statistical analyses. The analysis of proportion data utilised REML generalized linear mixed models which used logit-link functions and assumed binomial errors. The models generated used donor DNA as the random model when testing the treatment effects (non-stimulated vs. stimulated) as a fixed effect. Some models of chromosomal

abnormalities also used blastocyst stage/grade as a fixed effect. Analyses of the chromosomal abnormalities using the ICM and TE lineages were conducted separately to avoid inflating degrees of freedom; these lineages were later added as a fixed effect to the models. Analyses of chromosome-error classification used chi-square tests.

7.4 Results

A total of 783 COCs were retrieved by OPU, 359 from non-stimulated and 424 from stimulated cycles. Following IVM, 689 COCs were fertilised and yielded 459 Day 8 blastocysts, with only 152 selected for analysis of chromosome copy number due to being of a more advanced stage (IETS stages 7 to 9). Additionally, 20 *in vivo* produced embryos were recovered from five of the same donors to be analysed.

7.4.1 Specific Aim 5A: To Determine the Incidence of Chromosomal Abnormalities in Oocytes from Stimulated and Non-Stimulated Ovarian Cycles and Thereby Test the Hypothesis that Ovarian Stimulation Induces Increased Aneuploidy

The overall incidence of chromosomal abnormalities was 0.241 ± 0.0236 for the 152 OVU-IVP Day 8 blastocysts, with no significant differences were observed between stimulated and non-stimulated ovarian cycles (Figure 7.1A). However, a significant difference ($p < 0.001$) was detected when comparing *in vivo* and *in vitro* derived blastocysts using the 20 embryos obtained from the five common donors (Figure 7.1B). All 20 *in vivo* derived blastocysts were euploid, supporting the hypothesis that IVM and IVC processes are the primary cause of chromosomal abnormalities.

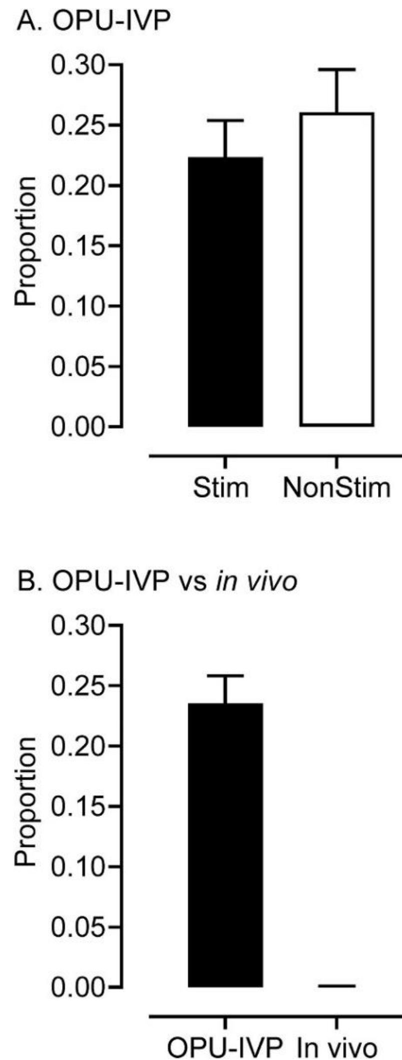

Figure 7.1: Aneuploidy incidence in Day 8 blastocysts from non-stimulated (N= 70) and stimulated (N= 82) OPU cycles. A) Aneuploidy incidence did not differ between each stimulation type. B) Incidence of aneuploidy differed (p= 0.004) between blastocysts derived from OPU-IVP cycles compared to *in vivo* derived blastocysts.

The more developmentally advanced Day 8 blastocysts (IETS stages 8 to 9) exhibited a lower incidence of chromosomal abnormalities ($p < 0.01$) in comparison to the less developmentally advanced blastocysts (IETS stage 7); a trend mirrored in embryo quality (Figure 7.2), with better quality embryos (Grade 1) showing a lower aneuploidy rate compared to lower quality embryos (Grade 2). There was, however, a link ($p < 0.05$) between the blastocyst stage/grade and ovarian stimulation treatment. Blastocysts from non-stimulated

cycles showed a decrease in the incidence of chromosomal abnormalities as the blastocyst stage/grade improved, though the incidence was greatest for expanded blastocysts of IETS stage 7 (Grade 1) from stimulated cycles.

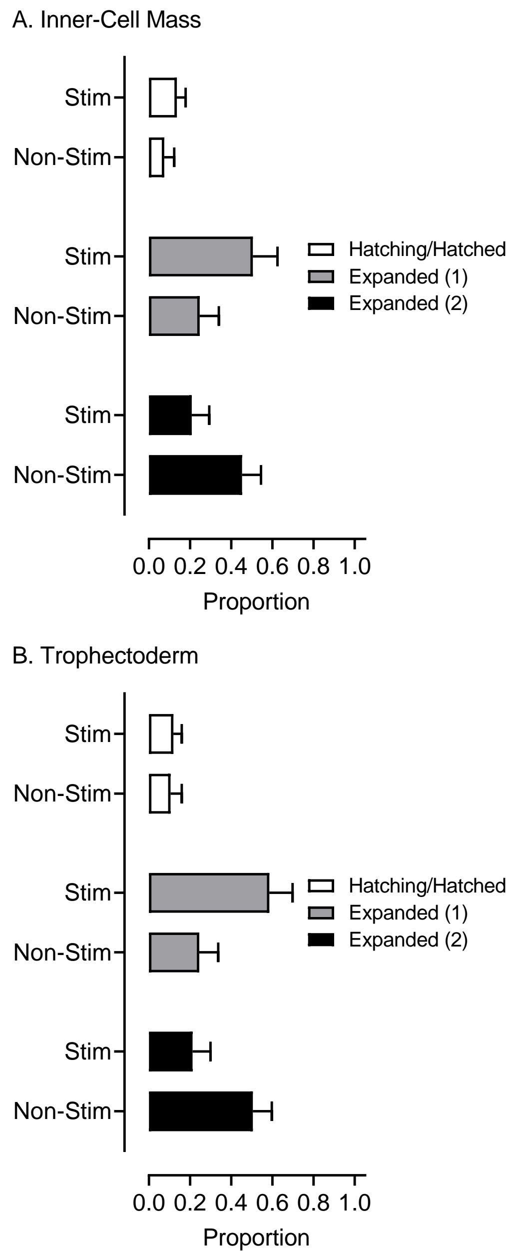

Figure 7.2: Aneuploidy incidence in Day 8 blastocysts from non-stimulated (N= 70) and stimulated (N= 82) OPU cycles. Graphs represent the proportion of hatching/hatched blastocysts (Grade 1) and expanded blastocysts (Grades 1 and 2) with aneuploidy in inner cell mass (A) and trophectoderm (B). Stim = stimulated. Non-Stim = non-stimulated.

