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Porcine oocyte quality, *in vitro* embryo production and storage

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Abstract

In the pig breeding industry, embryo transfer is a technique expected to significantly change export and import of genetics in the future. Besides genetic gain, an advantage of embryo transfer is increased biosecurity. However, limiting factors for embryo transfer are embryo production and storage, since non-optimal *in vitro* culture conditions are affecting embryo development and quality, and porcine embryos are highly sensitive to freezing. Gaining knowledge on what differentiates low- or high-quality oocytes is of significant value to understand and improve *in vitro* embryo production. The focus of this thesis was storage of porcine *in vitro* produced embryos and the identification of novel oocyte quality parameters. The aim of paper 1 was to assess embryo quality of *in vitro* produced porcine blastocysts after 3 h liquid storage at 37 °C in CO_2-free medium by evaluating morphology, *in vitro* developmental capacity and apoptosis. The study demonstrated that 3 h storage did not affect embryo quality as there was no significant difference between the storage and control group after 3 h storage and the further 24 h conventional incubation. Paper 2 aimed to identify novel parameters for porcine oocyte quality by examining expression of selected genes in cumulus cells (CCs) and oocytes, by employing the model assuming oocytes from adult, cycling animals being of higher quality compared to those from prepubertal animals. The study identified reduced expression of *BBOX1* and higher expression of *CPT2* in CCs before maturation and higher expression of *G6PD* and *ALDOA* after maturation as potential novel markers of oocyte quality. Paper 3 examined the abundance of transcripts supporting histone modifications during oocyte and early embryo development in oocytes of contrasting quality. This latter study required validation of the reference genes and identified the combined use of *ACTB* and *PFKP* as the most optimal normalisation for the porcine oocyte RT-qPCR data. Transcription did not appear to be silenced at the time of aspiration in oocytes collected from prepubertal gilts, and possibly not prior to resumption of meiosis. The results indicated delayed accumulation of transcripts supporting histone modifications associated with maternal imprinting, meiotic resumption, and embryonic genome activation in prepubertal gilt oocytes compared to sows. The results imply the investigated transcripts may have potential as markers of oocyte quality.

Sammendrag

I svineavlsindustrien er embryo-overføring en teknikk som forventes å endre eksport og import av genetikk betydelig i fremtiden. Foruten genetisk framgang, bidrar embryooverføring til økt biosikkerhet. Begrensende faktorer for bruk av embryooverføring er imidlertid produksjonen og lagring av embryo, siden suboptimale *in vitro* vekstforhold påvirker embryoutvikling og kvalitet, og svineembryo er svært sensitive for frysing. Økt kunnskap om hva som differensierer eggceller av lav eller høy kvalitet er av vesentlig verdi for å forstå og forbedre *in vitro* embryoproduksjon. Målet for denne avhandlingen var å studere lagring av *in vitro* produserte svineembryo og identifisere nye kvalitetsmarkører for eggceller. I artikkel 1 ble kvalitet til *in vitro* produserte svineblastocyster undersøkt etter 3 timer lagring i CO_2-fritt medium ved 37 °C, ved å evaluere morfologi, evne til å utvikle seg videre *in vitro* og apoptose. Studiet viste at 3 timer lagring ikke påvirket embryokvaliteten da det ikke var noen signifikante forskjeller mellom lagrings- og kontrollgruppen etter 3 timer lagring og ytterligere 24 timer konvensjonell inkubering. Målet til artikkel 2 var å identifisere nye markører for kvalitet av eggceller fra svin ved å undersøke uttrykk av utvalgte gener i kumulusceller og eggceller, ved å bruke en modell der eggceller fra voksne dyr antas å være av høyere kvalitet enn fra yngre, prepubertale dyr. Dette studiet identifiserte redusert uttrykk av *BBOX1* og høyere uttrykk av *CPT2* i kumulusceller før modning, og høyere uttrykk av *G6PD* og *ALDOA* etter modning som potensielle nye markører for eggcellekvalitet. Målet med artikkel 3 var å studere mengden av utvalgte epigenetikk-relaterte transkripter i eggceller av ulik kvalitet. Dette studiet krevde validering av referansegenene og identifiserte kombinasjonen av *ACTB* og *PFKP* som den optimale normaliseringen for RT-qPCR data fra eggcellene. Transkripsjon så ikke ut til å være stanset på aspirasjonstidspunktet i eggceller samlet fra prepubertale hunngris, og muligens ikke innen gjenopptak av meiose. Resultatene indikerte forsinket akkumulering av transkripter som støtter histonmodifikasjoner tilknyttet maternell imprinting, fortsettelse av meiose og embryonal genomaktivering i eggceller fra prepubertale gris sammenlignet med purker. Resultatene antyder at de undersøkte transkriptene kan ha potensiale som markører for eggcellekvalitet.

Preface

This thesis is a collaboration between Inland Norway University of Applied Sciences (INN) and Norsvin SA. The work was carried out at INN between September 2020 and December 2023.

After finishing my master's degree in ethology at NMBU, I worked for many years as an animal and transgenic technician at University College Dublin, Ireland, where I, amongst other things, assisted PhD students in their work. I got increasingly motivated for starting my own PhD, but first after moving back to Norway an opportunity came up. I still have the printed email which declared I was ranked as number one for the position! This has been a great journey both academically and personally.

Three people deserve special mentioning: My main supervisor Anne Hege Alm-Kristiansen; thank you so much for the great support you have given be. Both in supervision of this work, for motivating me to continue at a very challenging period of the PhD and as a friend. You keep an objective perspective when I get too involved. Reina Jochems; You were in the last year of your industrial PhD with Norsvin when I started. Thank you so much for training me in all the porcine IVEP procedures and your friendship. You are always very supportive, finding solutions to problems, and pretty much acted as my main supervisor for a period. Rob Wilson; without your support in relation to RT-qPCR the work for paper 2 and 3 would not have been possible. You were always patient, positive and helpful when I came asking questions again and again. And I have learnt a lot from you on scientific writing!

In addition, I would like to thank my other supervisors: Eli and Ann-Helen from Norsvin, thank you for your expert advice from the pig breeding section (along with Reina), and Ann-Helen for your always speedy reply with constructive advice! And from INN, Frøydis which was my main supervisor when I started, but had to withdraw after becoming the institute leader and Elisabeth with your extensive experience from research. Thanks to Teklu Zeremichael and Anne Bergljot Falck-Ytter for all their supporting services and all the students and other staff at INN for an excellent and supporting working environment. I have really enjoyed my time at INN with all of you!

My parents deserve a big thanks for minding the children every Tuesday early morning for the period when I was collecting ovaries. Thanks to friends for reminding me to stick true to myself, and my children for (sometimes) being patient, for being proud of me and reminding

me there are, after all, other things more important in life than this thesis. They have, nevertheless, developed a great interest for the lab and research!

Linda Marijke Haug,
Hamar, December 2023

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List of abbreviations

ACTB	Actin Beta
ALDOA	Aldolase, fructose-bisphosphate A
ATP	Adenosine triphosphate
ATP1A1	ATPase Na+/K+ transporting subunit alpha 1
BBOX1	Gamma-butyrobetaine hydroxylase 1
BRWD1	Bromodomain and WD repeat domain containing 1
cAMP	Cyclic adenosine monophosphate
CC	Cumulus cells
cDNA	Complementary DNA
COC	Cumulus-oocyte complex
CPT2	Carnitine palmitoyltransferase 2
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
EGA	Embryonic genome activation
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELF4	E74 like ETS transcription factor 4
ET	Embryo transfer
FAO	Fatty acid oxidation
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
G6PD	Glucose-6-Phosphate Dehydrogenase
gDNA	Genomic DNA
GREM1	Gremlin 1
GSH	Glutathione
H2A	Histone H2A
IVC	*In vitro* culture
IVD	*In vivo* derived
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* produced
KDM1B	Lysine demethylase 1B
KDM5A	Lysine demethylase 5A
LH	Luteinising hormone
PFKP	Phosphofructokinase, Platelet
PGC	Primordial germ cell
PPP	Pentose phosphate pathway
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative PCR
SALL4	Spalt-like transcription factor 4
SIRT1	Sirtuin1
SPF	Specific pathogen free
TCA	Tricarboxylic acid cycle
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
ZAR1	Zygote arrest 1

List of papers

I Haug, L. M., Jochems, R., Gaustad, A. H., Kommisrud, E., Myromslien, F. D., Grindflek, E., & Alm-Kristiansen, A. H. (2023). Liquid storage of porcine in vitro-produced blastocysts; a practical approach for short storage. *Zygote*, 1-10, 31. Doi: 10.1017/S0967199423000308.

II Haug, L. M., Wilson, R. C., Gaustad, A. H., Jochems, R., Kommisrud, E., Grindflek, E., et al. (2023). Cumulus Cell and Oocyte Gene Expression in Prepubertal Gilts and Sows Identifies Cumulus Cells as a Prime Informative Parameter of Oocyte Quality. *Biology (Basel).* 12, 1484. Doi: 10.3390/BIOLOGY12121484.

III Haug, L. M., Wilson, R. C., & Alm-Kristiansen, A. H. Epigenetic-related transcriptional reprogramming elucidated by identification and validation of a novel reference gene combination for RT-qPCR studies in porcine oocytes of contrasting quality. Accepted with minor revisions by Molecular Biology Reports.

1. Introduction

1.1. General background

Enhanced sustainability of meat production is essential for reaching the reduced emission goals and concurrently feeding the increasing world population (Fowler et al., 2018). Pigs display a lower feed conversion ratio and lower greenhouse gas emissions per kg produced meat than both beef and lamb (Fiala, 2008; Mottet et al., 2017) and may therefore serve as one of the more favourable meat sources. Porcine *in vitro* embryo production (IVEP) combined with embryo transfer (ET) technology can increase the sustainability of pig meat production by accelerating genetic gain. Applying ET facilitates selection of favourable production traits from both male and female gametes, i.e. 100% new genetics, compared to only 50% by use of artificial insemination (Grupen, 2014; Fowler et al., 2018; Chen et al., 2021). The use of oocytes collected from prepubertal gilts in IVEP has the potential of advancing this process by further decreasing the generation interval.

Progress has been made in porcine IVEP over the years, but the IVEP success rate and quality of embryos are still low compared to both their *in vivo* derived (IVD) counterparts (Sudano et al., 2013; Marsico et al., 2019) and *in vitro* produced (IVP) embryos of other livestock animals (Fowler 2018). This has primarily been related to incomplete cytoplasmic maturation in the porcine oocyte due to non-optimal *in vitro* culture conditions (Gilchrist and Thompson, 2007; Krisher et al., 2007; Prates et al., 2014; Gadea et al., 2020). Gaining knowledge of what differentiates a low- or high-quality oocyte or embryo could elucidate key processes involved in the acquisition of developmental competence. Therefore, additional novel quality parameters are required to further optimise *in vitro* culture conditions.

Quality parameters are also required to facilitate selection of embryos with the highest developmental capacity for an ET. Embryo transfer is expected to significantly change export and import of genetics in the future (Martinez et al., 2016). Besides genetic gain, the main advantage of ET is the biosecurity, as it leads to a minimal risk of pathogen introduction on farms when embryos instead of live animals can be distributed (Stringfellow and Givens, 2009; Fowler et al., 2018). Establishing specific pathogen-free (SPF) swine farms substantially contributes to reducing the climate footprint, as healthier animals will need less use of antibiotics and show a lower feed conversion ratio (Bonesmo and Enger, 2021). Norway

already has a considerable number of SPF farms, with more than 30% of the pigs classified as SPF, and the goal is to have all farms at SPF status within 2030 (Norsvin, 2023). In establishing and maintaining SPF farms, ET of "clean" IVP embryos is an invaluable tool (Fowler et al., 2018). Commercial use of ET also leads to less transport of live animals which enhances animal welfare, is better for the environment and decreases transportation and quarantine costs (Martinez et al., 2013; Fowler et al., 2018). However, apart from reduced quality of IVP embryos, another limiting factor for ET is embryo storage. To achieve acceptable farrowing rates, pigs require around 40 morulae or blastocyst stage IVD vitrified embryos for a non-surgical embryo transfer (Martinez et al., 2015). Little research has been conducted on IVP embryos in this category, but even higher numbers are likely required. Therefore, embryos need to be stored, under the best possible conditions, to obtain enough embryos at the desired stage of development, and to facilitate transport prior to transfer. Storage of porcine embryos has, however, proven challenging, mainly due to their high lipid content (Polge and Willadsen, 1978; Nagashima et al., 1994; Mandawala et al., 2016), and even more so with IVP than IVD porcine embryos (Maehara et al., 2012).

1.2. Porcine *in vitro* embryo production

Porcine IVEP still has lower success rates than most other species currently being employed in IVEP for either experimental or commercial purposes (Sturmey and Leese, 2003; Yoshioka et al., 2003; Prates et al., 2014; Gadea et al., 2020). In addition, porcine IVP embryos are of lower quality compared to their IVD counterparts (Macháty et al., 1998; Sudano et al., 2013; Marsico et al., 2019). Some remaining challenges are high rates of polyspermy, a developmental block at the four-cell stage; the time of porcine embryonic genome activation (EGA) (Cao et al., 2014; Bu et al., 2022; Zhang et al., 2022), and low blastocyst rates (Fowler et al., 2018). Incomplete oocyte cytoplasmic maturation has been suggested to explain most of these challenges (Gilchrist and Thompson, 2007; Krisher et al., 2007; Prates et al., 2014; Gadea et al., 2020). In addition, the high lipid content in porcine oocytes and embryos makes them particularly sensitive to oxidative stress and reduced temperatures, typical stressors of the IVEP process (Guérin et al., 2001).

Collecting *in vivo* matured oocytes by ovum pick-up is challenging in pigs, so most commonly ovaries are sampled from routinely slaughtered animals at the slaughterhouse.

Follicles of 3-8 mm diameter are collected, when the oocytes are expected to be fully grown and with the competence to resume meiosis (Hyttel et al., 1997; Hunter, 2000; Brevini Gandolfi and Gandolfi, 2001; Dvoran et al., 2022). Hence, oocytes need to complete maturation *in vitro* before fertilization. In addition to varying concentrations of different energy substrates and metabolites (Yoshida et al., 1992; Wang et al., 1997; Yoshioka et al., 2008), several supplements have demonstrated beneficial effects on porcine *in vitro* maturation (IVM), such as luteinizing hormone (LH), follicle stimulating hormone (FSH) (Mattioli et al., 1989), epidermal growth factor (EGF) (Abeydeera et al., 1997), dibutyryl cyclic adenosine monophosphate (cAMP) (Funahashi et al., 1997) and combining the three cytokines FGF2, LIF, and IGF1 (named FLI) (Yuan et al., 2017). However, as cytoplasmic maturation still remains unsatisfactory, the IVM media has commonly been supplemented with porcine follicular fluid (Wang et al., 1997; Yoshioka et al., 2003; Grupen, 2014; Romar et al., 2016). Follicular fluid collected from large sow follicles has proven superior in improving nuclear and cytoplasmic maturation and protecting the oocytes against oxidative stress compared to follicular fluid collected from prepubertal animals or smaller follicles (Grupen et al., 2003; Algriany et al., 2004; Ito et al., 2008). There are, however, concerns with the use of follicular fluid and other biological components, such as bovine serum albumin, in IVEP medium. The composition can vary between batches, leading to inconsistent results, and there is a potential risk of pathological contamination using biological material (Yoshioka et al., 2008; Krisher, 2013; Grupen, 2014). Therefore, chemically defined media, based on the composition of porcine oviductal fluid, has been developed (Yoshioka et al., 2008).

In vitro fertilization (IVF) is achieved by co-culturing oocytes with spermatozoa, where successful fertilization is the penetration of the oocyte's zona pellucida by one single spermatozoon (Fowler et al., 2018). However, 40% polyspermy is commonly reported in porcine IVF (Gadea et al., 2020). Several approaches have been explored for reducing the incidents of polyspermy, e.g. different coincubation times and sperm:oocyte ratios (Gil et al., 2007). The redistribution of cortical granules to the periphery of the oocyte during oocyte cytoplasmic maturation is an important preparation to prevent polyspermy (Jochems et al., 2021). Improved cytoplasmic maturation and quality of the oocyte will most likely reduce the extent of polyspermy, as it is mainly a problem with *in vitro* matured oocytes (Chen et al., 2021).

During *in vitro* culture (IVC) the presumptive zygotes are expected to start the process of cleavage, involving several rounds of cellular divisions, successfully undergo EGA at the 4-cell stage, and continue development to the blastocyst stage, which in porcine is usually reached on day 5-6 post fertilization. Lower blastocyst rates are achieved in pigs than in other species routinely used in IVEP (Sturmey and Leese, 2003; Yoshioka et al., 2003; Prates et al., 2014), as well as week-to-week variation in success rate (Fowler et al., 2018; Chen et al., 2021), possibly a result of varying quality of the oocyte material (Krey and Grifo, 2001; Krisher, 2013). Various supplements and composition of energy substrates have also been examined for the IVC medium (Kim et al., 2004; Medvedev et al., 2004; Mito et al., 2012; Redel et al., 2016; Chen et al., 2018a), in addition to different oxygen concentrations to reduce ROS (Booth et al., 2005). Supplementing the IVC media with reproductive fluids has shown to be beneficial (Canovas et al., 2017; París-Oller et al., 2021), but it is not as commonly applied in IVC as during IVM.

The chemically defined porcine zygote medium (PZM) was based on the components of the oviductal fluid and has demonstrated good blastocyst rates and litter sizes after ET (Yoshioka et al., 2003; Yuan et al., 2017) . However, an additional concern of porcine IVEP is the high incidence of chromosomal abnormalities, such as aneuploidy (McCauley et al., 2003; Jochems et al., 2023). Aneuploidy is also observed with IVD porcine embryos, but at a much lower rate than IVP embryos (Hornak et al., 2009; Jochems et al., 2023). Moreover, altered DNA-methylation and gene expression profiles observed in IVP blastocysts appeared to be affected by the inadequacy of chemically defined media, as supplementing the culture media with reproductive fluids did decrease these abnormalities (Canovas et al., 2017). Even when IVP embryos exhibit normal morphology, their quality may be compromised, and IVEP could have long term effects on offspring health and development due to these abnormalities (Krisher, 2004, 2013; Bauer et al., 2010). There have been substantial improvements in porcine IVEP through the years, but there are still challenges to overcome. Increased knowledge of the key processes involved in the acquisition of oocyte and embryo developmental competence could potentially provide insight for improving IVEP media and procedures, thereby reducing the differences between IVD and IVP embryos.

1.3. Oocyte development

Oocyte quality is acquired during oocyte growth and maturation and refers to the oocyte's potential for successful fertilization and embryo development (Krisher et al., 2007; Ledda et al., 2012). It is considered an intrinsic quality mainly determined by the follicular environment, with only limited ability to affect its developmental potential after the removal from the follicle (Ledda et al., 2012; Krisher, 2013; Leoni et al., 2015). Oocyte maturation can be divided in three categories, cytoplasmic, nuclear and epigenetic (Xu et al., 2017), all imperative for the oocyte's developmental competence. Cytoplasmic maturation entails biogenesis and arrangement of cytoplasmic organelles, redox homeostasis, changes in metabolism, and accumulation of mRNA, proteins and nutrients to sustain nuclear maturation, fertilization and early embryo development (Krisher, 2013; Jones and Shikanov, 2019). Nuclear maturation requires overcoming the arrest at meiosis prophase I, initiated with germinal vesicle breakdown and the assembly of the meiosis I spindle, and progressing meiosis to the extrusion of the first polar body and rearrest at metaphase II (Sutton-McDowall et al., 2010; Krisher, 2013; Fowler et al., 2018). There are two major epigenetic reprogramming events in gametes and early embryos. The first step is from somatic cells to gametes, deleting preceding DNA-methylation marks in the primordial germ cells (PGC), including imprinted regions, and re-establishing DNA methylation marks during oocyte growth. Secondly, after fertilization, erasing the gamete epigenetic marks and establish DNA methylation patterns to sustain embryo development (Mckay et al., 2021; Dvoran et al., 2022; Singh et al., 2023). Concerning epigenetic maturation, the oocyte must establish the correct epigenetic marks during oocyte growth to support progression through meiotic resumption, fertilization, and EGA (Viveiros and De La Fuente, 2019). Epigenetic modifications involve e.g. DNA methylation and histone modifications (Mckay et al., 2021; Dvoran et al., 2022; Singh et al., 2023), the latter being the focus in this thesis.

