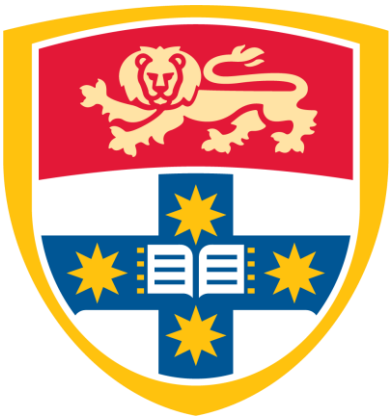
THE UNIVERSITY OF
SYDNEY

LIPID METABOLISM DURING THE IN VITRO PRODUCTION OF PORCINE EMBRYOS



Jenna Louise Lowe

A thesis submitted for the fulfilment of the requirements for the award of the degree of Doctor of Philosophy

Faculty of Veterinary Science
The University of Sydney

November 2014

DECLARATION

The studies presented within this thesis were planned and executed by the author, and have not been previously submitted for any other degree to a University or Institution. I certify that any assistance with the preparation of this thesis and other sources used have been acknowledged appropriately.

Jenna Louise Lowe

ACKNOWLEDGEMENTS

The course of my post-graduate study has been an extremely rewarding and enjoyable experience, and was made so by the contribution of a number of people both professionally and personally.

I would like to express my sincere thanks to my supervisors, Dr Christopher Grupen and Dr Roslyn Bathgate, for their guidance, support, and advice throughout the course of my candidature. Thank you to Peter Thomson for his endless patience in teaching me stats. For staining and confocal work, I would like to acknowledge the assistance of Ms Ellie Kable and Dr Minh Huynh from the Australian Centre for Microscopy and Microanalysis in developing staining protocols and learning confocal microscopy. Further thanks to Professor Kazuhiro Kikuchi and Dr Tamas Somfai from NARO Institute of Livestock and Grassland Science, Tsukuba, for letting me visit their lab to complete assays and staining. Huge thanks go to the staff of Wollondilly Abattoir, who supplied us with ovaries each week - a PhD is a great way to meet a really diverse group of people, and they were certainly among some of the best.

To my lab buddy and partner in crime, Louise Bartolac – thank you for sharing the media prep, abattoir runs and cleaning duties; for all the cake, cookies and lunch dates; and for always making lab time entertaining. I'm going to miss all your crazy stories, and I sincerely hope I have the opportunity to work with you again someday.

Personally, I was incredibly lucky to have an amazing support network, and I am grateful to all of my friends who were there through the best and the worst of it. Special thanks to Jess for her enduring fake friendship and for always knowing whether to hand over a cup of tea or a cup of concrete. Thank you to Ali, Natasa and Amy for sharing their lives and their desserts.

Finally, I would like to acknowledge my family for their support. Deepest thanks to Katie and Daniel Lowe, for loving science as much as I do. In a way, you both had

to live through this with me, and I apologise for all the dinner conversations revolving around sperm, embryos and abattoirs. My most heartfelt thanks are for my dad, Barry Lowe. You have given me so much love and encouragement from the very beginning. Even when I doubted myself, your belief in me was steadfast. Thank you for always telling me I could do absolutely anything I wanted in life, and for giving me the opportunities to do just that.

SUMMARY

Currently, the in vitro production (IVP) of porcine embryos suffers from low efficiency and reduced blastocyst quality. Poor outcomes from in vitro matured oocytes and in vitro fertilised embryos have limited the use of assisted reproductive technologies (ARTs) within commercial porcine herds, reducing the potential for global genetic improvement programs. It is believed that this reduced developmental competency compared to in vivo embryos is attributable to altered metabolism resulting from in vitro culture.

Improper or incomplete metabolic support from the culture media leads to production of inferior embryos. Much of the prior research centres on metabolism of carbohydrates by oocytes and embryos, with the formulation of media based on this knowledge. However, oocytes and embryos also contain endogenous lipid substrates, and there is a lack of understanding as to how and when these stores are utilised. Lipids are a dense form of energy storage, and there is evidence of their metabolism by oocytes and embryos for energy generation. Porcine oocytes and embryos have higher intracellular lipid content than other domestic livestock species, and this makes them an excellent model for studying aspects of lipid metabolism in vitro.

The aim of this study was to examine the impact of lipid metabolism on the acquisition of developmental competence during porcine IVP, and how this is affected by the presence or absence of exogenous carbohydrates. Stimulation or inhibition of the β-oxidation pathway was used to discern the importance of fatty acid oxidation to oocyte maturation and embryo development during in vitro maturation (IVM), in vitro fertilisation (IVF) and in vitro embryo culture (IVC).

During IVM, it was identified that porcine oocytes are capable of using different substrates to compensate for deficiencies in others. While pyruvate and glucose are preferentially utilised to support maturation, upregulation of β-oxidation can compensate for a low glucose concentration and an absence of pyruvate to support nuclear maturation. Although there was no discernible decrease in lipid

content associated with this, lipids provide such a dense energy reserve that any usage may have been beyond the limit of detection. Inhibition of β-oxidation in the absence of carbohydrates had a greater effect on nuclear maturation compared to inhibition in complete media. This indicates that lipid metabolism plays a minor role during oocyte maturation in the presence of carbohydrates and is likely to be more important when other substrates are deficient.

Energy generation prior to fertilisation is an important factor in the developmental outcomes of subsequent embryos. Upregulation of β-oxidation for the duration of IVF increased cleavage rates, but doses above 6mM L-carnitine led to decreased blastocyst development. This effect may be attributable to the antioxidant activity of L-carnitine, with low levels of reactive oxygen species (ROS) being required at fertilisation for normal sperm function and sperm-oocyte interactions. Oocyte incubation in media supplemented with 3mM L-carnitine for an hour prior to insemination increased cleavage and improved cryosurvival of Day 7 embryos after vitrification. While ATP content of oocytes did not increase over this period, it is unclear if lipid content was reduced. Previous studies have shown that L-carnitine treatment of oocytes and embryos decreased lipid content, thereby increasing cryotolerance. It would therefore appear that there is a limited role for β-oxidation during the IVF period itself, although upregulation immediately prior to fertilisation may have beneficial effects on metabolic processes and may provide antioxidant protection leading to improved development in early cleavage stage embryos.

During embryo culture, there was a greater effect of upregulating lipid metabolism seen in the absence of carbohydrate substrates than in complete media. However, this could not support embryo development to the same extent as carbohydrate substrates. Changing nutrient requirements of embryos has led to the development of sequential media, leading to the production of better quality IVP embryos. Upregulation of β-oxidation for the first three days of culture in a single media system increased embryo quality to the same extent as a sequential carbohydrate media system, implying there is some level of plasticity to embryo metabolism allowing for adaptability to different substrates. Inclusion of L-carnitine for either a three day period or the duration of culture increased cryosurvival,

suggesting decreased lipid content due to increased β-oxidation activity. Similarly for oocyte maturation, β-oxidation appears to be able to compensate for carbohydrate deficiencies during embryo culture to some extent, and oxidation of lipids has a greater role in promoting embryo quality over increasing production rates.

The findings reported in this thesis represent a contribution to the understanding of lipid metabolism during the in vitro production of porcine embryos. These results provide evidence to support a level of adaptability of porcine oocytes and embryos to different substrates available during maturation and culture. There is a preference shown for carbohydrates substrates, with the ability to utilise lipids to compensate for certain deficiencies. This would justify the inclusion of co-factors of lipid metabolism such as L-carnitine in culture media, to ensure that any deficiencies in other substrates might be corrected for and to promote higher embryo quality. Upregulation of β-oxidation also increased the cryosurvival of porcine embryos following vitrification, with this being a major development in the global movement of superior genetics for herd improvement programs. These findings will also have implications for improving in vitro culture of oocytes and embryos of other species, most notably advancements in human ARTs where research is predominantly limited to work in animal models. The understanding of how lipids are metabolised alongside exogenous carbohydrates will contribute to improving media formulations for better metabolic support in vitro, further to improving embryo production and quality.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ART	assisted reproductive technology
BSA	bovine serum albumin
CCs	cumulus cells
CoA	coenzyme A
COC	cumulus oocyte complex
CO_2	carbon dioxide
CPT1	carnitine palmitoyl transferase 1
d	days
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
FCS	foetal calf serum
FF	follicular fluid
GLUTs	glucose transporters
GSH	glutathione
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hours
hCG	human chorionic gonadotropin
HSL	hormone sensitive lipase
IVC	in vitro culture
IVF	in vitro fertilisation
IVM	in vitro maturation
IVP	in vitro production
LH	luteinising hormone
M	molar
min	minutes
ml	millilitres
mm	millimetres

mM	millimolar
MI	metaphase I
MII	metaphase II
NAD	nicotinamide adenine dinucleotide
NCSU-23	North Carolina State University medium 23
PFK	phosphofructokinase
pM	picomolar
POM	porcine oocyte medium
PPP	pentose phosphate pathways
PUFA	polyunsaturated fatty acid
PZM-3	porcine zygote medium-3
PVA	polyvinyl alcohol
ROS	reactive oxygen species
TALP-PVA	tyrode’s albumin lactate pyruvate polyvinyl alcohol
TCA	tricarboxylic acid
µM	micromolar
v/v	volume:volume ratio
w/v	weight:volume ratio

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Lowe, JL, Bathgate, R, Grupen, CG (2014) *Involvement of lipid metabolism during in vitro maturation of porcine oocytes*, Proceedings of the 18th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), Helsinki, Finland.

Lowe, JL, Bathgate, R, Grupen, CG (2013) *Stimulating lipid metabolism in porcine embryos: Effect of L-carnitine in a sequential media system*, Proceedings of the Annual Meeting of the Society for Reproductive Biology, Sydney, Australia.

Lowe, JL, Bathgate, R, Grupen, CG (2013) *Lipid and carbohydrate metabolism during in vitro culture of porcine embryos*, Proceedings of the 13th Annual Postgraduate Research Conference. Faculty of Veterinary Science, The University of Sydney, Australia.

Lowe, JL, Bathgate, R, Grupen, CG (2012) *Stimulating lipid metabolism during porcine oocyte maturation: Effect of L-carnitine in the absence of pyruvate and lactate*, Proceedings of the Annual Meeting of the Society for Reproductive Biology, Gold Coast, Australia.

Lowe, JL, Bathgate, R, Grupen, CG (2012) *Stimulating lipid metabolism during porcine oocyte maturation: Effect of L-carnitine in the absence of pyruvate and lactate*, Proceedings of the 12th Annual Postgraduate Research Conference. Faculty of Veterinary Science, The University of Sydney, Australia.

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CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

In vitro production of porcine embryos is currently limited to research applications in oocyte and embryo studies and the production of transgenic pigs for xenotransplantation. This is because the system is not effective or efficient enough to permit the application of ARTs, including embryo transfer, cloning and vitrification on a commercial scale. These ARTs are invaluable tools for facilitating genetic improvement and breeding programs of pig herds globally, and the ability to use them would be of great benefit to the industry. Quality differences between in vitro and in vivo derived embryos stem in part from poor metabolic support during in vitro culture, due to an incomplete understanding of the metabolic requirements of oocytes and embryos.

Porcine oocytes and embryos contain a large complement of lipid, the majority of which is stored as lipid droplets within the cytoplasm. There is evidence that lipids play an important metabolic role during oocyte maturation and embryo development in numerous species, including mice (Dunning et al. 2010a; Dunning et al. 2010b), cattle (Sutton-McDowall et al. 2012), and pigs (Somfai et al. 2011; Wu et al. 2011; You et al. 2012). However, the level of contribution to energy generation within the cell and the reasons for such high levels of intracellular lipid in porcine oocytes and embryos are still poorly understood.

The majority of research into oocyte and embryo metabolism has focussed on carbohydrate usage. Because of this, many current media formulations used during in vitro oocyte maturation and embryo culture are based predominantly on carbohydrate requirements, leading to potential inefficiencies as the metabolic requirements of the cells are only partially met. Comparatively little is known regarding lipid metabolism, as this is a recently emerging area of research (Romek et al. 2009; Romek et al. 2011b, a; Somfai et al. 2011; McKeegan and Sturmey 2012; You et al. 2012).

Specific metabolic processes and preferential substrate utilisation by oocytes and embryos appears to be species-specific. Understanding metabolic processes utilised by the oocyte and embryo for energy production will provide insight into substrate utilisation, critical to improving culture media conditions. By tailoring media to the needs of the cells, IVP protocols may be optimised to more closely match in vivo development and produce higher quality embryos, allowing this technology to be utilised in a commercial setting.

1.2. Embryo IVP

1.2.1. Significance of the technology

Further development of protocols for IVP of porcine embryos would be beneficial in providing the opportunity for the application of a wider range of ARTs to pig production. Genetic improvement programs are enhanced by the ability to create, store and transport embryos globally, allowing improvements in economically important traits. Embryo transfer programs using in vitro produced embryos facilitate gene flow between herds, as well as the ability to vitrify embryos for indefinite storage of desirable gene lines.

Porcine IVP also benefits human biomedical research. Pigs are particularly valuable models because of the similarities to human physiology, and the readily available nature of abattoir-sourced material. Development of ART treatments for infertile human couples relies largely upon animal models due to legislative restrictions on working with human tissue and limited availability of material. Further, animal cloning and production of transgenic animals is a growing research area with transgenic pigs used as models of human diseases (Kragh et al. 2009; Luo et al. 2011) and for research into xenotransplantation (Luo et al. 2012; Zinovieva et al. 2014). This research is creating potential options for treatments of certain diseases, such as islet cell transplantation in patients with diabetes mellitus (Nagaraju et al. 2013), demonstrating the value of developing porcine IVP technologies.

1.2.2. Limitations of the technology

These biotechnologies depend largely upon large quantities of good quality porcine embryos. Despite extensive and ongoing research into refining IVP protocols, these applications are currently limited due to inefficiencies inherent in IVP porcine embryos. Current porcine IVP systems still suffer from inefficient oocyte maturation and fertilisation, poor developmental capacity of in vitro produced embryos, and suboptimal IVC conditions (Abeydeera 2002; Grupen 2014).

Standard IVP systems include in vitro maturation, in vitro fertilisation and in vitro culture. During each in vitro stage, factors such as oxygen tension, temperature, humidity and composition of culture media all differ from conditions to which the oocytes and embryos are exposed in vivo, compromising the quality of the resulting embryos. The low efficiency of IVP in pigs, especially compared to that of other livestock species and of in vivo embryos, is a major limitation of this technology.

In vitro produced embryos have been shown to have altered metabolic processes compared with in vivo embryos, with differences seen in substrate utilisation and activity levels of metabolic pathways (Swain et al. 2002). Energy generation within the cell is a complex and important process required for proper growth and development, and this is likely a major contributor to the poorer quality of in vitro produced embryos. Further, improper or asynchronous cytoplasmic and nuclear maturation during IVM and a consequential high incidence of polyspermy following IVF, as well as sub-optimal embryo culture conditions, are also suspected to be major contributors to the low efficiency of IVP systems for porcine embryos. Porcine oocytes and embryos have high endogenous lipid reserves, stored predominantly as triglycerides within the cytoplasm, compared with other species. Negative effects of this increased lipid content include reduced cryotolerance (Dobrinsky and Johnson 1994; Abe et al. 2002; Sudano et al. 2011), making successful vitrification of these cells difficult.

1.2.3. In vitro maturation

In vitro maturation is a critical component of IVP systems, involving the harvesting of immature cumulus oocyte complexes from antral follicles and maturing them within the lab environment. Oocyte quality plays a major part in determining the yield and quality of resulting embryos (Krisher 2004), and therefore proper maturational support is a critical component of any IVP system. Oocyte quality is influenced by the nuclear and mitochondrial genome, as well as the surrounding environment supporting the oocyte, including factors such as in vitro culture media, temperature, humidity and oxygen tension. Because oocyte quality has such a marked effect on the quality of resulting embryos, ensuring optimal conditions throughout maturation is vitally important.

The cumulus oocyte complex (COC) refers to the oocyte itself as well as the surrounding cumulus cells (CCs). These are differentiated from follicular granulosa cells and act as support cells for the oocyte during growth and development. Interaction between CCs and oocytes is primarily through small transzonal projections from the cumulus cells which penetrate the zona pellucida of the oocyte to provide direct contact with the surface of the oolemma, forming gap junctional communication (Albertini et al. 2001). Oocytes also communicate with the cumulus cells via paracrine signalling, allowing oocyte-directed stimulation of processes within the cumulus cells. Bi-directional communication between the oocyte and the cumulus cells is critical for acquisition of oocyte developmental competence (Gilchrist et al. 2008).

Oocytes go through two periods of meiotic arrest. The first period of arrest is at the germinal vesicle (GV) stage following oogenesis within the immature follicle. In vivo, resumption of meiosis following arrest at the GV stage is triggered by the pre-ovulatory LH surge and gonadotrophin stimulation. This process occurs spontaneously in IVM systems following aspiration of the COC (Gilchrist and Thompson 2007). Following resumption of meiosis after the first arrest, the oocytes undergo germinal vesicle breakdown (GVBD), leading to arrangement of the chromatids on the spindle at metaphase I (MI) stage. Following MI, oocytes undergo anaphase and telophase to reach the stage of metaphase II (MII). This is

the second period of meiotic arrest, and can only be maintained for a few hours until cell quality irreversibly declines and cell death occurs. Sperm related factors trigger calcium oscillations within the oocyte, increasing calcium concentrations within the cell to reinitiate development and allow fertilisation to occur (Carroll 2001). Higher rates of in vitro nuclear maturation, pronuclear formation and blastocyst development are attained in oocytes derived from large follicles compared to those derived from small follicles (Yoon et al. 2000; Marchal et al. 2002). Oocytes obtained from adult pigs had higher developmental competence than those from gilts (Grupen et al. 2003; Bagg et al. 2007).

Concurrent with nuclear maturation, the oocyte undergoes cytoplasmic maturation. Cytoplasmic maturation involves rearrangement of the cytoplasmic structures, such as the endoplasmic reticulum and mitochondria, as well as accumulation of factors such as nutrients, substrates, mRNAs and de novo purines required for DNA synthesis and completion of maturation. The synthesis of gluthathione (GSH) plays a vital role in successful cytoplasmic maturation, with evidence for a role in maintenance of meiotic spindle morphology to ensure normal fertilisation (Zuelke et al. 1997). Sperm head decondensation, male pronuclear formation and development to the blastocyst stage are all quality markers of cytoplasmic maturation, and are positively correlated with GSH levels (Yoshida 1993; Abeydeera et al. 1998a). In vivo matured oocytes typically have higher levels of GSH than in vitro matured oocytes (Brad et al. 2003a), possibly contributing to quality differences.

The optimisation and synchronisation of nuclear and cytoplasmic maturation are major factors required for successful oocyte maturation. It is vital to ensure these factors are accounted for to ensure the mature oocyte has attained developmental competency prior to fertilisation.

1.2.4. In vitro fertilisation

Fertilisation marks the uniting of a mature MII oocyte with a spermatozoon to form a diploid zygote. There are numerous events necessary for normal, successful fertilisation; these include sperm penetration, sperm-oocyte fusion, oocyte

activation, expulsion of the second polar body, male and female pronuclear development and syngamy. Fertilisation media, sperm:oocyte ratio and co-incubation time all influence sperm penetration and fertilisation rates in vitro (Gil et al. 2010). The proportion of monospermic fertilisation in vitro from the total oocytes inseminated in pigs is around 30-50% (Abeydeera et al. 1998a; Kikuchi et al. 1999; Funahashi et al. 2000; Suzuki et al. 2000; Gil et al. 2003; Gil et al. 2004).

At fertilisation, spermatozoa undergo a series of reactions in order to bind to, and then penetrate, the oocyte. Acrosome-intact spermatozoa firstly bind in a species-specific manner to the zona pellucida of the oocyte, before undergoing the acrosome reaction to allow penetration of the zona pellucida and access to the perivitelline space. Following this, spermatozoa must bind to the plasma membrane of the oocyte. Oocyte activation is induced by fusion of the gamete membranes and entry into the ooplasm of a sperm cytoplasmic factor, phospholipase C-ζ (Fujimoto et al 2004), which causes an increase in intracellular calcium levels within 1-3 min (Lawrence et al. 1997; Carroll 2001). In mammalian oocytes, following the initial increase in calcium levels, transient fluctuations in calcium levels occur over the next several hours (Schultz and Kopf 1995; Carroll 2001; Whitaker and Larman 2001). It is likely that these oscillations play a role in regulating cell cycle transitions within the oocyte and embryo, with reciprocal regulation of calcium transients by mitotic kinases (Emerson et al. 2000; Carroll 2001; Whitaker and Larman 2001).

This elevation in intracellular calcium concentration induces alterations in the surface of the oocyte via exocytosis of cortical granules. This alteration prevents the penetration and binding of multiple spermatozoa, thus preventing polyspermy and ensuring formation of a normal diploid zygote. There is an increased adenosine triphosphate (ATP) requirement at this time to support calcium oscillations and exocytosis. Calcium increase also triggers completion of the second meiotic division, with the second polar body being extruded. Following completion of meiosis, the zygote contains two haploid pronuclei, one maternally- and one paternally-derived. The two pronuclei migrate to the centre of the zygote, where both nuclear envelopes break down and the condensed chromosomes of each align on a common spindle. Mitotic divisions begin to give rise to a two cell

embryo, each cell containing the diploid genome. These cells continue to undergo a series of mitotic cell divisions during embryo development.

1.2.5. In vitro culture

Following fertilisation, cell divisions within the embryo are associated with changing metabolic requirements to support new cell processes. Cleavage stages in the embryo include early cell divisions, from the two-cell stage through to the compacted morula, comprised of 8 to 16 cells. Within normal healthy embryos, cleavage divisions are uniform, producing blastomeres of approximately equal size; uneven cleavage or cell fragmentation is an indication of poor quality (Giorgetti et al. 1995). Compaction begins at the 8- to 16-cell stage, with an increase in cell-to-cell adherence through formation of tight junctions to form a morula.

From the morula, the cells located in the outer part of the embryo flatten out and form tight junctions with neighbouring cells to form the trophectoderm layer which eventually becomes placental tissue (Rossant and Croy 1985). The remainder of the cells are destined to become the inner cell mass, giving rise to the embryo proper (Rossant and Croy 1985). Fluid between the cells of the morula is increased to form a cavity which is then maintained through the action of the membrane sodium/potassium pump. This increases the internal salt concentration of the embryo, attracting water via osmosis. The fluid is concentrated into a centralised compartment known as the blastocoel, surrounded by the now tightly adhered trophectoderm cells. As fluid in the blastocoel increases, the number of cells also increases, leading to progressive growth in size of the blastocyst. Associated with this growth is a thinning of the zona pellucida, until the blastocyst hatches from the zona pellucida. In vivo, the hatched blastocyst would then implant within the uterus; in vitro culture systems cannot support development past this point.

As the embryo develops, the maternal genome inactivates as the embryonic genome assumes control of embryogenesis (Hsu et al. 2012). Embryonic genes become transcriptionally active at the late 4-cell stage in porcine embryos, with

the total levels of transcription increasing as the embryo develops. Subsequent cell divisions and protein synthesis are driven by the embryonic genome, and this can include changes in metabolic processes at this time (Hsu et al. 2012; Ostrup et al. 2013).

Compared with in vivo embryos, IVP porcine embryos typically have lower average cell numbers (Bryla et al. 2009), higher proportion of DNA fragmentation (Pomar et al. 2005; Bryla et al. 2009) and a greater number of blastocysts showing chromosomal aberrations (McCauley et al. 2003). Overall efficiency of IVP technology is still low, resulting in low embryo and pregnancy rates compared with in vivo systems and to results commonly achieved in other species. Rates for blastocyst development in porcine IVP systems are typically around 20% (Nguyen et al. 2015; Sakurai et al. 2015), with pregnancy and farrowing rates reported at 30-40% and 17-25% respectively (Yoshioka et al. 2012; Sakurai et al. 2015). Comparatively, blastocyst rates of around 50% are commonly seen in bovine IVP systems (Rizos et al. 2002; Sun et al. 2015), and both bovine and ovine systems have reported pregnancy rates of approximately 55% following transfer of IVP embryos (Papadopoulos et al. 2002; Machatkova et al. 2008).

This is partially attributable to a lack of understanding regarding the metabolic requirements of embryos, resulting in poor metabolic support during in vitro culture.

1.3. Energy requirements of oocytes and embryos

1.3.1. ATP synthesis and requirement throughout IVP

Metabolism refers to the chemical processes occurring within a living organism in order to maintain life. Included within these processes are the metabolic reactions occurring to produce energy within the cell, in the form of ATP. The ability to generate energy is important for all cells, as a requirement for growth, repair and production. Each stage of growth and development is energetically expensive for the cell, with ATP required for protein synthesis, homeostatic regulation and cellular functions throughout maturation, fertilisation and embryo development.

ATP is often used as a marker of cell viability, as it is present in all metabolically active cells and its concentration rapidly decreases following cell death. ATP production is self-regulated based on the ADP/ATP ratio within the cell, and therefore increased ATP synthesis reflects an increase in cell energy requirements.

The ATP accumulated during the oocyte maturation period is likely to be important during fertilisation events and embryo culture (Krisher et al. 2007). Higher rates of development and implantation are seen in human embryos derived from in vivo matured oocytes with an ATP content ≥2pM/oocyte (Van Blerkom et al. 1995). More bovine oocytes with high morphological quality had ATP levels between 2-3 pM/oocyte and increased blastocyst rates following IVF, compared with morphologically poorer quality oocytes which typically had lower ATP levels (Stojkovic et al. 2001). However, in porcine oocytes, ATP content did not differ between in vivo and in vitro matured oocytes (0.71-1.03pM/oocyte; Brad et al. 2003a) despite it being common to see developmental differences between these cohorts of oocytes. A later study found that while ATP content of porcine oocytes was shown to increase over the course of maturation (1.21pM/oocyte when immature to 3.08pM/oocyte at MII), the addition of 25% follicular fluid to maturation media did not affect final ATP content in mature oocytes (3.08 vs 3.60pM/oocyte; Brevini et al. 2005). Follicular fluid is known to improve developmental competence of oocytes (Algriany et al. 2004; Tatemoto et al. 2004; Ito et al. 2008) and this suggests that ATP content may not directly reflect porcine oocyte quality. Differences in ATP content between studies may reflect the sensitivity of porcine oocytes to media conditions and components, as well as the use of dbcAMP in one study (Brevini et al. 2005) but not the other (Brad et al. 2003a).

There is an increase in energy requirement at the blastocyst stage, corresponding to increased protein synthesis (Thompson 1997) and increased Na^+ / K^+ ATPase activity to create the blastocoel (Watson 1992). ATP content of Day 6 porcine embryos was higher when media contained glucose (0.635 vs 0.273pM/embryo; Mito et al. 2012), indicating a reliance on glucose substrates for energy production during embryo development. With ATP generation being a requirement for cell

growth and repair, it is vital that the metabolic pathways responsible for energy generation are properly supported with the required substrates.