In contrast to the lack of significance between stimulated and non-stimulated ovarian cycles, the number of blastocysts with chromosomal abnormalities differed significantly (p= 0.008) between donors but not the ICM and TE lineages (Figure 7.3). This is highlighted by the relatively high number of aneuploid blastocysts from Donor 835 in comparison to Donors 805 and 859 producing nearly all euploid blastocysts.

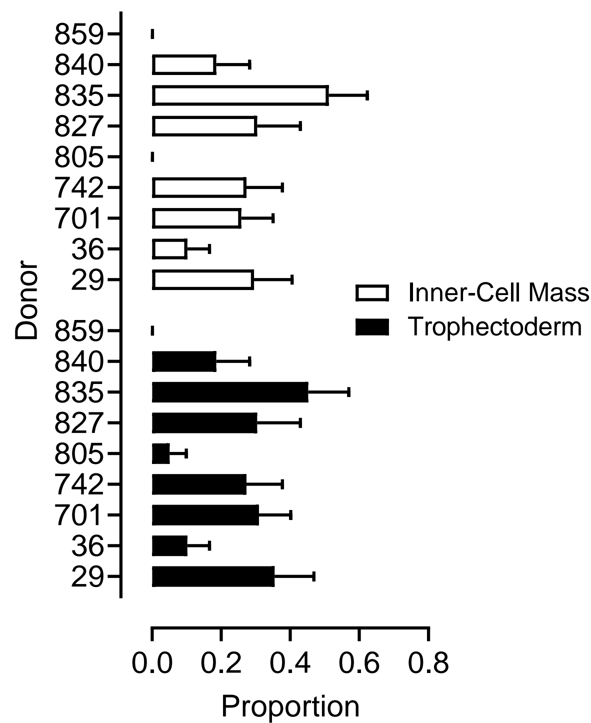

Figure 7.3: Incidence of aneuploidy for the inner cell mass and trophectoderm in Day 8 blastocysts (N= 152) by oocyte donor across non-stimulated and stimulated cycles.

7.4.2 Specific Aim 5B: To Determine the Precise Nature of Chromosomal Abnormalities in Aneuploid Bovine Embryos

Overall, chromosomal abnormalities were detected in at least one lineage for 40 OVU-IVP blastocysts. The majority of the abnormalities consisted of aneuploidy and triploidy, including double aneuploidy and hypotriploidy, and are summarised in Figure 7.4. Single maternally derived trisomies and maternally derived monosomies were most common, detected in 11 and 7 blastocysts, respectively. This was followed by double aneuploidies detected in 5 blastocysts, which can be further categorised as double monosomy, double trisomy, and three trisomy/monosomy. In contrast, no single or double paternally derived aneuploidies were detected in any of the blastocysts. Triploidy was detected in 5 blastocysts (1 paternal and 4 maternal) and hypotriploidy in 2 blastocysts (maternal origin only), and a single blastocyst comprised only of a maternal genome which is indicative of parthenogenesis. All of the blastocysts described above had the same karyotype between the TE and ICM lineages.

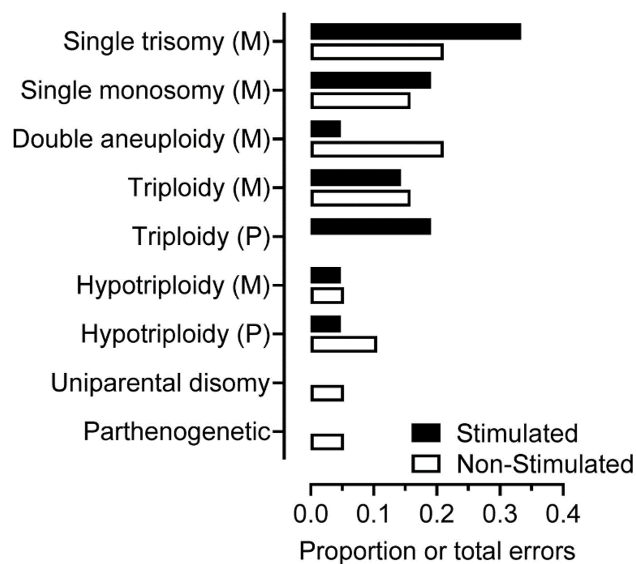

Figure 7.4: Nature of chromosomal errors in Day 8 blastocysts (N = 40). M= maternal origin. P= paternal origin. Double aneuploidy= combinations of maternal monosomy and/or maternal trisomy on two chromosomes.

The 9 remaining blastocysts were non-concordant, of which 4 were euploid in one lineage and triploid in the other, one exhibited UPD in the ICM but was euploid in the TE, and one was parthenogenic in the ICM and triploid in the TE. The other 3 blastocysts exhibited paternal hypotriploidy, with one blastocyst exhibiting mostly paternal chromosomes (two of which were lost in the TE) with indications of persistent maternal chromosomes in the ICM; the other 2 blastocysts were euploid in one lineage. The prevalence of specific categories of chromosomal abnormalities did not significantly differ between blastocysts generated from non-stimulated and stimulated cycles. However, blastocysts produced from stimulated and non-stimulated cycles had a similar overall incidence for the specific categories of chromosomal abnormalities.

Despite being unable to fully analyse the abnormalities on a chromosome-by-chromosome basis due to the small numbers, chromosomes 1 (4x), 4, (3x), 14 (3x), and 15 (5x) were noticeably overrepresented (Figure 7.5A). Though not statistically significant, the aneuploidy incidence appeared to be higher for the larger autosomes (Figure 7.5B).