In the ovary, the oocyte is enclosed within a follicle. Oogenesis, the development of a competent oocyte from the PGC pool (Desai et al., 2017), takes place concurrent with folliculogenesis, the development of the follicular unit (Jones and Shikanov, 2019). These processes are influenced by interactions between the somatic granulosa cells and the oocyte, composition of the follicular fluid, granulosa and theca cell secretions and in the second half of folliculogenesis; gonadotropins secreted by the pituitary gland (Hunter, 2000; Krisher, 2013; Desai et al., 2017).

The pool of oocytes is formed during the foetal period (Krisher, 2013). In pigs, the PGCs of the female embryos are generated around day 18 after mating (Hunter, 2000) and preceding epigenetic marks are removed to allow the reprogramming of a gamete (Dvoran et al., 2022). PGCs measure less than 30 µm (Hyttel et al., 1997) and are found in germ cell nests in the ovary (Wear et al., 2016). Primordial follicles are formed when these nests break down and flattened pre-granulosa cells form a single layer around individual oogonia (Wear et al., 2016). Meiosis may start 40 days after mating, during the foetal period, and all oogonia have then developed into primary oocytes by day 35 after birth. At that point they are arrested in prophase I of the first meiotic division, the germinal vesicle stage (Hunter, 2000). These follicles remain dormant until activated (Wear et al., 2016).

As follicle development is initiated and the follicle develops into the primary state, the pre-granulosa cells get a more cubic shape, as illustrated in Figure 1, and start to multiply (Jones and Shikanov, 2019). At the early stages, folliculogenesis is dependent on intrafollicular communication between the oocyte and the granulosa cells. In the primary follicle, gap junctions are found between granulosa cells, but not between granulosa cells and the oocyte, where signalling is mainly through endocytosis (Hyttel et al., 1997; Hyttel, 2011).

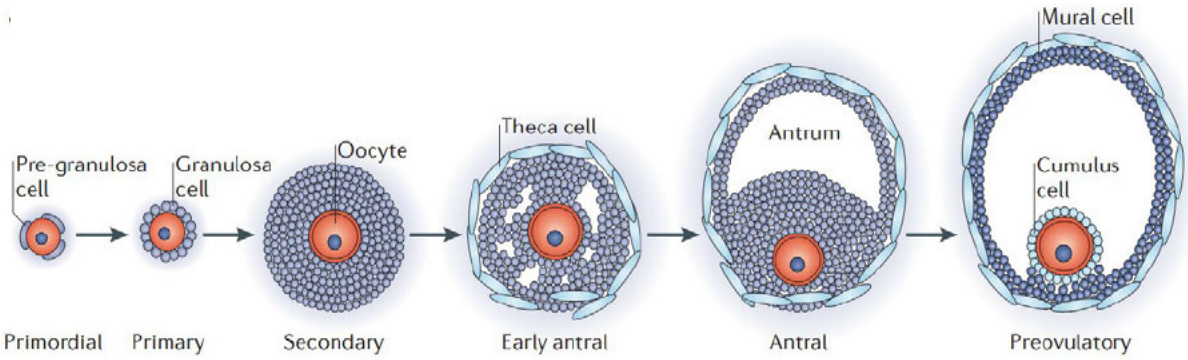

Figure 1. Follicle developmental stages. Oocyte development happens concurrently with folliculogenesis. As the follicles develop and granulosa cells proliferate, a cavity (antrum) filled with follicular fluid starts to form at the early antral stage. This divides the granulosa cells in cumulus cells surrounding the oocyte, and the outermost layer of mural granulosa cells (Li and Albertini, 2013). The figure is reproduced with permission from Li and Albertini (2013).

In the secondary follicle the granulosa cell layer surrounding the oocyte expands to approximately 6-7 layers (Araújo et al., 2014). The oocyte growth phase starts, during which time the oocyte diameter will increase from approximately 30 µm to 120 µm (Hunter, 2000). This takes several months (Hunter, 2000) and entails an increase in mitochondrial copy

number (Picton et al., 1998) and initiation of transcription for the synthesis and accumulation of mRNA, ribosomes, and proteins (Fair et al., 2002). Accumulation of these molecules is required for the time between meiotic resumption and EGA, when transcription is silenced (Leoni et al., 2007; Dvoran et al., 2022). The zona pellucida begins to form, and concurrently, transzonal projections from the granulosa cells with gap junctions at the end in direct contact with the oocyte cell membrane (Hyttel et al., 1997; Hunter, 2000; Sutton et al., 2003; Cui et al., 2009; Hyttel, 2011; Macaulay et al., 2016; Zhou et al., 2016). Apart from paracrine signalling, from this stage communication between the oocyte and granulosa cells shifts from endocytosis to direct contact through the gap junctions which can provide the oocyte with mRNAs and small metabolites to aid in the growth process (Hunter, 2000; Sutton et al., 2003; Hyttel, 2011; Auclair et al., 2013). Furthermore, maternal imprinting is reconstituted during oocyte growth. This process is completed by the time the oocytes reach the fully grown germinal vesicle stage (Dvoran et al., 2022). Additionally, both DNA-methylation and histone modifications are established to regulate transcription during the growth period and to facilitate correct gene expression during subsequent EGA (Dahl et al., 2016; Viveiros and De La Fuente, 2019).

Up to this point, follicle and oocyte development has relied on intrafollicular communication (Jones and Shikanov, 2019), but from the early (pre) antral follicle stage, development is primarily regulated by FSH, a gonadotropin produced by the pituitary gland (Li and Albertini, 2013). Theca cells are formed, and granulosa cells keep proliferating and develop FSH receptors (Jones and Shikanov, 2019). As puberty starts, the release of FSH at the follicular phase of each cycle will trigger the progression of early antral into antral follicles (Li and Albertini, 2013; Jones and Shikanov, 2019). Approximately 84 days after a PGC is activated it reaches the antral (aka tertiary) follicle stage (Morbeck et al., 1992), where several follicles will be selected to continue growth and development (Desai et al., 2017) and a cavity filled with follicular fluid, called the antrum, is formed amongst the granulosa cells (Araújo et al., 2014). The follicular fluid surrounds the oocyte and is required for follicular and oocyte development (Drummond, 2006). It contains hormones, enzymes, electrolytes, antioxidants and nutrients (Basuino and Silveira, 2016) from both the blood and secretions of the granulosa cells (Jones and Shikanov, 2019). The antrum divides the granulosa cells in the cumulus cells (CCs), the cells surrounding the oocyte, and the mural granulosa cells, which surround the antrum and line the follicular wall (Jones and Shikanov, 2019). With the influence of FSH,

granulosa cells can convert androgen, produced by theca cells, into oestrogen (Li and Albertini, 2013; Desai et al., 2017). This latter in turn further stimulates granulosa cell proliferation and progression of FSH receptors, making the follicle unit even more responsive to FSH (Desai et al., 2017). However, this reduces FSH secretion, and only follicles with high levels of FSH receptors will continue to develop (Desai et al., 2017). In 2-3 mm antral follicles the oocyte will have reached almost its full size, at which point accumulation of mRNAs is expected to be complete and transcription silenced (Hyttel et al., 1997; Hunter, 2000; Dvoran et al., 2022). Contingent upon completion of the epigenetic modifications involved in facilitating transcriptional silencing, chromosome stability and segregation (Xu et al., 2017; Viveiros and De La Fuente, 2019; He et al., 2021; Dvoran et al., 2022), the oocyte is now competent to resume meiosis (Hunter, 2000). However, cAMP produced by the CCs is transferred to the oocyte through gap junctions and inhibits the resumption of meiosis.

The pre-ovulatory/graafian follicle may expand up to 8-10 mm in diameter, while the oocyte size remains constant (Hunter, 2000). This is the fully developed follicle which will lead to ovulation of the oocyte. An LH wave induces a serial of processes, i.e. synthesis of EGF-like proteins by the mural granulosa cells (Li and Albertini, 2013), eventually resulting in ovulation (He et al., 2021). LH triggers CC expansion, a process which mediates the closure of gap junctions and withdrawal of the transzonal projections, leaving the oocyte more self-reliant (Sutton et al., 2003; Hyttel, 2011). When the transzonal projections withdraw, cAMP levels in the oocyte will be reduced and thereby its inhibitory effect on meiosis resumption will also decrease (Desai et al., 2017). Together with other regulatory stimuli, this triggers the resumption of meiosis, initiated by chromatin condensation and breakdown of the germinal vesicle (He et al., 2021). Meiosis will progress until it reaches metaphase II, where it again arrests until fertilization (Jones and Shikanov, 2019). After ovulation, the corpus luteum is formed by the remaining granulosa and theca cells, secreting in particular the hormone progesterone to prepare and sustain pregnancy (Li and Albertini, 2013; Jones and Shikanov, 2019). If there is no pregnancy, the corpus luteum will degenerate between day 12-15 after ovulation, and a new cycle will commence (Soede et al., 2011; Ziecik et al., 2018).

1.4. Metabolism in porcine oocytes and preimplantation embryos

The preferred energy substrates during early stages of preimplantation development in most species employed in IVEP are pyruvate and lactate (Swain et al., 2002). Porcine oocytes and cleavage stage embryos appear to have a distinct metabolism compared to most other species in that glucose is the preferred substrate, metabolised through glycolysis (Swain et al., 2002; Krisher et al., 2007). The pentose phosphate pathway (PPP), a branch from glycolysis which e.g. supports the cell's redox balance, appears to be of particular importance, while pyruvate is mainly converted to lactate, not metabolized through the tricarboxylic acid cycle (TCA) (Swain et al., 2002; Sturmey and Leese, 2003; Krisher, 2004). Concurrent with a higher energy demand, glucose metabolism through the TCA increases at the blastocyst stage, comparable to other species (Swain et al., 2002). Within the cumulus-oocyte complex (COC) (Figure 2) glucose is mainly metabolized by the CCs, which supplies the oocytes with its metabolites (Krisher, 2013). However, glucose metabolism by the oocyte is still essential and suggested to have regulatory functions during oocyte maturation (Herrick et al., 2006; Krisher et al., 2007; Krisher, 2013).

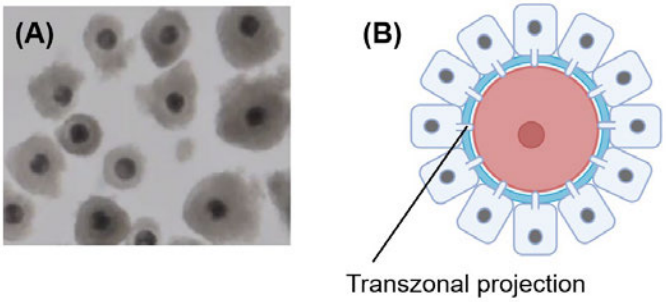

Figure 2. The cumulus-oocyte complex. (A) Oocytes surrounded by several layers of compact cumulus cells prior to *in vitro* maturation (L. Haug). (B) Schematic drawing of the innermost layer of cumulus cells surrounding the oocyte. The transzonal projections facilitate transfer of small molecules like RNAs, metabolites and cyclic AMP between the cumulus cells and the oocyte (Created in BioRender.com).

Cells can use two main sources of energy; exogenous or endogenous (Sturmey et al., 2009). Porcine oocytes and embryos also differ from those of other species by their high lipid content. The lipids are found as lipid droplets in the cytoplasm and mainly contain triglycerides (Sturmey and Leese, 2003). Mature porcine oocytes contain less triglycerides compared to immature oocytes and the intracellular lipids are suggested to play an important role in energy

metabolism and adenosine triphosphate (ATP) production during oocyte and early embryo development (Kikuchi et al., 2002; Sturmey and Leese, 2003; Sturmey et al., 2009; Krisher et al., 2012; Paczkowski et al., 2013; Prates et al., 2014).

Reactive oxygen species

IVP embryos are exposed to diverse stressors from the *in vitro* environment. Two of these are metabolic imbalance and increased oxidative stress (Leese, 2002). Reactive oxygen species (ROS) have an unpaired electron that can easily react with and damage other cellular molecules such as DNA, proteins and lipids (Leese et al., 2008; Bradley and Swann, 2019). Lipid peroxidation is a process where ROS steal electrons from free unsaturated fatty acids. This results in toxic lipid peroxides, and depending on the magnitude of the process, it may lead to lipotoxicity and cell death (Ayala et al., 2014; Bradley and Swann, 2019).

In the cell, metabolic activity in the mitochondria is the main source of ROS. Electron leakage from the electron transport chain may generate the highly reactive superoxide anion (O_2^-) which is easily converted to hydrogen peroxide (H_2O_2) (Bigarella et al., 2014; Bradley and Swann, 2019). By the transfer of electrons, antioxidants can neutralize ROS, e.g. H_2O_2 to H_2O and O_2. However, when ROS production is higher than the antioxidant capacity, it results in accumulation of ROS, which is known as oxidative stress (Bigarella et al., 2014). The redox state of the cell is mainly determined by the relative amounts of NAD(P)+/NAD(P)H and reduced glutathione (GSH)/oxidized glutathione (GSSG) (Figure 3), and it has been suggested that cytoplasmic maturation is regulated by the redox potential of the oocyte (Krisher, 2004).

ROS are also involved in cellular signalling (Krisher et al., 2007; Bigarella et al., 2014). Alterations in oxidative state can generate ROS-mediated communication between the nucleus and mitochondria, leading to altered gene expression to facilitate the most appropriate metabolism to sustain the redox balance (Bigarella et al., 2014). For the same purpose, ROS can cause functional changes to proteins by oxidation of amino acids, thereby modifying metabolic enzymes and influence the activity of different metabolic pathways (Bigarella et al., 2014).

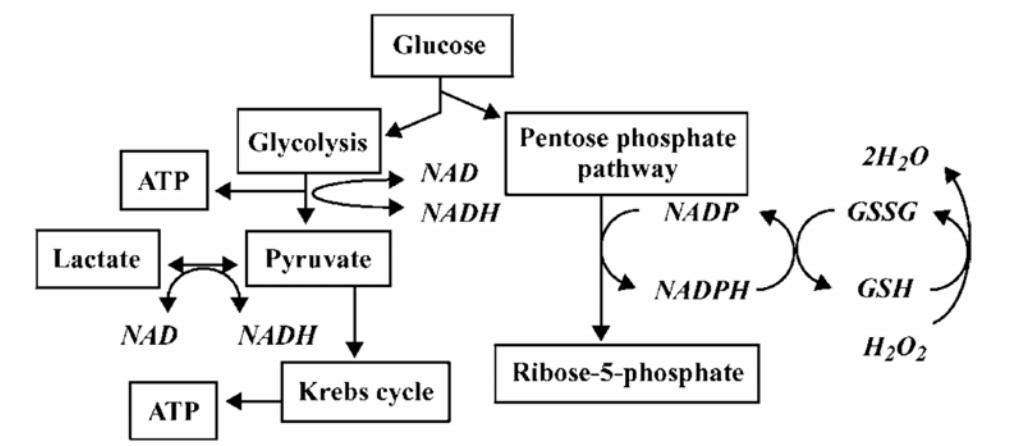

Figure 3. The association between glucose metabolism and redox potential within the cell. Molecules that have an impact on redox potential are italicized. GSH (reduced glutathione); GSSG (oxidized glutathione) (Krisher, 2004). The figure is reproduced with permission from Krisher (2004).

Alternative metabolic pathways

Porcine oocytes and preimplantation embryos have been suggested to share metabolic features to that of tumour cells (Krisher et al., 2012; Redel et al., 2012), known as the Warburg effect, named after the person who first recognised their distinctive metabolism (Warburg, 1956). This is characterized by increased glucose metabolism, but even when oxygen levels are abundant, most of the pyruvate is used for lactate production instead of maximising ATP production through the TCA cycle, concurrent with an increase in the PPP (Krisher et al., 2012; Redel et al., 2012). This implies the main purpose of glucose metabolism in these cells is not ATP production, but rather redox regulation and production of ribose-5-phophate for nucleic acid synthesis. In contrast to tumour cells, the energy demand of cleavage stage embryos is not very high, as their size do not increase until the blastocyst stage and there is no need to replicate all the cell components, only DNA and plasma membranes (Redel et al., 2012). However, both tumour cells and porcine oocytes and embryos require nucleic acid synthesis and redox control. The PPP produces NADPH, which is required for regeneration of GSH, an antioxidant protecting the cell against oxidative damage (Sturmey and Leese, 2003). This may be particularly important in porcine oocytes and embryos because of their high content of unsaturated lipids. Metabolic activity of the PPP is dependent on the rate of glycolysis (Swain et al., 2002; Herrick et al., 2006). Glycolysis requires NAD+ which is subsequently reduced to NADH. The purpose of reducing pyruvate to lactate may be to produce NAD+, required for continued glycolytic activity (Krisher et al., 2012; Redel et al., 2012).

Decreased flux of pyruvate throught the electron transport chain will reduce ROS production (Bigarella et al., 2014), both directly and indirectly, as more glucose would be available for the PPP and NADPH production (Krisher et al., 2012). Activity of the TCA cycle is important for oocyte maturation (Herrick et al., 2006), but porcine oocytes and preimplantation embryos might rely on FAO to produce ATP and TCA cycle intermediates (Krisher et al., 2012). The interspecies difference in intracellular lipid content is suggested to correlate to the importance of FAO during oocyte and early embryo development (Krisher et al., 2012; Bradley and Swann, 2019), and may also indicate the significance of the metabolic pathways described above (Krisher et al., 2012). Additionally, increased FAO may remove free fatty acids from the cytoplasm and reduce lipotoxicity (Bradley and Swann, 2019).

The quiet embryo hypothesis

According to the quiet embryo hypothesis, good quality embryos are expected to display a reduced metabolic rate compared to embryos of lower quality (Leese et al., 2008). This has two aspects. Oocytes and embryos with molecular or cellular damage might need to increase metabolism to sustain repairs, and it was found that cells with DNA damage had upregulated metabolism (Leese et al., 2008). In addition, more active metabolism per se was found to be associated with reduced viability, mainly caused by increased ROS, as ROS showed a stronger correlation to viability than metabolic rate (Leese, 2002). Hence, regulating metabolism to reduce the production of ROS is a key aspect of both the the Warburg effect and the quiet embryo hypothesis. In line with the quiet embryo hypothesis, nutrient provisions in the culture media should be relatively low, which again could lead to higher utilization of the endogenous energy reserves (Leese, 2002). It is further proposed that the oocytes and embryos not only need a quiet metabolism, but also an appropriate balance of the different metabolic pathways; a balance of pyruvate and FAO to sustain energy demands and at the same time avoid excessive accumulation of ROS (Bradley and Swann, 2019).

To sum up, capacity to regulate oxidative stress is particularly important for porcine oocytes and preimplantation embryos due to their high lipid content. To increase their redox potential, they appear to rely on FAO for ATP production and direct glucose metabolism towards the PPP and production of lactate instead of oxidation of pyruvate. It is still not established why the pig has such extensive energy reserves compared to other species, but interspecies

differences in lipid content is suggested to be correlated to litter size and preimplantation period (Sutton-McDowall et al., 2012; Prates et al., 2014; Bradley and Swann, 2019; Saint-Dizier et al., 2020). The high energy density of lipids makes them the ideal choice for energy reserve, and the cells have metabolically adapted *in vivo* to a high lipid content.