1.3.2. Metabolic pathways for energy generation

The majority of energy production within mammalian cells is via aerobic respiration. The complete breakdown of products through this pathway involves glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. The pentose phosphate pathway (PPP) produces intermediaries important for cell functions, although does not directly produce ATP. Lipogenesis and lipolysis are related to storage and utilisation of fatty acids. During maturation, oocytes have been shown to metabolise substrates via glycolysis, the TCA cycle in conjunction with oxidative phosphorylation, and the PPP, all to varying degrees (Durkin et al. 2001). In vivo matured porcine oocytes show unusually high levels of PPP activity (Durkin et al. 2001), indicating that this pathway may be of particular importance in this species. During culture, pre-implantation porcine embryos predominantly utilise the PPP, glycolysis and the TCA cycle (Flood and Wiebold 1988; Swain et al. 2002), although activity of these pathways is higher in in vivo-derived embryos than those produced in vitro (Swain et al. 2002). There is evidence supporting a role for lipolysis during both oocyte maturation and embryo development, although this is an area requiring further investigation.

1.3.2.1. Glycolysis (Figure 1.1)

Occurring in the cytoplasm, glycolysis is responsible for the formation of pyruvate from glucose via anabolic catabolism. The glycolytic pathway itself results in the production of 4 molecules of ATP and 2 of NADH but utilises 2 ATP molecules in the process, resulting in a net gain of 2 ATP and 2 NADH per glucose molecule entering the pathway. The pyruvate produced from glycolysis is utilised by the TCA cycle or the PPP.

Glycolysis is regulated by inhibition or activation of enzymes involved in certain steps of the pathway, thus increasing or decreasing flux through the pathway. The three regulatory enzymes of glycolysis are hexokinase, phosphofructokinase

(PFK), and pyruvate kinase. The reactions catalysed by these enzymes are effectively irreversible, serving as points of control for glycolytic flux. A high ATP:ADP ratio will serve to inhibit both PFK and pyruvate kinase activity, allowing for decreased glycolytic activity when energy is abundant.

1.3.2.2. The tricarboxylic acid cycle (Figure 1.2)

Also known as the citric acid cycle or the Krebs cycle, the tricarboxylic acid (TCA) cycle is an aerobic process which metabolises pyruvate to produce acetyl-CoA, NADH and $FADH_2$. The TCA cycle is closely linked to oxidative phosphorylation, where these products are fully metabolised to produce ATP. Further, many of the intermediaries produced through the TCA cycle are also utilised as precursors for other biomolecules, including for synthesis of amino acids and purine nucleotides required for growth and DNA synthesis. The TCA cycle is regulated via substrate availability, product inhibition, and feedback inhibition.

Several different metabolic pathways feed intermediaries into the TCA cycle, with the end goal of producing ATP. These include glycolysis, providing pyruvate; the β-oxidation cycle, providing acetyl-CoA, and propionyl Co-A for conversion to succinyl-CoA; and protein catabolism, whereby the carbon backbone of amino acids can be converted to acetyl-CoA for use in the TCA cycle.

Oxidative phosphorylation is a metabolic pathway occurring in the mitochondria, which utilises redox reactions to release energy in the form of ATP. Oxidative phosphorylation incorporates both the electron transport chain and chemiosmosis. The electron transport chain involves NADH and $FADH_2$, formed through other metabolic processes, acting as electron donors to oxygen, creating H_2O and liberating a large amount of free energy. Oxidative phosphorylation is a highly efficient means of producing ATP. It releases between 30-36 molecules of ATP from the complete hydrolysis of one molecule of glucose to CO_2 and water; compared with only 2 ATP molecules produced via glycolysis alone.

1.3.2.3. Pentose phosphate pathway (Figure 1.3)

While the pentose phosphate pathway itself does not directly generate ATP, it is an important metabolic pathway for the generation of NADPH and pentoses. This pathway acts as an alternative to glycolysis, playing a primarily anabolic role. The primary products of this pathway are reducing equivalents (NADPH), ribose-5-phosphate (R5P), and erythrose-4-phosphate (E4P). NADPH is used in reductive biosynthetic reactions within cells, for example fatty acid synthesis, as well as serving to reduce glutathione via glutathione reductase to reduce accumulation of H_2O_2 and prevent oxidative stress. R5P and E4P are required for the synthesis of nucleotides and nucleic acids and synthesis of aromatic amino acids, respectively; these are all requirements for cell growth and DNA synthesis.

The PPP is regulated by activity of the glucose-6-phosphate dehydrogenase enzyme. Activity of this enzyme is stimulated by increasing concentrations of $NADP^+$, which is formed from the use of NADPH in reductive biosynthetic pathways. The PPP then reduces $NADP^+$ to restore NADPH levels.

1.3.2.4. Lipogenesis and lipolysis

Lipogenesis is the process by which triglycerides are formed from acetyl-CoA, incorporating both the processes of fatty acid synthesis and triglyceride synthesis. The majority of acetyl-CoA within the cell results from pyruvate breakdown. In times of nutritional deficit, this acetyl-CoA will then feed into the TCA cycle to produce cellular energy. When there is sufficient energy available, the excess acetyl-CoA not required immediately for energy production is instead directed toward fatty acid synthesis, thus storing excess energy substrates as fatty acids for use when required.

The converse reaction, lipolysis, is the breakdown of lipids. The first step involves the hydrolysis of triglycerides by hormone sensitive lipase (HSL) to produce free fatty acids, which are subsequently metabolised to form a fatty acyl-CoA. Carnitine palmitoyl transferase 1 (CPT1) catalyses the exchange of the CoA for carnitine, forming fatty acyl-carnitine, which is capable of passing through the mitochondrial membrane. This process is known as the carnitine shuttle (Figure 1.4), and is the rate limiting step of lipolysis. Fatty acyl-CoA is reformed and

metabolised via the β-oxidation cycle. The end products of β-oxidation vary depending on fatty acid chain length, with one molecule each of $FADH_2$, NADH and acetyl-CoA formed for every two carbon atoms. These products are then further metabolised via the TCA cycle and oxidative phosphorylation to produce ATP.

The regulation of β-oxidation and fatty acid synthesis are closely coordinated, with both being under hormonal and allosteric regulation. Lipolysis is under precise regulation at multiple points by hormonal and biochemical signals which regulate the function of lipolytic enzymes (Duncan et al. 2007). Many of these regulation points are controls for the balance between lipolysis (when energy demands are high or other energy substrates are unavailable) and lipogenesis (when there is excess energy substrate and low energy demand). Regulation of HSL is primarily via glucagon and epinephrine, which activate lipolysis, and insulin, which inhibits fatty acid oxidation. Glucagon levels increase when blood glucose is low, and epinephrine production is increased during peak metabolic demand; in both instances, additional energy is required and the oxidation of fatty acids is subsequently increased to meet demand. In this case, glucagon and epinephrine can bind to G protein-coupled receptors, activating adenylate cyclase to increase cAMP production and protein kinase A activity. Following phosphorylation, protein kinase A activates HSL. When circulating glucose levels are high, insulin production is increased, inhibiting lipolysis; sufficient energy is available from glucose and fatty acid oxidation is not required. Protein phosphatase 2A is activated by insulin, and dephosphorylates HSL to inhibit its activity. Insulin also increased the activity of phosphodiesterases, which break down cAMP to prevent the phosphorylation of HSL by protein kinase A. Other control points include allosteric regulation by intermediaries from lipogenesis. For example, acetyl-CoA is carboxylated to form malonyl-CoA in the initial stages of lipogenesis, and inhibits β-oxidation at CPT1. High concentrations of malonyl-CoA indicate adequate energy substrate availability, as lipogenesis is occurring to store excess substrate, and therefore there is no requirement for β-oxidation.

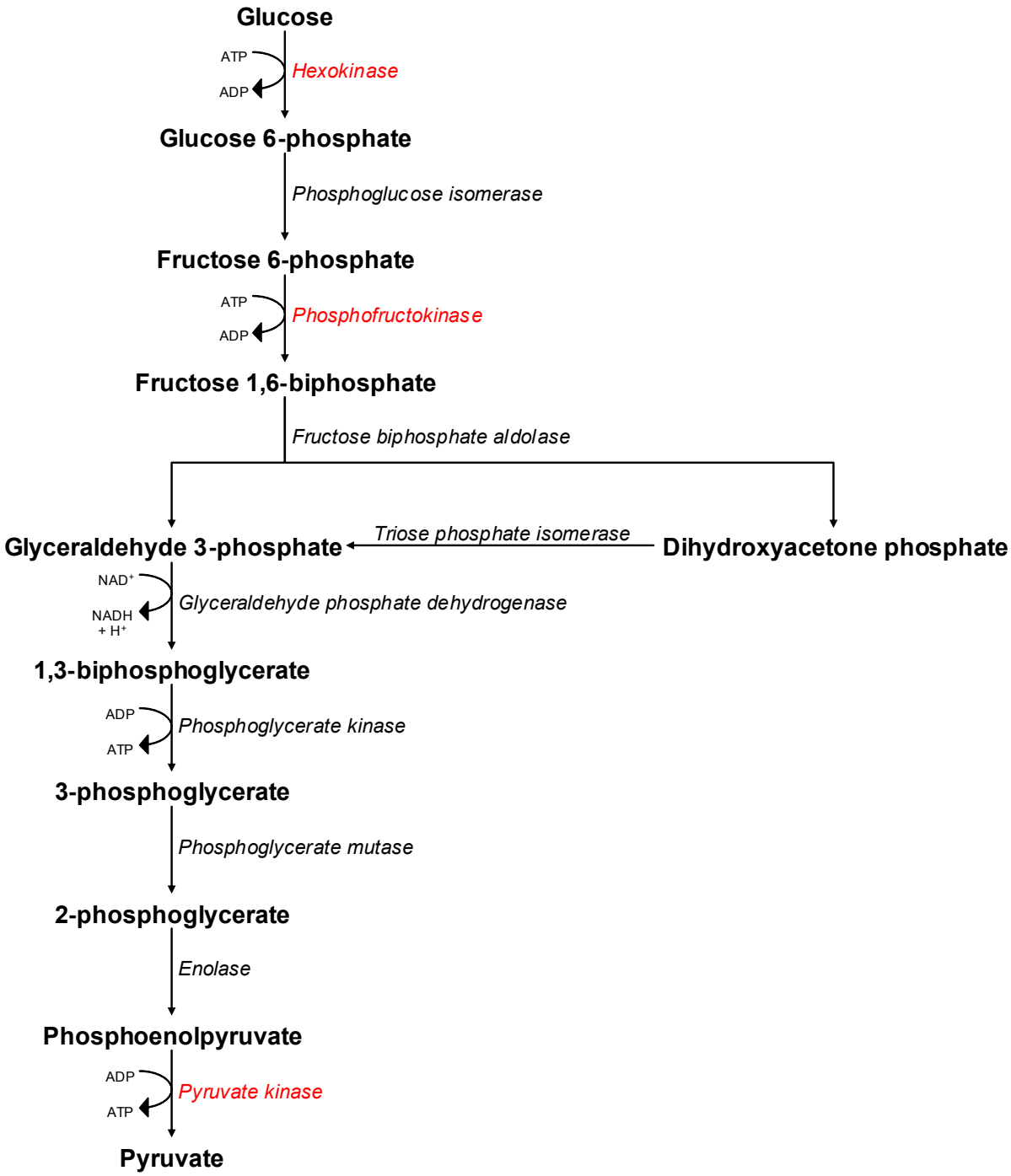


Figure 1.1. Glycolysis

Key regulator enzymes of the glycolytic pathway are highlighted in red.

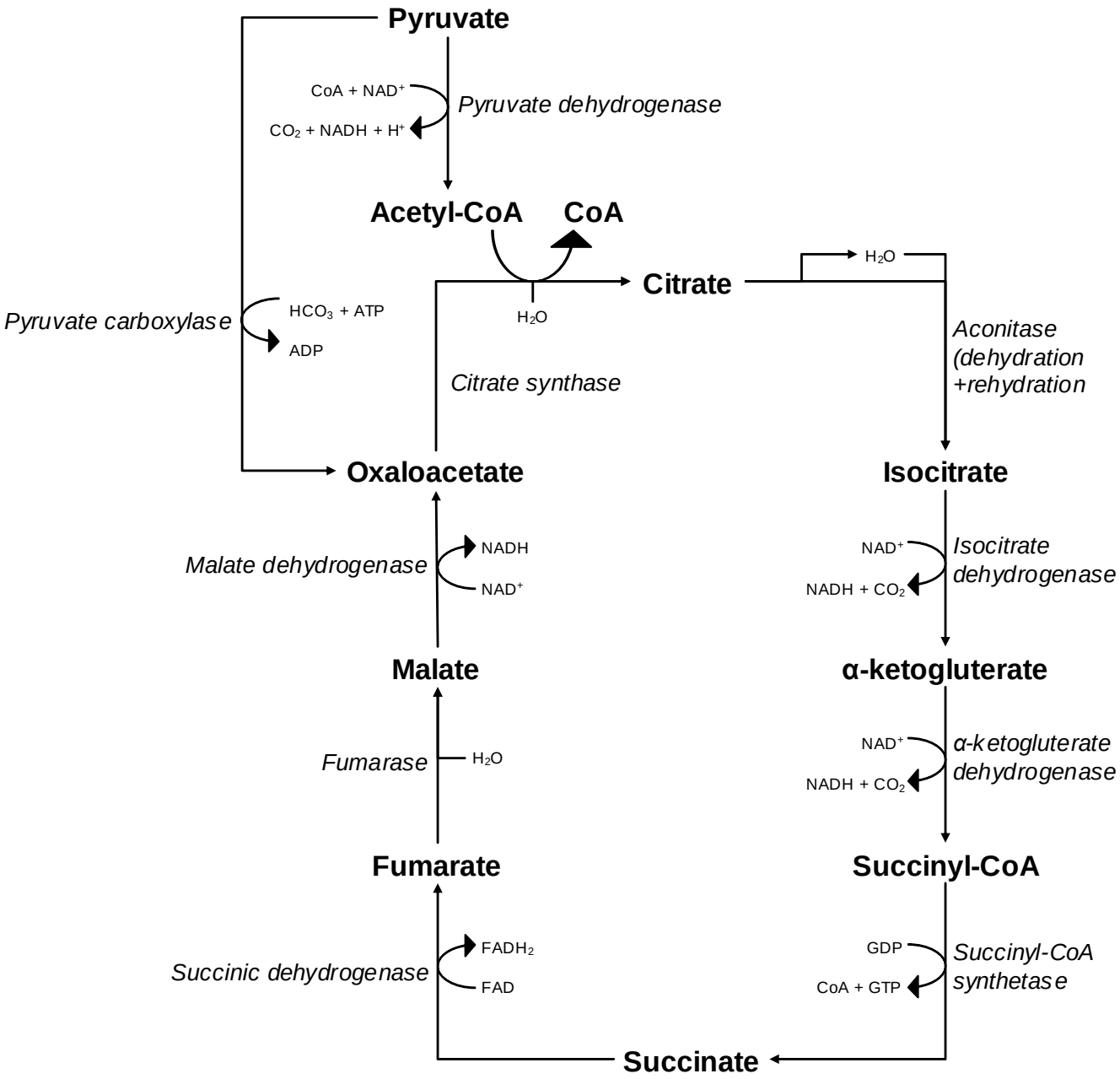


Figure 1.2. The tricarboxylic acid cycle

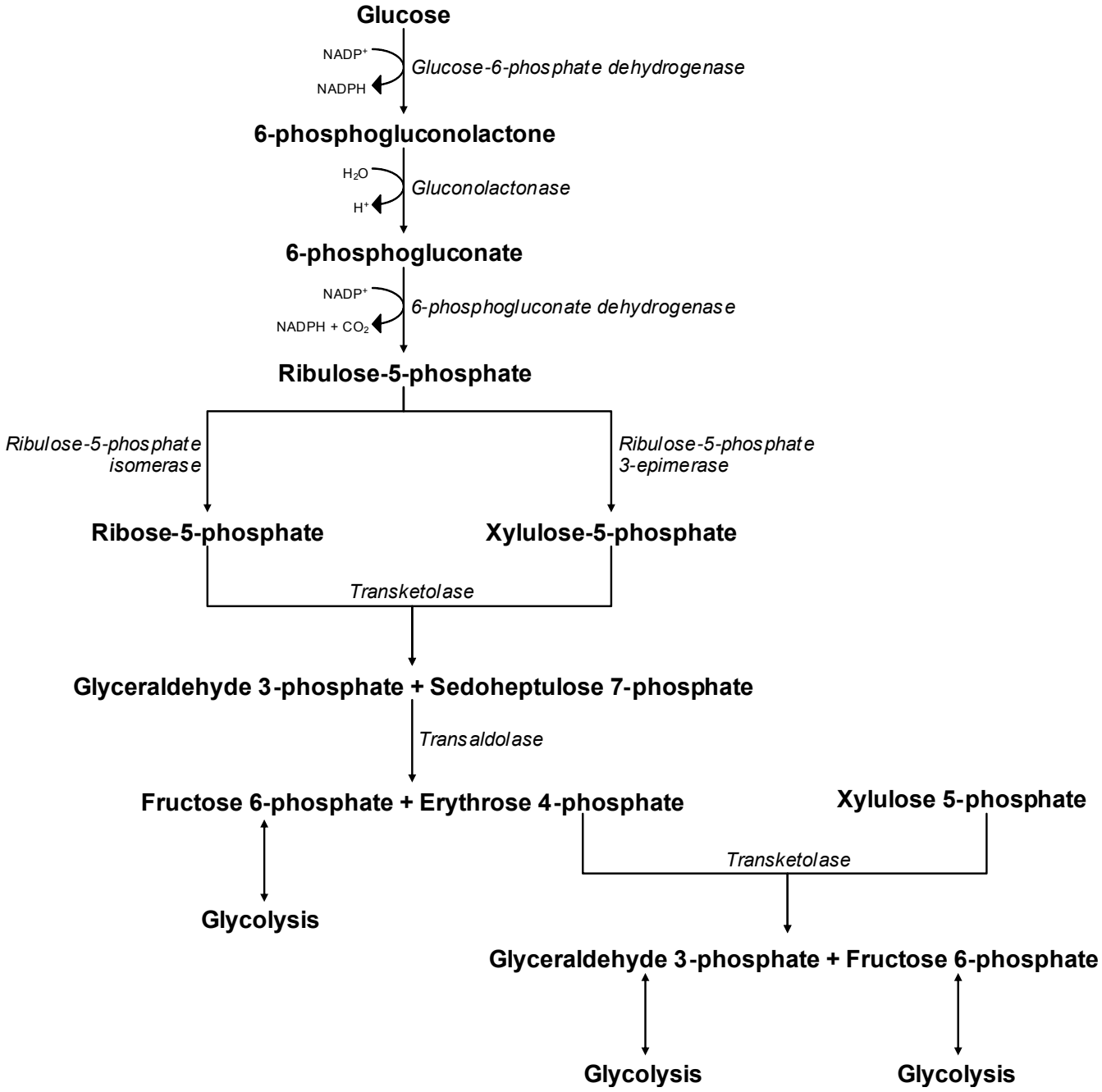


Figure 1.3. The pentose phosphate pathway

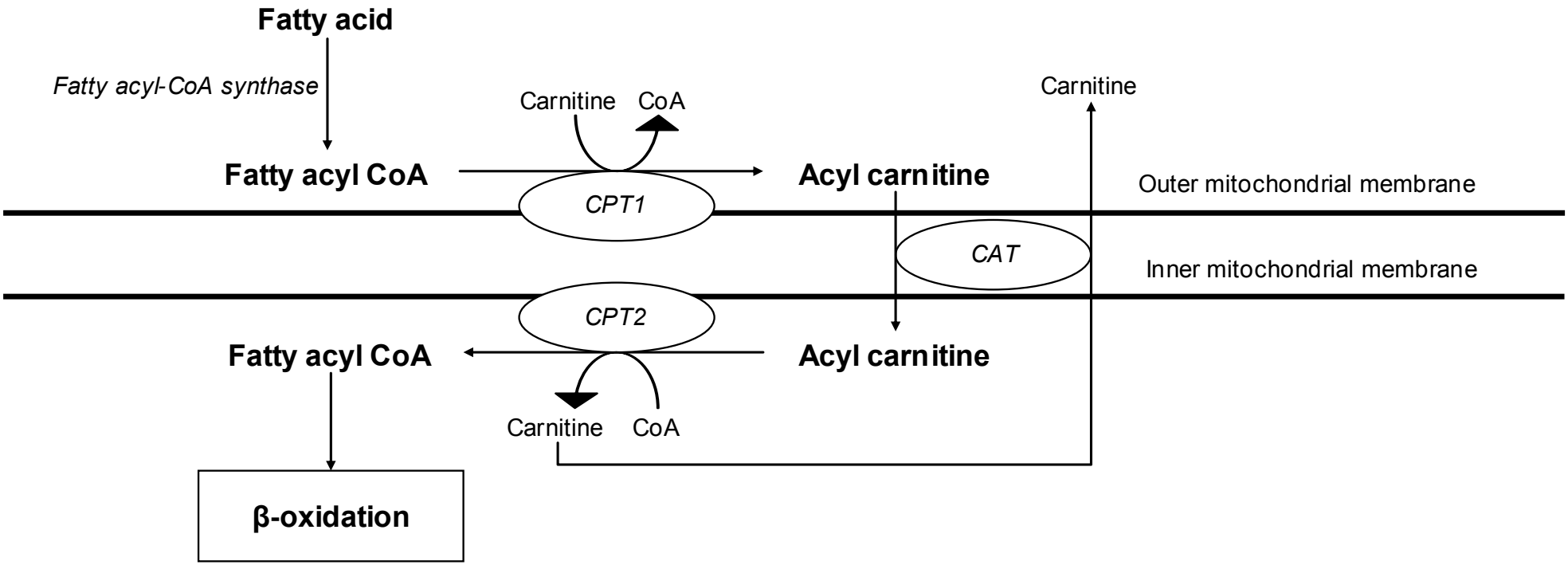


Figure 1.4. The carnitine shuttle

1.3.3. Energy substrates utilised by oocytes and embryos

1.3.3.1. Carbohydrates

There is still debate regarding the ability of oocytes to take up glucose directly (Downs et al. 1998; Clark et al. 2011), with evidence that cumulus cells are largely responsible for metabolism of exogenous glucose through glycolysis with provision of products to the oocyte via gap junctions (Biggers et al. 1967; Sugiura et al. 2005; Gilchrist et al. 2008; Sutton-McDowall et al. 2010; Clark et al. 2011; Wang et al. 2012). Cumulus cells also utilise glucose via the hexosamine biosynthesis pathway for production of hyaluron to support expansion of the cumulus matrix (Sutton et al. 2003b). Live fluorescent staining of glucose uptake in murine COCs has shown that glucose is taken up by cumulus cells via facilitative glucose transporters (GLUTs) and then transferred to the oocyte via gap junctions (Wang et al. 2012).

The oocyte itself appears to have a limited capacity for glucose utilisation (Biggers et al. 1967). The majority of glycolytic activity in the COC occurs within the cumulus cells, likely being utilised for cumulus cell functions such as cumulus expansion (Sutton-McDowall et al. 2004). Low levels of PFK activity are seen in denuded bovine oocytes, with a comparatively high level seen in the cumulus cells of the COCs (Cetica et al. 2002). Denuded oocytes also showed reduced glucose uptake compared to COCs in mice (Saito et al. 1994; Downs and Utecht 1999; Wang et al. 2012) and cattle (Zuelke and Brackett 1992). Further, the inhibition of GLUTs reduced glucose uptake in cumulus cells and oocytes of COCs, but had no effect on denuded oocytes (Wang et al. 2012), indicating differences in glucose transport pathways between CCs and oocytes. However, denuded porcine oocytes matured with 2.78mM glucose had greater nuclear maturation rates compared to those matured without glucose (Tsuzuki et al. 2008), indicating that glucose can still be utilised by denuded porcine oocytes to support nuclear maturation and this may be species specific.

Both glycolysis and the PPP appear to have pivotal roles in the resumption of meiosis (Krisher and Bavister 1998; Downs and Utecht 1999; Cetica et al. 2002;

Herrick et al. 2006; Wang et al. 2012); however, very high levels of glucose do not provide any additional benefits (Downs and Hudson 2000) and may even lead to decreased maturation rates (Downs and Mastropolo 1994; Kumar et al. 2012) in some species. Activity of both the glycolytic pathway and the PPP increase throughout the maturation period (Cetica et al. 2002), although it is unclear whether this is a cause or a consequence of progression of maturation.

The provision and concentration of glucose as an energy substrate has a major effect on the developmental capacity of porcine oocytes. The effect of glucose in maturation media is dose dependent. A low concentration of glucose (2mM) improved MII rates compared to oocytes matured without glucose (Tubman et al. 2005). The addition of 5.55mM glucose to maturation medium increased the proportion of porcine oocytes reaching MII compared to those matured in the absence of glucose (Funahashi et al. 2008; Tsuzuki et al. 2008; Wu et al. 2011). At glucose concentrations greater than 11mM, nuclear maturation rates began to decline (Tsuzuki et al. 2008). High glucose concentrations during IVM increase intracellular ROS formation and decrease intracellular glutathione levels, which leads to increased oxidative stress and impaired nuclear maturation (Hashimoto et al. 2000).

Glycolysis alone is insufficient to support proper maturation of porcine oocytes, with inhibition of the PPP reducing development to the MII stage (Herrick et al. 2006; Sato et al. 2007; Funahashi et al. 2008). Bovine oocytes have low activity levels of PFK and high activity levels of G6PDH (Cetica et al. 2002), indicating that the glucose is directed predominantly through the PPP in oocytes. Intermediaries of the PPP act as substrates for synthesis of purine nucleotides, which play a role in the regulation of meiosis (Downs and Mastropolo 1994; Downs et al. 1998; Downs and Hudson 2000; Herrick et al. 2006), as well as products required for maturation, such as NADPH (Funahashi et al. 2008). Supporting this, ATP content of porcine oocytes was similar regardless of the presence or absence of glucose (Tsuzuki et al. 2008), suggesting greater activity of the PPP than ATP production via glycolysis in this species.

Pyruvate is also considered a requirement for the resumption of meiosis and development of the cell (Gonzales-Figueroa and Gonzales-Molfino 2005), with a greater proportion of oocytes remaining at the GV stage in the absence of pyruvate (Funahashi et al. 2008). Mouse (Biggers et al. 1967) and cattle (Geshi et al. 2000) oocytes can successfully complete nuclear maturation in the presence of pyruvate alone. In porcine oocytes, the addition of pyruvate to a glucose-free medium improved maturation rates in a dose-dependent manner up to 5mM; however, MII rates were still significantly inferior when compared with media containing both glucose and pyruvate (Sato et al. 2007; Funahashi et al. 2008). This is likely because pyruvate supports energy production via the TCA cycle, allowing glucose to primarily be metabolised via the PPP at this stage.