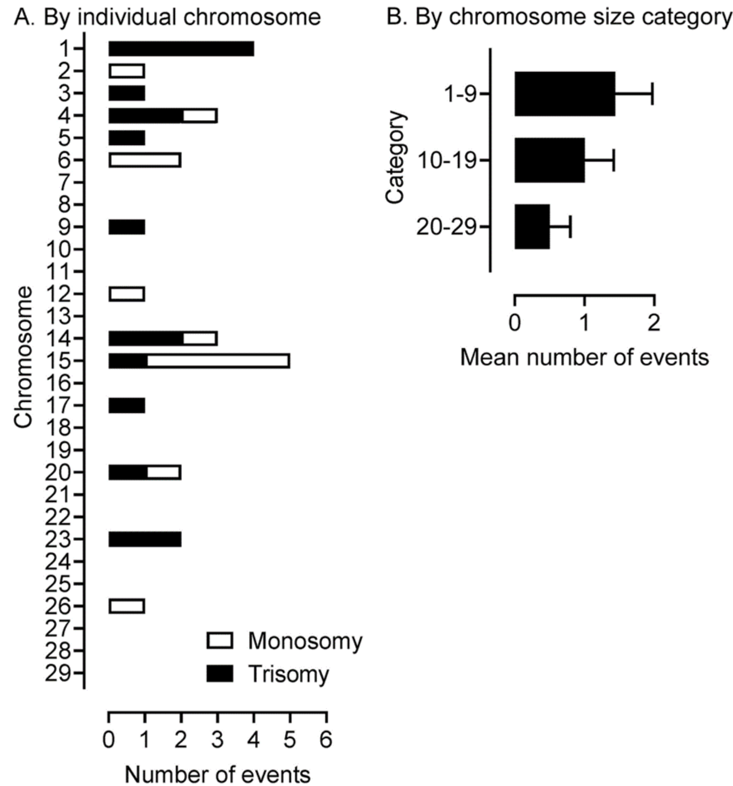

Figure 7.5: Chromosome-specific aneuploidy incidence in blastocysts (N= 152). A) Incidence of monosomy and trisomy in individual chromosomes. B) Chromosome-specific aneuploidy incidence categorised by small (20-29), medium (10-19) and large (1-9) chromosomes.

7.4.3 Specific Aim 5C: To Establish the Concordance of Chromosomal Abnormalities Between the ICM and TE Lineages

The TE and ICM lineages shared a similar incidence of aneuploidy. In total, 143 of the 152 Day 8 OVU-IVP blastocysts (70 non-stimulated, 82 stimulated) screened for chromosomal abnormalities were perfectly concordant, with an identical diagnosis between the ICM and TE lineages (Figure 7.6). For 2 of the 9 remaining blastocysts, these were "imperfectly concordant" and carried a different abnormality in the ICM and TE. An overview of the results is shown in Figure 7.7. Discordant results were detected in the other 7 blastocysts, meaning an abnormality was detected in one lineage but was euploid in the other.

Chromosomal abnormalities in the discordant embryos consisted mostly of maternal and paternal triploidy or paternal trisomy involving multiple chromosomes.

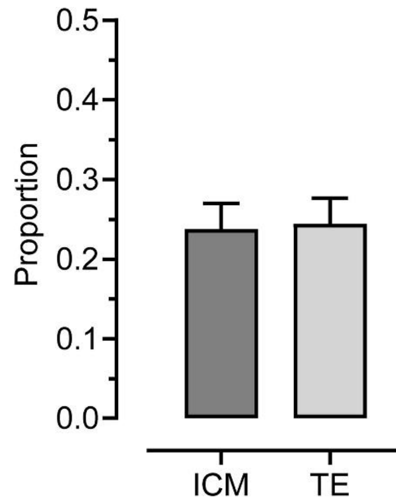

Figure 7.6: Chromosome-specific aneuploidy incidence in blastocysts (N= 152). A) Incidence of monosomy and trisomy in individual chromosomes. B) Chromosome-specific aneuploidy incidence categorised by small (20-29), medium (10-19) and large (1-9) chromosomes.

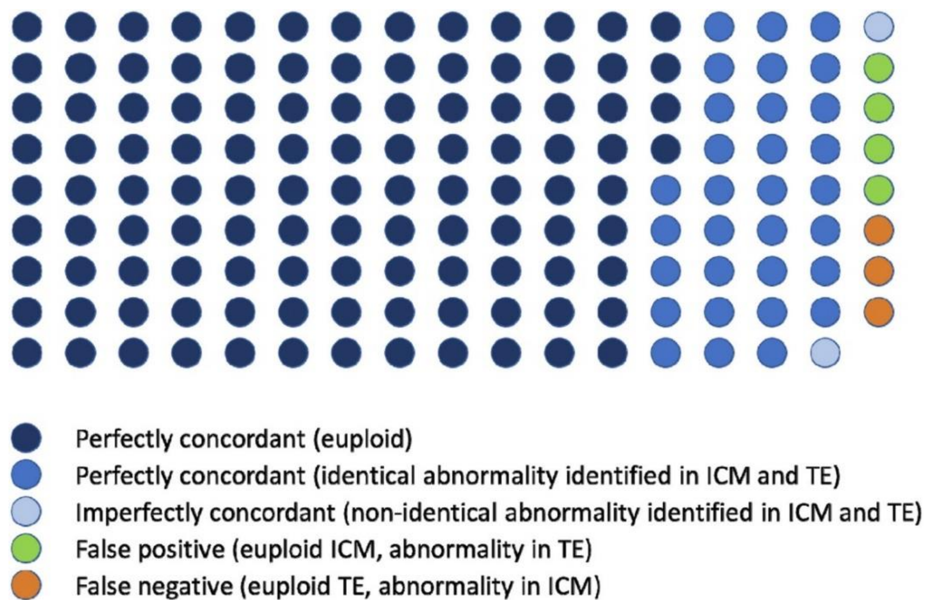

Figure 7.7: Depiction of concordance between the ICM and TE lineages of the 152 blastocysts analysed for chromosome abnormalities.

7.5 Discussion

7.5.1 The Incidence of Chromosomal Abnormalities in Blastocysts from Stimulated and Non-stimulated Ovarian Cycles

The use of ovarian stimulation did not increase the incidence of chromosomal abnormalities, yet the nature of chromosomal errors differed between stimulated and non-stimulated cycles. The incidence of multiple errors, consisting primarily of maternal monosomy and/or trisomy (often with paternal trisomy) on multiple chromosomes, was greater in blastocysts from non-stimulated cycles. On the other hand, blastocysts from stimulated cycles exhibited a greater incidence of maternal trisomy on single chromosomes. Unlike the OPU-IVP blastocysts, the *in vivo* derived blastocysts produced by the same donors were all euploid. The highly significant difference in aneuploidy incidence and the prevalence of maternal meiotic errors suggests that the 24-hour IVC period between OPU and insemination is the point in which most chromosomal abnormalities arise, but caution is advised when interpreting this data given the low numbers of *in vivo* derived blastocysts (N= 20).

It is clear that there was considerable variation in the incidence of chromosomal abnormalities between the different donors, which is particularly noticeable when comparing blastocysts from stimulated and non-stimulated cycles. Chromosomal abnormalities were detected in blastocysts produced almost entirely from non-stimulated cycles for donors 36, 805, and 859. Similarly, for donors 701 and 827, the incidence of chromosomal abnormalities was lower in stimulated cycles than in non-stimulated cycles. Conversely, donors 29, 835, and 840 showed an increase incidence with ovarian stimulation. The association between ovarian stimulation regimen and incidence of chromosomal

abnormalities between individual donors is unknown, despite the fact that in both humans (Broekmans, 2019) and cattle (Bó, 2014), variable donor responses to ovarian stimulation are well documented in terms of blastocyst yields.