1.5. Porcine oocyte and embryo quality parameters

Gaining knowledge on the quality of oocytes and embryos is of significant value to understand and improve IVEP, measure the effect of experimental procedures, and for selecting embryos for ET. The extent of mechanisms affecting oocyte and embryo viability is still not fully understood (Krisher, 2004), but some involved are gene expression, protein translation, metabolism and redox homeostasis (Krisher, 2013). Studies have revealed substantial differences between IVD and IVP embryos, making these differences potential quality parameters. Oocyte and embryo quality parameters can broadly be divided in non-invasive and invasive parameters, where non-invasive parameters will not affect oocyte or embryo viability, whereas the invasive will harm or require the endpoint of the oocytes or embryos.

Invasive quality parameters

Metabolism is one of the mechanisms most frequently compromised during IVEP, and the metabolism of porcine IVP embryos differ significantly from that of IVD embryos (Swain et al., 2002; Krisher et al., 2007). IVP embryos have shown impaired mitochondrial function (Ménézo et al., 2013) and reduced rates of glycolysis (Swain et al., 2002) and the PPP (Krisher et al., 2007) compared to IVD embryos. However, there was no divergence in ATP concentration between *in vivo* and *in vitro* matured oocytes (Brad et al., 2003) nor between high and low quality porcine oocytes collected from prepubertal and adult oocyte donors (Brevini et al., 2005), hence ATP content does not seem to be a reliable oocyte quality parameter.

A consequence of IVEP conditions is increased levels of ROS (Guérin et al., 2001; Wu et al., 2011). *In vitro* matured oocytes have considerably less GSH than their IVD counterparts (Brad et al., 2003), and GSH content in matured porcine oocytes has shown to be correlated with subsequent developmental success (Brad et al., 2003; Krisher, 2004; Luberda, 2005; Herrick et al., 2006).

The purpose of apoptosis, programmed cell death, in preimplantation embryos is to remove irreversibly damaged or abnormal cells (Ramos-Ibeas et al., 2020; Cambra et al., 2021). The extent of apoptosis is higher in IVP than IVD embryos (Rubio Pomar et al., 2005; Lloyd et al., 2009; Wu et al., 2011) and has shown to reliably predict porcine (Chen et al., 2018b) and human (Haouzi and Hamamah, 2009) embryo quality and developmental capacity. It is, however, not a reliable parameter until the later stages of IVEP, as apoptosis does not appear until after EGA, and in porcine embryos it was not detected by the staining assay terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) until day five post fertilization, at the blastocyst stage, (Hao et al., 2003).

In addition, IVP blastocysts show reduced total cell numbers (Macháty et al., 1998), trophectoderm cell count (Ahlstrom et al., 2011; Ebner et al., 2016) and a lower ratio of inner cell mass to trophectoderm cell count (Macháty et al., 1998), compared to IVD embryos.

To sum up, measures of oxidative stress (i.e. GSH), the activity of different metabolic pathways, mitochondrial function, apoptosis and blastocyst cell count can all give valuable information on the quality of oocytes and embryos. Most of these factors can be assessed by employing different assays, or alternatively, by evaluating gene expression. IVP embryos show altered gene expression compared to IVD embryos, in particular related to metabolism and transcriptional regulation (Whitworth et al., 2005; Miles et al., 2008). Hence, RNA-sequencing or gene expression of target genes can potentially identify novel markers of oocyte and embryo quality. An advantage of applying gene expression studies is that many different processes can be assessed at the same time with minor additional workload and cost.

Non-invasive quality parameters

Invasive quality parameters can give valuable information on diverse processes. However, non-invasive parameters are essential when e.g. selecting oocytes for IVEP or embryos for ET, and novel reliable non-invasive parameters of both oocyte and embryo quality are required. Commonly applied non-invasive parameters of oocyte quality are the age of the oocyte donors (Marchal et al., 2001; Grupen et al., 2003; Sherrer et al., 2004; Ledda et al., 2012), follicle size, the extent of CC layers and the colour of the oocyte's ooplasm (Krisher, 2013; Chen et al., 2021). The most applied non-invasive embryo parameters are kinetics of development and morphology. IVP embryos do in general display delayed development compared to IVD embryos (Macháty et al., 1998; Bauer et al., 2010), and fast-cleaving bovine embryos have

demonstrated superior developmental potential to late-cleaving ones (Van Soom et al., 1997). This could be related to incomplete cytoplasmic maturation in the late-cleaving embryos, with inadequate accumulation of transcripts, proteins and nutrients required for further development and EGA (Van Soom et al., 1997). In bovine embryos, timing of first cleavage was, however, not correlated to the timing of blastocyst development, which was found to be a more reliable predictor of embryo quality, correlating to blastocyst cell numbers and pregnancy rates following ET (Van Soom et al., 1997). The high lipid content of e.g. porcine and bovine oocytes and embryos makes it difficult to assess morphological structures inside the cells. Also, bovine embryos of low quality, with chromosomal or genetic aberrations, may still demonstrate normal morphology (Van Soom et al., 2003). Combining morphology and timing of development will increase the reliability in comparison to applying the parameters individually and can aid in identifying embryos of poor quality (Van Soom et al., 2003).

Transcript abundance in CCs, if sampled after fertilization, is a potential non-invasive measure of oocyte quality. In addition, they may demonstrate higher response to altered IVM conditions than oocytes, as transcription in oocytes is mainly completed at the time of follicle aspiration (Hyttel et al., 1997; Picton et al., 1998; Dvoran et al., 2022). Studies in bovine and human have identified the abundance of several transcript in CCs to be potential markers of oocyte quality (McKenzie et al., 2004; Assidi et al., 2008; Caixeta et al., 2009). Metabolomics of spent culture medium can give valuable information on the metabolic status of the embryos and presents another alternative for non-invasive quality assessment (Botros et al., 2008; Rødgaard et al., 2015). However, to give information on single embryos, they would need to be cultured individually. In addition, equally to gene expression in CCs, metabolomics is currently not practical for selecting oocytes or embryos “on the spot”. Staining assays exists for use on live oocytes and embryos, but since porcine oocytes and embryos are highly sensitive to oxidative stress and reduced temperatures, the procedure would most likely impair their developmental competence. Establishing novel invasive and non-invasive reliable oocyte and embryo quality parameters is essential for identifying the processes most influential on oocyte and embryo quality, improving IVEP and enabling selection of oocytes and embryos of the highest developmental competence.

1.6. Storage of porcine embryos

Commercial application of ET is dependent on the storage of embryos. For the preferred non-surgical ET it is recommended to transfer around 40 morulae or blastocysts with *in vitro* developmental capacity per recipient sow (Martinez et al., 2015, 2019b) and it can be difficult to obtain enough embryos at the right developmental stage from one IVEP round. In addition, transportation worldwide or even throughout one country takes time and therefore embryos must be stored and transported under the best possible conditions prior to ET. It is important that embryos survive without developing further and/or hatch during storage, as intact zona pellucida is required for ET (Martinez et al., 2019a). The most common preservation technique used in reproductive medicine is cryopreservation, which involves freezing cells and tissues at cryogenic temperatures. There are two principal techniques for cryopreservation: The slow freezing method and the more rapid freezing method of vitrification (Mandawala et al., 2016). During slow freezing, ice crystal formation in the extracellular solution dehydrates the cells. It requires a lower concentration of toxic cryoprotective agents than vitrification, however, the extracellular ice crystals damage the cells. During vitrification, the use of a medium of high viscosity prevents the formation of ice crystals (Mandawala et al., 2016). Nonetheless, vitrification requires a high concentration of toxic cryoprotective agents and is considered a more technically challenging procedure than slow freezing (Mandawala et al., 2016). Porcine embryos are highly sensitive to freezing due to their high lipid content (Polge and Willadsen, 1978; Nagashima et al., 1994; Prates et al., 2014; Martinez et al., 2019b) and they have demonstrated low survival rate with the slow freezing method (Berthelot et al., 2003). However, promising results have been made for embryo preservation involving vitrification (Berthelot et al., 2000; Cuello et al., 2004, 2008, 2016) and short-term liquid storage (Martinez et al., 2018, 2019a) for IVD embryos. Nonetheless, preservation of IVP pig embryos is more challenging. Survival rates of 51 % has been reported after vitrification of IVP embryos (Nohalez et al., 2018) compared to >90% with IVD embryos (Cuello et al., 2016), and more research is required to achieve high quality IVP embryos upon storage.

Short-term liquid storage involves keeping embryos in a storage medium at or close to physiological temperatures, eliminating the requirement for liquid nitrogen. Eppendorf or cryotubes containing the embryos and media can be kept in a portable transport box set at a distinct temperature. Promising results for IVD embryos have been achieved, with morula stored at 37 °C for two-three days demonstrating close to 100% survival rate. The majority of

the morula progressed to blastocysts during storage but without hatching (Martinez et al., 2018). However, applying physiological temperatures limits the storage time, as development will continue. Reducing the temperature down to 20 °C has been explored, which did prevent further development, but it also had negative effects on embryo quality (Martinez et al., 2019a; Lin et al., 2022). Nonetheless, in establishment of SPF farms short term liquid storage could be a good alternative as embryos often need to be transported for short distances only, and ET of fresh embryos would be advantageous.

As mentioned above, porcine embryos' high concentration of intracellular lipids negatively affects their cryotolerance. Temperatures below 15 °C cause irreversible damage to the embryos (Polge and Willadsen, 1978) through separation of membrane lipids (Edidin and Petit, 1977; Nagashima et al., 1994). These chilling injuries are affected by the composition of phospholipids in the cell membrane (Edidin, 2003; Sudano et al., 2013). Supplementing the culture media with specific unsaturated fatty acids and/or cholesterol demonstrated the ability to alter the lipid composition of the membrane and increase cryotolerance, as reviewed in Sudano et al. (2013).

Reducing the amount of intracellular lipid also has the potential of altering the membrane lipids and reduce chilling injury. The application of serum free media has shown to improve cryotolerance, as e.g. addition of foetal calf serum will increase the embryo lipid content (Sudano et al., 2013). Nagashima et al. (1994) found that delipidating porcine embryos by micromanipulation prior to vitrification resulted in significantly increased cryotolerance. Unfortunately, it also decreased subsequent blastocyst development and farrowing rates (Nagashima et al., 1995). In addition, there is a risk of contamination after lipid removal as the zona pellucida may get damaged (Takahashi et al., 2013). Less invasive methods have been examined, and inhibition of triglyceride synthesis in bovine embryos resulted in a reduction in the amount of intracellular lipid droplets, and significantly increased embryo survival after vitrification (Cañón-Beltrán et al., 2020). Further embryonic development was however not investigated.

The lipid content can also be reduced by stimulating FAO. Long chain fatty acids are dependent on L-carnitine for entry into the mitochondria, where they are metabolized through β-oxidation before entering the TCA (Sutton-McDowall et al., 2012). Several studies have investigated supplementing the media with L-carnitine to increase embryo quality and

cryotolerance, both by stimulating FAO and by improving the embryos redox balance (Somfai et al., 2011; Wu et al., 2011; Takahashi et al., 2013; Lowe et al., 2017a, 2017b). These studies have, however, given inconsistent results (Carrillo-González et al., 2020). Since L-carnitine is a strong antioxidant (Wu et al., 2011; Sutton-McDowall et al., 2012; Takahashi et al., 2013), L-carnitine supplementation has potential, but the effect of artificially increasing metabolism has not been examined and gives rise to concern, particularly in context with the quiet embryo hypothesis (Leese et al., 2008).

Porcine *in vivo* matured oocytes and IVD embryos also have substantial lipid stores (Kikuchi et al., 2002; Sturmey and Leese, 2003) and studies have reported the oocytes' high lipid content to be associated with increased developmental capacity (Auclair et al., 2013). The intracellular lipids are proposed to function as an energy reservoir (Sutton-McDowall et al., 2012; Prates et al., 2014; Bradley and Swann, 2019; Saint-Dizier et al., 2020), moreover, lipids are required for membrane synthesis (Bradley and Swann, 2019) and hormone production, hence the removal of lipids for the purpose of improving cryotolerance may have detrimental effects for further embryo development (Sutton-McDowall et al., 2012).

There are still obstacles to overcome before achieving good quality IVP embryos post storage, and optimal storage protocols remain to be established. Even so, the better quality of the embryos, the better they can withstand preservation (Marsico et al., 2019) and improving IVEP procedures would presumably increase the success rate after storage.

2. Aims of the thesis

Application of embryo transfer in the pig breeding industry can increase genetic gain in the breeding program, and even more by using oocytes from younger animals. In addition to distribute valuable genetics worldwide, transport of embryos instead of live animals strengthens animal welfare and lowers the risk of disease transmission. Embryo transfer is dependent on appropriate storage of the embryos prior to transfer. This has proven challenging with porcine embryos because of their high intracellular lipid content, and with *in vitro* produced porcine embryos in particular because of their reduced quality compared to *in vivo* derived embryos. Oocyte developmental competence is critical for *in vitro* embryo production. Identifying factors influencing oocyte quality is highly valuable for optimising oocyte *in vitro* maturation and thereby *in vitro* embryo production. Therefore, the overall aim of this thesis was to examine storage conditions for *in vitro* produced porcine embryos and to gain knowledge of oocyte quality parameters.

The following objectives were outlined:

- To examine how short-term liquid storage affects *in vitro* produced porcine embryo quality by evaluating morphology, *in vitro* developmental capacity and apoptosis (Paper 1).
- To identify novel porcine oocyte quality parameters through gene expression of cumulus- oocyte complexes collected from prepubertal gilts and sows with a focus on metabolic pathways (Paper 2).
- To examine gene expression of epigenetic-related genes as potential oocyte quality parameters using immature and *in vitro* matured oocytes collected from prepubertal gilts and sows (Paper 3).

3. Results: Summary of individual papers

Paper I: Liquid storage of porcine *in vitro*-produced blastocysts; a practical approach for short storage

Commercial application of embryo transfer in pig breeding is dependent on the storage of embryos. For a non-surgical embryo transfer it is recommended to transfer around 40 good quality morulae or blastocysts per recipient sow, and it can be difficult to obtain enough embryos from one IVEP round. In addition, transportation takes time. The aim of this study was to assess embryo quality of *in vitro* produced porcine blastocysts after 3 h liquid storage at 37 °C in CO_2-free medium by evaluating morphology, *in vitro* developmental capacity and apoptosis. A 3 h storage period was chosen as it is a time span that would enable handling and transportation to a local recipient porcine farm for planned embryo transfers. Blastocysts at days 5 and 6 post-fertilization were randomly allocated to the storage group (HEPES-buffered NCSU-23 medium including bovine serum albumin in a portable embryo transport incubator at 37 °C) or control group (porcine blastocyst medium in a conventional culture incubator). Blastocysts were evaluated for morphology at the start of the experiment, post 3 h storage and after further 24 h *in vitro* culture, and stained to assess apoptosis after the 3 h storage period or after a further 24 h conventional incubation. There was no significant difference between the storage and control group after 3 h storage and further 24 h conventional incubation for any of the parameters, nor for apoptosis after 3 h storage. Embryos that reached the blastocyst stage at day 5 showed less apoptosis (6.6% vs 10.9%, P = 0.01) and a trend for higher developmental capacity (70.6% vs 51.5%, P = 0.089) than embryos reaching the blastocyst stage on day 6. In conclusion, *in vitro* produced porcine blastocysts can be stored for 3 h at physiological temperature in transportable incubators using a CO_2-independent medium without compromising quality. In addition, the 3 h storage period can possibly identify embryos of lower quality as these are most likely more susceptible to undergo a strong collapse.

Paper II: Cumulus cell and oocyte gene expression in prepubertal gilts and sows identifies cumulus cells as a prime informative parameter of oocyte quality

Cumulus cells (CCs) can transfer metabolites and small molecules to the oocyte and have pivotal roles during oocyte growth and maturation through supporting e.g. metabolic processes and the capacity to regulate oxidative stress. The aim of this study was to identify novel marker genes for porcine oocyte quality by examining expression of selected genes in CCs and oocytes, collected from prepubertal gilts and sows to compare cumulus-oocyte complexes (COCs) of low and high developmental competence, respectively. For both prepubertal gilts and sows, total RNA was extracted from CCs and oocytes either directly after follicle aspiration or after 44 h *in vitro* maturation, followed by RT-qPCR. Immature gilt CCs accumulated *BBOX1* transcripts, encoding an enzyme involved in L-carnitine biosynthesis, to 14.8-fold higher level ($P<0.05$) relative to sows, while for transcripts encoding CPT2, participating in fatty acid oxidation, the level was 0.48 ($P<0.05$). After maturation there were no significant differences between gilt and sow CCs for these genes, while *CPT2* and *BBOX1* levels in oocytes were higher in gilts than sows both pre and post maturation. Transcripts encoding key enzymes of the pentose phosphate pathway (G6PD), and glycolysis (ALDOA), were present in gilt CCs at levels of 0.64 ($P=0.044$) and 0.49 ($P=0.070$), respectively, relative to those in sows, after maturation. The apparent delayed lipid metabolism and downregulation of glycolysis and the pentose phosphate pathway, suggests prepubertal gilt COCs have inadequate ATP stores and oxidative stress balance compared to sows at the end of maturation. Reduced expression of *BBOX1* and higher expression of *CPT2* in CCs before maturation and higher expression of *G6PD* and *ALDOA* after maturation are new potential markers of oocyte quality. The results of this study imply porcine COCs can differentially modulate L-carnitine synthesis, and additional supplementation of the media with L-carnitine should be exercised with caution.

Paper III: Epigenetic-related transcriptional reprogramming elucidated by identification and validation of a novel reference gene combination for RT-qPCR studies in porcine oocytes of contrasting quality

Reliable RT-qPCR results are dependent on appropriate normalisation. Oocyte maturation studies can be challenging in this respect, as they exhibit transcriptional silencing upon completion of the growth phase, up till embryonic genome activation at the 4 cell stage and the stage of development can distinctively affect reference gene transcript abundance. The aim of this study was to validate the use of reference genes in oocyte *in vitro* maturation RT-qPCR studies, and thereafter, examine the abundance of transcripts supporting histone modification during oocyte and early embryo development in oocytes of contrasting quality. For this purpose, prepubertal gilt and sow ovaries were collected after slaughter and follicles with a diameter of 2-6 mm aspirated. Total RNA from oocytes from prepubertal gilts and sows was extracted either directly succeeding follicle aspiration or after 44 h *in vitro* maturation, followed by RT-qPCR. The stability of a selection of potential reference genes, *YWHAG*, *HPRT1*, *ACTB*, *GAPDH*, *HMBS* and *PFKP*, was analysed by NormFinder and further cross-validated by assessing results generated following application of different combinations of potential reference genes for normalisation of the RT-qPCR data. Combining *ACTB* and *PFKP* generated high stability according to NormFinder and results in accordance with similar studies. Applying this normalisation, gilt derived oocytes displayed significantly higher abundance than oocytes from sows of almost all the epigenetic-related transcripts studied (*HDAC2*, *SIRT1*, *SALL4*, *KDM1A*, *KDM1B*, *KDM5A*), both before and after *in vitro* maturation. This study identified the combined use of *ACTB* and *PFKP* as the optimal normalisation for porcine oocyte RT-qPCR data. Transcription did not appear to be silenced in oocytes collected from prepubertal gilts at the point of aspiration, and possibly not prior to meiotic resumption. Moreover, accumulation appeared to be delayed of transcripts supporting histone modifications facilitating meiotic resumption and embryonic genome activation, which may perturb fertilization and further embryo development. Further investigations are required to establish the potential use of these transcripts as oocyte quality parameters.

4. Discussion

4.1. Methodological considerations

4.1.1. Experimental designs

Discussion of experimental design of paper 1

Three hours storage time is relatively short compared to other published storage experiments. However, the only other liquid storage study conducted with porcine IVP embryos (Lin et al., 2022), found that 24 h storage did reduce embryo quality in all conditions tested. Moreover, they tested different storage media to that employed in paper 1. We wanted to test the storage medium employed by Martinez et al. (2014, 2018) as it had demonstrated very good results with IVD porcine embryos where both 24 h and 48 h liquid storage at 37 °C gave similar viability as their untreated control groups. However, we naturally anticipated reduced survival rate after storage of porcine IVP embryos compared to IVD embryos. During preliminary studies, both 3 h and 6 h storage times were explored. Considerable variation was observed between experimental rounds, with occasionally very low survival and further *in vitro* development after 6 h storage. For this reason, liquid storage for 3 h was chosen. A longer storage time could have been beneficial as it would have made the study applicable for more users, which is a long-term goal. At the same time, examining 3 h storage time was valuable for the breeding company, and a starting point for embryo transfer of IVP embryos for short distances, which would cover many farms.