In vivo matured oocytes have been shown to metabolise greater amounts of glucose compared to their in vitro matured counterparts (Spindler et al. 2000; Durkin et al. 2001; Krisher et al. 2007). In porcine oocytes, the most distinct metabolic difference is the activity of the PPP, being almost seven times greater in in vivo matured oocytes than those matured in vitro (Durkin et al. 2001; Krisher et al. 2007). Given the role that the PPP plays in meiotic resumption and the acquisition of developmental competence, this could potentially explain the different developmental outcomes observed in oocytes from the two sources. Glycolytic activity was also significantly lower in in vitro matured porcine oocytes (Durkin et al. 2001; Krisher et al. 2007), but by a smaller magnitude than the difference in PPP activity. In feline oocytes, the level of glucose oxidation and glycolytic activity during in vitro maturation correlated with embryo development following in vitro fertilisation (Spindler et al. 2000). This suggests that metabolism of glucose is a determining factor of oocyte quality, agreeing with observations of lower glucose metabolism and decreased developmental competence of in vitro oocytes compared to in vivo. Pyruvate utilisation is also decreased in in vitro matured porcine oocytes compared to their in vivo matured counterparts (Krisher et al. 2007). Given that pyruvate is the predominant energy substrate utilised throughout maturation, this may indicate improper and insufficient energy production at this time.

Energy production within mammalian embryos changes greatly throughout development. The predominant energy substrates utilised during the culture period are glucose, pyruvate, lactate and glutamine (Dumollard et al. 2009); however, the levels of utilisation of each substrate varies with the stage of embryo development and species.

During early cleavage stages, the embryo relies predominantly on pyruvate, lactate and glutamine metabolism for ATP production (Leese 1995; Gardner 1998). Lactate can be converted to pyruvate, and along with exogenous pyruvate, is metabolised via the TCA cycle for ATP production. Early embryos are capable of producing ATP by the direct oxidation of glutamine in the mitochondria up to the morula stage (Dumollard et al. 2009).

Prior to compaction there is limited glucose metabolism (Biggers et al. 1967; Flood and Wiebold 1988) despite the presence of glycolytic and PPP enzymes (Cetica et al. 2002). High glucose utilisation at the early embryo stage was shown to result in impaired energy production and poor developmental outcomes (Lane and Gardner 1996; Gardner 1998). These poor outcomes have been attributed to abnormal glucose metabolism in in vitro produced embryos (Krisher and Bavister 1998; Gardner et al. 2000b), with increased activity of the glycolytic pathway (Bavister 1995; Khurana and Niemann 2000). Temporary inhibition of oxidative phosphorylation at the compaction stage in bovine and porcine embryos was found to be beneficial to blastocyst development and quality (Machaty et al. 2000; Thompson et al. 2000); this coincides with low oxygen tension seen for in vivo embryos in the reproductive tract (Fischer and Bavister 1993).

High glucose concentrations in media during early cleavage stages can compromise development (Flood and Wiebold 1988; Sturmey and Leese 2003). The combination of glucose and pyruvate in culture media significantly inhibits porcine embryo development to the morula and blastocyst stages (Iwasaki et al. 1999; Park et al. 2005). Porcine embryos cultured in sequential media with glucose for days 3-7 only had greater blastocyst rates and increased cell numbers than those cultured in glucose for the entire period (Kikuchi et al. 2002b; Karja et al. 2006). Higher H_2O_2 levels seen in embryos cultured with glucose for the 7 d

culture period (Karja et al. 2006) may indicate an increased metabolic rate and subsequent rise in ROS production, leading to compromised embryo development and quality.

As the embryo reaches the blastocyst stage, ATP demand increases due to greater activity of the sodium pump (Leese 1991; Donnay et al. 1999) and increased protein synthesis (Thompson et al. 1998). This leads to a decreased ATP:ADP ratio (Flood and Wiebold 1988; Dumollard et al. 2009), removing inhibition of PFK and increasing the glycolytic rate. At this stage, energy production shifts as glucose and glutamine are utilised as the predominant substrates (Flood and Wiebold 1988; Dumollard et al. 2009), and as such, attempts to culture porcine embryos for the entire culture period in media with lower glucose concentrations have been unsuccessful (Machaty et al. 1998). As a percentage of total glucose metabolised, PPP activity is the lowest at the blastocyst stage (Flood and Wiebold 1988), accounting for the majority of glucose being shunted through the glycolytic pathway for energy production. Oxygen consumption also increases (Leese 1995; Sturmey and Leese 2003), indicative of an overall increase in metabolic rate and energy production. Following expansion of the blastocoel, oxygen consumption decreases as the sodium pump is then required only to maintain homeostasis (Sturmey and Leese 2003).

The difference in quality between in vitro and in vivo cultured embryos is well documented. In vitro produced embryos typically show delayed development, decreased cell number, altered morphology and metabolism, and produce poorer outcomes following transfer (reviewed in Thompson 1997).

The glycolytic pathway is preferentially utilised at all stages by in vivo porcine embryos and after the two-cell stage in in vitro produced embryos, and activity increases throughout development in both in vitro and in vivo derived embryos, albeit earlier in in vitro produced embryos (Swain et al. 2002). In vitro culture caused an increase in the glycolytic rate in freshly flushed in vivo derived cattle (Khurana and Niemann 2000) and mouse (Gardner and Leese 1990) embryos, with lactate production (indicative of glycolytic activity) significantly lower in in vivo bovine embryos than those cultured in vitro (Khurana and Niemann 2000).

However, in porcine embryos, glucose utilisation was shown to be higher in in vivo embryos compared to in vitro produced embryos (Swain et al. 2002). This may be due to analysis techniques, where Khurana and Niemann (2000) assessed lactate production as a measure of glycolytic activity, while Swain et al. (2002) measured glucose uptake, which may not have accounted for any glucose utilised via the PPP. Alternatively, this may be due to species differences in embryonic metabolism. One possible explanation for increased glycolysis in in vitro produced embryos is stress-induced inactivation of ATP-consuming pathways. There are many stressors on cells cultured in vitro, and in response to stress 5'AMP-activated protein kinase (AMPK) inactivates ATP-consuming pathways and activates ATP-regenerating pathways, including glucose transport and glycolysis (Leese 2002).

While IVC is known to compromise embryo development (Kikuchi et al. 1999), IVP embryos are already predisposed to a range of inborn problems due to reduced cell quality following IVM and IVF. This can make it difficult to determine whether any quality differences are due to embryo culture differences, or perturbations in metabolic processes intrinsic to the oocyte resulting from IVM.

1.3.3.2. Amino acids

Amino acids serve a variety of roles within biological systems, including serving as precursors for proteins and nucleotides, acting as osmolytes, signalling molecules and energy substrates, protecting against oxidative stress, and regulating pH. A range of amino acids are present in both oviductal and uterine fluids at a range of concentrations (Iritani et al. 1974).

Intact COCs are capable of utilising amino acids as an energy substrate during IVM. In cattle oocytes, addition of non-essential and essential amino acids during IVM increased subsequent cleavage, blastocyst development rates and cell number, and resulted in higher oocyte maternal mRNA levels compared with media without amino acid or serum supplementation (Watson et al. 2000). In porcine oocytes, use of a commercial amino acid mix (Gibco) as the sole energy substrate during IVM increased meiosis and glycolytic rates compared to media

with 0.01mM pyruvate and 6mM lactate alone, but was not able to support subsequent blastocyst development to the extent of complete media (Tubman et al. 2005).

In pigs, IVM has improved greatly with the addition of cysteine to maturation media. Cysteine availability is a limiting factor of GSH synthesis, a requirement for proper cytoplasmic maturation and male pronuclear formation. Addition of cysteine during porcine oocyte maturation increased GSH content of oocytes (Abeydeera et al. 1999) as well as improving blastocyst formation rates following IVF (Grupen et al. 1995; Abeydeera et al. 1999). Owing to the increased GSH rates with cysteine supplementation, it is likely that the beneficial effects are due to the antioxidant activity of GSH protecting the oocytes from ROS damage, with there being no effect of cysteine supplementation on the ATP content of porcine oocytes (Tsuzuki et al. 2009).

During porcine embryo in vitro culture, the addition of a commercial mix of essential amino acids (Sigma) increased blastocyst formation rates, with the addition of a non-essential amino acids mix (Sigma) increasing blastocyst cell number (Suzuki and Yoshioka 2006). The essential amino acid mix includes L-arginine, L-cystine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, L-valine, while the non-essential amino acid mix contains L-alanine, L-asparagine, L-aspartic acid, L-glycine, L-serine, L-proline and L-glutamic acid. In mice, amino acid supplementation has been shown to stimulate hatching rates and attachment and growth following transfer, as well as contributing to the alleviation of in vitro culture blocks (Gardner et al. 1996). Sequential addition of non-essential amino acids during early cleavage stages and essential amino acids for the remainder of culture increased blastocyst cell number of porcine embryos (Beebe et al. 2007; Hashem et al. 2007). Concentration of amino acids was also found to influence their efficacy, with 20µl/ml essential amino acid mix and 10µl/ml non-essential amino acid mix shown to increase the mean number of ICM cells, but not total cell number (Hashem et al. 2007). Addition of amino acids had the most beneficial effect when added at a low concentration (1:100 dilution of commercial mix) to porcine embryo culture medium (Beebe et al. 2009). Amino acids degrade to form

ammonia, and at high concentrations may cause a toxic accumulation of ammonia in the media.

Glutamine has been shown to play a prominent role in embryo development, both as an energy substrate and for its role in regulatory processes. Glutamine metabolism has been shown to be highest at the early blastocyst stage in porcine embryos (Humpherson et al. 2005). Increased glutamine consumption at this stage may be linked to glucose metabolism, which also produces intermediaries required for high rates of DNA and RNA synthesis at this stage. Glutamine can be converted to glutamate, then α-ketogluterate which can then enter the TCA cycle for ATP production. Specific supplementation with 0.25-1mM glutamine during embryo culture increased blastocyst development rates relative to media with no glutamine (14.9-24.0% vs 2.9%), with 2-4mM glutamine showing higher blastocyst rates again (25.3-30.9%; Suzuki and Yoshioka 2006). Addition of 2mM glutamine also increased blastocyst cell number (42.9 average cells per blastocyst vs 20.3 in the control; Suzuki and Yoshioka 2006). In vitro produced porcine embryos cultured with 1mM glutamine metabolised constant amounts through the TCA cycle, while glutamine uptake increased throughout development of in vivo derived embryos (Swain et al. 2002). This indicates that the glutamine concentration may have been too low in the IVC medium, supporting the finding that 2-4mM glutamine was optimal for blastocyst development (Suzuki and Yoshioka 2006). However, 1- and 2-cell in vivo-derived porcine embryos were shown to be capable of forming blastocysts when cultured in vitro with 1mM glutamine as the sole energy substrate (Petters et al. 1990), suggesting that amino acid metabolism may be impaired in in vitro produced embryos.

1.3.3.3. Fatty acids

Fatty acids are a form of dense energy storage. The complete oxidation of palmitate, for example, generates 106 molecules of ATP, compared to oxidation of glucose which yields approximately 30 ATP molecules. The role of fatty acids as an energy substrate during oocyte maturation is contentious. The availability of lipid reserves and levels of lipase activity would suggest that the oocyte is able to use lipids as oxidative substrates after it is separated from the CCs during

maturation, however results in this area are widely varied, making it difficult to gain a definitive answer.

Endogenous lipid plays an as yet undetermined role during oocyte maturation. As early as the mid-1970s it was recognised that oocytes likely had at least partial reliance on endogenous substrates for energy generation during maturation in vitro (Zeilmake and Verhamme 1974), now believed to be intracellular lipids stored as triglycerides in cytoplasmic lipid droplets. Triglyceride levels within the ooplasm have been shown to decrease over the course of the maturation period (Sturmey and Leese 2003; Romek et al. 2011b) with a concomitant increase in oxygen consumption being correlated to triglyceride depletion (Sturmey and Leese 2003). A similar trend has been reported in cattle oocytes (Ferguson and Leese 1999). However, the upregulation of β-oxidation during maturation of porcine oocytes has shown varied results, with some studies reporting improved rates of nuclear maturation and blastocyst development (Somfai et al. 2011; Wu et al. 2011), and others reporting no effect (You et al. 2012). Although the precise reasons for these differences are unknown, it may be due to differences in the composition of media used. Inhibition of β-oxidation during porcine oocyte maturation impairs nuclear maturation and prevents cleavage and blastocyst development (Sturmey et al. 2006). The results of chemical alteration of fatty acid oxidation implies that this pathway plays an important role during oocyte maturation in a range of species, including mice (Dunning et al. 2010a; Dunning et al. 2011) and cattle (Ferguson and Leese 2006; Yamada et al. 2006).

Changes to lipid droplet size, arrangement and density, during in vitro maturation gives further evidence of a metabolic role for endogenous fatty acids. Oocytes treated with olocmoucine, an inhibitor of nuclear maturation, showed no reduction in the size of lipid droplets, whereas untreated oocytes displayed a reduction in size of large lipid droplets, which was correlated with meiotic progression (Niimura et al. 2002). The triglyceride, phospholipid and cholesterol content of lipid droplets have also been shown to decrease over the course of in vitro maturation, as determined by fluorescent staining (Romek et al. 2011b).

Oocytes of some species appear to be capable of developing in the absence of exogenous energy substrates. Bovine oocytes held in media without exogenous substrates showed no difference in oxygen consumption to those in media with energy substrates present (Ferguson and Leese 2006); however, when cultured in media lacking energy substrates with the addition of an inhibitor of fatty acid oxidation, oxygen consumption was significantly lower than those cultured in the absence of the inhibitor. Further, the activity of lipase enzymes in bovine oocytes was higher than that of either G6DPH or PFK (Cetica et al. 2002), indicating a greater rate of lipolysis than either PPP or glycolysis. Comparatively, murine oocytes are known to have a very small intracellular triglyceride complement compared with cattle oocytes (Loewenstein and Cohen 1964; McEvoy et al. 2000), and had significantly reduced maturation rates when cultured without exogenous substrates (Downs and Hudson 2000). Porcine oocytes are known to contain large amounts of triglyceride reserves (McEvoy et al. 2000), suggesting that, similarly to cattle oocytes, they are capable of utilising these endogenous reserves for energy production. When matured without glucose or pyruvate, the rate of progression to MII in porcine oocytes was significantly increased by the presence of a β-oxidation stimulant (Wu et al. 2011).

It has been suggested that exogenous fatty acids do not play a role in energy production. The addition of a commercial fatty acid solution to medium had no effect on maturation rates (Tubman et al. 2005). However, the fatty acid composition of this solution was not given, and potentially the fatty acids supplemented were not preferentially utilised by porcine oocytes.

In vitro maturation itself is known to alter metabolic processes. In vitro maturation of mouse COCs led to dysregulation of gene transcripts associated with lipid metabolism (Yuan et al. 2011) and reduced the rate of fatty acid oxidation by 2.8-fold (Dunning et al. 2014) compared to in vivo maturation. Furthermore, inhibition of lipid metabolism during IVM decreased the abundance of gene transcripts involved in fatty acid oxidation, glycolysis and oxidative stress in porcine oocytes (Paczkowski et al. 2013). In vitro matured cattle oocytes also showed significantly down-regulated expression of genes related to lipid metabolism, including Chol acyl-transferase (ACAT1) and fatty acid synthase (FASN; Gonzalez-Serrano et al.

2013), compared to in vivo matured oocytes. Interestingly, these genes were over-expressed in in vitro produced cattle blastocysts compared with in vivo sourced blastocysts, possibly accounting for the increased lipid seen in in vitro blastocysts.

There is a large body of evidence supporting a role for endogenous lipids in energy production throughout in vitro embryo culture. The embryos of numerous species have been reported to be capable of oxidising fatty acids for the production of energy during early cleavage stages, including rabbit (Khandoker and Tsujii 1998), mouse (Hillman and Flynn 1980) and cattle (Ferguson and Leese 1999, 2006). Embryos of some species have been shown to be capable of developing in the absence of exogenous energy substrates, suggesting reliance on endogenous reserves for ATP production. When cultured without exogenous energy substrates, almost half of IVP cattle zygotes underwent at least one cleavage division (Ferguson and Leese 1999). Further, oxygen consumption by 5- to 8-cell cattle embryos was similar whether cultured in the presence or absence of exogenous nutrients (Ferguson and Leese 2006), implying continued oxidation and metabolism of energy substrates likely from endogenous sources. Developmental rates decreased with the addition of a β-oxidation inhibitor (Ferguson and Leese 2006). In contrast, mouse zygotes were reported to arrest and degenerate early in culture when deprived of exogenous energy substrates (Manser and Leese unpublished, cited in Ferguson and Leese 2006). This difference may be due to cattle embryos containing a far greater amount of endogenous lipid than mouse embryos.

In porcine embryos, the success of culture using endogenous substrates alone appears to depend on upregulation of the β-oxidation pathway. When IVF zygotes were cultured in defined medium excluding exogenous energy substrates, cleavage rates were reduced and blastocyst formation was prevented, suggesting that lipids alone may be insufficient to support viable embryo development (Park et al. 2005). However, these developmental failures may be attributable to an inability to completely oxidise the products of β-oxidation for the full energy allowance. When porcine embryos were cultured in a defined media containing oxaloacetate in the absence of carbohydrates, blastocyst rates were similar to those of embryos cultured in media containing glucose; further, there was a

significant decrease in blastocyst triglyceride content (Park et al. 2005; Sturmey and Leese 2008). Oxaloacetate alone cannot generate ATP, but primes the TCA cycle to allow for complete oxidation of metabolic products; this further supports a metabolic role for triglyceride during embryo development.

Inhibition of β-oxidation during embryo culture causes developmental arrest in numerous species (Ferguson and Leese 2006; Sturmey and Leese 2008; Dunning et al. 2010a). Culture of porcine zygotes with a β-oxidation inhibitor led to increased glucose consumption and higher lactate production (Sturmey and Leese 2008), indicative of increased glycolytic rate and suggesting that other pathways can be upregulated to compensate for an inability to utilise lipids as an energy substrate. However, no embryos developed beyond the 1-cell stage when cultured with the inhibitor (Sturmey and Leese 2008), showing the importance of the β-oxidation pathway to embryo development.

There have been varying results regarding changes in oocyte and embryo fatty acid content during culture. Analysis of lipid content via the fluorescent probe Nile Red found that while fatty acid content remained constant from the zygote to morula stage, it decreased at the blastocyst stage and again at hatching (Romek et al. 2009; Romek et al. 2011b). Formation of the blastocoel and blastocyst hatching are two stages of development which are energetically expensive; thus decreased fatty acid content at these stages is consistent with a metabolic role. In contrast, an earlier study found that the triglyceride content of porcine embryos did not differ throughout embryo development, as measured via an assay based on the enzymatic hydrolysis of triglyceride, although there was considerable variation between individual embryos (Sturmey and Leese 2003). However, this does not preclude the use of fatty acids during embryo culture. Due to the energy-dense nature of lipid, only very small amounts would be required to produce large amounts of energy; potentially, significant differences in the volumes of lipid utilised may be below the limits of detection using the reported methods. The specific nature of the assay, only accounting for triglyceride degradation, may also account for discrepancies between studies, if other fatty acids are also utilised during embryo development.

1.4. Sources of energy substrates

1.4.1. In vivo systems

Between species, the concentrations of fatty acids within reproductive tract fluids differ, but the fatty acid composition is similar (Khandoker et al. 1997). This indicates that oocytes and embryos of different species have similar exogenous fatty acid requirements for maturation, fertilisation and development, and that the type of fatty acid is more important than the concentration present for oocyte maturation and embryo culture.

Oocyte and embryo composition changes depending on culture media, as substrate uptake and incorporation varies with nutrient availability (Aardema et al. 2011). Understanding the composition of reproductive tract fluid may help predict substrate utilisation by embryos in vivo and the effect on development.

1.4.1.1. Follicular fluid

Follicular fluid accumulates within antral follicles during the process of folliculogenesis. It is known to be composed of a variety of substances, including proteins, amino acids, sugars, enzymes, lipids, hormones (LH, FSH, prolactin, oestrogens, androgens and progestagens), and salts (Huang et al. 2002). Primary follicular fluid is secreted by granulosa cells during folliculogenesis, while secondary follicular fluid mainly transudes from the blood plasma and accumulates as the follicle matures (Huang et al. 2002). Due to the porous nature of the follicular epithelial cells, the fatty acid and protein profile of follicular fluid found in larger follicles generally reflects that of blood serum (Huang et al. 2002; Sutton et al. 2003a; Aardema et al. 2011); further, maternal nutrition can affect the substrate concentrations of the follicular fluid (Warzych et al. 2011).

The composition of follicular fluid is important owing to its role in oocyte maturation and development. As the arrested oocyte and surrounding granulosa cells are supported by follicular fluid, it plays a major role in the biochemical and metabolic processes during this time, being responsible for the provision of energy

substrates and nutrients, regulation of meiotic arrest and the resumption of meiosis (Hunter 2000).

Follicular glucose concentrations are positively correlated with ovarian follicle size in several species, including pigs, cattle, buffalo and sheep (Brad et al. 2003b; Leroy et al. 2004; Nandi et al. 2008; Sutton-McDowall et al. 2010; Ying et al. 2011; Bertoldo et al. 2013). Reports of concentrations of glucose in porcine follicular fluid vary, with 2.1-4.8mM reported in 3-6mm follicles up to the pre-ovulatory stage (Chang et al. 1976) but as low as 0.8mM being found in follicular fluid from small follicles and 1.0mM in large follicles (Bertoldo et al. 2013). Lactate concentrations also differed between follicular fluid from small (3-4mm) and large (5-8mm) follicles, reported at 12mM and 10mM respectively (Bertoldo et al. 2013). Pyruvate concentrations in follicular fluid were similar between small and large follicles at 0.01-0.02mM (Brad et al. 2003b).

Although it has been noted that there are no differences in total lipid concentrations between follicular fluid sourced from small, medium or large follicles (Chang et al. 1976; Huang et al. 2002), there is a general tendency observed for concentrations of triglycerides, phospholipids and total cholesterol to decrease as follicular maturation progresses (Chang et al. 1976). Transient changes in lipid concentration were observed following PMSG injections, with increased lipid concentrations consistent with steroidogenesis during growth of early follicles, and decreasing concentrations in larger follicles correlating with follicular degeneration (Huang et al. 2002).

There is a negative correlation between total concentrations of free fatty acids in follicular fluid and follicle size (Yao et al. 1980). The predominant fatty acids detected in follicular fluid were oleic (18:1), palmitic (16:0) and stearic (18:0) acids (Yao et al. 1980; Khandoker et al. 1997). Linolenic (18:3) and stearic acids as well as total polyunsaturated fatty acid (PUFA) concentrations were positively correlated with follicle size, while concentrations of palmitic and oleic acids decreased with increasing follicle size (Yao et al. 1980). Further, addition of follicular fluid derived from large follicles (5-8mm diameter) to media during porcine oocyte maturation had a greater beneficial effect on developmental

competence than follicular fluid from small follicles (2-4mm diameter; Algriany et al. 2004; Ito et al. 2008). Follicular fluid derived from sow ovaries was also more beneficial than follicular fluid from prepubertal gilts (Grupen et al. 2003). Specific fatty acids may play a role in the resumption and completion of meiosis, given the changes in concentrations with follicle size. Further, supplementation of maturation media with palmitic or stearic acid had detrimental effects on the developmental competence of bovine oocytes, while oleic acid improved blastocyst development, increased lipid storage, and could counteract the adverse effects of saturated fatty acids (Aardema et al. 2011). Supplementation of media with specific exogenous fatty acids to support maturation should be further examined.

1.4.1.2. Oviductal fluid

Oviductal fluid is secreted by the epithelial cells of the oviduct, and is the in vivo medium for fertilisation and early embryo development. Greater quantities of oviductal fluid are present around the time of ovulation and fertilisation, and declines as the embryo leaves the oviduct and enters the uterus (Iritani et al. 1974; Leese 1995).

Oviductal fluid contains numerous energy substrates, including glucose, lactate, pyruvate and fatty acids. Concentrations of glucose decrease following ovulation, from around 1.65mM to 0.43mM in unmated pigs (Nichol et al. 1992), but with no differences in glucose depending on the presence or absence of an embryo (Nichol et al. 1998). Pyruvate concentrations remained similar throughout pre- and post-ovulatory phases (Nichol et al. 1992; Nichol et al. 1998), while lactate concentrations were higher in oviductal fluid of mated animals compared with pre-ovulatory levels (6.83mM vs 3.86mM; Nichol et al. 1992) independent of whether an embryo was present or not (Nichol et al. 1998). Similarities in glucose, pyruvate and lactate concentrations between oviductal fluid from ovariectomised animals and those with embryos present (Nichol et al. 1998) suggest systemic regulations of concentrations rather than local effects of embryos. However, the changing concentrations do reflect a dynamic microenvironment for fertilisation and early embryo development in vivo.

The total lipid content of oviductal fluids during the oestrous period was shown to be 43.7±7.6mg/100ml (Iritani et al. 1974). The three main fatty acids found within oviductal secretions were oleic acid (36.24% of total fatty acids), palmitic acid (21.09%), and stearic acid (17.30%; Khandoker et al. 1997), following similar compositional trends for fatty acid content of uterine fluid, follicular fluid and oocytes. The predominant polyunsaturated fatty acids in oviductal fluid were linoleic acid, linolenic acid and arachidonic acid, present at 3.48%, 9.07% and 10.82% respectively (Khandoker et al. 1997). There is no known data available regarding changes in lipid and fatty acid concentrations of porcine oviductal fluid during pre- and post-ovulatory periods.

1.4.1.3. Uterine fluid

The uterine environment is important as the site of embryo development. The pre-implantation embryo relies upon substrates within the uterine tract fluid to support growth, with glucose, pyruvate, lactate, amino acids and free fatty acids present.

In pig uterine fluid, there was no significant variation in glucose concentrations recorded with stage of oestrus, from 18.4mg/100ml at diestrus to 27.5mg/100ml at oestrus (Iritani et al. 1974). During the oestrous period, total lipid content of uterine fluid was four-fold higher than that of oviductal fluid, with 177.7mg/100ml (Iritani et al. 1974). Oleic acid was the most common fatty acid, accounting for 34.91% of total fatty acids, followed by palmitic acid (23.79%) and stearic acid (14.03%) (Khandoker et al. 1997). The three predominant PUFAs were linoleic acid (4.38%), linolenic (9.85%) and arachidonic acid (8.61%; Yao et al. 1980; Khandoker et al. 1997). High concentrations of oleic and palmitic acids may serve as a storage pool of metabolic precursors, and as substrates for fatty acid elongation and desaturation in embryo development (Khandoker et al. 1997). The addition of specific fatty acids to embryo culture media has been shown to affect development. Supplementation of culture media with linoleic acid increased cleavage and blastocyst development of bovine embryos (Miyashita et al. 2012), while linolenic decreased blastocyst development and altered gene expression (Al Darwich et al. 2010). Given that in vivo embryos are exposed to these fatty acids

while in the tract, they are likely to play a role in embryo development and supplementation of media should be further examined.