Additionally, whilst the deleterious effects of ovarian stimulation have been reported in early animal (Fujimoto *et al.,* 1974; Vogel and Spielmann, 1992; Ma *et al.,* 1997) and subsequent human studies (Baart *et al.,* 2007; Rubio *et al.,* 2010), current ovarian stimulation protocols do not seem to increase the incidence of chromosomal abnormalities (Labarta *et al.,* 2012; Sekhon *et al.,* 2017; Hong *et al.,* 2019). Therefore, the variation in the nature and incidence of chromosomal abnormalities between the different donors following ovarian stimulation warrants further research.

7.5.2 The Nature of Chromosomal Abnormalities

The prevalence of any given chromosomal abnormality is determined by a balance of factors such as the differential survival at any given stage and the initial error rate in the gamete. For example, an extra copy of chromosome 16 is the most prevalent trisomy in humans, yet it is never detected in live births since it inevitably results in spontaneous abortion. The results show that bovine chromosomes 14 and 15 appeared to have an unusually high number of monosomies and trisomies. As there are more trisomies than monosomies, this indicates that the more lethal monosomies have been selected against by the blastocyst stage, particularly as the SNP-array based platform is limited to detecting both meiotic and mitotic monosomies and only meiotic trisomies.

7.5.3 Effect of Blastocyst Stage and Grade on the Nature and Incidence of Chromosomal Abnormalities

Day 8 blastocysts that were more developmentally advanced exhibited a lower incidence of chromosomal abnormalities, suggesting a link between aneuploidy levels and advanced development. Several human studies have reported relationships between the incidence of chromosomal abnormalities and morphological grade in both cleavage-stage (Magli *et al.,* 2007; Munné *et al.,* 2007; Del Carmen Nogales *et al.,* 2017) and blastocyst-stage embryos (Alfarawati *et al.,* 2011; Fragouli *et al.*, 2014; Capalbo *et al.,* 2014; Minasi *et al.,* 2016; Wang *et al.,* 2018), despite the fact that blastocyst stage and grade are typically regarded as poor predictors of euploidy in humans (Fragouli *et al.*, 2014; Gardner *et al.,* 2016). Conversely, the limited publications on bovine embryos which describe such relationships between the incidence of chromosomal abnormalities and morphological grade are conflicting and reliant on superseded technologies (King *et al.,* 1987; Dorland *et al.,* 1993). In this chapter, the decreasing incidence of chromosomal abnormalities with morphological stage is consistent with previous studies showing arrest (Fragouli *et al.,* 2019) or reduced rates of progression (Zaninovic *et al.,* 2017) in human embryos due to chromosomal abnormalities. In terms of morphological grade, qualitative differences were observed in that monosomies were more common (~70%) in the lower grade expanded blastocysts, whilst trisomies were the most common abnormality seen in higher grade expanding, hatching, and hatched blastocysts; a pattern also seen in human embryos (Alfarawati *et al.,* 2011).

7.5.4 Concordance Between TE and ICM Lineages

Overall, the incidence of chromosomal abnormalities had a high degree of perfect concordance (94.1%) between the ICM and TE, with only 9 blastocysts generating non-concordant cases. Of the 7 blastocysts with discordant results, having one euploid and one aneuploid lineage, 4 would have been considered a false positive within a diagnostic setting as the TE biopsy results would have diagnosed the blastocyst as aneuploid. All of the false positive results were triploid or hypotriploid in the TE despite the ICM being euploid, suggesting a minimal chance of normal pregnancy and live birth due to insufficiency of the aneuploid placenta. The remaining 3 blastocysts would have been considered a false negative within a diagnostic setting as TE biopsy results would have diagnosed the aneuploid blastocyst as euploid; for two of the three false negatives, the screening failed to detect triploid embryos which would result in the developmental failure of the embryos. The remaining false negative exhibited UPD, producing an effective misdiagnosis rate of only 2%. If the overarching goal of PGT-A is to detect abnormalities (and therefore advise whether to proceed with embryo transfer), it can be concluded that PGT-A was informative in these circumstances.

The incidence of chromosomal abnormalities in either the TE or ICM is example of confined mosaicism, which has been extensively reported in studies of human embryos (Liu *et al.,* 2012; Victor *et al.,* 2019); the high degree of concordance between the ICM and TE has also been observed and is near identical to that of human studies (Chuang *et al.,* 2018; Victor *et al.,* 2019). Chromosomal abnormalities of paternal origin have been observed in bovine cleavage-stage embryos, presenting as mosaic and considered to be a consequence of

dispermic fertilisation (Destouni *et al.,* 2016). Studies in mice and non-human primates have demonstrated that these abnormalities can be preferentially removed by apoptosis or fragmentation (Daughtry *et al.,* 2019), which can result in the over- or underrepresentation of abnormalities (Bolton *et al.,* 2016). Nevertheless, mosaicism in human embryos often decreases by the blastocyst stage, leading to the conclusion that PGT-A screening of TE biopsies is likely to provide a more trustworthy predictor of cytogenetic status (Fragouli *et al.,* 2019). Therefore, the findings presented in this chapter support the genomic evaluation of bovine blastocysts in commercial breeding using PGT-A in TE biopsies.

7.6 Conclusion

The data presented in this chapter indicates that PGT-A could be routinely employed in the cattle breeding industry given that trophectoderm biopsies reflect the ploidy status of the overall embryo. The significance of this is that it enables the simultaneous of a contemporary SNP-array platform to determine GEBVs, whilst also allowing for the assessment of future pregnancy outcomes in bovine blastocysts using PGT-A. The scope for future improvement is supported by the finding that chromosomal abnormalities in preimplantation embryos are primarily caused by IVM and IVC rather than ovarian stimulation cycles. Theoretically, a reduction in the number of aneuploid embryos transferred could be achieved by improving culture conditions to resemble the *in vivo* environment more closely.

Publications arising from this chapter, where I am first joint author:

Tutt, D.A.R., Silvestri, G., **Serrano Albal, M.,** Simmons, R.J., Kwong, W.Y., Guven-Ates, G., Canedo-Ribeiro, C., Labrecque, R., Blondin, P., Handyside, A.H., Griffin, D.K. and Sinclair, K.D, 2021. Analysis of bovine blastocysts indicates ovarian stimulation does not induce chromosome errors, nor discordance between inner-cell mass and trophectoderm lineages. *Theriogenology*, *161*, pp.108-119.