During the 3 h storage study the percentage of blastocysts progressing or collapsing showed a noticeable variation between different experimental rounds. However, as there were always both a storage and a control group, this variation was considered controlled for. IVEP for paper 1 was conducted over 9 different rounds, hence diluting the effect of inter-round variation.

Blastocyst stages and morphology were assessed at three different time points throughout the experiment. During preliminary studies the classification of blastocyst stages was discussed, and criteria decided. The microscope used to determine blastocyst stages did not have a heating plate, therefore, to reduce chilling, pictures were taken instead of evaluating the blastocysts in real time. Evaluating in real time has advantages, but there is always a concern of inconsistency and/or bias with manual assessment, and the pictures

served as a good control. The images also added the possibility of comparing assessments at the start of the experiment with later evaluations, and reanalysing if necessary.

The Fixed-3 h groups were applied for staining only, while the Fixed-24 h groups in addition were assessed for further developmental capacity and morphology. As these latter are non-invasive parameters, morphology data at the 3 h time point was collected from the Fixed-24 h groups, to enable evaluation of further development from the 3 h to the 24 h time point. Additionally, further *in vitro* developmental capacity and the end point analysis of apoptosis were considered the most paramount parameters, therefore a larger group size for the Fixed-24 h group was implemented.

Discussion of experimental design of paper 2 and 3

In paper 2 and 3, different age and maturity of the oocyte donors were applied to obtain oocytes of contrasting quality. As described in paper 2 and 3, oocytes collected from prepubertal animals have demonstrated reduced developmental capacity compared to those from adult animals (Marchal et al., 2001; Grupen et al., 2003; Sherrer et al., 2004; Leoni et al., 2006; Ledda et al., 2012; Fang et al., 2016). This has been employed as a model to study oocytes of low and high developmental competence in e.g. cows (Romar et al., 2011), sheep (Leoni et al., 2007, 2015) and pigs (Braga et al., 2019; Silva et al., 2023). It could be argued that alongside extracting RNA from oocytes for the gene expression studies, oocytes from both prepubertal gilts and sows should concurrently have been fertilized and cultured to the blastocyst stage to assess developmental capacity. However, the extensive studies which led to the establishment of the model did verify these differences and the model should hence be applicable without re-confirming this for each new study. Other porcine oocyte studies have employed the model the same way as in paper 2 and 3 of this thesis (Braga et al., 2019; Silva et al., 2023). Moreover, when we have previously used prepubertal gilt oocytes for IVEP, we experienced reduced blastocyst rates compared to oocytes collected from sows (own observations).

As mentioned in paper 2, adding an additional sampling point after 20 h of *in vitro* maturation, in addition to the start and end of maturation, may have given valuable information on transcriptional activity in the latter stage of maturation and should be included in future studies. However, another practical parameter influencing the experiments was that

the local slaughterhouse our research group has used for years (Nortura, Rudshøgda, Norway) stopped slaughtering pigs not long after the material for paper 2 and 3 was collected.

Statistics

In reverse transcription quantitative polymerase chain reaction (RT-qPCR) studies there were three biological replicates for all the experimental groups, containing oocytes or CCs from several animals. With three biological replicates, it was not possible to utilize non-parametric tests with rank-transformation. In retrospect, having more biological replicates per group could have been advantageous. However, data were log base 10 transformed and tested for normality applying the Shapiro-Wilk test, and the t-test has proven appropriate with very small sample sizes (De Winter, 2013).

4.1.2. *In vitro* embryo production

Very many factors influence IVEP success rates. IVEP is dependent upon completed oocyte cytoplasmic, epigenetic, and nuclear maturation, followed by fertilization of the oocyte by a single, good quality spermatozoon and suitable culture conditions up to the blastocyst stage. The IVEP protocol employed in this thesis is based on the methods described in Jochems et al. (2022), which were performed in the same IVEP lab as the experiments in this thesis. With the exception of exchanging polyvinyl alcohol for bovine serum albumin, chemically defined media were employed, as described in Yoshioka et al. (2008) and Mito and Hoshi (2019). Ovaries were collected from routinely slaughtered pigs at Nortura, Rudshøgda, immediately after slaughter. For paper 1, 3-8 mm in diameter sow follicles were aspirated, at which point the oocytes are expected to have completed the growth phase (Hyttel et al., 1997; Hunter, 2000). For paper 2 and 3, follicle sizes of 2-6 mm in diameter, from both sows and prepubertal gilts, were aspirated. The follicle size range was moderately adjusted to correspond to the generally smaller follicle sizes of prepubertal gilts. The IVM protocol adopted had demonstrated high nuclear maturation rates of around 90% (Jochems et al., 2021). However, there is no definite measure for completion of cytoplasmic maturation (Krisher, 2013).

In paper 1, IVM was followed by *in vitro* fertilization. To facilitate fertilization from a single ejaculate for the whole study, frozen semen was used. Post fertilization, presumptive zygotes were cultured in porcine zygote medium till day 4, at which point they were moved

into porcine blastocyst medium, which has demonstrated to yield increased blastocyst quality (Mito et al., 2012; Mito and Hoshi, 2019), for the remaining culture time. In the liquid storage experiment, blastocysts of good morphology commenced storage the day they reached the blastocyst stage, either on day 5 or 6 post fertilization. Later developing blastocysts are expected to be of lower quality compared to earlier developing blastocysts (Van Soom et al., 1997). During preliminary studies, blastocysts developed on day 7 post fertilization demonstrated very poor morphology and further developmental rates after storage and additional 24 h conventional culture. Day 7 blastocysts were therefore not included in paper 1.

A general concern with porcine IVEP is the variation in blastocyst rates between different IVEP rounds (Fowler et al., 2018). Observations from the experiments in paper 1 suggests the quality of individual blastocysts obtained from high success rate IVEP rounds, i.e. above 20% blastocyst rate, are most likely better than those from IVEP rounds of low success rate, below 20%. It is difficult determining the causative factors of these variations. One possibility is variable quality of the oocyte material collected from the slaughterhouse, which could affect subsequent oocyte maturation, fertilization and embryo development. Variation in IVEP media may be another explanation. Apart from the use of bovine serum albumin, the studies involved in this PhD project employed chemically defined media, a preference by the breeding company as it reduces the risk of pathogen contamination in conjunctions with future embryo transfer. All porcine IVEP medium was made in house, which takes a lot of time and can be a source of variation (Gadea et al., 2020). In the future, trying ready-made porcine IVEP medium with porcine follicular fluid incorporated, would be of interest, at least for research purposes. The addition of follicular fluid may aid embryo development from reduced quality oocytes, and the cost of ready-made medium may possibly be outweighed by the time saved and increased IVEP success rate.

4.1.3. Confocal microscopy

A laser scanning confocal microscope (LSCM) is a special type of fluorescent microscope which rejects out-of-focus light and constructs high resolution, focused images of fluorescent molecules from one distinctive focal point (Semwogerere and Weeks, n.d.; St Croix et al., 2005; Nwaneshiudu et al., 2012). Different fluorophores can absorb and emit energy at specific wavelengths, corresponding to different colours. Hence, a sample can be stained with

several fluorophores, which can be visualized separately (Semwogerere and Weeks, n.d.; Nwaneshiudu et al., 2012).

Application of confocal microscopy in paper 1

To calculate the percentage of apoptotic cells of the blastocysts in the short-term liquid storage experiment, total cell number and the number of apoptotic cells were assessed by confocal microscopy. Total cell numbers were visualised by staining the nuclei with Hoechst 33342 (Sigma-Aldrich, Oslo, Norway), and apoptotic cells by TUNEL TMR red (Roche, Mannheim, Germany). The intention was to also analyse lipid content in another storage study examening supplementation of L-carnitine. Hoechst emits blue light, Bodipy^{TM} (ThermoFisher, Oslo, Norway) lipid staining green, and a TUNEL kit of red colour was therefore chosen. Combining these three staining assays did, however, prove troublesome. To reach into the nuclei of the inner cell mass, TUNEL required a rather high permeabilization treatment of the blastocysts, with the optimum conditions found to be 1 h in 0.3% Triton X-100 supplemented with 0.1% sodium citrate. As positive controls for TUNEL, blastocysts were treated with DNAse I. During the optimalisation procedure, not all cells were stained by TUNEL, as shown in figure 4A, indicating the protocol required further optimising. After increasing the permeabilization treatment, TUNEL appeared to reach all nuclei of the blastocyst, as seen in figure 4B. A lower degree of permeabilization was necessary for Bodipy to work optimally, and 0.1% Triton X-100 for 10 min was found favourable. No published papers were found which had stained the same cells with TUNEL and Bodipy, most likely because these procedures are not very compatible. For paper 1, we prioritised to analyse apoptosis after storage by TUNEL.

TUNEL TMR red will label both double and single stranded DNA breaks, as a specialised DNA polymerase adds a fluorescence labelled nucleotide, TMR-dUTP, to the free 3'-OH end of DNA (Loo, 2011; Mirzayans and Murray, 2020). In paper 1, a total volume of 20 µl TUNEL mixture was applied. To avoid evaporation of this rather small volume, the TUNEL mixture and blastocysts were incubated in a closed Eppendorf tube. Hoechst 33342 binds to double stranded DNA, both intact and cleaved. Concentration and incubation time of Hoechst was optimised to correspond to the permeabilization treatment found optimal for TUNEL. When optimising the different steps of the procedure, negative and positive controls of TUNEL were included. However, when staining blastocysts from the experiment, these controls were omitted to save valuable blastocyst material.

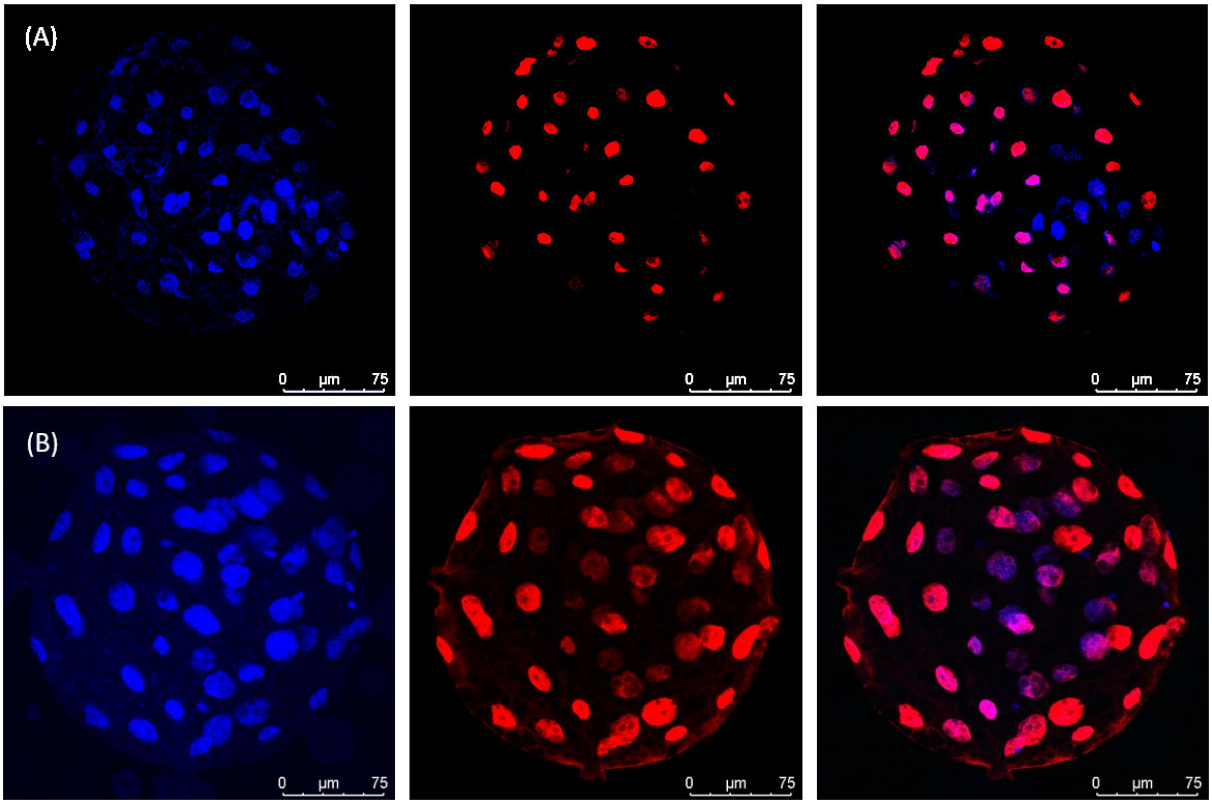

Figure 4. Optimalisation of TUNEL staining in porcine blastocysts. During optimising, blastocysts were treated with DNAse I prior to staining as positive controls for TUNEL. DNA was stained with Hoechst (blue colour) to visualise all nuclei and apoptotic nuclei were detected by TUNEL TMR red assay (red colour). Images on the right are Hoechst and TUNEL TMR red images merged. Panel (A) shows nuclei that has not been stained by TUNEL, while in (B), after further optimising the permeabilization treatment, all nuclei appear to be stained with TUNEL (L. Haug).

During optimising, the signal strength on the image monitor was set to prevent visualisation of background signalling for both TUNEL and Hoechst. During subsequent analysis, these settings were kept constant. To avoid exposing the specimens to extensive periods of light and hence photobleaching (Semwogerere and Weeks, n.d.; Nwaneshiudu et al., 2012), there were maximum 10 blastocysts per slide. Higher resolution requires longer time to scan the sample (St Croix et al., 2005) and considerations were made when setting the imaging parameters. In addition, mounting medium was applied to the stained blastocysts to reduce the speed of fading.

Both total and apoptotic nuclei were counted while manually scanning through separate planes of focus. Total cell nuclei were counted three times, and if there were only small differences, i.e. difference of 1 to 3 between counts, the average of these three counts were applied. If there were greater divergence, additional counts were made. To evaluate apoptotic cells, merged images of Hoechst and TUNEL TMR red were analysed on the monitor,

where true apoptotic nuclei would appear purple. Although the number of apoptotic nuclei were counted during microscopy, pictures were taken from different focal points for later reference.

Apoptotic nuclei can be fragmented and somewhat scattered, and it can be difficult to discern the boundary between different apoptotic nuclei if they are near each other. During optimising, criteria for how to judge these cases were discussed and decided. All subsequent assessments were conducted by the same person to reduce variation. TUNEL is not specific to apoptotic cells, and will also stain necrotic nuclei, in addition to cells undergoing DNA repair (Loo, 2011). As all these processes are associated with DNA damage and reduced embryo quality, it was decided not to discern the processes leading to DNA breaks, but to count and include all TUNEL positive nuclei.

4.1.4. Reverse transcription quantitative polymerase chain reaction

General principles of RT-qPCR

Reverse transcription quantitative polymerase chain reaction is a common technique to quantify specific RNA transcripts in gene expression studies. It is sensitive, i.e., it can detect low copy numbers of the specific target molecule, and it is relatively fast and cost effective (Leutenegger, 2001; Valasek and Repa, 2005). It entails RNA extraction, reverse transcription (RT) of RNA into complementary DNA (cDNA), which is subsequently applied as the template in qPCR reactions, where the amount of PCR product is monitored by fluorescent signals generated during each cycle (Valasek and Repa, 2005). Besides the inclusion of a fluorescent hydrolysis probe or intercalating dye, qPCR adheres to the same principles as PCR (Valasek and Repa, 2005).

In oocyte and embryo gene expression studies, very limited quantities of RNA are generally extracted. After RNA extraction, the samples are treated with DNAse I to remove contamination of genomic DNA (gDNA). However, assessing the presence of other contaminants and degree of RNA integrity are often only conducted during protocol optimisation, quality control steps that are left out when processing for the experimental samples, to save precious material. In addition, a fixed number of oocytes are often used instead of measuring RNA concentration (Bustin et al., 2009; Wang et al., 2017).

QPCR utilizes two main detection methods: Sequence-nonspecific and -specific. Nonspecific detection applies fluorophores, e.g., SYBR® Green or EvaGreen®, that emit substantially more fluorescence when intercalated into double-stranded DNA compared to unbound molecules in solution (Arya et al., 2005; Adams, 2020). The more double-stranded DNA, the higher the fluorescent signal (Valasek and Repa, 2005; Adams, 2020). However, the nonspecific fluorophores will bind to any double-stranded DNA sequence, and signals could be generated from both target and nontarget amplicons, including primer dimers (Ahmadipour et al., 2014). To assess amplification specificity and absence of primer-dimer interference, melting curve analysis can be performed following the amplification cycles (Adams, 2020). Of the specific detection methods, TaqMan® hydrolysis probes are most widely applied, consisting of an oligonucleotide with a 5'-coupled reporter fluorophore and a quencher fluorophore bound at the 3' end (Leutenegger, 2001; McGuigan and Ralston, 2002; Valasek and Repa, 2005). The oligonucleotide binds specifically to the target DNA sequence downstream of the primer (Leutenegger, 2001; McGuigan and Ralston, 2002). When in close proximity of each other on the same oligonucleotide probe, the fluorescence emitted by the fluorophore reporter is absorbed by the quencher. But, as Taq DNA polymerase extends the primer, it hydrolyses the probe from the 5' end, releasing the reporter from the quencher thus generating an increase in fluorescence. The detection of fluorescent signal increases in each consecutive cycle proportional to the amount of amplicon generated (Leutenegger, 2001; McGuigan and Ralston, 2002; Valasek and Repa, 2005). Applying sequence-specific detectors eliminates issues of non-specific amplification, e.g., primer-dimers, however, it is more expensive than utilising sequence-non-specific detection methods.

For both detection methods, the cycle at which the fluorescent signal becomes significantly higher than the background signal is referred to as the quantification cycle (Cq) which gives the the Cq value (Leutenegger, 2001; Bustin et al., 2009; Adams, 2020). The Cq value correlates to the copy number of the target sequence at the start of the reaction, the lower the Cq value, the greater target copy number was in the added cDNA sample and vice versa. At the end of PCR reactions, the amplification reaches a plateau. At this point the amount of PCR product will be similar independent of the amount of starting material and is why the Cq value is obtained at the earlier stage of PCR, at the commencement of exponential phase amplification (Soheili and Samiei, n.d.; Valasek and Repa, 2005).

Data analysis

Relative quantification of RNA relies on the appropriate use of reference genes for normalisation. Reference genes are applied to correct for any variation between samples concerning the amount of starting material and possible, sporadic errors during sample preparation. Furthermore, the reference genes should not be affected by experimental conditions and common reference genes are usually involved in processes essential for cell survival (Bustin et al., 2009; Kozera and Rapacz, 2013). For each new experiment the stability of a panel of carefully selected potential reference genes, based on their validated use in similar studies, should be assessed by one or more of the available algorithms, such as GeNorm (Vandesompele et al., 2002) or NormFinder (Andersen et al., 2004). GeNorm conducts a pairwise comparison between the analysed reference gene candidate and all the other included reference genes, where the stability measure, the M-value, is the average pairwise variation (Vandesompele et al., 2002). NormFinder calculates the individual stability of each reference gene using an ANOVA-based model, combining intra- and inter-group stability into a stability value (Andersen et al., 2004). For both approaches, lower values represent increased stability. GeNorm and NormFinder have been demonstrated to generate similar results (Zeng et al., 2014; Niu et al., 2017; Köhsler et al., 2020), but as they rely on different algorithms, applying both could be advantageous as a cross-validation. In particular, GeNorm can present a problem with co-regulated genes (Zeng et al., 2014) as it favours genes with the highest degree of similarity in expression profiles (Andersen et al., 2004). Co-regulated genes can more easily be identified by NormFinder (Zeng et al., 2014). To reduce potential errors, it is recommended to combine at least two reference genes of independent cellular functions for normalisation (Bär et al., 2009; Bustin et al., 2009; Kozera and Rapacz, 2013). These should be identified by one of the algorithms, because simply combining two genes can result in decreased stability (Andersen et al., 2004), as demonstrated in paper 3. NormFinder identifies the best combination of two reference genes. However, combining two might not generate acceptable stability, and GeNorm has the advantage of calculating the number of reference genes necessary to reach satisfactory stability levels (Vandesompele et al., 2002).