1.4.2. In vitro systems

Media for in vitro applications typically contain salts with one or more energy substrates provided, often in the form of glucose, lactate or pyruvate. The majority of the literature available on oocyte and embryo metabolism has predominantly focused on carbohydrate metabolism; as such, most current IVP media formulations, including those used for porcine embryos, were developed based on requirements for glucose, pyruvate and lactate as energy substrates throughout maturation and culture periods.

In standard protocols, embryos may be exposed to three discrete media during in vitro development, with a different medium used for each phase of IVP – ie. IVM, IVF and IVC. Effects on developmental potential as the embryo attempts to adjust to changes in osmolarity, pH and substrate availability indicates there are potential benefits to having a single base medium supplemented as appropriate for each stage (Yoshioka et al. 2008). Bovine oocytes can be matured, fertilised and cultured in a single basic medium appropriately supplemented for specific stages of development (Gandhi et al. 2000).

1.4.2.1. IVM medium

See Table 1.1 for common porcine IVM media formulations.

In vitro maturation medium was originally developed from medium designed for somatic cell or tissue culture. Maturation media are typically supplemented with follicular fluid or serum, as well as gonadotrophins and hormones. Following development of the North Carolina State University (NCSU) series of media in the 1990s (Petters and Wells 1993), these became the favoured media for porcine IVM. Subsequently, porcine oocyte medium (POM; Yoshioka et al. 2008) was developed as a fully defined medium modelled on the composition of oviductal fluid. Oocytes matured in POM with PVA had greater rates of normal penetration

and similar blastocyst rates to those matured in NCSU-37 supplemented with follicular fluid, but cell number was reduced, indicating a poorer quality of these blastocysts (Yoshioka et al. 2002).

Energy substrates supplied to oocytes in IVM media are generally glucose, pyruvate, lactate and amino acids, these having been identified as important metabolites for full embryo developmental potential. As events during oocyte maturation impact on early stages of embryo development, ensuring an appropriate and adequate supply of metabolic substrates to the COC during IVM is essential to support subsequent embryo development. Despite evidence of free fatty acids being present in oviductal fluid, none of these media supply a defined exogenous fatty acid source during IVM. Although follicular fluid and serum supplementation can provide fatty acids, these are undefined substances and variation between batches is unavoidable.

1.4.2.2. IVF medium

See Table 1.2 for common porcine IVF media formulations.

In vitro fertilisation medium has to meet the requirements for both oocytes and sperm at the time of fertilisation. The composition of IVF medium can affect a range of parameters including the acrosome reaction, sperm penetration, polyspermy and male pronucleus formation, as well as zona hardening and subsequent blastocyst formation (Coy et al. 2002). Commonly used media in pig IVF are tissue culture medium (TCM-199; Cheng 1987; Mattioli et al. 1989; Coy et al. 1999); Tyrode's albumin lactate pyruvate medium (TALP; Bavister 1989; Yoshida et al. 1993); and Tris-buffered medium (TBM; Abeydeera and Day 1997; Abeydeera et al. 1999).

It has been suggested that poor outcomes of in vitro produced porcine embryos has to do with the IVF procedure itself (Coy et al. 1999). While the three common IVF media used have resulted in the birth of piglets following embryo transfer (Cheng 1987; Mattioli et al. 1989; Yoshida et al. 1993; Abeydeera et al. 1998c; Rath et al. 1999), none of these media are considered to be optimal given the

current low efficiency of porcine IVF. Media formulations have been shown to affect the rates of polyspermic fertilisation, a common problem in pig IVF systems, as well as other factors including the acrosome reaction, cortical reaction and zona pellucida hardening, which all influence successful fertilisation (Coy et al. 2002). Further research is required to optimise the fertilisation conditions and ensure a high IVF efficiency is consistently achieved.

1.4.2.3. IVC medium

See Table 1.3 for common porcine IVC media formulations.

The culture medium has a substantial impact upon the success of in vitro embryo production. These include factors such as pH and osmolarity, as well as the composition of the medium. Energy substrate preferences change throughout maturation and culture, and media must be able to meet the needs of each stage of development to effectively support growth. There is evidence that the composition of culture medium influences not only substrate uptake, but also the metabolic pathways utilising these substrates in porcine oocytes and embryos at different stages of development (Gandhi et al. 2001).

The formulation of in vitro culture medium has a significant impact upon development, with porcine embryos held in culture medium for up to seven days. Early media was developed from somatic cell culture systems, but pre-implantation embryos cultured in vitro arrested at the four-cell stage. Development of semi-defined embryo-specific media based on the composition of oviductal fluid were beneficial for in vitro culture. NCSU-23 (Petters and Reed 1991; Reed et al. 1992; Petters and Wells 1993) was a commonly used medium, and was shown to consistently support higher blastocyst development rates in porcine embryos than other media available at the time. Several studies have shown that PZM is superior to other media, including NCSU media (Yoshioka et al. 2002; Im et al. 2004; Wang et al. 2009). The development of PZM-4 medium, with PVA replacing BSA, heralded the first report of live births from porcine embryos cultured in vitro in a fully defined media system (Yoshioka et al. 2002).

Sequential media systems were developed to adapt to the changing substrate preference of embryos. These systems involve culture for the first 48 to 72 hours in one medium before moving embryos into a different medium formulation, often with altered concentrations of glucose, pyruvate and lactate (Kikuchi et al. 2002b; Karja et al. 2004; Beebe et al. 2007). Sequential media systems were shown to have a beneficial effect on blastocyst development and quality, likely due to the metabolic support more closely resembling that of the in vivo environment.

Table 1.1. Formulations of media commonly used for porcine oocyte in vitro maturation. TCM-199 = Tissue Culture Medium 199; NCSU-23 = North Carolina State University medium 23; POM = Porcine Oocyte Medium.

Component (mM)	TCM-199[a]	NCSU-23[b]	POM[c]
NaCl	116.36	108.73	108.00
KCl	5.36	4.78	10.00
KH_2PO_4	-	1.19	0.35
$MgSO_4$	0.81	1.19	0.40
$NaHCO_3$	26.19	25.07	25.00
$CaCl_2$	1.80	1.70	-
Glucose	5.55	5.55	4.00
Sodium pyruvate	0.91	-	0.20
Calcium $(lactate)_2$	-	-	2.00
Glutamine	0.68	1.00	2.00
Taurine	-	7.00	-
Hypotaurine	-	5.00	5.00
Cysteine (µM)	0.57	-	600.00
BSA (mg/ml)	1.00	4.00	3.00

[a]TCM-199 (Gibco, catalogue number 11150), also contains a complex mixture of amino acids and vitamins

[b]Basic formulation (Petters and Wells 1993)

[c]Also contains commercial mixtures of essential and non-essential amino acids (Yoshioka et al. 2008)

Table 1.2. Formulations of media commonly used for porcine in vitro fertilisation. TCM-199 = Tissue Culture Medium 199; TALP = Tyrode's albumin lactate pyruvate; TBM = Tris-buffered medium; PGMtac4 = Porcine Gamete Medium, theophylline adenosine cysteine; Pig-FM = Pig fertilisation medium.

Component (mM)	TCM-199[a]	TALP[b]	TBM[c]	PGMtac4[d]	Pig-FM[e]
NaCl	116.36	114.00	113.10	108.00	90.00
KCl	5.36	3.16	3.00	10.00	12.00
KH2PO4	-	-	-	0.35	-
$MgSO_4$	0.81	0.50	-	0.40	0.50
$NaHCO_3$	26.19	25.00	-	25.00	25.00
HEPES	-	-	-	-	10.00
$CaCl_2$	1.80	2.00	7.50	-	8.00
NaH_2PO_4	1.01	0.35	-	-	0.50
Glucose	5.55	5.00	11.00	4.00	-
Sodium pyruvate	0.91	0.10	5.00	0.20	2.00
Calcium $(lactate)_2$	8.75	3.00	-	4.00	-
Sodium lactate	-	10.00		-	10.00
Glutamine	0.68	-	-	-	-
Caffeine	3.60	2.00	1.00	-	2.00
Tris	-	-	20.00	-	-
Cysteine (µM)	0.57	-	0.57	0.25	-
Adenosine (µM)	-	-	-	1.00	-
Theophylline	-	-	-	2.50	-
Phenol red (mg/ml)	0.02	0.01	-	-	-
BSA (mg/ml)	1.00	3.00	2.00	-	5.00
PVA (mg/ml)	-	1.00	-	3.00	-

[a]TCM-199 (Gibco, catalogue number 11150), also contains a complex mixture of amino acids and vitamins

[b]Bavister 1989

[c]Abeydeera and Day 1997

[d]Yoshioka et al. 2003

[e]Suzuki et al. 2000.

Table 1.3. Formulations of media commonly used for porcine embryo in vitro culture. NCSU = North Carolina State University; PZM = Porcine Zygote Medium.

Component (mM)	NCSU-23[a]	NCSU-37[a]	PZM-3[b]	PZM-4[b]	NCSU-23 sequential media[c]		NCSU-37 sequential media[d]	
					Media 1	Media 2	Media 1	Media 2
NaCl	108.73	108.73	108.00	108.00	108.73	108.73	108.73	108.73
KCl	4.78	4.78	10.00	10.00	4.78	4.78	4.78	4.78
KH_2PO_4	1.19	1.19	0.35	0.35	1.19	1.19	1.19	1.19
$MgSO_4$	1.19	1.19	0.40	0.40	1.19	1.19	1.19	1.19
$NaHCO_3$	25.07	25.07	25.07	25.07	25.07	25.07	25.07	25.07
$CaCl_2$	1.70	1.71	-	-	1.70	1.70	1.71	1.71
Glucose	5.55	5.55	-	-	-	5.55	-	5.55
Sodium pyruvate	-	-	0.20	0.20	0.20	-	0.17	-
Calcium $(lactate)_2$	-	-	2.00	2.00	5.70	-	-	-
Sodium lactate					-	-	2.73	-
Glutamine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Taurine	7.00	-	-	-	7.00	7.00	-	-
Hypotaurine	5.00	-	5.00	5.00	5.00	5.00	-	-
Sorbitol	-	12.00	-	-	-	-	12.00	12.00
BME amino acids (ml/l)	-	-	20.00	20.00	-	-	-	-
MEM non-essential amino acids (ml/l)	-	-	10.00	10.00	-	-	-	-
PVA (mg/ml)	-	-	-	3.00	-	-	-	-
BSA (mg/ml)	4.00	4.00	3.00	-	4.00	4.00	4.00	4.00

[a]Petters and Wells 1993

[b]Yoshioka et al. 2002

[c]Beebe et al. 2007

[d]Kikuchi et al. 2002, Karja et al. 2004

1.5. Lipid studies

'Lipid' is a general term referring to a broad range of substances, loosely defining any molecules that are insoluble in water and soluble in organic solvents. There is a wide range of lipid species, including glycerolipids, glycerophospholipids, sphingolipids, sterols, prenols, saccharolipids and polyketides. Due to their diverse nature, lipids fulfil a wide range of roles within biological systems. These include their main metabolic roles in energy storage and the formation of biological membranes, as well as having roles in cell signalling and the retention of fat-soluble vitamins and steroid hormones.

1.5.1. Endogenous lipids

One of the major functions of lipids is energy storage. Excess energy, from increased feed intake or decreased energy requirements, is stored as triglycerides, which are then broken down and metabolised at times when energy supplies are limited or during a spike in energy consumption. Metabolism of triglycerides for energy production yields a greater amount of energy per molecule compared with carbohydrate metabolism.

The majority of fatty acids within porcine oocytes are stored as triglycerides within cytoplasmic lipid droplets, with an average of 74ng present within the oocyte, accounting for 46% of the total lipid fraction (McEvoy et al. 2000). A later study reported the triglyceride content as almost double this, at 135ng in immature oocytes and 122ng following IVM (Sturmey and Leese 2003). Differences in analysis methods may have affected results, with one study using gas chromatography (McEvoy et al. 2000) compared with an assay based on enzymatic hydrolysis of triglycerides (Sturmey and Leese 2003). Triglycerides are comprised of a glycerol backbone with three fatty acid side chains, with properties varying depending on the identity of the fatty acids. The predominant fatty acids found in immature porcine oocytes are palmitic acid (34.9% of total fatty acids), oleic acid (21.7%) and stearic acid (14.4%; McEvoy et al. 2000). Bovine oocytes with higher developmental competency were found to contain oleic, linoleic and arachnidonic acids in higher proportions (Kim et al. 2001). The exact role these

specific fatty acids play during oocyte maturation is not fully understood, and the effect of exogenous supplementation is unknown.

Unsaturated fatty acids are more readily stored as triglycerides compared with saturated fatty acids (Listenberger et al. 2003), and can assist in improving the incorporation of saturated fatty acids to triglyceride stores. Increased intracellular lipid content in porcine oocytes may be due to higher levels of unsaturated fatty acids within oviductal fluid, with oleic acid comprising 36% of total free fatty acids compared to 22% in cattle oviductal fluid (Khandoker et al. 1997).

Evolution of the lipid component of oocytes begins within the primordial follicle. Oocytes at this stage have numerous dark, round lipid droplets within the cytoplasm (Silva et al. 2011). While lipid droplets within oocytes were abundant throughout follicular development (Silva et al. 2011), their changing morphology from homogenous dark droplets to a grey or streaked appearance indicates lipid utilisation throughout folliculogenesis (Isachenko et al. 2001; Silva et al. 2011). Immature oocytes contain the highest proportion of triglycerides, phospholipids and cholesterols than any other stage of development (Romek et al. 2011b). Cytoplasmic lipid droplets have been shown to translocate during in vitro maturation, following the patterns of mitochondrial rearrangement (Sturmey et al. 2006; Somfai et al. 2011), further supporting a role for their use as an energy substrate.

Lipid droplets tend to appear more homogenous and electron dense in in vivo oocytes and embryos compared to their in vitro counterparts (Kikuchi et al. 2002a). In regards to lipid utilisation, an early study found that the number of lipid droplets increased following resumption of meiosis in in vivo sourced oocytes (Cran 1985). A subsequent comparative study found that in vitro and in vivo matured oocytes contained similar numbers of small, medium and large sized lipid droplets (Niimura et al. 2002). In feline oocytes, which are similar to porcine oocytes in terms of the rich endogenous lipid content, in vivo matured oocytes oxidised greater amounts of palmitate than in vitro oocytes (Spindler et al. 2000). In vivo oocytes may be capable of incorporating or utilising exogenous fatty acids present in reproductive tract fluids, and thus any metabolic differences in fatty acid

oxidation between in vitro and in vivo matured oocytes may not be evident by comparing lipid droplet number and size alone.

Porcine embryo lipid content remains unchanged from early cleavage stages through to morula, before significantly decreasing at the blastocyst stage and again at the late blastocyst stage; this is seen in both in vitro and in vivo cultured embryos (Romek et al. 2009). There was no change in triglyceride content detected throughout embryo development, with triglyceride levels remaining constant at around 117ng from early cleavage onwards (Sturmey and Leese 2003). This suggests that there are potentially changes in other lipid species, such as phospholipids within membranes, during embryo culture.

While lipid droplet morphology and distribution was similar between in vivo embryos and those cultured in vitro in NCSU-23 medium, the triglyceride content of lipid droplets was significantly lower in in vivo cultured embryos (Romek et al. 2010). Potentially, the higher carbohydrate metabolic rates seen in in vitro cultured embryos may lead to a lower lipid utilisation, thus accounting for higher lipid levels in these cells when compared with in vivo embryos. At the early blastocyst stage, mitochondrial membrane potential (MMP) is lower in in vitro than in vivo embryos (Romek et al. 2011c). As the electron transport generates MMP, used to drive ATP synthesis through oxidative phosphorylation, MMP is a key indicator of cell metabolic activity relating to oxygen consumption and cellular energy needs. Increased intracellular lipid may also be due to abnormal lipid accumulation during in vitro culture, as suggested by the finding that culture in media containing serum increased intracellular lipid content (Ferguson and Leese 1999).

1.5.2. Addition of undefined fluids

The use of a fully defined medium is preferential in in vitro systems, allowing repeatable results in research applications and reducing disease risk in commercial settings. However, the addition of undefined biological fluids to maturation media is often beneficial to in vitro development.

The addition of follicular fluid to maturation medium has been shown to be advantageous in in vitro systems. Follicular fluid is known to be rich in nutrients, containing proteins, amino acids, sugars, enzymes, mucopolysaccharides, hormones, salts and fatty acids (Huang et al. 2002). Follicular fluid is released from the follicle at ovulation, with a portion transported into the oviduct along with the COC. However, it has been estimated that less than 1% of follicular fluid is retained in the oviduct following ovulation, with oviductal fluid being the medium of transport and maturation of in vivo ovulated oocytes (Leese 1988). Despite this, the addition of follicular fluid to maturation medium has been shown to have a range of beneficial effects. These include improved nuclear and cytoplasmic maturation rates, male pronucleus formation, cumulus cell expansion, male pronucleus formation, monospermic fertilisation, and greater cleavage and blastocyst rates following IVF (Naito et al. 1989; Rath et al. 1995; Algriany et al. 2004; Ito et al. 2008). The concentration of follicular fluid in maturation medium impacts on efficacy, with up to 30% found to be optimal depending on the IVM medium used while higher concentrations led to inhibited meiotic resumption (Kim et al. 1996).

There is a follicle size effect, with follicular fluid from larger follicles having a greater beneficial effect than that from small follicles (Algriany et al. 2004; Ito et al. 2008). It is likely that there is an accumulation of beneficial factors, such as steroids (Grupen et al. 2003), as well as decreasing concentrations of meiosis-inhibiting factors (Bertoldo et al. 2013), in follicular fluid from large follicles closer to ovulation compared with small follicles. Further to this, oocytes aspirated from large follicles have greater developmental competence than those from small follicles (Marchal et al. 2002; Algriany et al. 2004; Bagg et al. 2007). This suggests that the factors accumulated as the follicle increases in size are important for later stages of development, and can further aid in oocyte maturation and development.

However, the composition of follicular fluid has a marked effect on the outcome of its use in media systems. Mouse oocytes cultured in follicular fluid rich in fatty acids had poorer developmental outcomes than oocytes cultured in follicular fluid with low levels of fatty acids (Yang et al. 2012). In developing porcine ovarian

follicles, the fatty acid concentration of follicular fluid was found to decrease with increasing follicle size (Yao et al. 1980); it may be that supplementation with fatty acids at this stage, or the provision of specific fatty acids, can be detrimental to oocyte maturation. Variation between follicular fluid preparations and the undefined nature of the product can therefore have a variable effect on maturation.

Culture in certain undefined substances (such as serum) can lead to abnormal accumulation of lipid droplets (Ferguson and Leese 1999), as fatty acids from medium substrates are taken up and this excess energy substrate is used to synthesise lipids which are stored as lipid droplets in the cytoplasm. Because of this, the lipid profile of oocytes and embryos often mirrors that of serum products in culture media (Abe et al. 2002).

1.5.3. Addition of specific fatty acids

The fatty acid composition of low and high quality oocytes has been shown to differ (Aardema et al. 2011). Because oocyte composition often reflects the substrates available within the medium (Aardema et al. 2011), the supplementation of maturation medium with fatty acids has the potential to affect oocyte developmental potential. Experiments using radiolabelled fatty acids have shown that cattle oocytes uptake and incorporate exogenous fatty acids, with greater than 95% metabolised within the oocyte and 5% recovered in the free fatty acid component (Aardema et al. 2011). Oleic acid was found to be more efficiently incorporated into stored triglycerides, compared with relatively poor incorporation of palmitic or stearic acids. Addition of palmitic and stearic acids to the culture medium, either singly or in conjunction, resulted in poorer developmental outcomes (Aardema et al. 2011; McKeegan and Sturmey 2012). However, when oleic acid was added in conjunction with either palmitic or stearic or both, it counteracted the adverse effects and restored development to levels similar to controls with no fatty acid supplementation (Aardema et al. 2011).

Early studies worked to quantify the quantity and composition of fatty acids present in reproductive tract fluid (Iritani et al. 1974; Chang et al. 1976; Yao et al.

1980; Homa et al. 1986; Khandoker et al. 1997), and found a wide variety of fatty acids within tract fluid. Despite the large amount of research focussed on improving IVP through media composition refinement, the supplementation of media with specific fatty acids is not commonplace.

Saturated fatty acids are poorly incorporated into triglyceride reserves in non-adipose tissues and can cause lipotoxicity and increased apoptosis in cells (Listenberger et al. 2003). The addition of palmitic (16:0) or stearic (18:0) acids, either singly or in conjunction, to maturation medium for bovine oocytes reduced the cleavage and blastocyst rates following IVF, as well as reducing the number and size of lipid droplets in the oocytes, although nuclear maturation rates to MII were unaffected (Aardema et al. 2011; McKeegan and Sturmey 2012). Interestingly, in feline oocytes, oxidation of palmitic acid increased throughout GVBD (Spindler et al. 2000); further, it is the most abundant fatty acid found in porcine oocytes (Homa et al. 1986; Sturmey et al. 2009). It is possible that these fatty acids play a role in oocyte maturation of certain species, with varying responses to specific fatty acids. Oleic acid (18:1) supplementation counteracted adverse effects of palmitic and stearic acids in bovine oocytes (Aardema et al. 2011). The addition of 500μM oleic acid also increased both the number and size of lipid droplets in MII stage oocytes (Aardema et al. 2011). Greater developmental outcomes were seen from lipid rich porcine oocytes, and higher quality porcine oocytes were found to have greater proportions of oleic, linoleic (18:2) and arachidonic (20:4) acids than those in poorer quality oocytes (Kim et al. 2001). Unsaturated fatty acids also appear to serve a protective function against lipotoxicity and apoptosis by increasing triglyceride storage (Listenberger et al. 2003; Aardema et al. 2011).

Further, the addition of linolenic acid (18:3) to maturation medium improved MII development of cattle oocytes and resulted in higher cleavage and blastocyst rates following IVF, as well as increasing the activity of MAPK signalling pathways (Marei et al. 2009). In contrast, adding linoleic acid (18:2) inhibited CC expansion and reduced meiotic progression and cAMP levels, with a subsequent reduction of cleavage and blastocyst development rate, in a dose-dependent manner (Marei et al. 2010). The concentration of linolenic acid in follicular fluid was found to be

similar in small and large follicles, while the linoleic acid concentration decreased with increasing follicle size (Homa and Brown 1992). There is an obvious effect of supplementation with fatty acids at concentrations seen in in vivo tract fluids, and it is likely that they contribute to oocyte composition, which affects maturation and subsequent quality.

The supplementation of pig maturation medium with a commercial fatty acid mix (Gibco) had no effect on meiotic progression or blastocyst cell number (Tubman et al. 2005). Further, activity of glycolysis and the PPP in the resulting blastocysts were unaffected, suggesting a minimal role of exogenous fatty acids during porcine oocyte maturation. Elevated follicular free fatty acid levels have been correlated with poor COC morphology in humans (Jungheim et al. 2011) and altered mitochondrial distribution, increased ROS and morphologically abnormal zygotes in mice (Wakefield et al. 2008).

It is likely that current media formulations exclude fatty acids in an attempt to avoid excessive lipid accumulation. However, it is apparent that exogenous fatty acids do exert some beneficial effect on oocytes, and a greater understanding of their role in maturation is required. It is evident that further research is required to examine species specific effects and determine the optimal concentrations.

1.5.4. Alteration of lipid metabolism

Prior studies have also looked at the addition of metabolic regulators to maturation and culture media in an attempt to alter the in vitro metabolism of oocytes and embryos, so that it more closely resembles that of in vivo grown counterparts.

1.5.4.1. L-carnitine

The artificial upregulation of lipid metabolism in oocytes and embryos aims to increase the rate of lipid oxidation, attempting to increase the ATP available to the cell from utilisation of endogenous lipid stores as energy substrates. Because in vivo embryos have lower lipid levels than in vitro produced embryos, it is thought that metabolic aberrations in in vitro oocytes and embryos alter their substrate

preferences and that they are therefore not utilising lipid stores in a 'normal' manner.

The majority of studies examining the upregulation of lipid metabolism use L-carnitine, a co-factor involved in the entry of activated fatty acids from the cytosol into the mitochondrial matrix, which is the rate limiting step of fatty acid oxidation. Its addition to maturation medium has been shown to have beneficial effects across a number of species. In mice, the addition of 1mM L-carnitine during IVM increased the rate of β-oxidation and led to improved developmental competence following fertilisation in these oocytes (Dunning et al. 2010a). In cattle, oocyte maturation and blastocyst development rates were increased after the addition of L-carnitine during IVM (Yamada et al. 2006; Phongnimitr et al. 2013), as well as affecting the diffusion of mitochondria within the cytoplasm of oocytes (Yamada et al. 2006). However, a later study found no effect of L-carnitine on bovine oocyte maturation or embryo development (Chankitisakul et al. 2013). Differences in composition of media used between studies may account for the varied results seen, although further studies are required to confirm this.

In porcine oocyte maturation studies, the results of adding L-carnitine to IVM medium have been varied. One study reported that the addition of L-carnitine at doses between 3.04-25.30mM increased the proportion of mature oocytes at 44 h (Somfai et al. 2011). A subsequent study showed that 2.53mM L-carnitine hastened nuclear maturation, with a greater number of oocytes reaching MII by 36 h compared to the control, although similar maturation rates were achieved by 44 h of maturation (Wu et al. 2011). It was later shown that 10mM L-carnitine had no effect on nuclear maturation (You et al. 2012), although in this study the high proportions of oocytes reaching MII in the control group (91.6% at 44 h) may have masked any beneficial effects of the L-carnitine treatment. With regards to cytoplasmic maturation, the presence of 3.04-25.30mM L-carnitine during maturation improved cleavage rates following IVF (52.0-54.8% vs 41.7%), but blastocyst development was not improved (Somfai et al. 2011). In contrast, other studies showed that L-carnitine treatment did not alter the cleavage rate, but increased the blastocyst development rate following parthenogenetic activation (Wu et al. 2011; You et al. 2012). These conflicting results may be due to the

developmental differences between IVF and parthenogenetic embryos. Utilising IVF to produce blastocysts introduces a range of sperm factors which can influence developmental outcomes, potentially accounting for differences between these studies. All studies reported similar cell numbers between L-carnitine and control groups. Similar penetration rates, male pronuclear formation and monospermic fertilisation rates were reported, but the successful formation and extrusion of two polar bodies was significantly higher (92.0% vs 76.8%) in groups of L-carnitine treated oocytes (Somfai et al. 2011). Extrusion of both polar bodies may be related to rates of MII maturation, with MI oocytes being able to be fertilised but incapable of forming a second polar body. The presence of L-carnitine during maturation was also shown to reduce ROS levels and the proportion of apoptotic cells in resulting blastocysts (Wu et al. 2011), likely due to the antioxidant property of L-carnitine.