Personal contribution:

- Creation of Karyomaps
- Assessment of the ploidy of the embryos by Karyomapping, Gabriel-Griffin plots, log R ratio graphs, and B-allele frequency

8 General Discussion

The ultimate goal of this thesis was to improve the efficiency of pig *in vitro* production procedures and to demonstrate that the introduction of PGT-A tools in routine cattle embryo analysis can benefit the breeding industry. These goals were successfully achieved, contributing to the development of improved IVP techniques used for livestock animals.

Of the specific aims outlined in the introduction, this thesis was mostly successful in its pursuit of the following:

1. The addition of cytokines to IVM medium, specifically FLI, improves oocyte maturation in pigs and subsequently developmental competence through the synchronisation of nuclear and cytoplasmic maturation. In enhancing these cellular processes, higher quality embryos were produced to improve porcine *in vitro* production, demonstrating there is a benefit in using peri-pubertal gilts as a source of ovaries.

2. The comparison of MCS and DGS porcine sperm selection methods revealed that despite both methods yielding similar results for motility, morphology, and IVF outcomes, MCS offers a decreased rate of morphological abnormalities. However, as there was no discernible difference in IVF success rates, both are equally recommended for use in porcine IVP until further research can be conducted.

3. The modification of boar sperm vitrification protocols, including post-warming processes, was technically successful as it yielded similar results to slow freezing with regards to DNA damage and survivability. However, the use of mini-straws as a vitrification method is not a suitable

for boar sperm, and slow freezing still offers more benefits for preserving basic sperm parameters.

4. The implementation of PGT-A in bovine IVP not only allows for genomic evaluation and aneuploidy screening using SNP-array analyses but can also predict improved pregnancy and live birth rates.

5. The use of PGT-A to screen TE and ICM lineages in cattle embryos is a suitable method to identify chromosomal abnormalities and determine the ploidy status of the whole embryo, which is also accurately represented in the trophectoderm biopsy.

However, these outcomes could have yielded better results to have a greater impact in ART if the following factors could have been addressed differently.

8.1 COVID-19 Impact Statement

As a result of the COVID-19 pandemic, several lockdowns (stay-at home orders) were implemented by the UK Government in an effort to contain the spread of the disease. Lockdowns were implemented from March 2020 to June 2020, November 2020 to early December 2020, and mid-December 2020 (local Tier 4 restrictions in Kent) until March 2021. With all experiments requiring *in vitro* work and the use of specialist facilities, no experiments were conducted during the lockdowns and no data were readily available for *in silico* analyses (e.g., Karyomapping). Consequently, the lockdowns resulted in the inability to generate data for this thesis. Furthermore, entry into the abattoirs for collection of porcine ovaries was prohibited during the lockdowns. Upon the easing and/or lifting of the lockdown for the public, ovaries were still not collected as the

abattoir had stricter guidelines for which personnel could be onsite. In total, ovaries were not collected for ~1.5 years between March 2020 and September 2021. However, as the AI semen samples were still being collected and delivered to the University of Kent, the scope of research shifted to focus on sperm selection methods (chapters 5 and 6) from March 2021.

In addition to the stay-at-home orders and the limited availability of porcine ovaries, the University of Kent implemented limits of the number of research staff present within each laboratory, limiting the full return to work and the full use of all equipment. However, once these measures were lifted and research was allowed to resume as it was pre-pandemic, the increased presence of alcohol (ethanol for cleaning surfaces/shared equipment and alcohol-containing hand sanitisers) impacted IVF results as alcohol is embryo-toxic; this was seen in the sudden drop in the number of blastocysts yielded per experiment.

Prior to the COVID-19 pandemic, the plan was to continue with multiple rounds of porcine IVM, IVF, and IVC to have a significant sample size of mature oocytes and embryos. With an increased number of mature oocytes, initial plans were to conduct qPCR experiments for mitochondrial DNA quantification. By quantifying mitochondrial DNA, the aim was to further prove that the use of cytokines in IVM medium did result in higher embryo quality using factors other than visual embryo grading, as high mitochondrial DNA is considered to be a measurement of embryo quality. Additionally, in being unable to enter the University of Kent and with restrictions on the number of research staff in each facility, plans to use the confocal microscope for Mitotracker staining in mature oocytes and quantification of CG fluorescence were cancelled as it was unknown when

ovaries would be received to continue experiments and thus to prioritise newer research projects.

8.2 Source of Oocytes, Ovary Collection, and Transport for Testing IVP Methods

Producing IVP embryos from the gametes of elite animals with high genetic merit provides the opportunity for improving food production. However, it is commonly known that pig IVP is particularly difficult and has a low success rate compared to humans (Grupen, 2014; Fowler *et al.,* 2018). The findings presented in Specific Aim 1 shows an improved IVP protocol which results in the increased quality of blastocysts with the supplementation of FLI and provides a framework in which future experiments can utilise to continue the success. In trying to optimise IVP methods prior to disseminating for real world application within the breeding industry, it is incredibly important to have truly established protocols which have been repeated numerous times to verify the efficacy. In doing so, the source of oocytes and the collection process must be scrutinised.

The age of the animal is highly correlated with the developmental competence of oocytes (Bagg *et al.,* 2007; Grupen *et al*., 2003), which is reflected in the quality and number of cells per blastocyst, with significantly lower numbers of cells in blastocysts from gilts compared to blastocysts from sows (Bagg *et al.,* 2007). Gilt ovaries are more readily available due to the timing farmers have established for pork meat production. Furthermore, the developmental competence of oocytes from gilts is lower and gilt ovaries have a greater number of unstimulated follicles. The use of sow ovaries instead of those from young animals could have the potential to increase the efficiency of these IVP protocols, which in turn could reduce the number of repetitions per experiment

needed. Yet, the use of sow ovaries would not have reduced the variability in the results obtained between replicates, which is a problem that affects both young and adult animals.

The lack of blood irrigation in the ovaries after slaughter causes ischemic conditions, limiting oxygen and sources of energy. Due to this, the generation of reactive oxygen species within the follicles can increase, overloading the endogenous antioxidant systems and leading to oxidative stress and cell damage (Combelles *et al.*, 2009). The storage and duration of the ovaries in transport is key to ensuring the quality and developmental competence of the oocytes in order to undergo *in vitro* maturation and subsequent embryo development (Wongsrikeao *et al.*, 2005; Guibert *et al.*, 2011). Transporting preserved pig ovaries for 6 hours at 25-35°C maintains oocyte competence in IVM, however, the developmental competence of the oocytes is reduced when compared to a 3-hour transport interval (Wongsrikeao *et al.*, 2005). As there is little or no available pork slaughterhouses in Kent, obtaining porcine ovaries with minimal transport time was impossible. Therefore, the sponsors facilitated the distribution of the ovaries from other collaborating slaughterhouses. During the experiments presented in Specific Aim 1, ovaries were collected from Cranswick Country Foods (Hull, UK) and transported for around 5 hours until they arrived in the laboratory. The ovaries used for Specific Aim 2 were from C&K Meats Limited (Suffolk, UK) and their transport usually took around 2.5 hours. Since the extended transport periods are associated with a decrease in the efficiency of the IVP outcomes, closer sources of ovaries could have positively influenced the IVF outcomes in this thesis which would further verify the efficacy of the methods.