PCR reactions are exponential as amplicons generated in one cycle are employed as templates in subsequent cycles, thereby theoretically doubling the amount of PCR products per cycle (Valasek and Repa, 2005). The most widely applied method for calculating relative

mRNA transcript abundance has been the ΔΔCq method as outlined in Livak and Schmittgen (2001). However, these calculations assume invariant primer pair efficiencies of 100%, which can be incorrect (Bustin et al., 2009). Pfaffl (2001) describes a method which incorporates individual primer pair efficiencies. The final output of both methods is the fold change of the target gene relative to a control (Soheili and Samiei, n.d.). For example, with a fold change of 1.5 the transcript abundance was 150 % higher in the experimental group than in the control group, while a fold change of 0.3 corresponds to the transcript abundance being decreased by 70%, to 30% of that observed in the control group.

Application of RT-qPCR in this thesis

For the gene expression studies in papers 2 and 3, the enzymatic RNA extraction kit RNAGEM™ Tissue PLUS (ZyGEM, Hamilton, New Zealand) was employed, since it is described to be especially suited for RNA extraction from low cell numbers. DNAse I was included in the extraction kit, but the protocol for its use required optimising as gDNA was still detected after adhering to the manufacturer's guidelines. This was accomplished by performing the DNAse I treatment followed by cDNA synthesis, including a no reverse transcription (-RT) control along with the RT reaction. The -RT reaction includes all the components of the RT reaction with the exception of the RT enzyme, hence, no cDNA can be generated in the -RT sample (Taylor et al., 2018). Amplification using the -RT sample as template would indicate gDNA contamination and the requirement for further optimisation of DNAse I treatment. For the RNAGEM kit, both DNAse I incubation time and concentration had to be increased before the prepared sample appeared free of gDNA contamination. The optimised DNAse I protocol was subsequently applied in all the following sample preparations, where all the -RT controls were found to be negative. Concentration of RNA was measured using a Qubit™ fluorometer applying the Qubit™ RNA High Sensitivity assay kit (Invitrogen, Oslo, Norway). Similar amounts of RNA were reverse transcribed in both the oocyte and CC samples, as recommended in Bustin et al. (2009).

In preliminary experiments the non-specific fluorophore EvaGreen® (SolisBiodyne, Tartu, Estonia) demonstrated insufficient sensitivity with less than 100 ng RNA starting material. As we were optimising the procedures with both oocyte and embryo gene expression studies in mind, it was decided to test whether TaqMan hydrolysis probes were applicable with the lower amounts of RNA instead of increasing the number of oocytes per

sample. TaqMan probes were applied as assays, which contain both the probe and primers for the gene of interest. TaqMan® Gene Expression FAM-labelled Assays (Applied Biosystems, Foster City, California) demonstrated increased sensitivity compared to the non-specific fluorophore EvaGreen and were hence applied for both paper 2 and 3. To minimise potential pipetting errors, the recommended 60:40 ratio of qPCR master mix to cDNA was employed, by further diluting the cDNA samples (Taylor et al., 2018). When possible, all the different samples and assays were run on the same PCR plate, with technical replicates on separate plates, to control for inter-plate variability (Taylor et al., 2018).

Appropriate normalisation of data is of increased importance in situations with only subtle differences in gene expression (Bär et al., 2009), as was observed in papers 2 and 3. Validating reference genes can be particularly challenging in oocyte maturation studies, as the stage of development can be expected to affect reference gene expression (Kumar et al., 2012; Kozera and Rapacz, 2013; O'Connor et al., 2013). In paper 2, five potential reference genes were analysed by NormFinder. Actin beta (*ACTB*) was identified as the most stable reference transcript for both oocytes and CCs and was subsequently applied for normalisation of the RT-qPCR data in paper 2.

In paper 3, *ACTB* was first identified as the single most stable reference transcript, and *ACTB* and *HPRT1* as the best combination with increased stability compared to *ACTB* applied alone. However, normalising the oocyte RT-qPCR data for the epigenetic-related genes to *ACTB* and *HPRT1* generated results in disagreement with similar studies, which triggered further experimentation to validate the reference genes. In pursuit of the most appropriate normalisation, other genes were also considered. NormFinder recognised phosphofructokinase, platelet (*PFKP*) as the single most stable reference transcript, and *ACTB* and *PFKP* as the best combination, demonstrating higher stability than *PFKP* alone. The results generated after normalising to *ACTB* and *PFKP* combined was in concordance with similar studies and was applied to normalise the oocyte RT-qPCR data in paper 3. To further assess *PFKP* as a reference gene, *PFKP* was included in stability analysis for the CC data in paper 2. NormFinder identified *ACTB* as the single most stable gene and the combination of *ACTB* and *PFKP* as the best combination, demonstrating better stability than applying *ACTB* alone. Reanalysing the RT-qPCR data from paper 2, applying the combination of *ACTB* and *PFKP* for normalisation, generated the same trends as when normalising to *ACTB* alone.

4.2. Storage of porcine embryos

Short term liquid storage with IVP porcine embryos was investigated in paper 1. Embryos were evaluated for morphology, further *in vitro* developmental potential and degree of apoptosis after storage. The conclusion of the study was that 3 h liquid storage in CO_2 independent media did not compromise embryo quality. Additionally, the study showed that faster developing embryos (reaching the blastocyst stage on day 5 post fertilization) tolerated storage better, demonstrated through less apoptosis and higher developmental capacity, than slower developing embryos (reaching the blastocyst stage on day 6 post fertilization). This supports that the timing of blastocyst development is a reliable predictor of embryo quality (Van Soom et al., 1997). Embryos of both the storage and control group did, however, display increased rates of collapsing than what is observed during IVEP with no experimental treatment (own observations). This demonstrates the sensitivity of IVP embryos, where merely the increased time outside the incubator affects embryo viability. The inclusion of a control group where the embryos were only subjected to standard IVEP procedures could have been beneficial. However, the experimental design employed elucidated exclusively the effect of CO_2 free media and the transport incubator, which was the aim of paper 1. Since 3 h storage in CO_2 independent media did not compromise quality, studies with longer storage times can be conducted. Simultaneously, embryo transfer experiments with IVP embryos stored for up to three hours can be implemented by the breeding company using the method in paper 1. However, liquid storage studies for longer time periods with IVP embryos are required.

As mentioned under experimental designs, there were substantial variations between different experimental rounds in how many embryos progressed development and how many collapsed. The variation appeared to correlate with IVEP success rate, i.e. more blastocysts progressed during the experiment in rounds with high IVEP success rate (more than 20% blastocyst development) (Table 1). This implies embryos from high blastocyst rate IVEP rounds were of increased quality, which suggests IVEP blastocyst rate could be an additional parameter of embryo quality. This would be a general parameter for all the embryos of the same IVEP round, and more research is needed to identify what differentiates the embryos from low and high IVEP rounds. Moreover, the process of storage can be considered a stress test, and the embryos' morphology post storage can aid in identifying embryos of poor quality before ET (Van Soom et al., 2003; Haug et al., 2023).

Table 1. Rate of progression and collapsing as a factor of IVEP blastocyst rate. The proportion of porcine blastocysts that progressed or collapsed after the 3 h storage period and further 24 h conventional incubation in paper 1. The control (3 h in conventional incubator and conventional medium) and storage (3 h in portable incubator in storage medium) groups were merged in the high IVEP (blastocyst rates above 20%, N=62) and low IVEP (blastocyst rates below 20%, N=40) categories. Data was analysed using Fisher's exact test. Results are shown as number (n) and percentage of progressed and collapsed blastocysts.

	High IVEP	Low IVEP	p-value
Progressed	(41) 66.1 %	(18) 45.0 %	0.04
Collapsed	(15) 24.2 %	(16) 40.0 %	0.12

In paper 1, bovine serum albumin was supplemented to the IVEP media. However, for vitrification, serum free media should be applied (Sudano et al., 2013). In addition to optimising storage procedures, identifying methods to increase IVP embryo quality appears to be the most critical factor influencing the success rate of both liquid storage and vitrification. Supplementing both the culture media and storage media with an antioxidant may be beneficial, and L-carnitine supplementation has been shown to increase embryo survival after cryopreservation (Chankitisakul et al., 2013; Takahashi et al., 2013). Following the 3 h storage study, we have initiated an 18 h liquid storage study, applying the same storage media and transport incubator as in paper 1. Two experimental groups were compared, 18 h storage with or without L-carnitine supplementation during *in vitro* embryo culture. Different L-carnitine concentrations were tested during optimising, starting at 2.5 mM, according to the results of similar studies (Wu et al., 2011; Chankitisakul et al., 2013; Takahashi et al., 2013; Lowe et al., 2017b). However, blastocyst and success rate after storage was found to be impaired with this L-carnitine concentration. Gradually lowering the L-carnitine concentration, 1,5 mM was adopted. It did not appear to improve embryo quality, but also not impair it. In retrospect, an even lower concentration would possibly have been advantageous, if any supplementation of L-carnitine at all. The results from paper 2 suggests porcine COCs can differentially express genes encoding enzymes involved in L-carnitine biosynthesis, which implies porcine oocytes have the ability to regulate L-carnitine biosynthesis. Blastocysts also demonstrated expression of the same two genes involved in L-carnitine biosynthesis (own unpublished data), which indicates L-carnitine supplementation should be done with caution to avoid excess concentrations. Additionally, the L-carnitine concentrations reported in other studies were based on IVEP with oocytes collected from prepubertal gilts. Paper 2 implies L-carnitine supplementation would be more beneficial for gilt than sow oocytes, in agreement

with Carrillo-González et al. (2020). This could possibly explain why the L-carnitine concentration had to be reduced for blastocysts obtained from IVEP of sow oocytes. It could be interesting to compare the effect of L-carnitine during storage on IVP embryos derived from oocytes from prepubertal gilts versus sows. Nonetheless, although porcine oocytes and embryos appear to be capable of regulating L-carnitine biosynthesis, there might be a delay which makes their own synthesis inadequate with sudden exposure to raised oxidative stress. Additionally, the antioxidant effect of L-carnitine might be particularly effective in reducing oxidative stress generated by metabolism, because of its location in the mitochondria. Hence, there might be other antioxidants than L-carnitine more beneficial for reducing oxidative stress experienced during storage, which should be explored in future studies. In regards to vitrification, the approach of altering the lipid composition of the membrane to increase the embryos' cryotolerance appear to have potential and would be interesting to pursue.

4.3. Potential novel porcine oocyte quality parameters

In paper 2 and 3, COCs collected from gilts and sows were applied as a model to compare oocytes of low and high quality, respectively (Marchal et al., 2001; Grupen et al., 2003; Sherrer et al., 2004; Ledda et al., 2012). To identify processes affecting oocyte quality, it is valuable to investigate both oocytes and CCs because of their close inter-dependency (Krisher, 2004). Few studies have examined gene expression in porcine CCs with the aim of identifying oocyte quality markers. To select the experimental genes for paper 2 and 3, a thorough literature search was conducted to pinpoint processes or specific transcripts identified to affect oocyte quality in porcine or other species. There was little information to be found on porcine CCs, but supplementary RNA-sequencing data from Bu et al. (2022) and the webtool BioGPS pigatlas (http://biogps.org/pigatlas/#goto=welcome) were used to obtain information on expression levels in oocytes for the genes of interest.

Potential oocyte quality parameters in paper 2

Because of the high lipid content in porcine oocytes and their vulnerability to oxidative stress, genes related to lipid and glucose metabolism, in particular the PPP, was of interest. Transcripts involved in lipid metabolism had previously been examined in porcine oocytes applying the gilt/sow model (Yuan et al., 2011; Braga et al., 2019), demonstrating higher

abundance in gilt oocytes compared to sows after maturation, explained by delayed metabolism in gilt derived oocytes. To examine associated mechanisms of lipid metabolism, paper 2 assessed the transcript abundance of two genes encoding enzymes involved in L-carnitine biosynthesis, along with evaluating the rate of lipid metabolism. Transcript abundance of the metabolism related genes was analysed in both oocytes and CCs. In CCs, apoptosis has been suggested to be associated with oocyte quality, albeit different hypothesis exists concerning the causative mechanisms (Torner et al., 2004; Yuan et al., 2005; Pawlak et al., 2016). In addition, four genes were selected whose expression in CCs had been suggested as potential oocyte quality markers in other species (McKenzie et al., 2004; Assidi et al., 2008; Caixeta et al., 2009). Of these four, we could not detect amplification of Gremlin 1 (*GREM1*) in porcine CCs, and it is not presented in paper 2. A new *GREM1* TaqMan assay was tested, however, generating the same negative result. *GREM1* affects the final processes of oocyte maturation (McKenzie et al., 2004), and the expression of *GREM1* in CCs has been suggested as a marker of oocyte developmental competence in both cow (Assidi et al., 2008) and human (McKenzie et al., 2004). Why there was no expression of *GREM1* in porcine CCs is difficult to explain, and it would be interesting to see whether other studies with porcine CCs will get amplification of this gene.

Potential markers of porcine oocyte quality identified in paper 2 was lower expression in CCs of gamma-butyrobetaine hydroxylase 1 (*BBOX1*) and higher expression of carnitine palmitoyltransferase 2 (*CPT2*) prior to maturation, and higher expression of glucose-6-phosphate dehydrogenase (*G6PD*) and fructose-bisphosphate A (*ALDOA*) post maturation. In addition to delayed lipid metabolism, these results imply downregulation of glycolysis and the PPP in gilt derived oocytes. In accordance with the quiet embryo hypothesis it could be argued gilt oocytes showed a more adaptive metabolism than sow oocytes. However, metabolism should not be too low, but work at an optimal level to sustain the cellular requirements while keeping production of ROS to a minimum (Bradley and Swann, 2019). Oocytes need to accumulate nutrients and other molecules. The reduced abundance of transcripts involved in glucose metabolism observed in gilt oocytes probably reflect their delayed development, and an inadequate, not an adaptive, metabolism.

Epidermal growth factor receptor (*EGFR*) showed a tendency for being higher expressed in gilt derived compared to sow derived oocytes after maturation, in addition to a trend for higher expression after IVM as compared to before IVM within the gilt oocytes.

Further investigation into lower expression of *EGFR* in CCs after IVM as a potential parameter of oocyte quality should be conducted. The activation of *EGFR* is influenced by FSH (Sugimura et al., 2015), and the abundance of follicle stimulating hormone receptor (*FSHR*) in CCs has also been suggested as a marker of oocyte competence (Caixeta et al., 2009). Assessing the abundance of *FSHR* in conjunction with *EGFR* in CCs could be elucidative.

Transcripts involved in oxidative stress response, such as glutathione synthetase, glutathione regeneration and superoxide dismutase, in addition to genes associated with TCA activity, could have added valuable information to the study. As a follow up to paper 2, to confirm actual protein levels, protein expression of the transcripts suggested as quality parameters should be assessed, in addition to adopting functional assays to evaluate activity of the relevant metabolic pathways.

Potential oocyte quality parameters in paper 3

Transcripts directly involved in DNA-methylation had previously been investigated in porcine oocytes (Braga et al., 2019), therefore, the selected genes in paper 3 were related to histone modifications. Additionally, since both de novo DNA-methylation (Xu et al., 2017) and the establishment of H3K4me3 marks (Sha et al., 2020; Bu et al., 2022) is expected to be completed during the oocyte's growth phase, accumulation of transcripts involved in the removal of H3K4-methylation marks were explored. However, as gilt oocytes may not have finished the establishment of the H3K4me3 marks at aspiration, it could have been valuable to have included genes involved in this process as well.

Transcripts accumulated during the oocyte's growth phase but acting at different stages during oocyte and early embryo development, were selected for paper 3, e.g. lysine demethylase 1B (*KDM1B*), a H3K4me2 demethylase required for de novo DNA methylation at imprinted genes during oocyte growth (Sha et al., 2020; Mitchell, 2022), and lysine demethylase 5A (*KDM5A*), required for removing H3K4me3 marks prior to EGA (Dahl et al., 2016; Bu et al., 2022). Besides the H3K4 demethylases, a transcription factor, Spalt-like transcription factor 4 (*SALL4*), regulating the expression of several histone demethylases and described as pivotal for oocyte maturation and resumption of meiosis (Xu et al., 2017), in addition to genes encoding proteins required for histone deacetylation were selected.

Regulating both epigenetics, oxidative stress and metabolism, Sirtuin1 (*SIRT1*), a NAD+ -dependent histone deacetylase, was included in paper 3 as it has been suggested as a

potential marker of oocyte quality (Tatone et al., 2018; He et al., 2021). However, related genes for the different processes may need to be included in future studies to identify which processes are the main factors influencing oocyte quality.

All the epigenetic related genes in paper 3 demonstrated similar profiles of higher abundance in oocytes from gilts as compared to sows, both before and after maturation. Results of paper 3 did suggest that gilt oocytes had not completed the accumulation of these transcripts at the point of aspiration, possibly not silenced transcription prior to meiotic resumption and were presumably delayed in the epigenetic processes influenced by these genes. Further studies are, however, required to determine associated preconditions for the potential use of these transcripts as oocyte quality markers. Additionally, as transcripts can be transferred from CCs to oocytes, and paper 2 suggested transcript abundance in CCs could be a more informative parameter of oocyte quality than transcript abundance in oocytes, it could have been valuable to have analysed the abundance of these epigenetic-related transcripts also in CCs.

In addition to examining genes related to other epigenetic processes, several other transcripts identified as essential for oocyte maturation or EGA could be interesting to investigate as potential markers of porcine oocyte quality. Examples are histone H2A (*H2A*) (Caixeta et al., 2009), bromodomain and WD repeat domain containing 1 (*BRWD1*) (Bianchi and Sette, 2011), E74 like ETS transcription factor 4 (*ELF4*) (De Sousa et al., 1998; Liu et al., 2016; Shi et al., 2022), zygote arrest 1 (*ZAR1*) (Wu et al., 2003; Pennetier et al., 2004; Zuccotti et al., 2011) and ATPase Na+/K+ transporting subunit alpha 1 (*ATP1A1*) (De Sousa et al., 1998; Leoni et al., 2007). However, the selected genes in paper 2 and 3 generated valuable insight and a basis for further investigations.

4.4. How can we improve IVEP procedures?

Results from paper 2 and 3 indicate that oocytes originating from prepubertal gilts have not completed their growth phase at the time of aspiration and have reduced glucose and delayed lipid metabolism, affecting ATP stores and oxidative stress response. In addition, transcription does not appear to be silenced at aspiration, and the delayed accumulation of epigenetic related transcripts suggests delayed epigenetic reprogramming involving these histone

modifications. Delayed accumulation of *HDAC2*, as demonstrated in paper 3, could affect HDAC2 facilitated deacetylation of H4K14, essential for chromosome segregation (Ma and Schultz, 2013; He et al., 2021). It is tempting to speculate whether a decreased level of HDAC2 could affect the separation of chromosomes and partly explain the increased rates of aneuploidy in oocytes derived from gilts (Lechniak et al., 2007).