Comparisons of these three studies is difficult owing to differences in media composition and maturation conditions, and further research is required to examine how these variables, such as the presence or absence of dibutyryl cAMP, follicular fluid, BSA and synthetic macromolecules, interact with lipid metabolism to affect maturation.

Further effects of L-carnitine have been reported on mitochondrial and lipid droplet distribution. The addition of 7.4mM L-carnitine to maturation medium was reported to increase the density of active mitochondria within the central and peripheral areas of the oocyte, and result in translocation of lower density lipid droplets to the centre of the ooplasm (Somfai et al. 2011). The co-localisation of lipid droplets and mitochondria has previously been documented for porcine oocytes (Kikuchi et al. 2002a; Sturmey et al. 2006), as has the translocation of active mitochondria (Sun et al. 2001; Krisher et al. 2007) and lipid droplets (Sturmey et al. 2006) from peripheral to central during IVM. As the movement of active mitochondria and lipid droplets during in vivo maturation has yet to be examined in detail, the significance of these findings is unclear.

L-carnitine is also known to act as an antioxidant, having been shown to alter the redox state via mitochondrial pathways in animal somatic cells (Pillich et al. 2005;

Ye et al. 2010). There is a consensus between studies that the addition of L-carnitine to media at a range of concentrations reduced intracellular ROS levels (Somfai et al. 2011; Wu et al. 2011; You et al. 2012), while also increasing GSH concentrations within the oocytes (Wu et al. 2011; You et al. 2012) and reducing the proportion of apoptotic cells in blastocysts (Hashimoto et al. 2008; Wu et al. 2011). Addition of 9-25μM L-carnitine to medium supplemented with H_2O_2 decreased intracellular hydrogen peroxide and lipid peroxide levels and increased nuclear maturation rates in porcine oocytes (Yazaki et al. 2013). Given the induced oxidative stress in these cells, a lower dose of L-carnitine was found to improve maturation parameters compared with other reports. It is undetermined whether L-carnitine treatment has a dual beneficial effect as an antioxidant and a regulator of lipolysis or if there is a single effect leading to beneficial outcomes following IVM. The effects of adding other sources of antioxidants have been varied, with cysteamine showing improved maturation (Grupen et al. 1995), while other antioxidants have led to no improvement (Tatemoto et al. 2004; Choe et al. 2010). The decrease in lipid content following application of lipolytic stimulators (Fu et al. 2011; Romek et al. 2011a; Somfai et al. 2011) would infer that lipid is indeed being used to a greater extent in these oocytes. Further, the rate of β-oxidation was increased by the addition of L-carnitine to maturation medium in mouse oocytes (Dunning et al. 2010a), providing evidence that lipolysis is the primary effect.

To date, there are no studies regarding the effects of the addition of metabolic regulators during the IVF period on oocyte developmental potential. The use of L-carnitine in sperm medium has been shown to increase intracellular GSH levels in the sperm, leading to improved fertilisation rates (Luberda 2005). There is an increased demand for ATP during fertilisation, and oocytes may be capable of utilising endogenous lipid substrates at this stage to meet energy requirements.

The use of lipolytic stimulators in embryo culture media has shown improvements in development to the blastocyst stage and a reduction in ROS formation in mice (Abdelrazik et al. 2009) and cattle (Sutton-McDowall et al. 2012) embryos. The lipid content of bovine embryos cultured with L-carnitine was decreased (Sutton-McDowall et al. 2012), and cryosurvival increased in these cells. In pigs, the

addition of L-carnitine to culture medium had no effect on cleavage rates, blastocyst development rates or cell number, or intracellular GSH content of parthenogenetic embryos (Wu et al. 2011), despite the intracellular ROS levels and incidence of apoptosis in blastocyst cells being reduced. Continued research into the use of lipolytic stimulators is required to fully understand the role of endogenous lipids during oocyte maturation and embryo culture, further to developing culture media to better support this aspect of metabolism.

1.5.4.2. Inhibitors

Several studies have examined the effect of inhibiting lipid metabolism in oocytes and embryos, with the aim of understanding the level of contribution of this pathway to energy generation and cell development. In mice, inhibition of lipolysis prevented meiotic resumption in COCs and denuded oocytes, hypothesised to be due to interference with the AMPK-induced meiotic induction pathway (Downs et al. 2009). A subsequent study showed that inhibition of CPT1 in mouse COCs using 100µM etomoxir did not inhibit development to MII, but significantly inhibited activity of the β-oxidation pathway (Dunning et al. 2010a). Following IVF of these oocytes, embryo development was significantly reduced, suggesting that lipid metabolism may also have a role in cytoplasmic maturation of mouse oocytes. Similarly in cattle oocytes, addition of 1 or 5mM methyl palmoxirate during IVM reduced blastocyst development compared with the control group (14%, 7% and 22% respectively; Ferguson and Leese 2006). While 250µM etomoxir was required to inhibit nuclear maturation in mouse oocytes, doses of only 100µM and 10µM were required to have the same effect in bovine and porcine oocytes, respectively (Paczkowski et al. 2013). Given the differing lipid content in oocytes of these species, this strongly suggests that the use of endogenous lipids is species-specific. Furthermore, in both mice and cattle, increased glucose consumption corresponded with a decrease in lipolysis (Paczkowski et al. 2013).

In cattle embryos, culture with 1 to 5mM methyl palmoxirate decreased blastocyst development (11-13% vs 22% control), while doses as low as 0.5mM also led to a reduction in blastocyst cell number (Ferguson and Leese 2006). There was a corresponding reduction in O_2 consumption during culture with 1 to 5mM methyl

palmoxirate, implying that energy generation from endogenous lipids plays a necessary role in embryo development.

In pigs, the reported effects of inhibition of lipolysis vary. Addition of 0.5 to 1.0mM methyl palmoxirate during IVM prevented any subsequent cleavage and blastocyst development (Sturmey et al. 2006). Doses of 0.1 to 1.0mM of mercaptoacetate during IVM had no effect on cleavage or blastocyst development, while a 10mM dose inhibited blastocyst formation (Sturmey et al. 2006). Differences in responses may be attributable to the type of inhibitors used. Methyl palmoxirate works by inhibiting the activity of the carnitine shuttle, preventing entry of free fatty acids into the mitochondria for β-oxidation. Mercaptoacetate is a competitive inhibitor of 3-hydroxyacyl CoA dehydrogenase, a key enzyme in the β-oxidation pathway. Lower response with mercaptoacetate is likely due to competitive inhibition, whereby higher concentrations of substrates would lead to lower effectiveness of the inhibitor.

The addition of 10μM etomoxir was sufficient to inhibit nuclear maturation in porcine oocytes, with the majority of oocytes arresting at MI following treatment with 10 to 100μM etomoxir; at doses of 250μM, oocytes arrested primarily at the GVBD stage (Paczkowski et al. 2013). Etomoxir has a similar mode of action as methyl palmoxirate, whereby activity of the carnitine shuttle is inhibited. However, this study used TCM-199 medium, which contains 5mM glucose and 0.01mM pyruvate. As pyruvate is an important factor for the resumption of meiosis, the low concentration present in this medium may not have supported meiotic progression adequately. Further, exposure to etomoxir during IVM decreased the relative abundance of genes involved in fatty acid oxidation and glycolysis (Paczkowski et al. 2013). In contrast to mice and cattle, there was no increase in glucose consumption in porcine oocytes treated with etomoxir (Paczkowski et al. 2013). Interestingly, there was also no change in lipid content or ADP/ATP ratio following etomoxir treatment (Paczkowski et al. 2013), suggesting lipid metabolism was a requirement for cell viability. Given the high lipid content in porcine oocytes, this pathway may be of greater importance in this species than in others.

1.6. Concluding remarks and objectives of the present study

The low efficiency of IVP and the poor quality of resulting embryos is a major impediment to the application of ARTs in pig production. The purpose of the high lipid content of porcine oocytes and embryos remains unknown, despite this being a major hurdle to improving IVP outcomes in pigs. The discrepancies in quality and development between in vivo- and in vitro-derived embryos are likely attributable to altered metabolism resulting from the in vitro conditions. Supporting this notion is the progress made so far in improving the media formulations – as these have been refined to more closely resemble the composition of the reproductive tract fluids, blastocyst quality and development rates have increased together with a rise in live birth rates following transfer. However, there is still a lack of understanding regarding the contribution of lipid substrates to energy generation in porcine oocytes and embryos.

The limited studies in pigs that have examined IVM or IVC have used completely different media, some of which are undefined. Therefore there is little knowledge about the interactions of carbohydrate and lipid metabolism at these stages of IVP. There are variable reports regarding the effects of lipolytic stimulants and inhibitors, suggesting that other factors during culture, including carbohydrate composition of the media, may affect the use of lipid stores. There are no known studies examining the involvement of lipids during porcine IVF. This thesis examines lipid metabolism at each stage of the IVP process in detail using a semi-defined medium, and explores the interaction of carbohydrate and lipid metabolism. It is hypothesised that porcine oocytes and embryos utilise endogenous lipid substrates during the IVP process, and that changes in concentrations of carbohydrate substrates will impact upon use of lipids. Further, given the changing nutrient requirements seen throughout the stages of IVP, it is hypothesised that the level of lipid usage will vary with stage of development.

To this end, the overall aim of this thesis was to identify the role of lipid metabolism during in vitro production of porcine embryos. The specific aims of the thesis studies were:

i. To determine the effects of upregulating and inhibiting lipolysis in a defined medium on oocyte nuclear maturation and developmental competence during IVM
ii. To determine the effects of upregulating and inhibiting lipolysis in a carbohydrate deficient medium on oocyte nuclear maturation and developmental competence during IVM
iii. To determine the effects of upregulating lipolysis during IVF on fertilisation and embryo development
iv. To determine the effects of upregulating lipolysis during IVF on embryo survival following vitrification
v. To determine the effects of upregulating lipolysis in defined single and sequential media systems on embryo development during IVC
vi. To determine the effects of upregulating lipolysis in a carbohydrate-deficient media on embryo development during IVC
vii. To determine the effects of upregulation of lipolysis in a defined media system on embryo survival following vitrification

CHAPTER 2. MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.1. In vitro maturation

Ovaries from prepubertal gilts were collected immediately after slaughter at a local abattoir (Wollondilly Abattoir Pty Ltd, Picton NSW) and transported to the laboratory at 34-38^{o}C in 0.9% NaCl (Baxter, Deerfield, IL) supplemented with an antibiotic/antimycotic solution (100 IU penicillin G, 0.25µg/ml streptomycin sulphate and 0.85% amphotericin B; Gibco) within 1 h. Cumulus oocyte complexes (COCs) were aspirated from follicles 3-6mm in diameter through a 21-gauge needle under constant suction (1L/min). Cellular debris and COCs were allowed to sediment, then aspirated and spread over the bottom of a 10cm Petri dish (Falcon; Becton-Dickinson, NJ, USA). Under a stereomicroscope, oocytes were collected by mouth pipette attached to a modified glass Pasteur pipette. Collected cumulus-oocyte complexes (COCs) were washed twice in HEPES-buffered porcine X medium (PMX; 108mM NaCl, 10mM KCl, 0.35mM KH_2PO_4, 0.40mM $MgSO_4$, 5mM $NaHCO_3$, 25mM Hepes, 0.2mM Na-pyruvate, 2mM Ca-$(lactate)_2$, 3.00 mg/ml PVA; Yoshioka et al. 2008). Oocytes with an evenly granulated cytoplasm and at least three layers of compact cumulus cells were selected and washed in pre-equilibrated porcine oocyte media (POM; Yoshioka et al. 2008), supplemented with 3mg/ml fatty acid-free bovine serum albumin (BSA; IVP grade gamma irradiated, MP Biomedicals, Auckland, New Zealand), and 10ng/ml epidermal growth factor (EGF), 10 IU/ml equine chorionic gonadotropin (eCG) and 10 IU/ml human chorionic gonadotropin (hCG). Washed COCs were transferred to 4-well dishes (Nunc) containing 500µl maturation media covered with mineral oil, and cultured at 38.5^{o}C in a humidified atmosphere of 6% CO_2 in air.

2.2. In vitro fertilisation

After 44 h maturation, oocytes were partially denuded by gentle pipetting in PMX after brief exposure (<1 min) to 0.5mg/ml hyaluronidase. Oocytes were washed and transferred into Nunc wells containing 500µl TALP-PVA media (Bavister 1989), under 250µl mineral oil pre-equilibrated in 6% CO_2 in air at 38.5^{o}C. Meanwhile, frozen spermatozoa were thawed by agitating in a water bath at 42^{o}C for 20 s. Spermatozoa were then processed through a 45%:90% PureSperm (Nidacon) gradient at 720 x g for 10 min using TALP medium (114mM NaCl, 3.16mM KCl, 5mM glucose, 0.5mM $MgCL_2$, 0.35mM NaH_2PO_4, 25mM $NaHCO_3$, 4.72mM $CaCl_2$, 1mg/ml PVA, 0.002mg/ml phenol red, 0.0075mg/ml penicillin-G, 0.0125mg/ml streptomycin sulfate) supplemented with 0.05% BSA (spermTALP). Following this, the pellet was gently aspirated, and made up to 1ml in volume with spermTALP medium and centrifuged at 310 x g for 5 min. The supernatant was removed and the pellet was gently resuspended in 400µl medium. Motility and concentration were assessed. Spermatozoa were added to fertilisation wells at a concentration of 200 motile sperm/oocyte. Gametes were co-incubated for 30 min at 38.5^{o}C in 6% CO_2 in air, after which oocytes and bound spermatozoa were placed into fresh, pre-equilibrated TALP-PVA medium for a further 5 h, for a total 5.5 h co-incubation (Grupen and Nottle 2000; Gil et al. 2004).

2.3. In vitro culture

Presumptive zygotes were denuded of remaining cumulus cells and loosely bound spermatozoa, then placed in 50µl droplets (maximum 15 zygotes/droplet) of porcine zygote medium-3 (PZM-3; Yoshioka et al. 2002) and cultured at 38.5^{o}C in 6% CO_2, 5% O_2 in nitrogen. Media dishes were equilibrated for a minimum of 3 h prior to use. On Day 4, cleavage was assessed, with embryos expected to be at the morula stage, although those at earlier stages were still counted as cleaved. Uncleaved embryos were removed from culture, and 10% foetal calf serum (FCS; heat inactivated, qualified, Australian origin, Gibco) was added to each droplet. Addition of serum to culture medium during late cleavage stages has been shown to improve blastocyst development and hatching (Pollard et al. 1995; Dobrinsky et al. 1996; Koo et al. 1997). While standard PZM-3 does not include the addition of FCS, in these experiments FCS was added to culture medium to promote optimum blastocyst development to ensure blastocyst numbers were sufficient to

see experimental treatment effects. The blastocyst development rate, determined as a percentage of cleaved embryos, and embryo cell number were assessed on Day 7.

2.4. Determination of blastocyst cell number

Blastocysts were removed from culture, washed in PMX medium supplemented with 1mg/ml PVA and transferred into 0.3mg/ml Hoechst 33342 in ethanol. After staining for 30 min, blastocysts were transferred into absolute ethanol and fixed overnight in the dark at 4^{o}C. Embryos were slide mounted and assessed using fluorescent microscopy (Olympus BX61; Olympus, Tokyo, Japan) with excitation by UV light (350nm) and emission measured at 460-490nm. An image of each blastocyst was captured and cell numbers were determined using ImageJ software (v. 1.46r, National Institutes of Health, USA).

2.5. ATP assay

The total ATP content of oocytes was measured using a commercial assay kit (FL-ASC; Sigma). Briefly, oocytes were completely denuded of cumulus cells, and washed three times in PBS supplemented with 3mg/ml PVP. Oocytes were transferred in groups of ten into Eppendorf tubes containing 50µL ice-cold PBS and stored at -80^{o}C until analysis. For analysis, sample tubes were thawed on ice protected from light. Ice-cold somatic cell reagent (100µl) was added to each tube, briefly centrifuged, and incubated on ice for 5 min. Next, ice-cold assay mix solution (diluted 1:25 with ATP assay mix dilution buffer; 100µl) was added, and each tube was briefly centrifuged and incubated on ice for 5 min. For experiments in chapter 3, the ATP content was measured using a TD-20/20 single-tube luminometer (Turner Biosystems; California, USA) with high sensitivity (0.01pM/tube). For experiments in chapter 4, the ATP content was measured in a flat bottom 96-well plate (Greiner Bio-One, Frickenhausen, Germany) using a microplate reader with luminescence optics (POLARstar Optima; BMG Labtech, Ortenburg, Germany). A seven-point standard curve (0-60pM/tube) was included in each assay to allow calculation of ATP content of samples.

2.6. Embryo vitrification and thawing

Day 7 grade 1 blastocysts (Gardner et al. 2000a) were vitrified according to the methods of Vajta et al. (1998). All manipulations were conducted at room temperature. Briefly, in groups of up to 10, blastocysts were washed in holding medium (HEPES-buffered Medium 199 supplemented with 20% FCS) for 5 min. Blastocysts were then transferred into equilibration medium (holding medium supplemented with 7.5% ethylene glycol and 7.5% DMSO) and held for 3 min before being moved into vitrification medium (holding medium supplemented with 17% ethylene glycol, 17% DMSO, 0.4M sucrose) for 30 s. Embryos were then loaded into a super-fine open-pulled straw (SOPS; Minitube, Tiefenbach, Germany) within 30 s and immediately plunged into liquid nitrogen. To thaw, the open end of the SOPS was placed directly into thaw medium 1 (holding medium supplemented with 0.14M sucrose), and blastocysts were held in this solution for 6 min before a 5 min hold in each of thaw medium 2 (holding medium supplemented with 0.075M sucrose) and holding medium. Blastocysts were then washed in culture medium (PZM-3 supplemented with 20% FCS), and transferred into 50µl culture droplets under mineral oil pre-equilibrated at 38.5^{o}C in 6% CO_2, 5% O_2 and balanced nitrogen. Survival was assessed after 24 h of post-thaw culture, with surviving blastocysts classified as those in which the blastocoel had re-expanded.

CHAPTER 3. INVOLVEMENT OF LIPID METABOLISM DURING IN VITRO MATURATION OF PORCINE OOCYTES

3.1. Abstract

Porcine oocytes contain a large amount of endogenous lipid, which is thought to function as an intracellular source of energy. This study was conducted to determine the effects of upregulating or inhibiting lipid metabolism using L-carnitine or etomoxir, respectively, on the maturational competence of porcine oocytes cultured in the presence or absence of carbohydrates. When matured in POM, the addition of 0-12mM L-carnitine had no effect on nuclear maturation rates ($P>0.05$). Removal of glucose from maturation medium increased the proportion of oocytes arresting at MI after 44 h maturation ($P<0.05$). The addition of 12mM L-carnitine to medium had no effect on nuclear maturation, cleavage or blastocyst development with either 1.5 or 4mM glucose. Cleavage was reduced in oocytes matured without glucose regardless of L-carnitine supplementation ($P<0.05$). In the absence of pyruvate and lactate and with 1.5mM glucose, supplementation of media with 12mM L-carnitine significantly increased nuclear development to MII ($P<0.05$). Exclusion of pyruvate and lactate in maturation medium resulted in decreased oocyte ATP content at 44 h. When matured with 1.5mM glucose, 10μM etomoxir was required to significantly inhibit nuclear maturation ($P<0.05$) compared to maturation in 4mM glucose, where development to MII was only reduced with the addition of 250μM etomoxir ($P<0.05$). These results show that carbohydrate substrates are necessary for oocyte maturation, and that stimulating lipid metabolism can compensate to some extent for insufficient exogenous carbohydrates.

3.2. Introduction

In vitro maturation (IVM) is a key process in the in vitro production of embryos. It allows for the recovery and use of immature oocytes, increasing the available pool

of mature oocytes for research and commercial applications. Oocytes matured in vitro have reduced developmental capacity compared to in vivo matured oocytes (Krisher 2013), and resulting embryos are of poorer quality with altered metabolism (Swain et al. 2002). Optimal in vitro medium conditions are required to ensure adequate metabolic support and attainment of optimal oocyte quality.

The majority of research into the provision of exogenous energy substrates during IVM has focussed on carbohydrate utilisation. Consequently, media formulations are predominantly based on carbohydrate requirements. In porcine oocytes, both glucose and pyruvate have been shown to be essential for the resumption of meiosis and acquisition of developmental competence (Tubman et al. 2005; Funahashi et al. 2008; Tsuzuki et al. 2008; Wu et al. 2011). Only recently has attention focussed on lipid metabolism. Porcine oocytes contain large stores of endogenous lipids compared with those of other domestic livestock species (McEvoy et al. 2000). However, the contribution to energy generation and the reason for such high amounts of intracellular lipids is still poorly understood. It is unknown whether endogenous lipid substrates can be utilised in place of exogenous carbohydrates by porcine oocytes.

Lipid substrates can be used for ATP production via β-oxidation, and there is strong evidence supporting a metabolic role for endogenous triglyceride reserves during oocyte maturation. The triglyceride fraction in porcine oocytes decreases during the course of IVM (Romek et al. 2011b) with a concomitant increase in oxygen consumption (Sturmey and Leese 2003). Cattle oocytes have been shown to be capable of resuming meiosis and reaching MII without exogenous carbohydrates present in media (Ferguson and Leese 1999), suggesting reliance on endogenous substrates for ATP production. Inhibition of the β-oxidation pathway in mouse and cattle oocytes has been shown to negatively impact upon maturation and subsequent developmental outcomes (Ferguson and Leese 2006; Dunning et al. 2010a), while stimulation of fatty acid oxidation has led to increased rates of nuclear maturation in mouse oocytes (Dunning et al. 2010a). In pigs, the addition of lipolytic stimulants has had a variable response in regards to nuclear maturation and embryo development (Somfai et al. 2011; Wu et al. 2011; You et al. 2012) and translocation of lipid droplets and mitochondria (Sun et al. 2001;

Kikuchi et al. 2002a; Sturmey et al. 2006; Krisher et al. 2007). Comparisons between studies are difficult due to variations in carbohydrate composition of the base culture media used, which may have masked the impact of energy generation by lipid metabolism.

The aims of this study were to examine the role that lipid metabolism plays in energy generation during porcine oocyte maturation and subsequent blastocyst development, and determine the influence of carbohydrates on this role. L-carnitine, a co-factor required for the entry of fatty acids into the mitochondrial matrix for metabolic breakdown through the β-oxidation pathway, and etomoxir, an inhibitor of the carnitine shuttle at CPT1, were used to upregulate or block fatty acid oxidation respectively, to examine its role during in vitro maturation.

3.3. Materials and methods

3.3.1. Assessment of cumulus expansion

Oocytes were matured in vitro as described in section 2.1, with IVM medium altered as per experimental design. After 22 h of maturation, a digital image was taken of each oocyte (Nikon Digital Sight DS-L2; Nikon, Tokyo, Japan) and used to assess cumulus expansion. Individual oocytes were scored on a subjective scale of 0 to 4 based on expansion of the cumulus mass. The mean score (0.0 to 4.0) for each group per replicate was calculated to give the cumulus expansion index (Vanderhyden et al. 1990).

3.3.2. Evaluation of oocyte nuclear status

To assess nuclear maturation, oocytes were denuded of cumulus cells and fixed in acetic acid:ethanol (1:3) for a minimum of 24 h at room temperature. Following fixation, oocytes were slide mounted and stained with 1% (w/v) orcein in acetic acid for 1 h, destained with glycerol:acetic acid:water (1:1:3) and examined using phase-contrast microscopy at 400x magnification. Oocytes were classified as being at either the germinal vesicle (GV), metaphase-I (MI; including diakenesis

stage) or metaphase-II (MII; including the anaphase-I and telophase-I stages) stage based on descriptions from Motlik and Fulka (1976).

3.3.3. Lipid staining

Oocytes were completely denuded of cumulus cells then washed through 1, 2, 3 and 4% solutions of paraformaldehyde (PFA) in PBS supplemented with 2mg/ml PVP (PBS-PVP). Oocytes were fixed overnight at 4^{o}C in 4% PFA, protected from light. Fixed oocytes were washed three times for 15 min each in PBS-PVP, and then stained with Nile Red (10µg/ml in 0.9% NaCl supplemented with 1mg/ml PVP) for 1 h in the dark at room temperature. Following staining, oocytes were washed twice in PBS-PVP and twice in antifade solution (SlowFade Component B; Invitrogen). Oocytes were mounted between paraffin lines on glass slides under coverslips with minimum pressure in antifade solution supplemented with 10µg/ml Hoechst for nuclear staining. Coverslips were sealed with nail varnish, and slides were stored in the dark and assessed within 6 h.

Slides were examined under a laser-scanning confocal microscope (Nikon D-Eclipse C1; Nikon, Tokyo, Japan) equipped with an argon-krypton and helium-neon ion laser using a 543nm excitation filter. An image was taken at the equatorial plane of each oocyte using identical gain and offset settings for each sample. Images were analysed for relative lipid content and distribution of lipid droplets as described by Chankitisakul et al. (2013). Lipid content was analysed using ImageJ (v.1.46r, National Institutes of Health, USA). Original files were converted into 8-bit greyscale images. Relative lipid content of oocytes was determined by the mean intensity of Nile Red-positive pixels in total area of the equatorial section.

Lipid droplet distribution was assessed after 22 and 44 h IVM, with the stage of nuclear maturation recorded (as confirmed by nuclear staining). Oocytes were classed as having one of three lipid droplet distributions: ‘peripheral’, where lipid droplets were localised in the peripheral region of the oocyte (Figure 3.1a); ‘semi-peripheral’, characterised by lipid droplets found predominantly in the periphery of the oocyte with a low abundance of lipid droplets in the centre of the oocyte

(Figure 3.1b); and ‘diffuse’, with lipid droplets dispersed evenly throughout the cytoplasm (Figure 3.1c).