8.3 Improving the Techniques Used to Assess Sperm

Sperm motility has traditionally been evaluated manually using phase contrast microscopy. However, this assessment is subjective because it depends on the criteria used by each evaluator and their skill level. On the other hand, the use of a CASA system reduces this subjectively, diminishing the bias during the evaluation and generates a data output free from human error (Amann and Waberski, 2014; O´Meara *et al.*, 2022).

In Specific Aim 3, sperm motility evaluations of the frozen-thawed and vitrified-warmed samples were performed manually due the absence of a CASA system. Lately, the acquisition of the iSperm system allowed the evaluation of motility using this device (as presented in Specific Aim 2). However, this device has its limitations since its purpose is to be used in a farm. It is quite sensitive to low sperm concentrations and requires highly concentrated samples to provide an accurate measure, reducing the suitability for use in a laboratory. This was not a problem when fresh samples in extender were used but becomes increasingly complicated when testing samples which have undergone selection. Furthermore, the way the chip needed to be handled is prone to make bubbles, interfering in the assessment of the samples and requiring a skilled evaluator to load the sample successfully. These limitations could have been bypassed with the use of a CASA system integrated in one of our microscopes to offer a precise evaluation of sperm kinetic variables.

8.4 Optimising Vitrification Protocols

As previously mentioned, the increase in global pork consumption is driving improvements in the pig breeding industry to be more efficient and environmentally sustainable (Kumar *et al.*, 2021). Of the livestock species

transported in Europe, pigs are the highest transported species with an average of ~31 million animals transported per year (EFSA Panel on Animal Health and Welfare, 2022). In optimising protocols to facilitate the transport of genetics, whilst also reducing the welfare consequences on animals and the impact to the environment, the cryopreservation of sperm is key to achieving this.

By optimising vitrification methods to study the impact on sperm, we have opened a new line of investigation to improve the efficacy of vitrification by demonstrating that these techniques are available for boar sperm cryopreservation. The key limitation in using the vitrification protocol in this thesis is the low concentration of the samples in vitrification groups which may limit the application of this approach for intrauterine insemination (IUI). This, however, would not be an issue for other *in vitro* methods such as IVF or ICSI (Gil *et al.,* 2008). There is also the possibility of increasing the concentration as Saravia *et al*. did in 2005 and 2011. There is also the possibility of increasing the initial volume of fresh/extender sperm since this protocol only uses 2 mL extender semen. Furthermore, the sperm preparation prior to cryopreservation could have performed in a less invasive manner by swapping DGS for a single centrifugation step or using the MCS device in Specific Aim 2 since this selection method does not require centrifugation.

8.5 The Effect of Oocyte and Embryo Developmental Competence on IVP Outcomes

The inconsistent and poor development rates achieved from porcine IVP have been linked to the quality of the oocytes and inadequate IVF efficiency (Romar *et al.,* 2019). The quality of oocyte maturation has been shown to affect the developmental competence of the oocyte, which in turn effects IVF efficiencies

and the developmental competence of the embryo. The key to solving this is ensuring the maturation of oocytes to the correct meiotic stage (*in vivo* and *in vitro*) prior to fertilisation. Oocyte maturation is only successfully accomplished when the maturation of the nucleus and the cytoplasm are synchronised (Channing *et al.*, 1980; Eppig, 1996), with the distribution in the cytoplasm of cortical granules and mitochondria are great indicators to assessed cytoplasmic maturation (Liu *et al.,* 2010).

In the Specific Aim 1, the results from the assessment of CG distribution were key to discovering the effect of FLI in gilt oocytes during maturation. The use of FLI highlighted the importance of cytokine supplementation in IVM media to enhance the oocyte and embryo developmental competence by promoting the synchrony of the nucleus and cytoplasmic maturation. Though, cytoplasmic maturation was only assessed by the distribution of the cortical granules since the development of a protocol using Mitotracker Red CMXRos (Invitrogen™, Inchinnan, United Kingdom) to stain mitochondria failed. Several dye concentrations were assessed, however, all resulted in overstained samples that did not allow for clear identification of the mitochondria. Furthermore, other approaches were evaluated to assessed mitochondrial activity, such the measurement of mitochondrial DNA in the oocytes using qPCR (Read *et al.,* 2021), but was quickly dismissed after repeated failures trials to establish a protocol.

8.6 The Impact of the External Environment on IVP Process

Embryo *in vitro* culture is the longest and most crucial part of IVP process. In this period the embryos are very sensitive to external conditions, requiring an appropriate environment for the early embryo to undergo a series of cleavage

divisions and eventually form a blastocyst. As the most critical period is between the 2-4 cell and blastocyst stage (Cao *et al.*, 2014), the embryos were not evaluated for cleavage or subjected to media changes to prevent additional stress to the embryos. However, every time the incubator was opened for other experiments, the fluctuation of the temperature and the gas proportion could have drastically affected embryo development. The use of other incubators models (such as those with multiple chambers of smaller volumes) could have improved the results of these thesis since the embryos would face less disturbances. Moreover, the small volume of the chambers would facilitate a faster gas and temperature recovery, reducing environmental stress (Swain, 2020).

8.6.1 Laboratory Conditions

One of the challenges faced throughout all experiments was the inconsistent results obtained within the same experiment. The success of IVP is also determined by environmental conditions, chemicals, and equipment used (Lane *et al.*, 2007). The gathering of several different researchers in the same laboratory has made it impossible to maintain an IVF only laboratory. For example, the use of other compounds (such as ethanol), plastics or nitrile gloves may have adversely affected my results (Nijs *et al.*, 2009). Furthermore, the use of the same incubators by students inexperienced in ART, alongside the introduction of second-hand laboratory equipment that did not fit the quality standards of required for an IVF lab, could also contribute to the inconsistency in creating reproducible results. (Gardner *et al.*, 2005). These factors can cause a sudden decrease in oocyte maturation or blastulation rates as a result of embryotoxicity (Nijs *et al.*, 2009), which means that any of the tested

mediums/methods which were dismissed as failures could have yielded positive results in a different setting. In addition, due to the COVID-19 pandemic, new hygiene measures implemented by the University, such as the use of hand sanitisers, could also have been reflected in the presented results.