Although intrinsic oocyte quality prior to maturation is recognized as the most important factor determining its developmental potential, additional steps during IVM could potentially increase the developmental capacity of low quality oocytes. Cytoplasmic, more so than nuclear, maturation mainly influences the developmental potential of oocytes (Tatemoto et al., 2000; Liu et al., 2010; Auclair et al., 2013). CCs support the oocytes during growth and maturation. As CC transzonal projections start to withdraw from the oocyte around the time of meiotic resumption, and cAMP inhibits the resumption of meiosis, increasing the duration of exposure to cAMP analogue supplements in the IVM medium could potentially enhance COC competence by prolonging the period of functional gap junctions (Luciano et al., 2014). This pre-maturation could offer the gilt oocytes additional time to complete their RNA, protein and nutrient stores and continue the epigenetic programming. Pre-maturation, in conjunction with FSH and EGF supplementation, enhanced bovine oocyte developmental competence, demonstrated through significantly increased blastocyst yield and quality (Albuz et al., 2010). In porcine, the standard IVM protocol is to include dibutyryl cAMP together with FSH, LH and EGF to the medium for the first 20 h. Future studies should investigate whether a preceding period with dibutyryl cAMP, FSH and EGF supplementation, possibly in conjunction with an antioxidant (Lin et al., 2018), could increase the developmental competence of poorer quality porcine oocytes. Pre-maturation would, however, require careful judgement and flexibility, as pre-maturation of more advanced bovine COCs showed negative effects (Fair et al., 2002). Currently, follicle size is perhaps the most applied non-invasive parameter of oocyte developmental stage, although follicles of the same size can still display differences in oocyte competence (Hunter, 2000). Reliable, non-invasive parameters of oocyte developmental stage need to be validated to further determine their cultural requirements.

Results of paper 2 did indicate gilt derived oocytes are less competent in regulating oxidative stress at the end of IVM than oocytes from sows, through the implied reduced rate of the PPP. Increased activity of the PPP could improve the cellular redox balance by increased regeneration of GSH. However, stimulating the PPP did not improve porcine oocyte quality

nor increase further embryo development (Herrick et al., 2006). Oocytes and embryos are exposed to much higher oxidative stress *in vitro* than *in vivo*, and gilt oocytes would presumably benefit from supplementation of an antioxidant. L-carnitine has been suggested as a supplement to increase oocyte and embryo quality because of its dual action as an antioxidant and facilitating increased FAO (Somfai et al., 2011; Wu et al., 2011; Takahashi et al., 2013; Xu et al., 2020). From the results of paper 2, L-carnitine appear to have potential as a supplement for gilt oocytes during the first part of IVM. However, this should be done with care as to not disrupt the cellular L-carnitine balance, and the addition of a different antioxidant such as melatonin (Lin et al., 2018) or ascorbic acid (Nohalez et al., 2018) could be explored.

It is, however, critical to recognize that suggestions for adjusted IVEP procedures based on the requirements of gilt derived oocytes might not improve, or could possibly rather reduce, the quality of oocytes from sows.

5. General conclusions

The focus of this thesis was examining storage of porcine embryos and identifying oocyte quality parameters. The results of paper 1 demonstrated that liquid-storage in HEPES buffered medium for 3 h did not compromise porcine embryo quality. Blastocysts developed on day 5 post fertilization demonstrated less apoptosis and higher *in vitro* developmental capacity after storage than blastocysts developed on day 6. To select the best quality embryos for ET, day 5 blastocysts should be applied, and blastocysts undergoing a strong collapse during the 3 h storage should be avoided as they are most likely of reduced quality.

Applying gene expression in CCs to predict oocyte quality do have advantages compared to evaluating the transcript abundance in oocytes. From the follicles reach approximately 3 mm in diameter till the time of EGA, there is hardly any transcription in the oocyte. Transcript abundance in CCs may be easier to interpret than in oocytes, as it does not involve transcriptional silencing. Additionally, gene expression in CCs can potentially exhibit greater response to environmental changes during COC maturation than oocytes, RNA is more abundant in CCs, and it has potential as a non-invasive quality parameter if collected after fertilization. Paper 2 identified lower expression in CCs of *BBOX1* and higher expression of *CPT2* prior to maturation, and higher expression of *G6PD* and *ALDOA* post maturation as potential novel oocyte quality parameters. Results of paper 2 suggest porcine COCs can differentially regulate L-carnitine synthesis. Hence, any supplementation of L-carnitine should be done with caution, as too high concentrations may have adverse effects. L-carnitine may have potential as a supplement for gilt derived oocytes during the first part of IVM. Increased FAO can increase ATP production and rendering glucose available for the PPP, which could increase the oocytes' capacity to regulate oxidative stress, which appeared reduced in gilt derived oocytes.

Appropriate normalisation is critical to obtain reliable results from RT-qPCR studies, which is particularly challenging in oocyte maturation studies as the quantity of RNA, including the reference genes, is affected by the stage of development. Paper 3 validated the use of a novel reference gene combination, *ACTB* and *PFKP,* for normalisation of RT-qPCR oocyte data. Paper 2 and 3 implied that gilt derived oocytes had not completed accumulation of RNA and silenced transcription prior to IVM, and possibly not before the resumption of meiosis. Moreover, gilt oocytes did exhibit delayed and/or reduced metabolism (paper 2) and were

most likely delayed in the epigenetic processes involving histone modifications (paper 3) required for successful fertilization and further embryo development.

6. Future perspectives

For the pig breeding industry to offer a competitive sustainable product in the future, increased quality and success rate of both IVEP and embryo storage, is of great importance. Liquid storage of porcine IVP embryos for longer time periods should be investigated, in addition to methods for improving survival rate and embryo quality post vitrification. An approach could be altering the lipid composition of the membrane to improve the cryotolerance of porcine embryos. Additionally, other antioxidants than L-carnitine, such as melatonin (Lin et al., 2018) or ascorbic acid (Nohalez et al., 2018), could be explored as supplements for both IVEP and storage procedures. However, in addition to identifying the optimal storage conditions, improving the quality of IVP embryos will most likely increase their survival and quality post storage.

Incomplete cytoplasmic maturation has been suggested as the major causative factor of low porcine IVEP success rate, and even more so in gilt than sow derived oocytes. Improving *in vitro* cytoplasmic maturation is, however, not an easy task and numerous studies have put a lot of efforts into the matter. IVEP can potentially be further improved by identification of the most critical components of follicular fluid, and when possible, implementing these to the chemically defined maturation medium. Moreover, apart from further developmental potential, there is currently no definite measure of completed cytoplasmic maturation. Technologies such as metabolomics and RNA-sequencing, comparing IVP and IVD embryos or applying oocytes collected from prepubertal gilts and sows, can potentially elucidate processes which need to be stimulated to improve cytoplasmic maturation and achieve higher IVP embryo quality. Elucidating these processes could at the same time aid in identifying novel oocyte and embryo quality parameters. However, in case of artificially stimulating metabolism, it should be thoroughly investigated how this affects the oocytes or embryos. Additionally, as blastocyst rate appeared to be correlated with embryo quality in the liquid storage study (Table 1), it could be interesting to examine what differentiates embryos obtained from high and low blastocyst rate IVEP rounds, and the potential of applying this as a general quality parameter.

With the anticipated increased use of IVP porcine embryos in ET in the future, knowledge of their epigenetic aberrations and how this affects long term health in pigs is of essence. Further, to improve quality of gilt derived oocytes, pre-maturation in conjunction

with antioxidant supplementation could be examined. However, initially, reliable, non-invasive parameters of the oocytes' developmental stage need to be established to further determine their cultural requirements. To sum up, improving the quality of IVP embryos should have primary focus as this will, most likely, increase survival rate after storage and pregnancy rates after ET.

7. References

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Dissertation articles

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Article

Cumulus Cell and Oocyte Gene Expression in Prepubertal Gilts and Sows Identifies Cumulus Cells as a Prime Informative Parameter of Oocyte Quality

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Simple Summary: Progress has been made in pig in vitro embryo production (IVEP) over the years, but some of the remaining challenges are low embryo production rates compared to many other species. Oocyte quality is an important factor influencing IVEP. Cumulus cells (CCs) surround and support the oocyte during growth and maturation, but few studies have examined gene expression in pig CCs. This study aimed to identify novel markers of oocyte quality by examining gene expression in oocytes and CCs employing the model of oocytes collected from prepubertal animals being of lower quality than those from adult animals. This knowledge could be applied in genetic selection for oocyte quality and improve IVEP procedures, quality and success rates, particularly when using oocytes from gilts, which would be valuable in increasing genetic gain through their use in embryo transfer. New markers for oocyte quality were identified, with functions related to redox potential, L-carnitine biosynthesis, and fatty acid and glucose metabolism, where transcript abundance in CCs appeared to be more reliable parameters of oocyte quality than levels in the oocyte. A potential approach for increasing oocyte competence might be prolonging the period of support from CCs.

Abstract: Cumulus cells (CCs) are pivotal during oocyte development. This study aimed to identify novel marker genes for porcine oocyte quality by examining the expression of selected genes in CCs and oocytes, employing the model of oocytes from prepubertal animals being of reduced quality compared to those from adult animals. Total RNA was extracted either directly after follicle aspiration or after in vitro maturation, followed by RT-qPCR. Immature gilt CCs accumulated *BBOX1* transcripts, involved in L-carnitine biosynthesis, to a 14.8-fold higher level ($p < 0.05$) relative to sows, while for *CPT2*, participating in fatty acid oxidation, the level was 0.48 ($p < 0.05$). While showing no differences between gilt and sow CCs after maturation, *CPT2* and *BBOX1* levels in oocytes were higher in gilts at both time points. The apparent delayed lipid metabolism and reduced accumulation of *ALDOA* and *G6PD* transcripts in gilt CCs after maturation, implying downregulation of glycolysis and the pentose phosphate pathway, suggest gilt cumulus–oocyte complexes have inadequate ATP stores and oxidative stress balance compared to sows at the end of maturation. Reduced expression of *BBOX1* and higher expression of *CPT2* in CCs before maturation and higher expression of *G6PD* and *ALDOA* after maturation are new potential markers of oocyte quality.

Keywords: cumulus–oocyte; metabolism; in vitro maturation; porcine; fatty acid oxidation; oxidative stress; L-carnitine

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1. Introduction

Oocyte quality refers to an oocyte's potential for successful fertilization, implantation and ultimately developing into a healthy offspring [1] and is possibly the predominant

factor influencing in vitro embryo production (IVEP) [2]. During oocyte growth, which is continuous until the follicle reaches a diameter of 2–3 mm, the oocyte progressively acquires developmental competence by synthesizing and storing molecules that are necessary for sustaining meiotic maturation, fertilization and early embryo development [1,3–6].

Cumulus cells (CCs) are granulosa cells that surround the oocyte and play pivotal roles during oocyte growth and maturation [7]. Transzonal projections from the CCs penetrate the zona pellucida and are in direct contact with the oocyte cell membrane where gap junctions at the end of the transzonal projections allow for bidirectional paracrine signaling and the transfer of small molecules [8–10]. In this way, CCs support the oocyte in, e.g., metabolic processes and the capacity to regulate oxidative stress [9,11].

Large amounts of energy are required for the oocyte to progress through maturation and fertilization [12]. Glucose metabolism is crucial for oocyte maturation and early embryo development [9,11,12], but immature and growing oocytes have a very low capacity to metabolize glucose. Thus, this is mainly performed by CCs [9,11], with subsequent transfer of both glycolysis and the pentose phosphate pathway (PPP) metabolites to the oocyte [12]. Previous studies have demonstrated that cumulus–oocyte complexes (COCs) from prepubertal gilts showed lower levels of both transcripts and proteins [9,13] involved in glycolysis compared to COCs from sows. In addition, in vitro-produced porcine oocytes exhibit reduced glucose metabolism compared to those derived in vivo [9]. Hence, the capacity of the oocyte to metabolize glucose is suggested as a marker of oocyte developmental competence [9,13]. This relationship has, however, not been investigated separately in porcine CCs. The PPP is possibly more crucial for oocytes to acquire developmental competence than glycolysis, as the PPP, in addition to supplying energy, also plays a vital role in the prevention of cellular damage from reactive oxygen species and produces ribose 5-phosphate for purine synthesis [2,9,12]. For glucose metabolism, glucose-6-phosphate dehydrogenase (*G6PD*), encoding a rate-limiting enzyme for the PPP, and transcripts encoding two key enzymes in glycolysis, namely the platelet isoform of phosphofructokinase (PFKP) and fructose-bisphosphate A (ALDOA), could be potential biomarkers for oocyte quality.

Porcine oocytes contain a larger total volume of cytoplasmic lipid droplets compared to other species [12,14,15]. Mature porcine oocytes contain less triglycerides, the main lipid droplet components, compared to immature oocytes, and it has been demonstrated that inhibition of lipid metabolism during in vitro maturation (IVM) resulted in decreased developmental potential [16]. The intracellular lipid store has therefore been proposed to be of special importance for adenosine triphosphate (ATP) production during porcine oocyte maturation [11,17–19]. Increased lipid metabolism for ATP production could, in addition, make glucose accessible for other functions than energy production, such as the PPP [17]. Long-chain fatty acids are dependent on the molecule L-carnitine for entry into the mitochondria where they are metabolized through β-oxidation before entering the tricarboxylic acid (TCA) cycle for further ATP production [20]. L-carnitine is in addition an antioxidant [20–22] and is found in both follicular [23] and oviduct fluids [24]. As L-carnitine is not a standard inclusion in IVM medium, it would be of interest to analyze the expression of trimethyllysine hydroxylase, epsilon (*TMLHE*), and gamma-butyrobetaine hydroxylase 1 (*BBOX1*), encoding the enzymes catalyzing the first and last step of the L-carnitine biosynthesis pathway [25], respectively, to investigate whether porcine COCs can modulate L-carnitine biosynthesis; to our knowledge, this has not yet been studied. In conjunction with the expression of carnitine palmitoyltransferase 2 (*CPT2*), an indicator of the rate of fatty acid oxidation (FAO) [26], this could contribute valuable knowledge about regulatory mechanisms of lipid metabolism in COCs and its relation to oocyte quality.

Apoptosis has previously been suggested as a marker of oocyte developmental potential. However, studies have concluded that apoptosis in oocytes is not a reliable measure of the oocyte's quality [27–29], while apoptosis in CCs before maturation is proposed to be an indicator of oocyte developmental competence both in humans [30] and cattle [29]. Hussein et al. [31] demonstrated that bovine oocytes could affect apoptosis in CCs by modulating the expression of apoptosis-related genes; thus, transcript levels of pro-apoptotic

bcl2 associated x (*BAX*) and anti-apoptotic bcl2 like 1 (*BCL2L1*) in CCs before and after in vitro maturation could serve as possible indicators of oocyte quality.

Furthermore, higher expression of epidermal growth factor receptor (*EGFR*) [32,33], growth hormone receptor (*GHR*) [33] and prostaglandin-endoperoxide synthase 2 (*PTGS2*) [32,34] in CCs has led to these three genes being proposed as markers of competence in bovine and human oocytes. These gene products act through, and influence, CC function, oocyte maturation and developmental competence. Hence, their transcript abundances could be potential markers of oocyte quality in porcine CCs.

Besides genetic factors, the intrinsic developmental potential of oocytes is primarily determined by the follicle environment [4,14,35], and oocytes collected from prepubertal animals have demonstrated reduced developmental capacity compared to those from adult animals [5,36–40]. Based on this, the model of prepubertal and adult oocyte donors has previously been employed to study oocytes of low versus high developmental competence in, e.g., cows [41], sheep [35,42] and pigs [27,43].

Increased knowledge of the interplay between oocytes and CCs could provide insight for improving IVEP procedures and consequently lead to increased oocyte and embryo quality. Improving the quality and IVEP success rate of gilt oocytes would be valuable both for their use in research and in commercial embryo transfer. Gilt oocytes are more accessible from the slaughterhouse than sow oocytes, and the transfer of embryos after IVP with gilt oocytes would lead to higher genetic gain in a breeding program. Besides evaluating oocyte quality, e.g., after altered maturation protocols, new genetic markers of porcine oocyte quality could potentially identify differences in oocyte quality between individuals and be applied in genetic selection for oocyte quality.

The aim of this study was to identify novel markers of oocyte quality. This was performed by analyzing gene expression in immature and in vitro matured CCs and oocytes collected from prepubertal gilts and sows, focusing on genes related to metabolism, apoptosis and markers of oocyte competence identified in other species.

2. Materials and Methods

2.1. Chemicals and Media

All chemicals and reagents were purchased from Sigma-Aldrich (Oslo, Norway) unless otherwise stated. Washing of COCs was performed with porcine X medium (PXM), and maturation was performed in porcine oocyte medium (POM) [44]. Minor changes were made to the POM medium, and the final composition was 108 mM NaCl, 10 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.35 mM KH_2PO_4, 25 mM $NaHCO_3$, 5.0 mM glucose, 0.2 mM Na-pyruvate, 2.0 mM L-glutamine, 2.0 mM Ca-$(lactate)_2 \cdot 5H_2O$, 5.0 mM hypotaurine, 0.6 mM L-cysteine, 20 mL/L BME amino acids, 10.0 mL/L MEM non-essential amino acids, 0.01 mg/mL gentamicin, 4.0 mg/mL BSA, FLI (FGF2 40 ng/mL, LIF 20 ng/mL, IGF1 20 ng/mL), 10 ng/mL epidermal growth factor and 50 µM β-mercaptoethanol (Gibco, Fisher Scientific AS, Oslo, Norway).

2.2. Animal Material and Ethics

Sow and gilt ovaries were collected at a commercial abattoir, originating from random herds. Material was collected from animals that were routinely slaughtered; therefore, no ethical approval was required. In Norway, swine are cared for according to internationally recognized guidelines and regulations for keeping pigs in Norway (The Animal Welfare Act, 10 July 2009, https://www.regjeringen.no/en/dokumenter/animal-welfare-act/id571188/, accessed on 12 April 2023 and Regulations for keeping pigs in Norway, 18 February 2003, https://lovdata.no/dokument/LTI/forskrift/2003-02-18-175, accessed on 12 April 2023). Ovaries were collected from May to August 2022.

2.3. Experimental Design

Prepubertal gilts are pigs that have not yet produced a litter, and their ovaries have follicles of smaller size and show no signs of ovulation (no preovulatory follicles, corpora

lutea or corpora albicantia) [45], in contrast to ovaries of cycling gilts which have follicles of different sizes along with corpora lutea or corpora albicantia. The prepubertal gilts were around 5 months of age. The term sows refers to older pigs that have produced at least one litter. Sow ovaries that had follicles along with corpora lutea or corpora albicantia from previous cycles were collected [45]. At the abattoir, gilts, both prepubertal and cycling, were slaughtered separately from sows, and ovaries from sows and prepubertal gilts were collected and kept apart. Immature COCs from prepubertal gilts and sows were randomly placed into the immature or in vitro maturation groups, where total RNA was extracted from CCs and oocytes separately, either directly following aspiration or after 44 h in vitro maturation, respectively.

There were 8 experimental groups in total: 4 groups with oocytes (prepubertal gilts before and after IVM and sows before and after IVM) and 4 groups with CCs (prepubertal gilts before and after IVM and sows before and after IVM). For all groups, triplicate pools of 50–60 oocytes or their corresponding CCs were used for RNA extraction and downstream analyses.

2.4. Selection of Target Genes

To select the experimental genes, a thorough literature search was conducted to pinpoint processes that previous studies had identified as having implications for oocyte quality in porcine or other species. Glucose and lipid metabolism in both oocytes and CCs and apoptosis in CCs were found to be associated with oocyte quality, as described in the introduction section. For each of the processes of interest, rate-limiting genes and/or genes where transcript abundance had been recognized in other studies to affect oocyte quality were selected. In addition, relating to lipid metabolism, two genes involved in L-carnitine biosynthesis were selected. This study did not involve supplementation of the media with L-carnitine, but it investigated whether COCs from prepubertal gilts and sows exhibit different expression levels of genes encoding enzymes involved in L-carnitine biosynthesis. Finally, expression in CCs of a selection of three genes (*EGFR*, *GHR* and *PTGS2*), which previous studies in other species had identified as potential markers of oocyte quality, was selected. Supplementary RNA-sequencing data from Bu et al. [46] and the webtool BioGPS pigatlas (http://biogps.org/pigatlas/#goto=welcome (accessed on 1 September 2022)) were used to assess the expression levels of the potential genes in oocytes. There was little information to be found on the expression levels of these genes in porcine CCs; hence, the genes were selected based on their expression in oocytes.