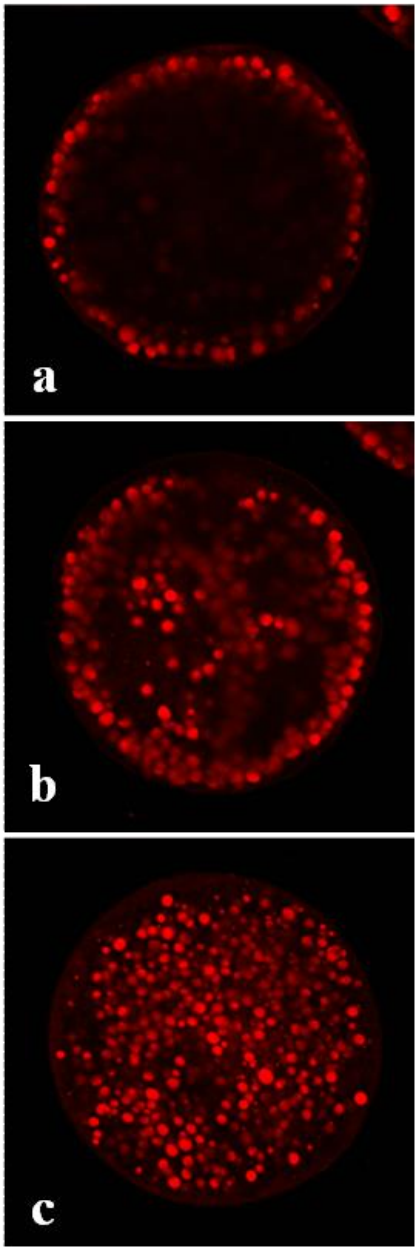


Figure 3.1. Patterns of lipid droplet distribution in IVM porcine oocytes stained with Nile red. Lipid droplets showed peripheral (a), semi-peripheral (b), or diffuse (c) patterns of distribution. Images were taken at the equatorial section of each oocyte using laser scanning confocal microscopy.

3.3.4. Experimental design

Experiments 1, 2, 3, 5 and 6 were conducted at the Faculty of Veterinary Science, The University of Sydney. Experiment 4 was conducted at the National Agriculture and Food Research Organisation Institute of Livestock and Grassland Science, Animal Breeding and Reproduction Research Division, Tsukuba, Japan.

Experiment 1: L-carnitine dose response in complete medium

Oocytes were matured in complete POM (4mM glucose, 0.2mM pyruvate, 2mM lactate) and treated with 0, 1.5, 3, 6 or 12mM L-carnitine. Oocytes were fixed and assessed for stage of nuclear maturation at 40, 44 and 48 h maturation. Three replicate experiments were performed with 10 to 16 oocytes assessed per treatment group at each time within each replicate.

Experiment 2a: Effect of L-carnitine and glucose in the presence of pyruvate and lactate on nuclear maturation and cumulus expansion

Oocytes were matured in POM (0.2mM pyruvate, 2mM lactate) with 0, 1.5 or 4mM glucose, and treated with 0 or 12mM L-carnitine. Cumulus expansion was assessed after 22 h of maturation. Oocytes were fixed and assessed for stage of nuclear maturation after 22 and 44 h maturation. Three replicate experiments were performed with 13 to 20 oocytes assessed per treatment group at each time within each replicate.

Experiment 2b: Effect of L-carnitine and glucose in the presence of pyruvate and lactate on developmental competence

Oocytes were matured in POM (0.2mM pyruvate, 2mM lactate) with 1.5 or 4mM glucose, and treated with 0 or 12mM L-carnitine. IVF was performed after 44 h maturation. Cleavage was assessed on Day 4 of IVC, and blastocyst development and cell number were assessed on Day 7. Four replicate experiments were performed with 30 to 40 oocytes inseminated per treatment group within each replicate.

Experiment 3: Effect of L-carnitine and glucose in the absence of pyruvate and lactate on nuclear maturation and cumulus expansion

Oocytes were matured in modified POM (0mM pyruvate, 0mM lactate) with 0, 1.5 or 4mM glucose, and treated with 0 or 12mM L-carnitine. Cumulus expansion was assessed after 22 h of maturation. Oocytes were fixed and assessed for stage of nuclear maturation after 22 and 44 h maturation. Three replicate experiments were performed with 10 to 18 oocytes assessed per treatment group at each time within each replicate.

Experiment 4: Effect of L-carnitine and glucose in the absence of pyruvate and lactate on ATP content, lipid droplet distribution and fluorescence intensity of Nile Red staining

This experiment was conducted at the National Agriculture and Food Research Organisation Institute of Livestock and Grassland Science, Animal Breeding and Reproduction Research Division, Tsukuba, Japan. Ovaries from pre-pubertal gilts were collected from a local slaughterhouse and transported to the laboratory within 1 h in dPBS (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan). Cumulus oocyte complexes were collected by slicing open follicles 3-6mm in diameter with a scalpel blade. The aspirated fluid was collected into a tube and cellular debris and COCs were allowed to sediment for ten minutes. The sediment was then aspirated into a 35mm Falcon dish and COCs were isolated and washed in PMX medium. Oocytes were matured in Nunc wells with 50 oocytes per well, in modified POM that contained 1.5mM glucose, with or without pyruvate and lactate, and treated with 0 or 12mM L-carnitine. Oocytes were assessed for ATP content, lipid droplet distribution and fluorescence intensity prior to IVM (0 h), and after 22 and 44 h maturation. For ATP measurements, six replicate experiments were performed with 10 oocytes pooled at each time point for analysis. For lipid staining, four replicate experiments were performed, with 5 to 10 oocytes assessed per treatment group at each time within each replicate.

Experiment 5: Etomoxir dose response in complete medium

Oocytes were matured in complete POM (4mM glucose, 0.2mM pyruvate, 2mM lactate) supplemented with 0, 62, 125 or 250µM etomoxir. Oocytes were fixed and assessed for stage of nuclear maturation at 22 and 44 h maturation. Three replicate experiments were performed with 12 to 18 oocytes assessed per treatment group at each time within each replicate.

Experiment 6: Interaction between etomoxir and glucose in the absence of pyruvate and lactate

Oocytes were matured in modified POM without pyruvate and lactate, with 1.5 or 4mM glucose and 0, 10 or 250μM etomoxir. Oocytes were fixed and assessed for stage of nuclear maturation at 22 and 44 h maturation. Three replicate experiments were performed, with 10 to 20 oocytes assessed per treatment group at each time within each replicate.

3.3.5. Statistical analysis

All analyses were conducted using GenStat (v.16, VSN International Ltd). Nuclear maturation, cleavage, blastocyst development and embryo cell number data were analysed by one-way analysis of variance (ANOVA). Fisher's protected LSD post-hoc test was used when significant differences between treatments were detected. Fluorescence intensity and ATP assay data were log transformed and analysed via a restricted maximum likelihood (REML) model. Lipid distribution data were log transformed and analysed by general linear mixed model (GLMM). For all analyses, $P<0.05$ was considered significant.

3.4. Results

Experiment 1: L-carnitine dose response in complete medium

There was no effect of L-carnitine dose on nuclear maturation at 40, 44 or 48 h maturation ($P>0.05$, Table 3.1).

Experiment 2a: Effect of L-carnitine and glucose in the presence of pyruvate and lactate on nuclear maturation and cumulus expansion

Cumulus expansion was reduced in groups matured without glucose (1.11-1.22) compared with groups matured in the presence of 1.5 or 4mM glucose (2.63-2.74; $P<0.05$, Figure 3.2a). L-carnitine supplementation did not affect cumulus expansion ($P>0.05$). After 22 h maturation, oocytes matured without glucose had the highest proportions of oocytes remaining at GV (69.11-82.40%) compared with oocytes matured in the presence of 1.5 or 4mM glucose (12.86-22.34%;

P<0.05, data not shown). After 44 h maturation, a higher proportion of oocytes matured without glucose remained at the MI stage (32.26-37.17%) compared with those matured in the presence of 1.5 or 4mM glucose (3.63-11.96%; P<0.05, Figure 3.3a). Oocytes matured with 4mM glucose without L-carnitine, and those matured with 1.5 or 4mM glucose in the presence of L-carnitine, had higher rates of development to MII compared to those matured without glucose (P<0.05). Supplementation of 12mM L-carnitine had no effect on nuclear maturation (P>0.05).

Experiment 2b: Effect of L-carnitine and glucose in the presence of pyruvate and lactate on developmental competence

Following IVF, oocytes matured without glucose had reduced cleavage rates (63.75-68.13%) compared with oocytes matured with no L-carnitine and either 1.5mM or 4mM glucose (81.73-83.27%; P<0.05, Figure 3.3b). Further, in groups treated with 12mM L-carnitine, the cleavage rate was reduced in oocytes matured with no glucose (63.75%) compared with those matured with 1.5mM glucose (80.65%; P<0.05). There was no effect of IVM treatment on blastocyst development rate (P>0.05, Figure 3.3b). There was no significant difference in embryo cell number between treatments (P>0.05), although there was a strong trend for a decreased cell number in embryos derived from oocytes matured with 4mM glucose and 12mM L-carnitine (25.45 average cell number) compared with those matured with 0mM L-carnitine and 1.5 or 4mM glucose (46.75-58.15 average cell number, P=0.06).

Experiment 3: Effect of L-carnitine and glucose in the absence of pyruvate and lactate on nuclear maturation and cumulus expansion

There was no effect of treatment on cumulus expansion (1.23-1.46; P>0.05, Figure 3.2b). After 22 h maturation, GV and MI rates were similar among treatments (P>0.05). MII rates did not differ for all levels of glucose when there was no L-carnitine present (2.22-8.28%; P>0.05). When supplemented with 12mM L-carnitine, MII rates were greater in the 4mM glucose group compared to the 0mM glucose group (15.85% vs 0%; P<0.05). After 44 h maturation, the rate of maturation to MII was greater in oocytes matured with 4mM glucose (41.03%) than in those matured in 0mM (10.83%) and 1.5mM (9.58%) glucose when L-

carnitine was not added (P<0.05, Figure 3.4). The addition of 12mM L-carnitine increased the MII rate of oocytes matured in 1.5mM glucose (35.88% v 9.58%; P<0.05, Figure 3.4), but had no effect on oocytes matured in 0mM or 4mM glucose (P>0.05).

Experiment 4: Effect of L-carnitine and glucose in the absence of pyruvate and lactate on ATP content, lipid droplet distribution and fluorescence intensity of Nile Red staining

There was no effect of treatment, stage of nuclear maturation, or time on oocyte fluorescence intensity (P>0.05). There was no effect of L-carnitine treatment on the distribution of lipids within oocytes (P>0.05). The relative frequency of peripheral distribution increased at 44 h compared with 22 h (15.46% vs 3.55% respectively; P<0.05), with no detectable changes in the relative frequency of disperse or semi-peripheral distribution over the two times (P>0.05). Further, MII stage oocytes tended to have a higher occurrence of peripheral distribution of lipid droplets compared to GV or MI stage oocytes (P=0.07). Maturation in medium with pyruvate and lactate decreased the relative frequency of peripheral lipid distribution in MII oocytes after 44 h IVM (3.10-4.59% vs 12.74-16.65%; P<0.05, Figure 3.5).

In oocytes matured in medium with pyruvate and lactate and treated with 12mM L-carnitine, the ATP content was greater at 44 h than at 0 and 22 h (P<0.05, Figure 3.6). The ATP content did not change significantly over the 44 h maturation interval for the other groups. At 44 h IVM, regardless of L-carnitine supplementation, oocytes matured with pyruvate and lactate had a higher ATP content (2.01-2.42pM ATP/oocyte) compared with oocytes matured without pyruvate and lactate (1.46-1.66pM ATP/oocyte; P<0.05, Figure 3.6).

Experiment 5: Etomoxir dose response in complete medium

All doses of etomoxir increased the proportion of oocytes remaining at GV after 22 h of maturation (33.33-50.37%) compared with oocytes matured without etomoxir (17.02%; P<0.05, Figure 3.7a). Progression to MI was lower with 250µM etomoxir (34.80%) than with 0 or 62µM etomoxir (59.74 and 58.97% respectively; P<0.05). After 44 h of maturation, a greater proportion of oocytes remained at MI in oocytes

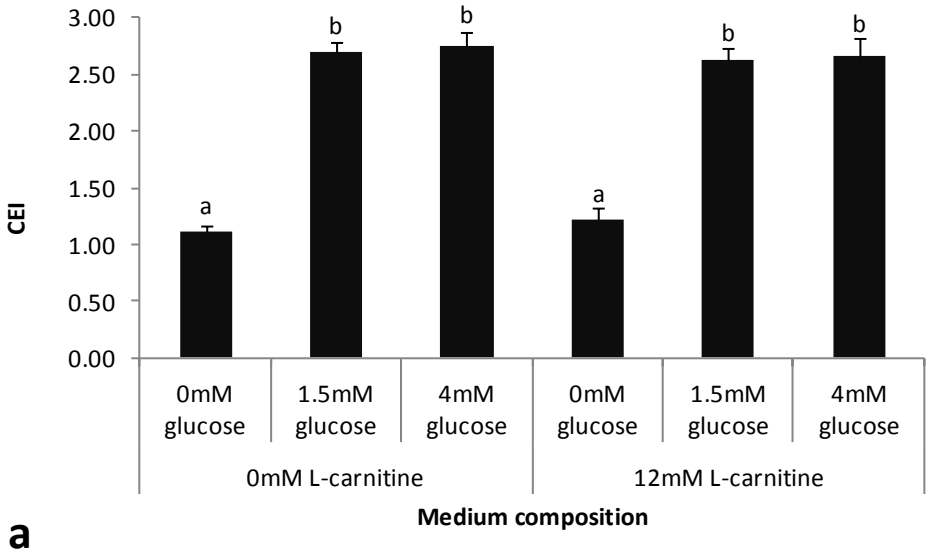
matured with etomoxir (32.77-50.11%) compared with those matured without etomoxir (10.46%; P<0.05, Figure 3.7b).

Experiment 6: Interaction between etomoxir and glucose in the absence of pyruvate and lactate

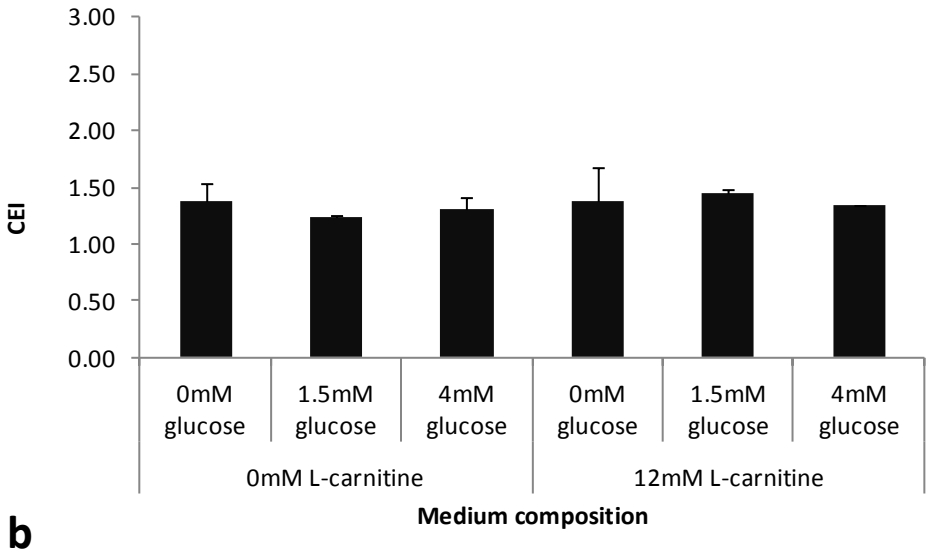
After 22 h maturation, the proportion of oocytes reaching MI was lowest when medium contained 250μM etomoxir and either 1.5mM or 4mM glucose (12.11 and 12.17% respectively), compared with groups matured with no etomoxir and 1.5mM or 4mM glucose (34.85% and 47.98% respectively; P<0.05), or 10μM etomoxir with 4mM glucose (41.28%; P<0.05, Figure 3.8a). A decreased proportion of oocytes reached MII stage after 44 h of maturation when matured with 1.5mM glucose and 10 or 250μM etomoxir (2.38% and 17.73% respectively) or 4mM glucose and 250μM glucose (9.95%) compared with other treatments (P<0.05, Figure 3.8b).

Table 3.1. Nuclear maturation after 40, 44 and 48 h IVM supplemented with 0, 1.5, 3, 6 or 12mM L-carnitine. Mean ± SEM maturation to MII after 40, 44 and 48 h IVM. Oocytes were matured in complete POM (4mM glucose, 0.2mM pyruvate, 2mM lactate), and supplemented with either 0, 1.5, 3, 6 or 12mM L-carnitine.

L-carnitine (mM)	% MII oocytes		
	40 h	44 h	48 h
0	100 ± 0.0	90 ± 6.3	88 ± 7.9
1.5	96 ± 2.2	93 ± 0.3	87 ± 10.2
3	87 ± 5.0	92 ± 4.3	94 ± 5.6
6	96 ± 4.2	90 ± 5.1	91 ± 4.3
12	96 ± 2.2	85 ± 5.3	95 ± 2.3



a



b

Figure 3.2. Mean ± SEM cumulus expansion index (CEI) after 22 h IVM. Individual oocytes were scored on a subjective scale of 0 to 4 based on expansion of the cumulus mass, and the mean score (0.0 to 4.0) for each group per replicate was calculated to give the CEI. Oocytes were matured in medium formulated with (a) or without (b) 0.2mM pyruvate and 2mM lactate, and supplemented with 0, 1.5 or 4mM glucose and 0 or 12mM L-carnitine. Groups within each graph represented by bars without a common letter differ significantly ($P<0.05$).

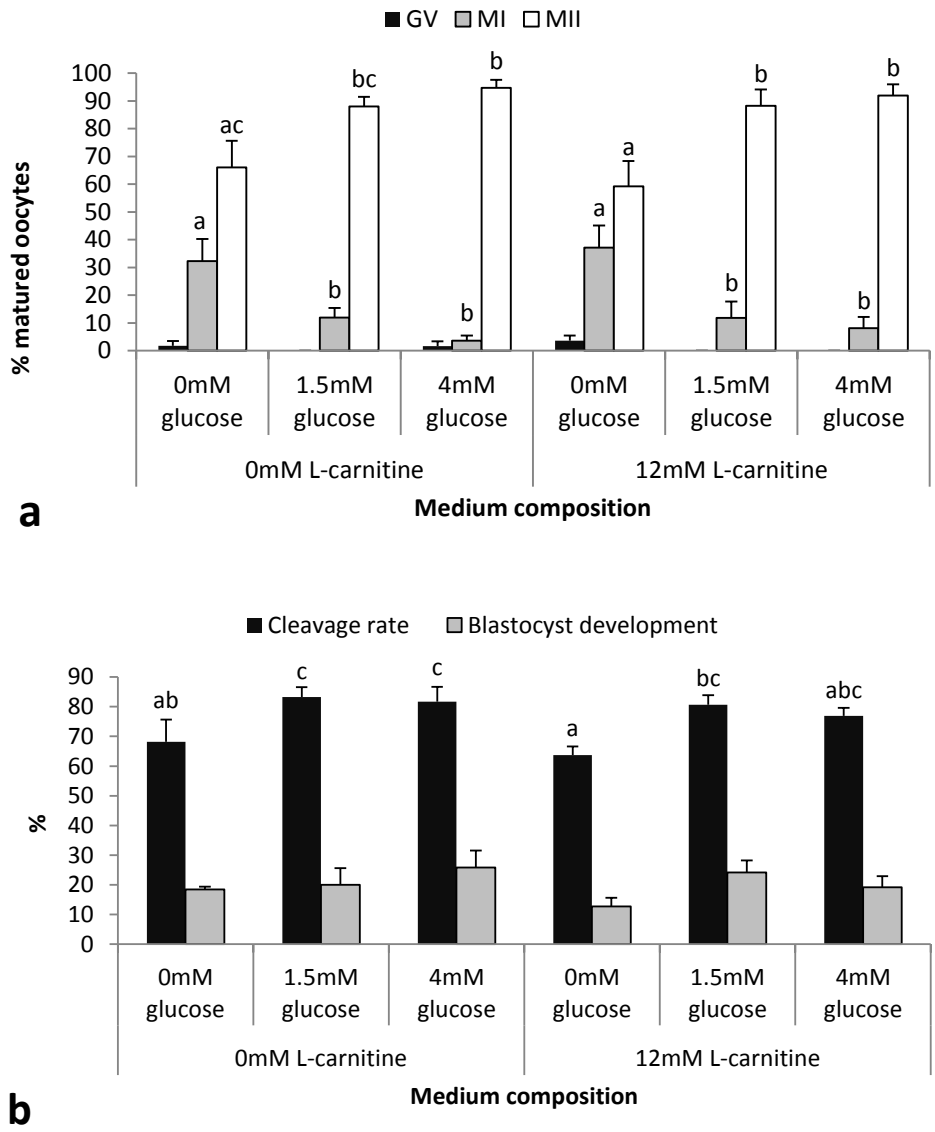


Figure 3.3. Nuclear maturation, cleavage rates and blastocyst development after IVM with pyruvate and lactate, supplemented with 0, 1.5 or 4mM glucose and 0 or 12mM L-carnitine. Mean ± SEM rate of nuclear maturation after 44 h IVM (a), and cleavage and blastocyst development rates following IVF (b). Oocytes were matured in POM with 0.2mM pyruvate and 2mM lactate, supplemented with 0, 1.5 or 4mM glucose and 0 or 12mM L-carnitine. Cleavage data is presented as a percentage of total oocytes inseminated; blastocyst development is presented as a percentage of cleaved embryos. Within GV, MI and MII (a) and cleavage rates and blastocyst development (b), groups represented by bars without a common letter differ significantly ($P<0.05$). Groups without letters have no significant differences ($P>0.05$).

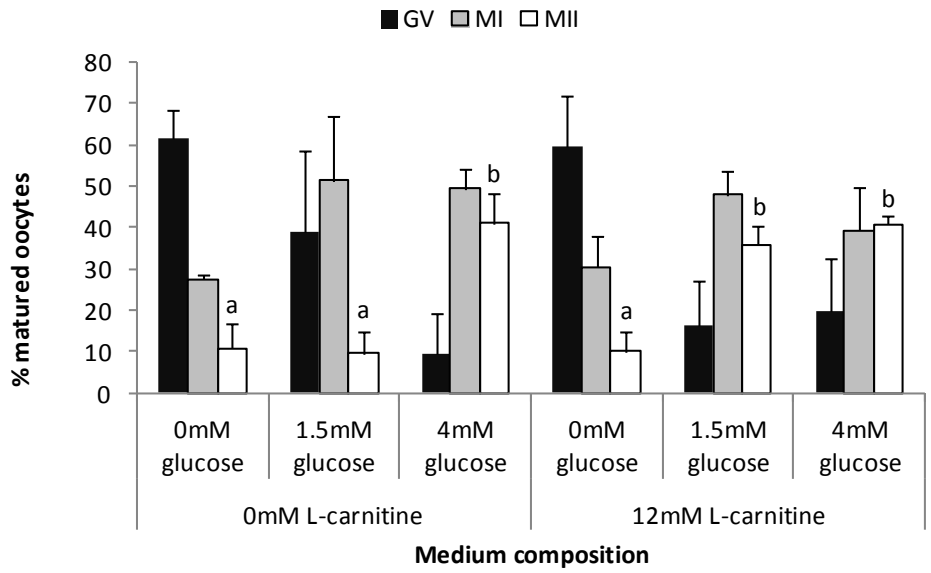


Figure 3.4. Nuclear maturation after 44 h IVM without pyruvate and lactate, supplemented with 0, 1.5 or 4mM glucose and 0 or 12mM L-carnitine. Mean ± SEM rate of nuclear maturation after 44 h IVM. Oocytes were matured in POM without pyruvate and lactate, supplemented with 0, 1.5 or 4mM glucose and 0 or 12mM L-carnitine. Within GV, MI and MII, groups represented by bars without a common letter differ significantly ($P<0.05$).

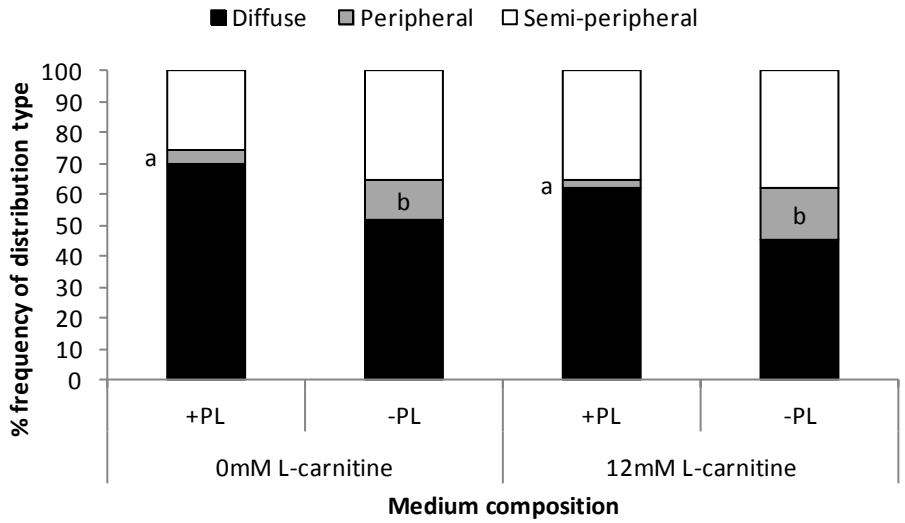


Figure 3.5. Mean frequency of lipid droplet distribution type in MII oocytes after 44 h IVM with 1.5mM glucose, with or without pyruvate and lactate, and treated with 0 or 12mM L-carnitine. Oocytes were matured in modified POM (1.5mM glucose), with (+PL) or without (-PL) 0.2mM pyruvate and 2mM lactate, and treated with 0 or 12mM L-carnitine. Lipid droplets within oocytes were classified as being 'peripheral' (P), where lipid droplets were localised in the peripheral region of the oocyte; 'semi-peripheral' (SP), characterised by lipid droplets found predominantly in the periphery of the oocyte with a low abundance of lipid droplets in the centre of the oocyte; or 'diffuse' (D), with lipid droplets dispersed evenly throughout the cytoplasm. Within P, SP and D, groups without a common letter differ significantly ($P<0.05$).