8.7 Evaluating the Effectiveness of PGT-A

Despite the benefits that PGT-A offers to improve IVF success and reduce the risk of miscarriage, its use continues to be very controversial; the existence of mosaicism and detection of false positives are the key factors used to negate the suitability of PGT-A testing in human clinics (Griffin and Ogur, 2018). Nevertheless, studies have shown that PGT-A improves live birth rates, reduces the chance of transferring aneuploid embryos, and reduces the economic losses of breeding companies (Tutt *et al.,* 2021; Silvestri *et al.,* 2021). However, the methods used in Specific Aims 4 and 5 have limited applicability. Biopsied blastocysts must be cryopreserved until a diagnosis is made, which has no adverse effect within the animal breeding industry but delays the use of the embryo in human IVF, which may be time critical for some patients. Furthermore, biopsies are usually collected from ~5 TE cells, making it impossible to determine the genetic content of each embryonic cell that forms the embryo.

Moreover, the bovine embryos investigated in Specific Aim 4 were already biopsied and screened for a commercial need, but the embryos used in Specific Aim 5 were dissected to isolate the ICM from the TE, making the embryo unusable for transfer. The use of other non-invasive methods could be employed for screening the embryo without being biopsied/dissected, such as the analysis of cell-free DNA in spent culture medium, which has been employed as a method

to predict embryo sex and viability and could also be used as a PGT-A method (Orvieto *et al.*, 2021).

8.8 Future Work

Further research is recommended to optimise the protocols that have been established to increase the developmental competence of oocytes from young animals. Despite the addition of FLI to the POM media, this media is still considered simple. Increasing the complexity of POM through the addition of more supplements with antioxidant effects, such as melatonin (Liang *et al.*, 2017) or lycopene (Kang *et al.*, 2021), would help to reduce the oxidative stress of the oocytes during IVM.

The focus on the improvement of boar sperm vitrification protocols is highly necessary in order to improve the sperm parameters affected by thermal and osmotic shock during vitrification. Vitrification is a potential tool which would be useful to protect the reproductive efficiency of cryopreserved sperm, but it requires additional exploration to identify a more efficient and less invasive sperm selection method. If the breeding industry is hesitant in shifting from the standard method of DGS for sperm selection, a reduction in the viscosity of the density gradient solution and the centrifugation time should be studied in order to reduce some morphological damage. Additionally, the combination of the MCS devices with other techniques, such as thermotaxis, could facilitate sperm migration within the device (Pérez-Cerezales *et al.*, 2018).

Furthermore, for Specific Aims 3 and 4, the evaluation of sperm fertilising capacity and polyspermy rates would have been beneficial; these are good indicators of the ability for sperm to penetrate the oocyte and quality of the

IVM/IVF protocols in place to reduce polyspermy (Suzuki *et al.*, 2003; Romar *et al.*, 2019). However, due to the COVID-19 pandemic, the number of ovary collections was very limited (no collections for ~1.5 years) since access to the abattoir was restricted, meaning these tests could not be performed.

With all the above taken into consideration, the studies carried out in Specific Aims 2 and 3 could be adapted to cattle IVP to enhance the efficiency of the established systems in order to reduce the production of chromosomally abnormal embryos. The selection of bull sperm currently uses DGS, which as seen in pigs, can have an adverse effect on many sperm parameters and could ultimately impact IVP outcomes. Furthermore, the vitrification of bull sperm is not established and there is very little published data to support its use.

In developing a vitrification protocol for bull sperm, this would open up the same commercial benefits which has previously been noted for pigs. In a similar fashion, the studies carried out in Specific Aims 4 and 5 could be adapted for use in porcine IVP. The introduction of PGT-A to our IVP protocol would be beneficial to study the incidence of chromosomal abnormalities in IVP porcine embryos and the effect of aneuploidy on their developmental competence, as has been shown with cattle embryos (Tutt *et al.*, 2021). In addition to this, the use of a time lapse system during embryogenesis would facilitate the assessment of abnormal or asymmetrical patterns of cell division, which could be associated with aneuploidy. It would also allow for the monitoring of cell reabsorption when apoptosis reacts to suboptimal developmental conditions, stress or chromosomal abnormalities, that could be missed if only blastocyst morphology is evaluated (Hardy,1997; Bets and King, 2001).

As a final point, to truly evaluate the efficiency of the methods employed in this thesis, embryo transfers should be conducted to determine whether the main goal of porcine IVP can be achieved: the birth of live born piglets. Non-surgical embryo transfers have been achieved by other groups, but the litter sizes are small with 4-12 piglets (Suzuki *et al*., 2004; Martinez *et al*., 2013). Therefore, improving the likelihood of successful embryo transfers is not only beneficial for the porcine breeding industry, but is a requirement.

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10 Appendix

Supplementary Table S1. Composition of each component stock solution used to prepare the different media. All solutions were dissolved in H_2O and all aliquots were stored for up to 4 months.

Component	Concentration (mM)	Volume (mL)	Quantity (g)	Aliquot Volume (µL)
Ca-lactate 5H2O	200	15	0.9248	110 and 400
Na-pyruvate	100	5	0.055	200 and 40
Hypotaurine	500	6	0.3273	110
L-Cysteine	100	4	0.4884	70
L-glutamine	200	6	0.1754	110
Glucose	500	6	0.54048	110
db-cAMP	50	5.7	0.1	50

Supplementary Table S2. Composition of the mix solution of hormones LH/FSH for IVM. All solutions were dissolved in H_2O and all aliquots were stored for up to 4 months.

LH/ FSH Solution					
Component	**Quantity**	**Final Concentration**	**50x Stock Concentration**	**Volume**	**Final Solution (40 mL)**
LH	100 iu	0.05 iu/mL	2.5 iu/mL	20 mL 0.1% BSA H_2O	20 mL LH + 20 mL FSH
FSH	100 iu	0.05 iu/mL	2.5 iu/mL	20 mL 0.1% BSA H_2O	

Supplementary Table S3. Composition of EGF stock solution. All solutions were dissolved in H_2O and all aliquots were stored for up to 4 months.

Component	Quantity	Final Concentration	1000x Stock Concentration	Volume
EGF	100 µg	10 ng/mL	10 µg/mL	10 mL 0.1% BSA H_2O

Supplementary Table S4. Composition of FLI stock solution. All solutions were dissolved in H_2O and all aliquots were stored for up to 4 months.