2.5. Cumulus–Oocyte Complex Collection and In Vitro Maturation

COC collection and in vitro maturation were performed as described by Haug et al. [47]. Sow and gilt ovaries in different phases of the estrus cycle were collected and transported to the laboratory in 0.9% NaCl at 32–36 °C within 2 h of slaughter. On arrival, ovaries were washed with 0.9% NaCl supplemented with 2.5 µg/mL kanamycin and placed in a beaker in a water bath at 34–35 °C until follicle aspiration. Follicles with a diameter of 2 to 6 mm were aspirated with an 18-gauge needle and 10 mL syringe. Oocytes with several layers of compact cumulus and evenly granulated cytoplasm, as determined by microscopy, were selected. For in vitro maturation, 25–30 COCs were washed three times in PXM and once in POM medium and transferred into each well of a Nunc® four-well dish containing 500 µL of pre-equilibrated POM medium. For the first 20 h, COCs were matured in POM supplemented with 0.1 mM dibutyryl-cAMP (dbcAMP) and 0.05 IU/mL porcine FSH and LH (Insight Biotechnology Ltd., Wembley, UK). Subsequently, COCs were matured for another 24 h in POM without dbcAMP and hormones. COCs were cultured for a total of 44 h at 38.8 °C in a humified atmosphere containing 6% CO_2 in air.

2.6. RNA Extraction and cDNA Synthesis

To separate the CCs from the oocytes, COCs were transferred to a 1.5 mL Eppendorf tube and vortexed for 1 min. The remaining CCs were removed by pipetting, and any

oocytes with CCs still attached were discarded. Oocytes were subsequently washed with 6 drops of PXM and then 3 drops of diethyl pyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA) (dPBS/PVA) before being transferred in 2 µL dPBS/PVA to the RNA extraction solution. Total RNA was extracted using RNAGEM^{TM} Tissue PLUS (ZyGEM, Hamilton, New Zealand) and treated with DNase I (at 80 units/mL for 10 min, a minor deviation from the manufacturer's instructions). CCs were transferred to a 1.5 mL Eppendorf tube within 0.5 mL of dPBS/PVA and centrifuged at 1000× *g* for 4 min. The supernatant was removed, and total RNA from the cell pellets was extracted as described for oocytes. RNA concentrations were measured in all samples using a Qubit fluorometer with the Qubit^{TM} RNA High Sensitivity assay kit (Invitrogen, Oslo, Norway). RNA samples were subsequently stored at −80 °C until further use.

Total RNA from each pool of oocytes (ca. 70 ng) and 100 ng RNA from each pool of CCs was reverse-transcribed into cDNA using SuperScript IV VILO Master Mix (Invitrogen, Oslo, Norway), containing both random and oligo(dT) primers, according to manufacturer's instructions, and then diluted 1:20 with PCR-grade water. One RNA sample from each preparation was processed without reverse transcriptase (-RT) to provide a negative control for subsequent gene expression analyses. cDNA was stored at −20 °C until further use.

2.7. Quantitative PCR

Gene expression was assessed by quantitative polymerase chain reaction (qPCR) using FAM-labeled TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) available on the ThermoFisher website (http://www.thermofisher.com (accessed on 1 October 2022)). Details for each gene are listed in Table 1 and Appendix A (Table A1). Each qPCR reaction mix contained 5 µL of (2×) TaqMan Fast Advanced Master Mix (Applied Biosystems catalog No. 4444556), 0.5 µL (20×) TaqMan Assay, 0.5 µL dH_2O and 4.0 µL cDNA template (equivalent to 1 ng RNA per reaction), giving a final reaction volume of 10 µL. All PCR reactions were performed in triplicate. QPCR was run on a 7500 Fast Real-Time PCR System (Applied Biosystems) in fast cycling mode. The qPCR reaction mixture was subjected to an initial UNG incubation at 50 °C for 2 min and then enzyme activation at 95 °C for 20 s. This was followed by 45 cycles of denaturation at 95 °C for 3 s and annealing and elongation at 60 °C for 30 s. Negative controls included -RT samples to check for genomic DNA contamination, and PCR reactions consisting of the qPCR reaction mixture without an added sample cDNA template were always included to ensure the absence of nucleic acid contamination. All -RT samples and no template controls came up negative.

A panel of reference genes, previously validated and/or used as reliable reference genes in similar studies, namely Actin beta (*ACTB*) [27,48–51], glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [27,46,49,52], hydroxymethylbilane synthase (*HMBS* aka *PBGD*) [53–55], hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) [56] and tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Gamma (*YWHAG*) [49] were compared using NormFinder [57], which identified *ACTB* as the most stable reference gene for both CCs and oocytes. The transcript abundance from each gene was hence normalized against the abundance of the reference transcript *ACTB*. The relative expression of each gene was calculated using the ΔCt method with efficiency correction [58]. Mean efficiency values for each primer set were calculated from the amplification profiles of individual samples with LinRegPCR software (version 2021.2; https://medischebiologie.nl/files/ (accessed on 10 December 2022)) [59,60]. Cq values obtained from LinRegPCR were employed for all calculations. To analyze the relative change in gene expression during COC in vitro maturation, transcript abundance of in vitro matured oocytes or CCs was compared to that of immature (immature as calibrator) oocytes or CCs for gilts and sows separately. To analyze the difference in gene expression between gilts and sows of the same stage of maturation, transcript abundance in gilt oocytes or CCs was compared to that of sows (sow as calibrator) for the immature and in vitro matured groups separately.

Table 1. Summary of genes analyzed by reverse transcription quantitative PCR in porcine oocytes and cumulus cells.

Gene Symbol	Function	Accession Number	TaqMan® Assay
G6PD	Pentose phosphate pathway	XM_003360515.5 ††	Ss02690824_g1
PFKP	Glycolysis	XM_021065066.1	Ss06887532_m1
ALDOA	Glycolysis	XM_021087995.1 ††	Ss06920688_m1
TMLHE	Carnitine biosynthesis	XM_003135511.4 ††	Ss06886117_m1
BBOX1	Carnitine biosynthesis	XM_021083234.1 ††	Ss06906097_m1
CPT2	Fatty acid metabolism	NM_001246243.1	Ss04322743_m1
GHR †	Growth hormone receptor	NM_214254.2 ††	Ss03383662_u1
EGFR †	Epidermal growth factor receptor	NM_214007.1	Ss03393423_u1
PTGS2 †	Cumulus cell expansion and function	NM_214321.1	Ss03394694_m1
BAX †	Pro-apoptotic	XM_003127290.5 ††	Ss03375842_u1
BCL2L1 †	Anti-apoptotic	NM_214285.1 ††	Ss03383783_s1
ACTB	Cytoskeletal structural protein	XM_003124280.5 ††	Ss03376563_uH
GAPDH	Glycolysis	NM_001206359.1 ††	Ss03375629_u1
HMBS	Heme biosynthesis	NM_001097412.1 ††	Ss03388782_g1
HPRT1	Recycling of purines	NM_001032376.2	Ss03388274_m1
YWHAG	Signal transduction	XM_005661962.3	Ss06938931_s1

† These transcripts were only analyzed in cumulus cells. †† TaqMan® Assay targets specific transcript variants, see Appendix A (Table A1) for details.

2.8. Statistical Analysis

Statistical analysis was performed using RStudio version 4.1.2 (1 November 2021). Gene expression ratios were log base 10 transformed and then tested for normality using the Shapiro–Wilk test. Normally distributed data were analyzed using a two-sample t-test assuming unequal variance. The results were considered statistically significant when $p \leq 0.05$. Graphs were plotted using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

3. Results

CCs displayed greater changes in gene expression, in gilts more so than sows, than oocytes, when comparing mRNA transcript abundance of selected genes in CCs and oocytes at the time of follicle aspiration and after 44 h COC in vitro maturation.

3.1. Gene Expression in Porcine Cumulus Cells

Relative to before maturation, transcript levels of *G6PD* in CCs showed a fold increase of 3.11 (p = 0.002) in gilts and 3.66 (p = 0.002) in sows after maturation (Figure 1A). Accumulation of *CPT2* transcripts also increased for both gilts and sows, exhibiting a fold increase of 3.78 (p = 0.022) in gilts and 1.61 (p = 0.088) in sows, while *BBOX1* transcript abundance changed in gilts only, being expressed at a level of 0.04 (p = 0.019) post-maturation. Following maturation, *GHR* was expressed at a lower level in both gilts and sows (0.34, p = 0.028 and 0.14, p = 0.038, respectively), and there was a trend for higher expression of *EGFR* in gilts (1.75, p = 0.060), relative to pre-IVM. Both genes related to apoptosis were expressed at higher levels after maturation relative to before maturation in gilt CCs (Figure 1A), while *BCL2L1* displayed a higher fold increase (3.20, p = 0.002) than *BAX* (1.71, p = 0.015). However, comparing the ratio of *BCL2L1* to *BAX*, *BCL2L1* was expressed at a significantly lower level as compared to *BAX* for both gilts and sows both before and after IVM (Figure 2).

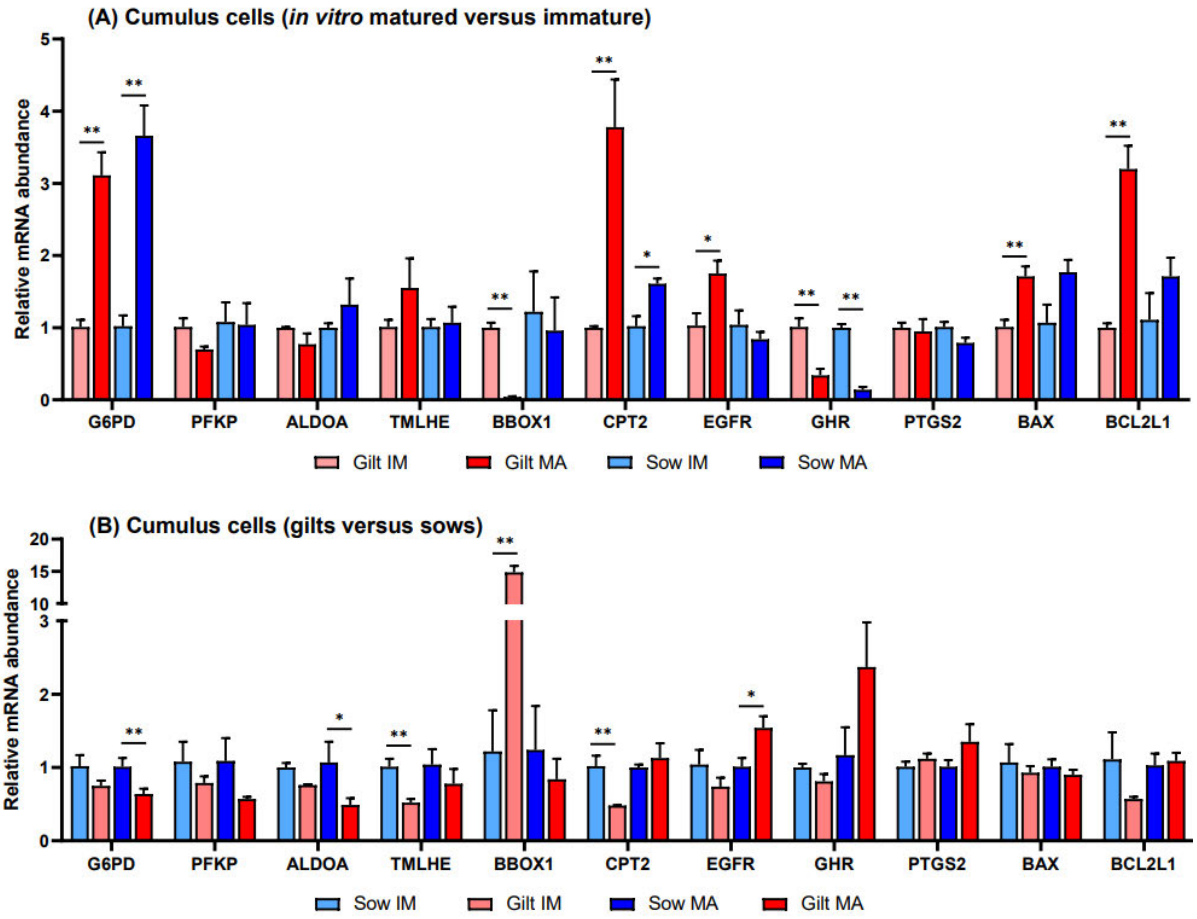

Figure 1. Relative transcript abundance of selected target genes in cumulus cells isolated from immature (IM) and in vitro matured (MA) cumulus oocyte complexes derived from prepubertal gilts and sows as determined by reverse transcription quantitative PCR. Data are expressed as mean ± SEM of three biological replicates per group with (**A**) in vitro matured transcript levels expressed relative to those in immature cumulus cells and (**B**) gilt transcript levels expressed relative to those in sow cumulus cells. Gene expression ratios were calculated applying the ΔCq method with efficiency correction after normalization against *ACTB*. ** indicates significant difference between bars ($p < 0.05$) while * indicates $p < 0.10$.

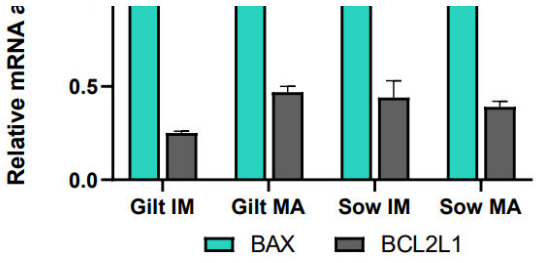

Figure 2. Transcript levels of *BCL2L1* relative to *BAX* in cumulus cells isolated from immature (IM) and in vitro matured (MA) cumulus–oocyte complexes derived from prepubertal gilts and sows as determined by reverse transcription quantitative PCR. Data are expressed as mean ± SEM from three biological replicates per group. Gene expression ratios were calculated by applying the ΔCq method with efficiency correction after normalization against *ACTB*. ** indicates significant difference between bars ($p < 0.05$) while * indicates $p < 0.10$.

3.2. Gene Expression in Porcine Oocytes

There were no significant differences in transcript abundance when comparing in vitro matured vs. immature oocytes derived from gilts or sows (Figure 3A). Investigating gene expression in gilts relative to sows (Figure 3B), transcripts of two genes related to

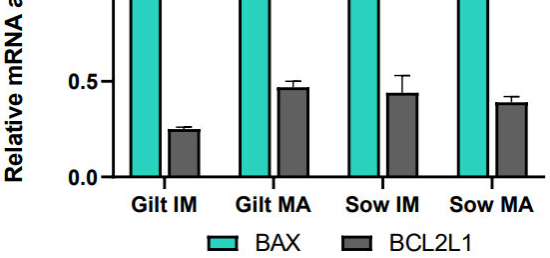

Figure 2. Transcript levels of *BCL2L1* relative to *BAX* in cumulus cells isolated from immature (IM) and in vitro matured (MA) cumulus–oocyte complexes derived from prepubertal gilts and sows as determined by reverse transcription quantitative PCR. Data are expressed as mean ± SEM from three biological replicates per group. Gene expression ratios were calculated by applying the ΔCq method with efficiency correction after normalization against *ACTB*. ** indicates significant difference between bars ($p < 0.05$) while * indicates $p < 0.10$.

Prior to in vitro maturation, gilt CCs showed a 14.8-fold higher level ($p = 0.026$) of *BBOX1* transcripts relative to CCs from sows, while *TMLHE* and *CPT2* exhibited levels of 0.52 ($p = 0.010$) and 0.48 ($p = 0.037$), respectively (Figure 1B). After maturation there were no significant differences between gilts and sows in the transcript levels of genes involved in FAO, while transcripts encoding key enzymes in glucose metabolism, *G6PD* and *ALDOA*, were present in gilt CCs at levels of 0.64 ($p = 0.044$) and 0.49 ($p = 0.070$), respectively, relative to those in sows. In addition, *EGFR* displayed a 1.54-fold ($p = 0.058$) elevated level after maturation in gilt CCs as compared to sows.

3.2. Gene Expression in Porcine Oocytes

There were no significant differences in transcript abundance when comparing in vitro matured vs. immature oocytes derived from gilts or sows (Figure 3A). Investigating gene expression in gilts relative to sows (Figure 3B), transcripts of two genes related to FAO exhibited higher relative transcript abundance in gilt oocytes, with *BBOX1* showing a 1.69-fold ($p = 0.027$) level before and a 3.38-fold ($p = 0.036$) elevated level after maturation and *CPT2* showing a 1.80-fold ($p = 0.044$) level before and a 2.35-fold ($p = 0.058$) higher relative level after maturation. None of the differences observed in gene transcripts related to glucose metabolism were significant.

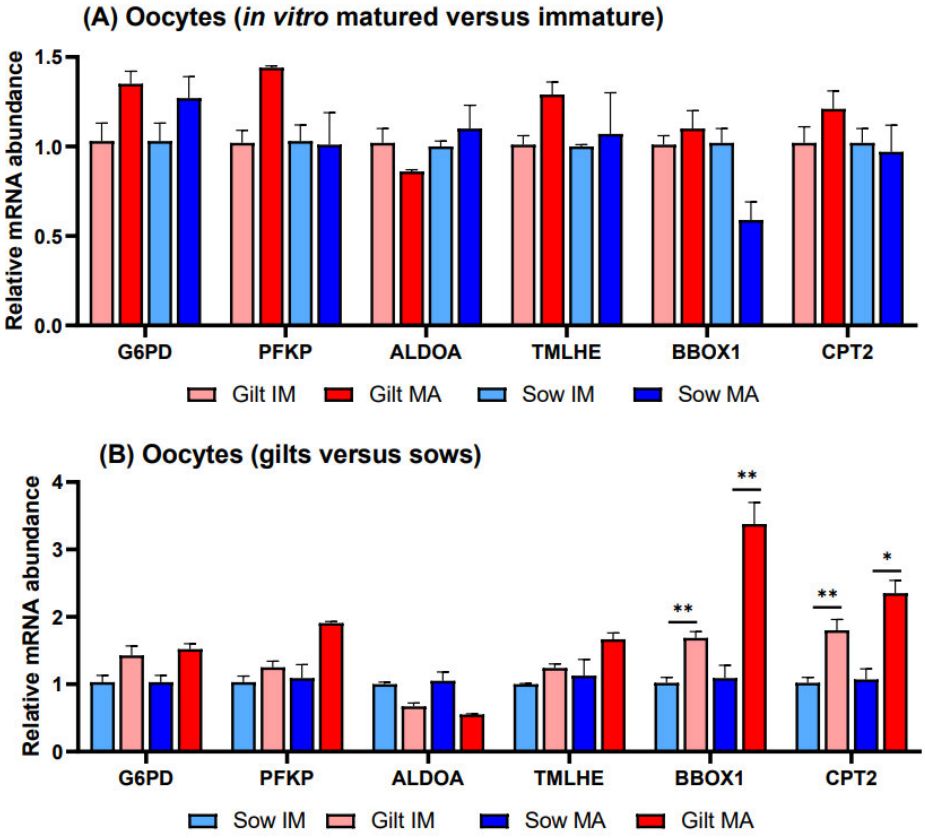

Figure 3. Relative transcript abundance of selected target genes in oocytes isolated from immature (IM) and in vitro matured (MA) cumulus–oocyte complexes derived from prepubertal gilts and sows as determined by reverse transcription quantitative PCR. Data are expressed as mean ± SEM of three biological replicates per group with (**A**) in vitro matured transcript levels expressed relative to those in immature oocytes and (**B**) gilt transcript levels expressed relative to those in sow oocytes. Gene expression ratios were calculated by applying the ΔCq method with efficiency correction after normalization against *ACTB*. ** indicates significant difference between bars ($p < 0.05$) while * indicates $p < 0.10$.