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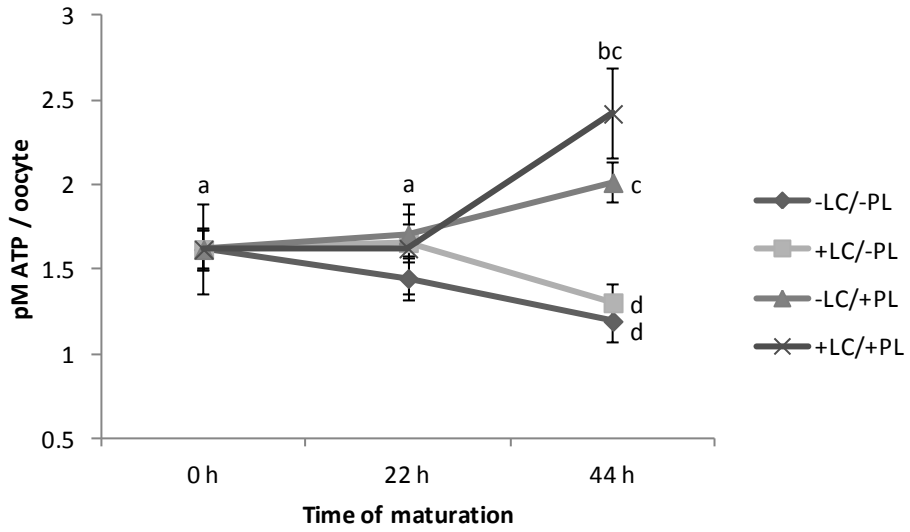

Figure 3.6. ATP concentration in oocytes after 0, 22 and 44 h IVM with 1.5mM glucose, with or without pyruvate and lactate, and treated with 0 or 12mM L-carnitine. Mean ± SEM pM ATP/oocyte at 0, 22 and 44 h IVM. Oocytes were matured in modified POM (1.5mM glucose), with (+PL) or without (-PL) 0.2mM pyruvate and 2mM lactate, and treated with 0 (-LC) or 12mM (+LC) L-carnitine for the duration of maturation. Significant differences (P<0.05) between times within treatments are denoted with the letters a and b. Significant differences (P<0.05) between treatments within times are denoted with the letters c and d.

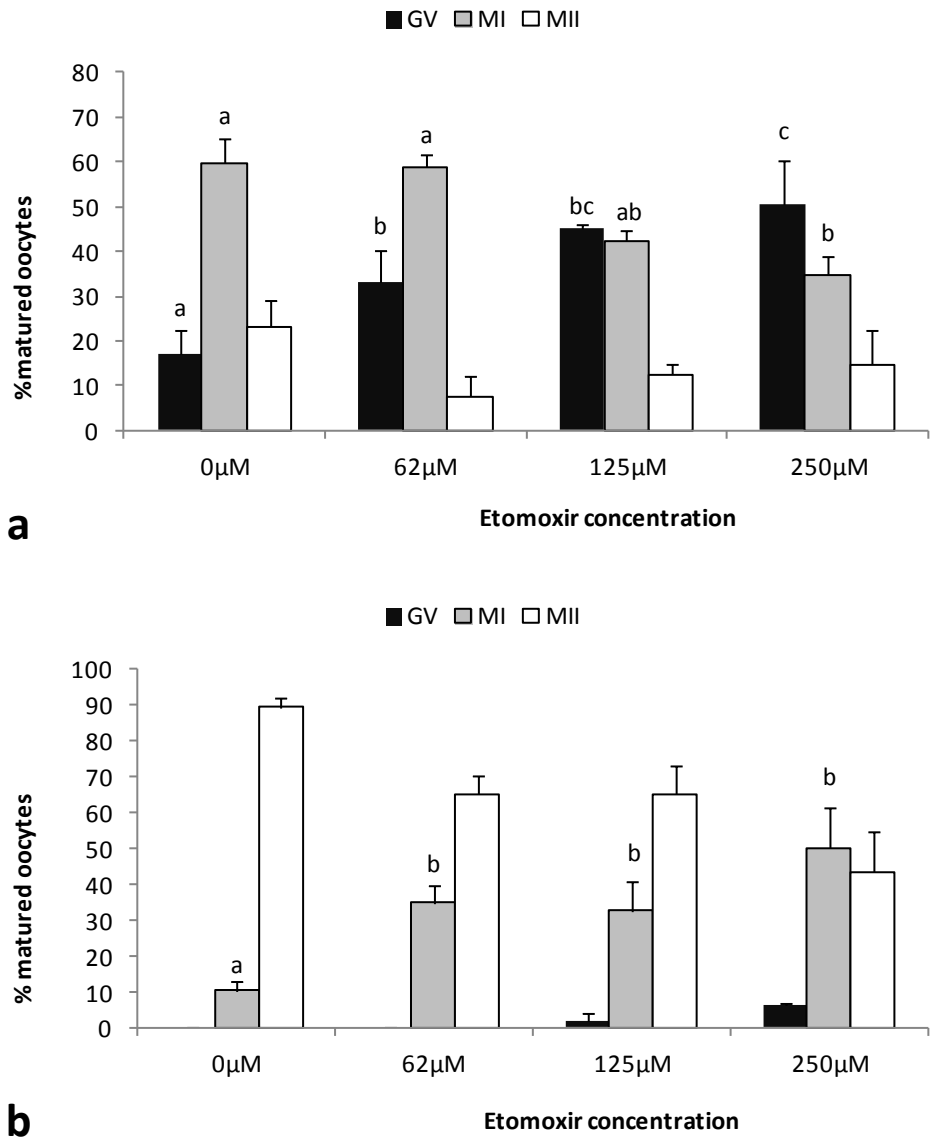

Figure 3.7. Nuclear maturation after 22 and 44 h IVM supplemented with 0, 62, 125 or 250µM etomoxir. Mean ± SEM rate of nuclear maturation after 22 h (a) and 44 h (b) IVM. Oocytes were matured in complete POM (4mM glucose, 0.2mM pyruvate, 2mM lactate), supplemented with 0, 62, 125 or 250µM etomoxir. Within GV, MI and MII, groups represented by bars without a common letter differ significantly ($P<0.05$).

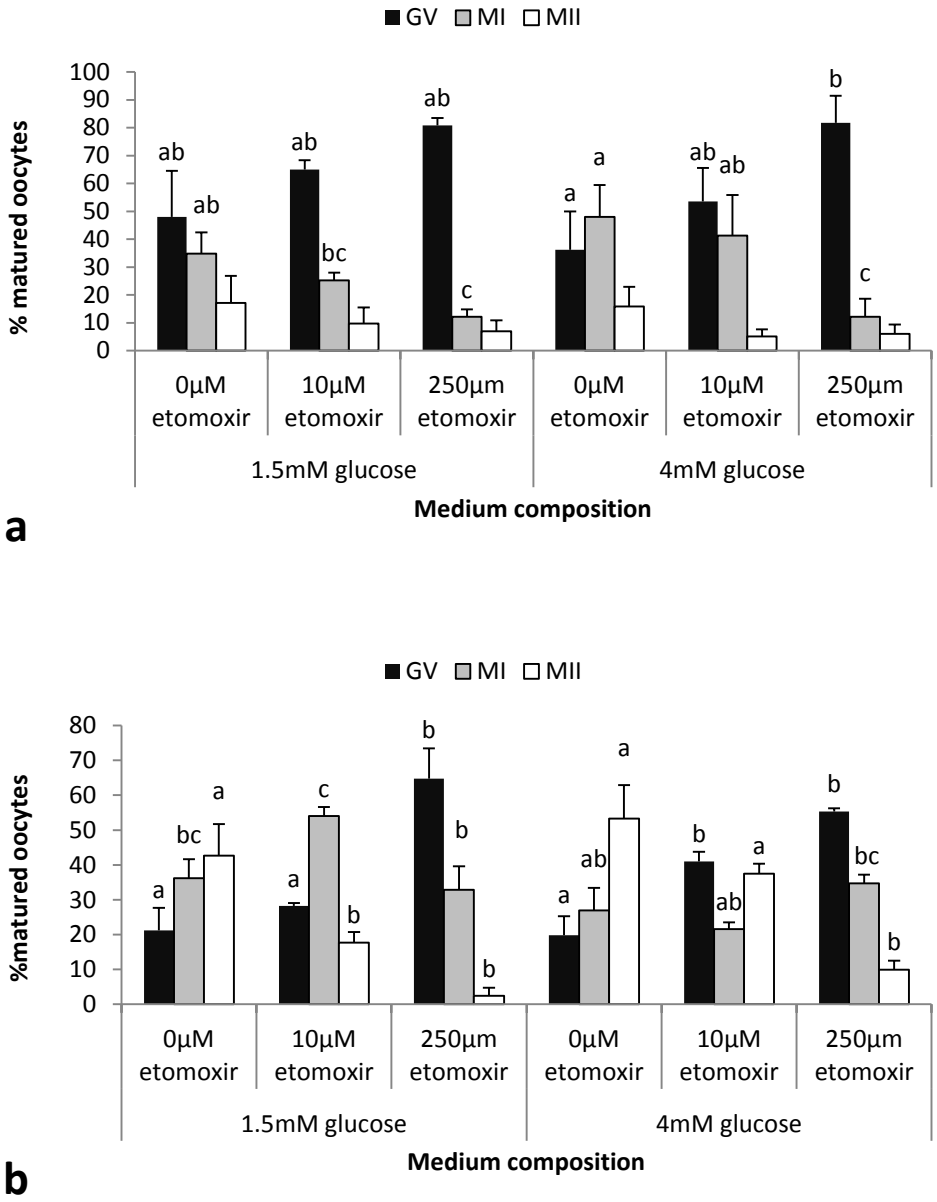

Figure 3.8. Nuclear maturation after 22 and 44 h IVM without pyruvate and lactate, supplemented with 1.5 or 4mM glucose and 0, 10 or 250µM etomoxir. Mean ± SEM rate of nuclear maturation after 22 h (a) and 44 h (b) IVM. Oocytes were matured in POM without pyruvate and lactate, supplemented with 1.5 or 4mM glucose and 0, 10 or 250µM etomoxir. Within GV, MI and MII, groups represented by bars without a common letter differ significantly ($P<0.05$).

3.5. Discussion

This study examined the role of lipid metabolism in energy generation during the in vitro maturation of porcine oocytes. Previous studies have suggested a metabolic role for cytoplasmic lipids (Sturmey and Leese 2003), but the contribution of lipids to ATP production in the presence and absence of carbohydrate substrates has not been examined. The results show that fatty acid oxidation can compensate for insufficient levels of carbohydrate substrates to some degree, although carbohydrate substrates are required for optimal nuclear maturation.

In complete POM containing 4mM glucose, 0.2mM pyruvate and 2mM lactate, upregulation of lipid metabolism with L-carnitine, at concentrations varying from 1.5-12mM, showed no effect on the progression of nuclear maturation. This contrasts with the findings of prior studies (Somfai et al. 2011; Wu et al. 2011) that reported positive effects of L-carnitine on nuclear progression at doses of 3.72-31.02mM and 3.10mM respectively. These two studies utilised NCSU-37 medium, which is formulated without pyruvate and lactate, and with the sole carbohydrate substrate being 5.55mM glucose. A further study (You et al. 2012) showed no effect of 10mM L-carnitine on nuclear progression using TCM-199 medium, which contains 0.91mM pyruvate and 5.55mM glucose. Supporting these results, when pyruvate and lactate were removed from POM, and glucose was supplied at a low (1.5mM) concentration, the addition of 12mM L-carnitine improved meiotic progression to MII. Therefore, in IVM medium that does not contain pyruvate and lactate, such as NCSU-37, treatment with L-carnitine exerts a beneficial effect.

Previous studies on oocyte metabolism and substrate utilisation have shown that both pyruvate and glucose are essential for the resumption of meiosis and acquisition of developmental competence (Krisher and Bavister 1998; Downs and Utecht 1999; Cetica et al. 2002; Herrick et al. 2006; Wang et al. 2012). The roles played by these substrates are not limited to energy production. Activity of the pentose phosphate pathway (PPP) increases throughout IVM in porcine oocytes (Cetica et al. 2002), and provides intermediaries of other metabolic pathways. In cattle oocytes, increasing glucose consumption with constant lactate production

suggests that glycolysis may not be the primary metabolic fate of glucose during maturation (Sutton et al. 2003b). In porcine COCs, there was no increase in glucose consumption observed after treatment with an inhibitor of lipid metabolism (Paczkowski et al. 2013), further supporting that glucose is not metabolised via glycolysis for ATP production at this time. It is likely that there is a higher utilisation of these substrates through the PPP, with a similar ATP content in porcine oocytes regardless of the presence or absence of glucose (Tsuzuki et al. 2008). Further, mouse oocytes exposed to dehydroepiandrosterone (DHEA), an inhibitor of the PPP, had reduced lipid content, citrate levels, and glucose-6-phosphate dehydrogenase activity compared with untreated control oocytes (Jimenez et al. 2013), indicating that metabolism via the TCA cycle is altered in mouse oocytes in which the PPP is inhibited. Given the effect of L-carnitine was only evident in the absence of glucose or pyruvate, this suggests that in vitro matured porcine oocytes require these carbohydrate substrates for the acquisition of developmental competence, despite the partial compensatory contribution of lipid substrates to ATP production. However, while the level of ATP in the cell gives an indication of the available energy, this measurement alone is limited in that it does not indicate the energy requirement of the cell. Measurement of ATP with reciprocal ADP levels provides a measure of energy demand of the cell, and indicates cell viability. Potentially, low energy demand led to unchanged levels of ATP, and future studies should also examine the ATP:ADP ratio.

In POM with 1.5mM glucose and without pyruvate and lactate, 10µM etomoxir inhibited nuclear maturation. This is consistent with the results of an earlier study, in which an inhibitory dose of 10µM etomoxir was observed when using media containing 5mM glucose and 0.01mM pyruvate (Paczkowski et al. 2013). When oocytes were matured in complete POM with 4mM glucose, 0.2mM pyruvate and 2mM lactate, inhibition of lipid metabolism had a minimal effect on nuclear progression. Etomoxir doses of 100µM and 250µM resulted in inhibition of nuclear maturation in cattle and mouse oocytes respectively (Paczkowski et al. 2013). The minimum effective inhibitory doses correlate negatively with the amount of lipid within the ooplasm in these species (Loewenstein and Cohen 1964; McEvoy et al. 2000), suggesting the level of reliance on lipid metabolism differs between species. Further, glucose consumption was increased in both mouse and cattle

COCs, while there was no change in glucose uptake by porcine COCs, in response to the etomoxir treatments (Paczkowski et al. 2013). In that study, however, glucose consumption was only measured in the final 18 h of maturation in porcine oocytes. Progression to MI after 22 h IVM with 10μM etomoxir treatment was 1.6 times higher in groups matured with 4mM glucose compared to 1.5mM glucose, although this was not significantly different. This suggests that glucose utilisation may be greater during the initial 24 h of IVM. Further, treatment with 10μM etomoxir in POM without pyruvate and lactate had a lesser effect in the presence of 4mM glucose compared to 1.5mM glucose. This implies that carbohydrate substrates are required for resumption of meiosis, and that there is a heavier reliance on lipid metabolism when these substrates are deficient.

Cumulus expansion was observed to be reduced in COCs matured without pyruvate or lactate, regardless of glucose supplementation. When pyruvate and lactate were included in the maturation medium, cumulus expansion was reduced only in groups matured without glucose. Cumulus cells metabolise glucose via the hexosamine biosynthesis pathway to produce hyaluronic acid that supports cumulus expansion (Sutton-McDowall et al. 2010), accounting for the poor cumulus expansion seen in oocytes matured in the absence of glucose. Although there are conflicting results regarding the correlation between morphology and expansion of the cumulus mass with nuclear and cytoplasmic maturation (Ali and Sirard 2002; Qian et al. 2003; Luciano et al. 2004; Somfai et al. 2004; Alvarez et al. 2009), cumulus cells are known to be responsible for providing the oocyte with nutrients and other regulatory signals to promote maturation (Sutton-McDowall et al. 2010). Oocytes have a limited ability to utilise glucose, with cumulus cells metabolising the majority of glucose to supply metabolic intermediaries to the oocyte (Biggers et al. 1967). Owing to the metabolic substrates and pathways utilised by cumulus cells, lipids appear to be unable to compensate for carbohydrate deficiencies to support cumulus cell health, even when lipid metabolism is upregulated. Appropriate metabolic support for the cumulus cell mass should also be considered in medium formulations due to their vital relationship with the oocyte.

In POM with 1.5mM glucose containing 0.2mM pyruvate and 2mM lactate, inclusion of L-carnitine promoted a significant increase in oocyte ATP content over the IVM period. During IVM of mouse oocytes, the addition of L-carnitine to media increased the β-oxidation rate (Dunning et al. 2010a). Bovine oocytes morphologically graded as 'good' had significantly higher levels of ATP and developed into blastocysts at higher rates with greater cell numbers than morphologically poor oocytes (Stojkovic et al. 2001). In humans, oocytes with ≥2pM ATP displayed higher rates of embryonic development and implantation (Van Blerkom et al. 1995). Although not seen in this study, previous reports have shown that the lipid content of porcine oocytes decreases throughout maturation (Fu et al. 2011; Romek et al. 2011b; Somfai et al. 2011). Lipids are an energy dense substrate, producing several times the ATP that is generated from the complete oxidation of glucose. In the present study, it is possible that only small amounts of lipid were utilised to increase the ATP content of oocytes, with the decrease in lipid content being outside the range of detection in this study.

After 44 h IVM, oocytes matured with pyruvate and lactate had a higher ATP content than those matured without pyruvate and lactate, despite both groups of oocytes having similar ATP levels after 22 h of IVM. Generation of ATP plays an important role in determining oocyte quality and subsequent blastocyst development. Despite similar levels of ATP after 22 h of IVM, there were two- to three-fold higher rates of oocytes remaining at GV when they were matured without pyruvate and lactate, supporting the proposal that pyruvate is required for the resumption of meiosis (Gonzales-Figueroa and Gonzales-Molfino 2005; Funahashi et al. 2008). There was no effect of L-carnitine treatment during IVM on oocyte ATP content. Gene transcripts relating to lipid metabolism were dysregulated in in vitro matured mouse COCs and the rate of fatty acid oxidation was reduced by 2.8-fold compared to in vivo matured COCs (Dunning et al 2014). Further, inhibition of lipid metabolism in porcine oocytes decreased the relative abundance of gene transcripts involved in fatty acid oxidation, glycolysis and oxidative stress (Paczkowski et al. 2013). This suggests that lipid metabolism is compromised in IVM oocytes, and perturbation of this pathway has negative effects on other metabolic functions within the oocyte. Oocytes matured in vitro

may be unable to utilise lipids effectively to compensate for deficiencies in energy generation caused by insufficient provision of carbohydrate substrates.

In this study, maturation in medium with pyruvate and lactate decreased the proportion of oocytes with peripheral distribution of lipid droplets. This distribution type was also more commonly found at 44 h than at 22 h. These results are consistent with those of prior studies, where oocytes matured in NCSU-23 (5.55mM glucose, 0mM pyruvate and 0mM lactate) had more lipid droplets located in peripheral areas with low abundance in central areas after 44 h IVM (Somfai et al. 2011). Maturation in TCM-199 (5.55mM glucose, 0.91mM pyruvate, 0mM lactate) also showed a similar pattern at 44 h (Sturmey et al. 2006). Supplementation of maturation medium with 12mM L-carnitine had no effect on lipid droplet distribution in this study. In contrast, the addition of L-carnitine increased the proportion of oocytes with lipid droplets located within the central region in porcine oocytes matured in NCSU-23 (Somfai et al. 2011) and cattle oocytes matured in TCM-199 (Chankitisakul et al. 2013). Oocytes with a central distribution of lipid droplets at the end of maturation, rather than dispersed throughout the cytoplasm, had a higher blastocyst development rate following IVF (Hiraga et al. 2013). Analysis using fluorescence resonance energy transfer suggests a molecular association between lipid droplets and mitochondria (Sturmey et al. 2006). In porcine oocytes, active mitochondria translocate from peripheral to central regions of the oocytes during meiotic progression (Sun et al. 2001; Krisher et al. 2007), indicating that centrally distributed lipid droplets may be more closely associated with mitochondria. Inhibition of the PPP inhibited migration of mitochondria in cattle oocytes (Gutnisky et al. 2013), while in porcine oocytes glucose metabolism through the PPP has been shown to be a stimulus for meiotic resumption and progression of nuclear maturation (Tubman et al. 2005; Funahashi et al. 2008). With the low glucose concentration (1.5mM) used in this study, PPP rate may have been reduced, resulting in improper maturation and leading to altered mitochondrial and lipid droplet translocation. Further studies should examine the effect of glucose and pyruvate concentrations on lipid droplet and mitochondrial distribution and activity.

This study has demonstrated that the carbohydrate composition of media alters the effect of L-carnitine treatment, which stimulates lipid metabolism, on oocyte maturation. The differential effects of L-carnitine supplementation observed in the various experimental groups of the present study, in which glucose, pyruvate and lactate levels were manipulated in POM, explains the inconsistent findings of previous studies reporting either a positive or negligible effect of L-carnitine on porcine oocyte maturation. When L-carnitine was added to media containing standard levels of pyruvate, lactate and glucose, there was no effect on nuclear maturation or subsequent embryo development. However, L-carnitine supplementation in media without pyruvate and lactate and a low glucose concentration improved nuclear maturation and led to a greater increase in oocyte ATP content over the course of IVM. In the absence of glucose, there was no effect of L-carnitine treatment, regardless of the presence or absence of pyruvate and lactate. Inhibiting lipid metabolism had a greater detrimental effect on nuclear maturation in the absence of pyruvate and lactate when glucose was supplied at a low concentration. Together, these findings suggest that endogenous lipid substrates can to some extent compensate for carbohydrate deficiencies, and the effect of upregulating lipid metabolism varies with the presence or absence of carbohydrate substrates. Prior studies have identified glucose and pyruvate as required media components for porcine oocytes (Funahashi et al. 2008), and this conclusion is supported by this study. These results suggest optimal IVM media composition should contain a minimum 4mM glucose and 0.2mM pyruvate, as supplied in POM, to meet metabolic requirements to support oocyte maturation. Treatment with L-carnitine appears to only be effective in media with carbohydrate concentrations lower than these levels. Understanding the roles that lipid and carbohydrate substrates play in energy generation during porcine oocyte maturation will allow for continuing refinements to culture media formulations that better support oocyte metabolic requirements in vitro.

CHAPTER 4. THE EFFECT OF L-CARNITINE IMMEDIATELY PRIOR TO AND DURING IN VITRO FERTILISATION ON PORCINE EMBRYO DEVELOPMENT AND CRYOSURVIVAL

4.1. Abstract

The ability of oocytes to generate sufficient ATP is a critical factor to ensure proper fertilisation and embryo development, as decreased ATP content at fertilisation is detrimental to developmental competency. This study utilised L-carnitine, a co-factor of β-oxidation, to examine the role of lipid metabolism immediately prior to and during fertilisation. In vitro matured oocytes were fertilised in IVF medium (TALP) supplemented with 3, 6, 12 or 24mM L-carnitine for the duration of co-culture with spermatozoa. The proportion of oocytes fertilised was higher in the control group compared with the 6-24mM groups ($P<0.05$). Cleavage was similar amongst groups ($P>0.05$), while blastocyst development was reduced with 6-24mM L-carnitine. Subsequently, 3mM L-carnitine was included for either 1 h incubation of oocytes prior to co-culture; during the first 30 min of gamete co-culture; during the subsequent 5 h co-culture; for the entire co-culture period; included in the medium used for spermatozoa preparation; or not added for the duration of IVF (control). All groups had similar rates of fertilisation ($P>0.05$). The cleavage rate was increased in the group incubated in medium supplemented with L-carnitine for 1 h prior to insemination ($P<0.05$) compared with the other oocyte treatment groups, while blastocyst development rate and cell number were similar amongst groups ($P>0.05$). Finally, ATP content and post-thaw survival following vitrification were compared between groups incubated with or without 3mM L-carnitine for 1 h prior to insemination. The ATP content of oocytes was similar among groups following the 1 h incubation ($P>0.05$), and this was similar to the ATP content seen prior to incubation ($P>0.05$). Post-thaw survival was increased in embryos derived from oocytes incubated in L-carnitine ($P<0.05$). In conclusion, incubation of oocytes in

fertilisation medium supplemented with 3mM L-carnitine for 1 h prior to insemination increased cleavage rates, but not blastocyst development, and improved cryosurvival following vitrification. Further studies are required to determine if this is due to increased lipid metabolism within oocytes, or attributable to the antioxidant properties of L-carnitine.

4.2. Introduction

At the time of fertilisation, there is an increased adenosine triphosphate (ATP) requirement to support calcium oscillations and calcium homeostasis (Dumollard et al. 2004). In aged oocytes, noted for having poor developmental outcomes, deficient ATP content at fertilisation is believed to lead to altered calcium oscillation patterns and homeostasis (Igarashi et al. 2005). Sufficient ATP is also required to support cellular events triggered by fertilisation, including polymerisation of microtubules, cell cycle regulation, segregation of chromosomes and membrane biosynthesis (Chappel 2013). ATP production and accumulation during oocyte maturation is likely to be an important factor for fertilisation. Dysfunctional or low numbers of mitochondria, resulting in lower ATP content of oocytes at fertilisation, has been shown to lead to a higher incidence of fertilisation failure (Van Blerkom et al. 1995; May-Panloup et al. 2005; Swain and Pool 2008; Chappel 2013). Cattle oocytes morphologically graded as being of higher quality had a higher ATP content following IVM and had increased blastocyst development following IVF (Stojkovic et al. 2001). Further, the ATP content of both mice and human oocytes has been positively correlated with embryo viability (Ginsberg and Hillman 1973; Quinn and Wales 1973; Van Blerkom et al. 1995). In mouse oocytes, ATP content peaks at the time of extrusion of the first polar body, with MII stage oocytes consuming greater amounts of ATP than those arrested at GV (Dalton et al. 2014). Further, in aged porcine oocytes, the electron density of lipid droplets decreased over time to 56 h IVM (Hao et al. 2009). This higher consumption of ATP by MII stage oocytes, coupled with decreasing lipid stores, suggests that oocytes may be utilising lipid substrates during MII arrest prior to fertilisation.

In toad eggs, fertilisation triggers a decrease in triglycerides, hypothesised to be due to metabolic breakdown of lipid substrates for ATP production to meet increased energy requirements (Alonso et al. 1986). Porcine oocytes contain large amounts of endogenous lipid (McEvoy et al. 2000), and although they differ physiologically from toad eggs, this lipid fraction may play a similar role. Also, following sperm penetration of porcine oocytes, electron density of lipid droplets decreases, implying a reduction of lipid content at this time (Kikuchi et al. 2002a). Given that lipids are a very efficient fuel source, it may be that the β-oxidation pathway is utilised during fertilisation.

The impact of supplementing IVF medium with metabolic regulators is poorly understood. L-carnitine is a key co-factor involved in the carnitine shuttle, with this being the rate-limiting step in the entry of activated fatty acids to the mitochondrial matrix for metabolic breakdown, and is also known to have strong antioxidant properties. L-carnitine has also been shown to have an effect on in vitro sperm parameters. The addition of 1.76mM L-carnitine to testicular mouse spermatozoa increased motility and chromatin quality following 30-180 min incubations at room temperature (Aliabadi et al. 2012). Further, inclusion of 3.1mM L-carnitine led to higher human sperm motility after 2 h incubation at 37^{o}C, although there was no effect on DNA oxidation or sperm viability (Banihani et al. 2012). The positive effects on sperm are believed to be due to the antioxidant activity of L-carnitine. Accumulation of reactive oxygen species (ROS) in spermatozoa causes ATP depletion, lipid peroxidation and axonemal phosphorylation (Dokmeci 2005), affecting sperm motility.