FLI Solution					
Components	**Quantity**	**Final Concentration**	**50x Stock Concentration**	**Volume**	**Final Solution (5 mL)**
FGF2	10 µg	20 ng/mL	2 µg/mL	100 µL 0.1% BSA H_2O	100 µL FGF2 + 50 µL LIF + 50 µL IGF1 + 4800 µL 0.1% BSA H_2O
LIF	5 µg	20 ng/mL	1 µg/mL	50 µL 0.1% BSA H_2O	
IGF1	20 µg	20 ng/mL	1 µg/mL	200 µL 0.1% BSA H_2O	
Aliquots of 25 µL					

Supplementary Table S5. Composition of TAL-c stock solution. All solutions were dissolved in H_2O and all aliquots were stored for up to 4 months.

TAL-c Stock Solution					
Components	**15x Stock Concentration (mM)**	**Solution 1**		**Solution 2 (Dilution)**	**Final Solution (30 mL)**
		Quantity (g)	**Volume (mL)**		
Theophilline	37.5	0.2032	29.325	N/A	29.325 mL Theophilline Solution 1
Adenosine	0.015	0.0267	10	1 mL Adenosine Solution 1 + 9 mL water	0.45 mL Adenosine Solution 2
L-cysteine	0.00375	0.0121	20	1 mL L-cysteine Solution 1 + 9 mL water	0.225 mL L-cysteine Solution 2
Aliquots of 1.1 mL					

Supplementary Table S6. Composition of base stock used to prepare all IVP medias (PXM, POM, PGM, and PZM). All solutions were dissolved in H_2O, and all stocks were stored at 4 °C for up to 4 weeks.

Base Stock 4x (250 mL)		
Components	**Concentration**	**Quantity (g)**
NaCl	1080	15.7788
KCl	100	1.86375
KH2PO4	3.5	0.11907875
MgSO4 7H2O	4	0.24647

Supplementary Table S7. Composition of bicarbonate stock solution. Solutions were dissolved in H_2O, and all stocks were stored at 4 °C for up to 4 weeks.

Bicarbonate Stock 4x (100 mL)		
Component	**Concentration**	**Quantity (g)**
NaHCO3	100	0.8401

Supplementary Table S8. Composition of HEPES stock solution. Solutions were dissolved in H_2O, and all stocks were stored at 4 °C for up to 4 weeks.

HEPES Stock 4x (100 mL)		
Component	**Concentration**	**Quantity (g)**
HEPES	100	2.3830

Supplementary Table S9. Trisomy analysis determined by Gabriel-Griffin plots. M= Maternal. P= Paternal. MI= Meiosis I. MII= Meiosis II. MT= Mitotic

Embryo Number	Diagnosis	Chr	Parental Origin	Error Origin
37	26MT	26	M	MI
44	14MT	14	M	MI
46	14MT 15MT	14	M	MI
		15	M	MI
52	14MT	14	M	MI
53	3MT 20UPD	3	M	MI
54	4MM 11T 18qDel	11	-	MT
69	XMT	X	M	MI
104	4MT 26MM 23T	4	M	MI
		23	-	MT
105	14MT	14	M	MI
132	14MT	14	M	MI
142	15MT 16MM	15	M	MI
146	15MT 23MT	15	M	MI
		23	M	MI
151	13MT	13	M	MI
172	12MT	12	M	MI
224	24MT	24	M	MI
251	14MT	14	M	MI
268	16MT 17MM 18MT	16	M	MI
		18	M	MI
274	6MT 14MT	6	M	MI
		14	M	MI
360	24MT	24	M	MI
397	12T	12	-	MT
431	14T	14	-	MT
485	4MT	4	M	MII
497	23MT	23	M	MI
499	14MT	14	M	MI
516	14MT 15MM	14	M	MI
518	26MT	26	M	MI
528	13MT	13	M	MI
530	19MT	19	M	MI
534	1MT	1	M	MI
537	3MT	3	M	MI
545	23MT	23	M	MI
560	11MT	11	M	MI
566	4MT	4	M	MI
601	27MT	27	M	MI
604	13MT	13	M	MI

Embryo Number	Diagnosis	Chr	Parental Origin	Error Origin
605	15MT	15	M	MI
613	4T	4	-	MT
615	24MT	24	M	MI
633	25MM 28MT	28	M	MI
664	2MT 16MM	2	M	MI
667	5MT	5	M	MI
680	6MT	6	M	MI
681	13MT	13	M	MI
695	25MT	25	M	MI
701	8T	8	-	MT
710	14MT 27MT	14	M	MI
		27	M	MI
737	23MT	23	M	MII
766	13MT 26MT	13	M	MI
		26	M	MI
767	14MT	14	M	MI
773	13T	13	-	MT
778	1T 3T 16T 20T 23T	1	-	MT
		3	-	MT
		16	-	MT
		20	-	MT
		23	-	MT
782	20MT	20	M	MI
841	14MT 16MM, 1dup	14	M	MI
916	19T	19	-	MT
920	15MT	15	M	MI
924	1MT	1	M	MI
934	12MT	12	M	MI
961	13MT	13	M	MI
984	14MT	14	M	MI
1006	24MT	24	M	MII
1014	29MT	29	M	MI
1020	14MT XMM	14	M	MI
1039	19T	19	-	MT
1050	17MT	17	M	MI
1074	14MT	14	M	MII
1075	14MT	14	M	MII
1082	9MT	9	M	MI
1105	12p&qDel 23PT	23	P	MI
1121	24MT	24	M	MI
1127	27MT	27	M	MII
1139	20MT	20	M	MI
1141	23T 27MT	23	-	MT

Embryo Number	Diagnosis	Chr	Parental Origin	Error Origin
		27	M	MI
1153	24MT	24	M	MI
1154	13MT	13	M	MI
1157	1MT 14MT 29MT	1	M	MI
		14	M	MI
		29	M	MI
1159	19MT 20MM	19	M	MI
1160	13MT	13	M	MI
1173	4T	4	-	MT
1180	11MT	11	M	MI
1203	14MT	14	M	MI
1215	6MM 14MT 15MM 29MM	14	M	MI
1324	23MT	23	M	MII
1341	9MM 11T 18qDel	11	-	MT
1352	25T	25	-	MT
1419	20MT	20	M	MI
1437	26MT	26	M	MI
1458	4MM 20MT 25MM	20	M	MI
1490	13T	13	-	MT
1491	4MM 14MT 25MM	14	M	MI
1502	20MT	20	M	MI
1524	6T	6	-	MT
1546	12MT	12	M	MI
1578	10MT	10	M	MI
1600	24MT	24	M	MI
1603	27T	27	-	MT
1611	3MT	3	M	MI
1620	24MT	24	M	MI
1622	26T	26	-	MT
1642	23MT	23	M	MI