3.3. The Rate of the Pentose Phosphate Pathway Relative to Glycolysis

To elucidate the relative rate of the PPP compared to glycolysis, transcript levels of *G6PD* were analyzed relative to *ALDOA*. In cumulus cells, *G6PD* transcripts accumulated to a significantly lower level relative to *ALDOA* for all the experimental groups, as shown in Figure 4A. In gilt oocytes (Figure 4B), transcript abundance of *G6PD* was nearly equivalent to that of *ALDOA* prior to maturation, while it was 1.46-fold ($p = 0.06$) higher compared to *ALDOA* after maturation. Sow oocytes demonstrated the opposite relationship, where *G6PD* was expressed at lower levels both before (0.45, $p = 0.036$) and after (0.53, $p = 0.087$) maturation, relative to *ALDOA*.

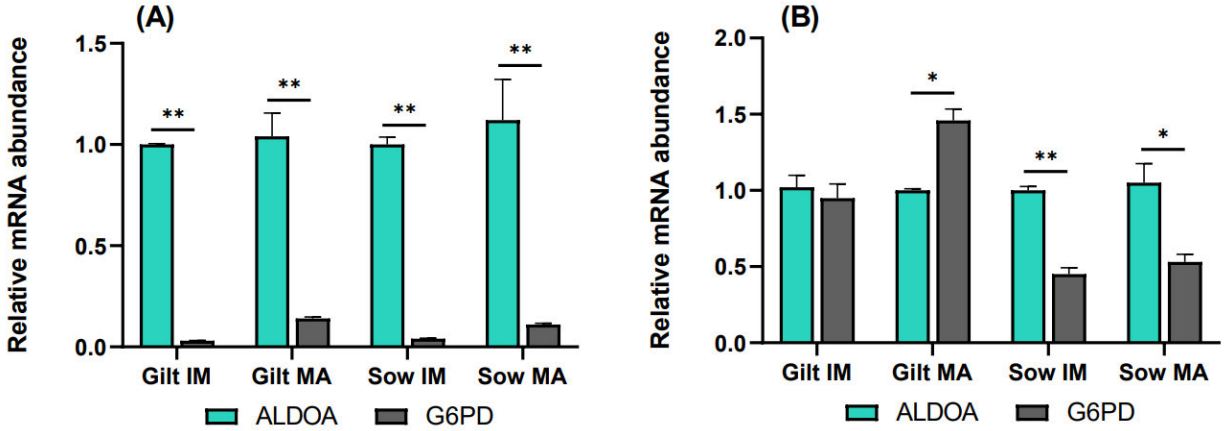

Figure 4. Transcript levels of *G6PD* relative to *ALDOA* in (**A**) cumulus cells and (**B**) oocytes isolated from immature (IM) and in vitro matured (MA) cumulus–oocyte complexes derived from prepubertal gilts and sows as determined by reverse transcription quantitative PCR. Data are expressed as mean ± SEM from three biological replicates per group. Gene expression ratios were calculated by applying the ΔCq method with efficiency correction after normalization against *ACTB*. ** indicates significant difference between bars ($p < 0.05$) while * indicates $p < 0.10$.

4. Discussion

In this study, the expression of genes involved in glucose and lipid metabolism was evaluated in both oocytes and CCs from gilts and sows. In addition, the expression of genes associated with apoptosis and oocyte quality in other species was evaluated in CCs.

4.1. Glucose Metabolism

Oocytes stimulate glycolysis in CCs, but fully grown oocytes will promote higher levels of glycolysis than those not having completed the growth phase [61]. In the current study, reduced transcript levels of *ALDOA* in gilt CCs after maturation indicate glycolysis was downregulated in gilt CCs as compared to sows, suggesting gilt oocytes had not finished the growth phase. Hence, higher expression of *ALDOA* in CCs after maturation is a potential marker of oocyte quality. Although the same pattern of transcript abundance for *ALDOA* was observed in oocytes, the differences were not significant. It might be that more significant differences would have been detected if a higher number of biological replicates had been included.

In vitro cultured oocytes and embryos are usually exposed to higher levels of oxidative stress than those derived in vivo [15,61,62]. The PPP supports cellular redox balance by producing NADPH, which is involved, e.g., in converting oxidized glutathione into reduced glutathione, an antioxidant critical for the developmental potential of oocytes [9,63]. NADPH can be transferred from CCs to oocytes via gap junctions, and for both gilt and sow CCs, *G6PD* levels indicated significant upregulation of the PPP after maturation, supporting a vital role for the PPP in COC maturation [2,17,64]. The *G6PD* transcript abundance suggested, however, at the same time, downregulation of the PPP in gilt compared to sow CCs, which suggests that gilt COCs might be less competent in regulating oxidative stress.

Elevated expression of *G6PD* in more competent porcine oocytes has been reported [65], but this was not observed in the current study, nor by Yuan et al. [52]. Although no significant difference in *G6PD* expression was observed between gilt and sow oocytes, the total COC contribution towards supporting the redox balance through the PPP was reduced in gilt COCs. Abundant expression of *G6PD* might be a more reliable marker of oocyte quality when measured in CCs than in oocytes, but this would need further investigation.

To compare the rate of the PPP to glycolysis, *ALDOA* was preferred over *PFKP* as a measure of the rate of glycolysis as more significant differences were detected between the groups for *ALDOA*, and therefore *ALDOA* did, in this study, appear more rate-limiting for glycolysis than *PFKP*. The results suggest a stronger relative contribution from the PPP in porcine oocytes than in CCs, which was also observed in bovine COCs [9,62]. The main contribution of CCs in glucose metabolism is through supplying oocytes with pyruvate and lactate, further validating the use of elevated expression of *ALDOA* in CCs as a marker of oocyte quality. Further metabolism of pyruvate will cause increased production of reactive oxygen species [14,66,67], and it could be speculated whether high rates of glycolysis in fully grown oocytes are regulated by their redox state, as only fully grown COCs can sustain a higher oxidative stress response.

4.2. Fatty Acid Oxidation

The amount of ATP in oocytes has been linked to its further developmental potential [17–19,23,68]. Since glycolysis appeared to be downregulated in gilt CCs in this study, gilt COCs would have to rely more on lipid metabolism as a supply of energy. In accordance with this, gilt oocytes displayed a higher transcript abundance of genes involved in FAO as compared to sow oocytes, which corroborates previous studies on porcine oocytes [27,52]. As discussed in a study on oocytes from prepubertal and cycling gilts [69] and bovine oocytes of contrasting quality [70], this most likely reflects prepubertal gilt oocytes not having finished the growth phase and therefore needing a higher expression of the transcripts encoding enzymes involved in ATP synthesis. In contrast, sow oocytes have completed the necessary accumulation of ATP and show a lower more stable expression of these transcripts. In gilt CCs, transcript abundance of *CPT2* suggested FAO was significantly downregulated before maturation compared to sows, but no difference in *CPT2* was detected after maturation. This apparent delay in FAO in both gilt oocytes and CCs, together with the lower rate of glycolysis in CCs, implies gilt oocytes might not have adequate stores of ATP at the end of IVM. Studies of amino acid metabolism could provide additional information and would be of interest to include in the future. Excessive fatty acid levels in the follicular fluid or medium can also cause increased FAO in CCs to protect oocytes from lipotoxicity [71]. But seeing a similar pattern in both CCs and oocytes before and after maturation suggests the FAO in CCs observed in this study rather reflects the need for ATP in the COCs.

CC transcript abundance needs to be interpreted differently from that in oocytes, as transcript levels in CCs reflect protein synthesis demands in COCs, while those in oocytes also reflect their stage of development and maturation. When oocytes have completed the growth phase, transcription ceases [72,73]. From meiotic resumption and until embryonic genome activation, oocyte and embryonic development relies on post-transcriptional regulation for modulating protein synthesis [42,72]. Consequently, after transcription is silenced, there is a gradual decline in transcript abundance observed during oocyte maturation [74–76]. In porcine IVM, the standard is to include dbcAMP in the medium for the first 20 h to prevent meiotic resumption, followed by 24 h without dbcAMP supplementation [44,77]. Hence, if the oocyte was not fully grown at aspiration, transcription could continue during the first 20 h of IVM. This might further explain the differences observed between gilt and sow oocytes in transcript abundance of, e.g., *CPT2*, as gilt oocytes probably still had active transcription in the first part of IVM, while the fully grown sow oocytes would have minimal transcription and rely on the stored supply of RNA. In hindsight, analyzing transcript abundance also after 20 h maturation would have added valuable information on transcriptional activity in the space of time between meiotic resumption and the end of maturation.

4.3. L-Carnitine Biosynthesis

The most striking result in this study is perhaps the interplay between the L-carnitine biosynthesis pathway gene *BBOX1* and *CPT2*, a measure of the rate of FAO. This is most clearly demonstrated in the CCs where the level of *CPT2* transcripts suggests that FAO was downregulated in gilt CCs at the time of aspiration. We hypothesize this was caused at least in part by an insufficiency in L-carnitine as indicated by elevated levels of *BBOX1* transcripts, as a proxy for enhanced BBOX1 protein amounts. Building on this hypothesis, which needs to be further validated by functional assays, after maturation, there appears to be sufficient L-carnitine to sustain a similar level of FAO in gilt CCs to that seen in sows. This hypothesized delay in L-carnitine biosynthesis, and hence the rate of FAO, appeared to be more pronounced in gilt oocytes in contrast to CCs, with a difference in *BBOX1* and *CPT2* transcript abundance still being present in gilt oocytes after maturation, relative to sows. This hypothesis is supported by the study of Godárová et al. [78], which demonstrated that insufficient levels of L-carnitine caused a reduction in *CPT2* transcript abundance, while the abundance of *CPT2* increased significantly within 48 h of L-carnitine supplementation. The results from the current study indicate that *BBOX1* encodes the more rate-limiting enzyme for L-carnitine biosynthesis than *TMLHE* in both CCs and oocytes as more significant differences in transcript abundance were observed for *BBOX1*.

In contrast to this study in pigs, Montjean et al. [23], although employing different methods, found no expression of genes encoding enzymes in L-carnitine biosynthesis in human oocytes. A correlation has been proposed between the amount of fatty acids in an oocyte and the importance of FAO for oocyte and preimplantation embryo development [14,17]. As porcine oocytes contain a very large amount of fatty acids compared to most other species, approximately 156 ng fatty acid per porcine oocyte as compared to, e.g., 4 ng per murine oocyte [14], it should perhaps not be surprising that porcine oocytes exhibit expression of *TMLHE* and *BBOX1*. RNAs can travel from CCs to oocytes through gap junctions [72,79], but the high level of *BBOX1* in oocytes after maturation, when the gap junctions are supposed to be closed [80], suggests *TMLHE* and *BBOX1* are also transcribed by the oocytes. Nevertheless, this study indicates, for the first time, that porcine COCs can differentially express the genes involved in the synthesis of L-carnitine.

The current study did not examine the effect of L-carnitine supplementation. Multiple other studies have, however, investigated the effect of supplementing either the in vitro maturation or culture medium with L-carnitine, and it has been reported to have the potential to accelerate nuclear maturation [81], increase blastocyst development [21,22] and increase embryo survival after cryopreservation [21,82]. There is, however, a lack of consensus between studies regarding both optimal concentrations and experimental outcomes, as reviewed in the work of Carrillo-González et al. [83] which found no effect of L-carnitine supplementation on embryo production or post-vitrification survival rate. These inconclusive results might be explained by the varying levels of L-carnitine synthesized by COCs.

In addition to increasing lipid metabolism, L-carnitine is an antioxidant, and there seems to be agreement on its ability to decrease the level of reactive oxygen species [21,22,81,84]. If our hypothesis concerning the delay in L-carnitine biosynthesis in gilt COCs is correct, this may affect both their rate of FAO and redox potential, and gilt COCs could possibly benefit from L-carnitine supplementation during the first part of IVM. This is in accordance with the findings of Carrillo-González et al. [83], who suggested L-carnitine supplementation would predominantly have a positive effect when applied to poor-quality oocytes. Additional L-carnitine supplementation should, however, be applied with caution as the total concentration of L-carnitine will vary depending on amounts synthesized by the COCs, and excessive concentrations have demonstrated negative effects [22].

4.4. Apoptosis

Apoptosis is another potential indicator of COC quality. When anti-apoptotic *BCL2L1* is expressed at a lower level than pro-apoptotic *BAX*, it could promote apoptosis [85]. This was observed both before and after maturation in both gilt and sow CCs and could possibly

be explained as a reaction to oxidative stress experienced during ovary collection and follicle aspiration [29]. Pawlak et al. [69] proposed that CCs from prepubertal gilts would display less apoptosis after IVM than those from cycling gilts, as prepubertal gilt oocytes would not have completed maturation and would therefore still need their CCs for support. In this study, *BCL2L1* was expressed at a higher level in gilt CCs after IVM as compared to before IVM, albeit at a similar level to that seen in sows, and *BCL2L1* was still expressed at a lower level relative to that of *BAX*. It is difficult to draw any conclusions regarding the function of apoptosis from the current study. According to Yuan et al. [29], CC apoptosis is rather common in the periphery of COCs, and an analysis of oocyte-proximal and -distal CC populations could have potentially added insight to this study.

4.5. Gene Expression of PTGS2, GHR and EGFR

Although higher expression of *PTGS2* in CCs has previously been correlated to a higher oocyte developmental competence in cows [32] and humans [34], no such relationship was seen in the current study. A possible explanation could be that transcription of *PTGS2* was completed at an earlier stage of development, as suggested by Regassa et al. [13], who also failed to see a relationship between *PTGS2* expression and oocyte quality in their study of bovine COCs.

The follicular fluid contains growth hormone (GH), but it was not included in the IVM medium. Regardless, the levels of *GHR* transcripts were measured both pre- and post-IVM since an increase in abundance following maturation could indicate the COCs still required GH. Both gilt and sow CCs did, however, demonstrate reduced accumulation of *GHR* transcripts post-IVM. Even so, high variability was observed, with CCs from one biological sample from prepubertal gilts displaying increased *GHR* expression following maturation, which could indicate this specific group of COCs would have benefited from GH being included in the IVM medium. Higher variability between the biological replicates in prepubertal animals has also been reported in other studies [41]. There were no significant differences observed in *GHR* transcript abundance between gilts and sows, neither for CCs nor for oocytes, and, therefore, *GHR* expression in COCs did not seem to be a marker of oocyte quality in this study.

The increased level of *EGFR* expression observed in gilt CCs after maturation could be explained by delayed development of these receptors in less competent oocytes [80]. Consequently, gilt COCs would have responded poorly to epidermal growth factors (EGFs) while *EGFR* transcript abundance was low. It can be speculated whether *EGFR* expression was even lower compared to sows at an earlier stage of development, prior to aspiration. However, cumulus cell transzonal projections begin to withdraw from the oocyte around the time of meiotic resumption [9], but EGF signaling can prolong gap-junctional communication [86]. Therefore, the increased abundance of *EGFR* transcripts could also be a response from the COCs to reduce the withdrawal of transzonal projections while there was still a need for the exchange of factors between the gilt CCs and oocytes. Prolonging the period of functional gap junctions during IVM could potentially enhance COC quality [87]. It was not the intention of this study to characterize the mechanism of *EGFR* per se. Still, it would have been interesting to have analyzed the levels of follicle-stimulating hormone receptor (*FSHR*), as follicle-stimulating hormone (FSH) influences the activation of *EGFR* [80].

This study did not document a correlation between higher transcript abundance of *PTGS2*, *GHR* and *EGFR* and enhanced oocyte quality. It is possible that the expression of these genes needs to be measured at an earlier stage of development for their meaningful use as markers of oocyte quality in pigs. This would, however, not be practicable as it would mean analyzing oocytes from follicles of less than 2 mm diameter, when they have not yet finished the growth phase.

5. Conclusions

To identify novel markers of oocyte quality, the close association between CCs and oocytes makes it crucial to examine gene expression in both cell types. For several genes, transcript abundance in CCs seemed to be a more reliable parameter of oocyte quality than

levels in oocytes. In addition, gene expression in CCs can potentially exhibit a greater response to environmental conditions during maturation than that in oocytes. Reduced expression of *BBOX1* and higher expression of *CPT2* in CCs before maturation and transcript abundance for both *G6PD* and *ALDOA* after maturation were found to be potential markers of oocyte quality. The results from the current study indicate growth and maturation of oocytes are delayed in gilt COCs, through the downregulation of genes encoding enzymes important for glycolysis and the PPP, and an apparent delay in FAO in both oocytes and CCs, in addition to delayed EGFR signaling in gilt CCs. Hence, gilt COCs might not have adequate stores of ATP and are less competent in responding to oxidative stress compared to sow COCs at the end of maturation. Other studies have explored supplementing media with L-carnitine to increase oocyte quality, but we hypothesize porcine oocytes and CCs have the potential to differentially regulate L-carnitine biosynthesis. Further studies are needed to verify these indications and hypotheses by measuring actual levels of ATP, oxidative stress and L-carnitine in gilt and sow oocytes.

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Appendix A

Table A1. Complete list where the TaqMan® Assay will detect specific transcript variants.

Gene Symbol	TaqMan® Assay	Accession Numbers of Transcript Variants
G6PD	Ss02690824_g1	XM_021080744.1 (X1), XM_003360515.5 (X2)
ALDOA	Ss06920688_m1	XM_021087995.1 (X1), XM_021087996.1 (X2), XM_021087997.1 (X3), XM_021087998.1 (X4), XM_021088000.1 (X6), XM_021088001.1 (X7), XM_021088002.1 (X8), XM_021088004.1 (X9)
TMLHE	Ss06886117_m1	XM_003135511.4 (X1), XM_021080447.1 (X2), XM_021080448.1 (X3), XM_021080450.1 (X4), XM_005674022.3 (X5)
BBOX1	Ss06906097_m1	XM_021083234.1 (X1), XM_021083235.1 (X2), XM_021083236.1 (X3)
GHR	Ss03383662_u1	XM_021076567.1 (X1), XM_013990636.2 (X2), XM_021076568.1 (X3), XM_021076569.1 (X4), XM_021076570.1 (X5), XM_021076571.1 (X6), XM_021076572.1 (X7), XM_021076573.1 (X8), XM_021076574.1 (X9), XM_013990637.2 (X10), XM_021076575.1 (X11)
BAX	Ss03375842_u1	XM_003127290.5 (X1), XM_013998624.2 (X2)
BCL2L1	Ss03383783_s1	XM_021077292.1 (X1), XM_021077293.1 (X2), XM_021077294.1 (X3), XM_021077295.1 (X4), XM_021077296.1 (X5), XM_021077297.1 (X6), XM_021077298.1 (X7)
ACTB	Ss03376563_uH	XM_003124280.5 (X1), XM_021086047.1 (X2)
GAPDH	Ss03375629_u1	XM_021091114.1 (X2)
HMBS	Ss03388782_g1	XM_021102144.1 (X1), XM_021102146.1 (X2), XM_021102147.1 (X3), XM_021102148.1 (X4)
HPRT1	Ss03388274_m1	XM_021079503.1 (X1), XM_021079504.1 (X2)

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In the pig breeding industry embryo transfer can increase genetic gain, biosecurity and animal welfare. However, limiting factors for embryo transfer are embryo production and storage, since non-optimal *in vitro* culture conditions are affecting embryo development and quality, and porcine embryos are highly sensitive to freezing. The focus of this thesis was storage of porcine *in vitro* produced embryos and identification of novel oocyte quality parameters to increase knowledge of *in vitro* oocyte maturation.

Paper 1 assessed embryo quality of *in vitro* produced porcine blastocysts after 3 hours liquid storage at 37 °C in CO_2-free medium by evaluating morphology, *in vitro* developmental capacity and apoptosis. The study demonstrated that 3 hours storage did not significantly affect embryo quality.

Paper 2 aimed to distinguish parameters for porcine oocyte quality by examining expression of selected genes in cumulus cells and oocytes, by employing the model assuming oocytes from adult, cycling animals being of higher quality than those from prepubertal animals. The study detected expression in cumulus cells of four genes related to metabolism as potential novel markers of oocyte quality.

Paper 3 validated the use of reference genes and identified the combined use of *ACTB* and *PFKP* as the most optimal normalisation for the porcine oocyte RT-qPCR data. Results further indicated delayed accumulation of transcripts supporting histone modifications during oocyte and early embryo development in prepubertal gilt oocytes compared to sows. These transcripts may also have potential as oocyte quality markers.