Upregulation of the β-oxidation pathway by using L-carnitine during IVM has been shown to improve rates of nuclear maturation in mice (Dunning et al. 2010a), cattle (Ferguson and Leese 2006) and porcine oocytes (Somfai et al. 2011; Wu et al. 2011). Inclusion of L-carnitine in culture media decreased lipid content and increased cryosurvival in cattle embryos (Sutton-McDowall et al. 2012). It is believed that by stimulating lipid metabolism, greater amounts of lipid stores are used to generate ATP to support development while also improving cryotolerance due to decreased lipid content. Potentially, stimulating lipid metabolism during fertilisation may increase ATP levels to support requirements during fertilisation.

Any beneficial effect seen from inclusion of L-carnitine during IVF would need to be clarified as affecting spermatozoa or oocytes.

The aim of this study was to determine if L-carnitine supplementation either during or immediately prior to fertilisation of IVM porcine oocytes enhances fertilisation and developmental outcomes. Further, this study examined the effect of L-carnitine supplementation in IVF medium on embryo cryosurvival following vitrification.

4.3. Materials and methods

4.3.1. In vitro fertilisation

Oocytes were matured in vitro as described in section 2.1. Mature oocytes were in vitro fertilised as described in section 2.2. For experiments with a pre-IVF incubation period, oocytes were held in treatment medium for 1 h, and then transferred to the fertilisation medium with or without L-carnitine (as per experimental design) immediately prior to insemination.

4.3.2. Evaluation of fertilisation status

To assess fertilisation, presumptive zygotes were fixed in acetic acid:ethanol (1:3) 12 h after insemination for a minimum 3 d at room temperature. Following fixation, zygotes were slide mounted and stained with 1% (w/v) orcein in 45% acetic acid for 1 h, destained with glycerol:acetic acid:water (1:1:3) and examined using phase-contrast microscopy at 400x magnification. Zygotes were classified as unfertilised (meiotic spindle and one polar body present), polyspermic (multiple pronuclei and/or multiple decondensed sperm heads present), penetrated (one pronuclei and/or decondensed sperm head present), or fertilised (two pronuclei with two polar bodies present).

4.3.3. Experimental design

Experiment 1: L-carnitine dose-response during gamete co-incubation

TALP-PVA fertilisation medium was supplemented with 0, 3, 6, 12 or 24mM L-carnitine for the entire 5.5 h duration of co-culture. Approximately 15 presumptive zygotes per treatment group were fixed 12 h after insemination, and stained to assess sperm penetration and pronuclear formation. The remaining zygotes were cultured and assessed for cleavage, blastocyst development and embryo cell number. Four replicate experiments were performed with 30 to 45 presumptive zygotes used per treatment group within each replicate.

Experiment 2: Effect of L-carnitine during sperm pre-IVF treatment and gamete co-incubation

IVF was conducted with the addition of 3mM L-carnitine for defined periods. This dose was selected based on the results of experiment 1. Oocytes were subjected to a 1 h incubation prior to insemination, and then co-incubated with spermatozoa, either with or without L-carnitine supplementation as defined in Table 4.1. Spermatozoa were prepared in two batches, in standard spermTALP or spermTALP supplemented with 3mM L-carnitine. Approximately 15 presumptive zygotes per treatment group were fixed 12 h after insemination, and stained to assess fertilisation. The remaining zygotes were cultured and assessed for cleavage, blastocyst development and embryo cell number. Four replicate experiments were performed with 30 to 45 presumptive zygotes used per treatment group within each replicate.

Experiment 3: Effect of L-carnitine treatment on ATP content of oocytes and post-thaw survival of resulting blastocysts

Oocytes were fertilised in vitro after a 1 h incubation in TALP-PVA supplemented with 3mM L-carnitine followed by co-incubation in standard TALP, or without L-carnitine supplementation for the entire duration. Oocytes were removed and assayed to determine ATP content after 44 h maturation, and again following the 1 h incubation. The remaining oocytes were fertilised, cultured and assessed for cleavage and blastocyst development, with grade 1 blastocysts vitrified on Day 7. Survival was assessed 24 h after thawing. Four replicate experiments were performed with n=44 and n=37 embryos vitrified for the L-carnitine treated and untreated control groups respectively.

4.3.4. Statistical analysis

All analyses were conducted using GenStat (v. 16, VSN International Ltd). ATP content, cleavage, blastocyst development and embryo cell number data were analysed by one-way analysis of variance (ANOVA). Fisher's protected LSD post-hoc test was used when significant differences between treatments were detected. Survival data was analysed by Generalised Linear Mixed Model (GLMM). For all analyses, $P<0.05$ was considered significant.

Table 4.1. Experiment 2 design. LC = supplementation of media with 3mM L-carnitine; 0 = media used as standard. Sperm = sperm preparation in spermTALP media. All oocyte incubations and gamete co-incubations were conducted in TALP-PVA media.

Group	Sperm	1h incubation	30m co-incubation	5h co-incubation
1	LC	0	0	0
2	0	LC	0	0
3	0	0	LC	0
4	0	0	0	0
5	0	LC	LC	LC

4.4. Results

Experiment 1: L-carnitine dose-response during gamete co-incubation

The proportion of fertilised oocytes was higher in the control group compared with the groups fertilised in medium supplemented with 6-24mM L-carnitine (41.43% vs 0-25.00%; $P<0.05$). Cleavage rates were similar amongst treatments ($P>0.05$). Assessment of fertilisation by staining at 12 h post-insemination coincides with the time of pronuclear formation in normally fertilised oocytes. While 0% fertilisation was observed in certain treatment groups, this more correctly means that 0% of oocytes had formed pronuclei normally 12 h after IVF. Cleavage rates, as noted in Figure 4.1, suggests that delayed or abnormal fertilisation and pronuclear formation subsequently occurred in some of the oocytes cultured for 7 d. Further,

a small proportion of oocytes may have undergone cytoplasmic fragmentation, which is difficult to distinguish from early embryonic cleavage without staining. Blastocyst development was reduced with the addition of 6-24mM L-carnitine relative to the control ($P<0.05$, Figure 4.1). Blastocyst cell numbers did not differ with treatment (36-72 cells/blastocyst; $P>0.05$).

Based on these results, 3mM L-carnitine was selected for use in subsequent experiments, as higher doses had a detrimental effect on fertilisation and blastocyst development rates.

Experiment 2: Effect of L-carnitine during sperm pre-IVF treatment and gamete co-incubation

The period of L-carnitine supplementation during IVF had no effect on the fertilisation status of oocytes (10.36-30.36%; $P>0.05$). The cleavage rate of oocytes treated with a 1 h pre-IVF incubation in 3mM L-carnitine tended to be higher than those of oocytes in other treatment groups ($P=0.10$, Figure 4.2). Blastocyst development rates (7.40-21.00%, Figure 4.2) and average cell numbers (61-103 cells per blastocyst) were similar amongst groups ($P>0.05$).

Experiment 3: Effect of L-carnitine treatment on ATP content of oocytes and post-thaw survival of resulting blastocysts

ATP content was similar between oocytes before incubation and after incubation ($P>0.05$, Figure 4.3). There was no difference in ATP content of oocytes incubated with or without L-carnitine ($P>0.05$, Figure 4.3). Post-thaw survival was increased in embryos derived from oocytes pre-incubated in medium with L-carnitine prior to IVF (42%, n=44 embryos vitrified) compared to embryos derived from oocytes pre-incubated without L-carnitine (25%, n=37 embryos vitrified; $P<0.05$).

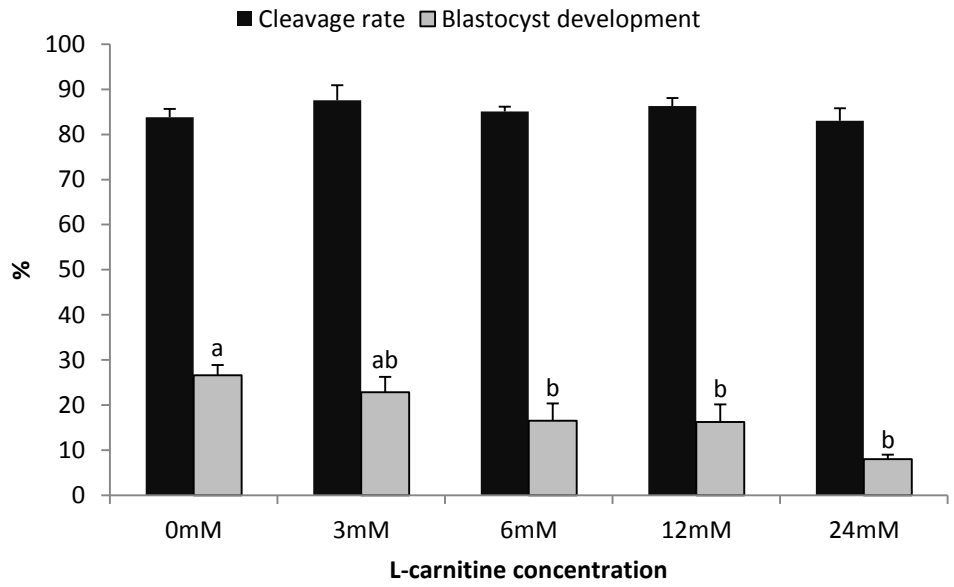

Figure 4.1. Mean ± SEM rates of cleavage and blastocyst development for porcine oocytes fertilised in vitro in TALP-PVA medium supplemented with 0, 3, 6, 12 or 24mM L-carnitine for the duration of gamete co-culture. Cleavage data is presented as a percentage of total oocytes inseminated; blastocyst development is presented as a percentage of cleaved embryos. Within cleavage rate and blastocyst development, groups represented by bars without a common letter differ significantly ($P<0.05$).

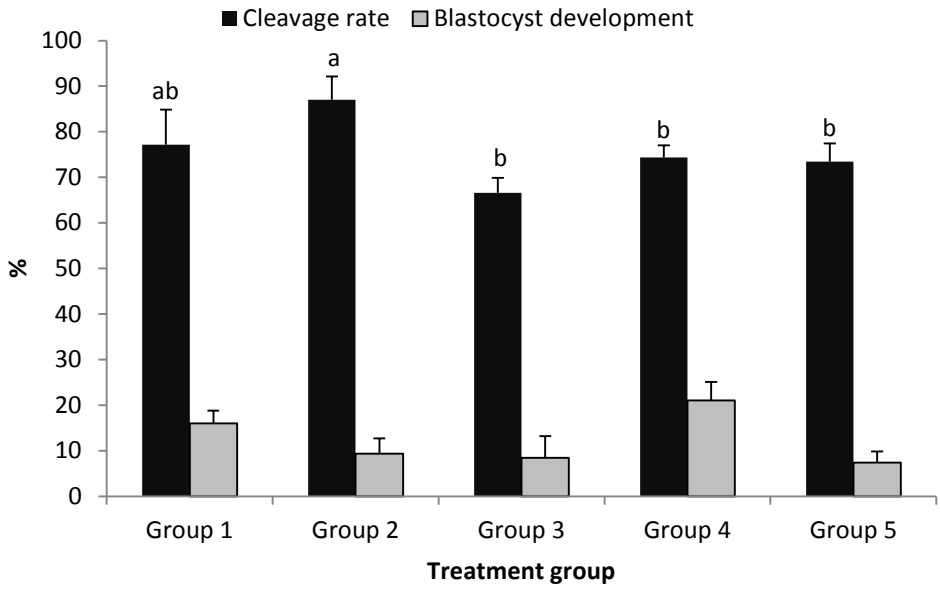

Figure 4.2. Mean ± SEM cleavage rates and blastocyst development of porcine oocytes fertilised in vitro in TALP-PVA medium with L-carnitine supplementation either for 1 h prior to co-culture; the initial 30 min of gamete co-culture; for the duration of gamete co-culture; no L-carnitine supplementation; or L-carnitine supplementation of sperm preparation medium. Group 1 = 3mM L-carnitine supplementation of sperm preparation medium; Group 2 = 3mM L-carnitine supplementation of TALP-PVA for 1 h prior to insemination; Group 3 = 3mM L-carnitine supplementation during the initial 30 min co-culture with spermatozoa; Group 4 = no L-carnitine supplementation; Group 5 = 3mM L-carnitine supplementation for the duration of co-culture. Cleavage data is presented as a percentage of total oocytes inseminated; blastocyst development is presented as a percentage of cleaved embryos. Within cleavage rate and blastocyst development, groups represented by bars without a common letter differ significantly (P=0.10).

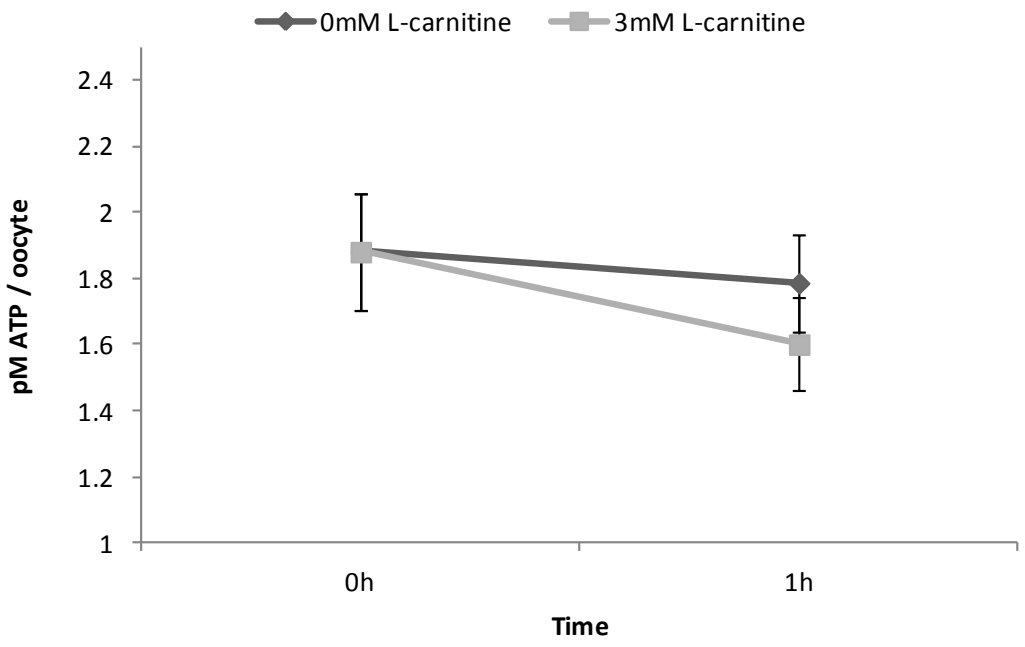

Figure 4.3. ATP concentration in oocytes before and after a 1 h incubation in fertilisation medium supplemented with or without L-carnitine. Mean ± SEM pM ATP/oocyte before and after incubation in TALP-PVA medium supplemented with or without 3mM L-carnitine for 1 h.

4.5. Discussion

This study examined the potential of L-carnitine treatments immediately prior to and during IVF to elevate ATP levels in porcine oocytes and thereby improve fertilisation outcomes. The capacity of oocytes and early stage embryos to generate ATP has been correlated with developmental competence and embryo development in cattle, mice and humans (Ginsberg and Hillman 1973; Quinn and Wales 1973; Van Blerkom et al. 1995; Stojkovic et al. 2001; Nagano et al. 2006). L-carnitine treatments during in vitro maturation have previously been found to increase ATP levels in mouse oocytes (Dunning et al. 2010a). However, the results of this study indicate that the relatively brief L-carnitine treatment did not alter ATP levels significantly or improve fertilisation. Rather, when included for the duration of gamete co-culture, supplementation of 6-24mM L-carnitine exerted detrimental effects on fertilisation.

Incubation of oocytes in fertilisation medium supplemented with L-carnitine for 1 h prior to gamete co-incubation increased the post-thaw survival of vitrified Day 7 blastocysts. Porcine oocytes and embryos have large amounts of intracellular lipid droplets (Youngs et al. 1994; McEvoy et al. 2000), which is known to be the reason for their poor cryotolerance (Dobrinsky and Johnson 1994; Nagashima et al 1995; Abe et al. 2002; Sudano et al. 2011). Prior studies have shown that inclusion of L-carnitine during bovine embryo culture decreased lipid content (Sutton-McDowall et al. 2012) and increased cryosurvival (Ruiz et al. 2013; Takahashi et al. 2013). The results of this study show that only a short exposure to L-carnitine significantly improved the cryotolerance of resulting blastocysts. Future studies are required to determine if a decrease in lipid content is the reason for the improved cryotolerence, and the optimum L-carnitine incubation time to maximise post-thaw survival.

Embryo cleavage rates were increased following IVF of oocytes incubated with L-carnitine for 1 h prior to the addition of spermatozoa. Mitochondrial ATP is essential for sustaining calcium oscillations triggered by fertilisation events, which then subsequently upregulate ATP production to satisfy energy demands (Dumollard et al. 2004; Campbell and Swann 2006). In humans, total carnitine

content of follicular fluid was not correlated to embryo developmental outcomes following IVF, although enzymes involved in β-oxidation were strongly expressed in mature oocytes and the expression of enzymes involved in carnitine synthesis was not detected (Montjean et al. 2012). Given this, the addition of exogenous L-carnitine may aid in upregulating β-oxidation rates in the oocyte. Higher ATP content in mature oocytes has been correlated to greater developmental success (Van Blerkom et al. 1995; Stojkovic et al. 2001), with the majority of ATP derived from oxidative phosphorylation (Dumollard et al. 2004; Igarashi et al. 2005; Dalton et al. 2014). Acetyl-CoA, produced via β-oxidation, is metabolised through the TCA cycle to produce NADH and $FADH_2$, both of which are then fully metabolised via oxidative phosphorylation to produce ATP. However, in this study, there was no associated increase in ATP content of oocytes following incubation in medium supplemented with L-carnitine.

Other studies have found no correlation between ATP content of oocytes and developmental competence (Combelles and Albertini 2003; Brevini et al. 2005). In some homozygous mouse strains, excessive ATP production and increased cytoplasmic lipid deposition is associated with early cleavage arrest (Ginsberg and Hillman 1975). It is possible that rather than a critical level of ATP being required to support fertilisation, the higher ATP content is indicative of a healthy, functional mitochondria population necessary for embryo development. Measurement of ATP:ADP ratio would be beneficial for future studies to indicate cellular energy demand and cell viability. Current media formulations do not contain co-factors to support β-oxidation. In humans, IVF patients with higher reproductive potential have decreased levels of total carnitine in serum and follicular fluid, suggesting upregulation of lipolytic pathways and depletion of carnitine stores in these patients (Varnagy et al. 2013). Expression of carnitine synthesis enzymes is low in human oocytes and early embryos, while β-oxidation enzymes are strongly expressed (Montjean et al. 2012), supporting a role for the supplementation of exogenous carnitine in embryo culture media.

Alternatively, the beneficial effect of L-carnitine may be attributable to its strong antioxidant properties. Markers of oxidative stress in human follicular fluid have been correlated to fertilisation and pregnancy outcomes following IVF (Sabatini et

al. 1999; Oyawoye et al. 2003; Pasqualotto et al. 2004), with higher fertilisation success seen in oocytes recovered from follicular fluid with higher antioxidant levels (Oyawoye et al. 2003). Addition of L-carnitine to medium for the duration of porcine oocyte maturation decreased levels of intracellular ROS, hydrogen peroxides and lipid peroxides, reduced apoptosis, and increased GSH concentrations (Hashimoto et al. 2008; Somfai et al. 2011; Wu et al. 2011; You et al. 2012; Yazaki et al. 2013). Oxidative stress is proposed to be the catalyst for post-ovulatory aging (Lord and Aitken 2013), which is associated with reduced fertilisation rates and poor embryo quality. Certain compounds with antioxidative activity have been shown to delay signs of post-ovulatory aging (Kuo et al. 2000; Goud et al. 2008; Lord et al. 2013). The antioxidative properties of L-carnitine may have assisted in maintaining oocyte developmental competence prior to fertilisation. However, fertilisation rates were decreased following inclusion of 6-24mM L-carnitine for the duration of gamete co-culture. High concentrations of antioxidants included in medium during bovine IVF decreased cleavage and blastocyst rates (Ali et al. 2003). Reactive oxygen species are required for fertilisation (Miesel et al. 1993; Fujii and Tsunoda 2011), playing a role in sperm function, sperm-oocyte interactions and sperm capacitation (de Lamirande et al. 1998; de Lamirande and O'Flaherty 2008). This indicates that higher doses or longer exposure to L-carnitine may be detrimental during IVF due to its antioxidant properties suppressing the concentrations of vital ROS. Further research is required to determine the optimal concentration and incubation time to maximise the beneficial effects of L-carnitine at fertilisation.

Although there was no beneficial effect on fertilisation, cleavage or blastocyst development rate when sperm preparation medium was supplemented with L-carnitine, prior research suggests that this is an area for future research. Spermatozoa have a low antioxidant defence capacity and are particularly susceptible to oxidative damage due to a high lipid content of membranes (Saleh and Agarwal 2002). Incubation in L-carnitine has been shown to increase motility and chromatin quality of testicular mouse spermatozoa (Aliabadi et al. 2012), as well as motility in human spermatozoa (Banihani et al. 2012). However, given that cleavage rates were increased in oocytes incubated in L-carnitine prior to fertilisation with spermatozoa prepared without L-carnitine, there is clearly a

beneficial effect of L-carnitine on oocytes as well as spermatozoa, and the individual effects of L-carnitine on male and female gametes needs to be further clarified with future studies.

In conclusion, incubation of oocytes in fertilisation medium supplemented with 3mM L-carnitine for 1 h prior to co-culture with sperm increased oocyte developmental competency and post-thaw survival of blastocysts. Inclusion of L-carnitine for any period of gamete co-culture had no effect on fertilisation rates or further development. Fertilising ability of spermatozoa was unaffected by L-carnitine supplementation during preparation. These findings indicate that there is little benefit of L-carnitine supplementation immediately prior to or during IVF in terms of ATP levels. Therefore, it appears that the antioxidant properties of L-carnitine are beneficial for the maintenance of oocyte viability, although not necessarily for sperm functionality, during the pre-insemination period. Detailed in vitro assessments are needed to determine the influence of the L-carnitine treatment on sperm parameters.

CHAPTER 5. L-CARNITINE SUPPLEMENTATION DURING IN VITRO CULTURE OF PORCINE EMBRYOS IMPROVES BLASTOCYST QUALITY AND CRYOSURVIVAL

5.1. Abstract

Although porcine oocytes and embryos contain large amounts of endogenous lipid, little is known regarding its metabolic role during development. The objective of this study was to investigate the role of lipid metabolism and the interaction between carbohydrate and lipid substrates in pre-implantation porcine embryos. Abattoir-derived porcine COCs were cultured and fertilised in vitro. Post-fertilisation, embryos were transferred into PZM-3 medium supplemented with L-carnitine, a co-factor in entry of fatty acids into mitochondrial matrix for metabolic breakdown via β-oxidation. In standard PZM-3 medium, 3mM L-carnitine improved cleavage rates ($P<0.05$); when supplemented in modified PZM-3 medium with no carbohydrate substrates, all doses from 1.5-12mM L-carnitine increased cleavage rates ($P<0.05$). Embryos cultured in standard PZM-3 medium had lower blastocyst cell numbers when compared to those cultured in sequential culture medium ($P<0.05$). The addition of 3mM L-carnitine for the first 3 d of culture in standard PZM-3 restored blastocyst cell numbers to the levels observed in the sequential medium. Post-thaw survival was increased when embryos were cultured in a single medium containing L-carnitine for 7 d, and a sequential media containing L-carnitine for the first 3 d only, compared with the single medium, untreated control ($P<0.05$). In conclusion, a greater effect of L-carnitine was seen in the absence of carbohydrates, suggesting a preference for carbohydrate substrates for metabolic requirements in pre-implantation porcine embryos. Supplementation of L-carnitine for the first 3 d of embryo culture improved embryo quality and improved cryosurvival, suggesting that the treatment decreased intracellular lipid content via increased β-oxidation.

5.2. Introduction

The in vitro production (IVP) of porcine embryos is an important technology for biomedical research purposes, as well as permitting the application of advanced reproductive technologies (ARTs), including vitrification and embryo transfer, to support commercial animal breeding programs. However, in vitro derived porcine embryos display low developmental rates and are generally of poorer quality compared with in vivo-derived embryos (Thompson 1997; Leese et al. 1998; Machaty et al. 2000; Yoshioka et al. 2002), limiting these applications. Major differences seen between in vitro and in vivo embryos include altered metabolic processes (Thompson 1997; Durkin et al. 2001) and changes in lipid droplet morphology, size and number (Plante and King 1994; Kikuchi et al. 2002a). These differences are likely to contribute to variations in developmental competence and quality.

While common media preparations include exogenous carbohydrate substrates to support energy requirements, there is evidence that embryos are capable of utilising endogenous lipid reserves for ATP production. There is a decrease in fatty acid content (Romek et al. 2009; Romek et al. 2011a), with a corresponding increase in O_2 consumption (Sturmey and Leese 2003) during development of porcine embryos to the blastocyst stage. Further, the addition of L-carnitine, a stimulant of lipid metabolism, to murine (Dunning et al. 2010a) and bovine (Sutton-McDowall et al. 2012) embryo culture media improved embryo development. Porcine embryos have a higher intracellular lipid content compared to those of other production animal species (McEvoy et al. 2000), with in vitro produced embryos having a greater lipid content than in vivo derived embryos (Kikuchi et al. 2002a). Furthermore, lipid content is correlated with poor cryosurvival (Abe et al. 2002) limiting the application of vitrification in porcine embryos.

Embryos have changing nutrient preferences associated with different developmental stages. Many culture systems rely on single medium formulations, with energy substrates unchanged for the duration of culture. While glucose, pyruvate and lactate have been identified as key carbohydrate substrates

supporting embryo development (Leese 1995; Gardner et al. 1998), there is evidence that substrate preference changes with the stage of development. In porcine embryos, glucose inhibits development when included in culture medium during early cleavage stages, while glucose consumption increases at the blastocyst stage (Flood and Wiebold 1988; Sturmey and Leese 2003). Sequential medium systems have been developed to better meet the energy requirements of the embryo, with altered concentrations of carbohydrate substrates provided for different stages of development. Such systems have led to increased blastocyst yields and improved blastocyst quality by including pyruvate and lactate for the first 48 h following fertilisation, and glucose for the remainder of the culture period (Kikuchi et al. 2002b; Kim et al. 2004; Beebe et al. 2007).

Previous studies in mouse and cattle embryos have suggested a role for lipid metabolism during in vitro embryo culture. In the absence of exogenous glucose, pyruvate, lactate or an extracellular protein source, the addition of 5mM L-carnitine to culture medium increased zygote cleavage to the 2-cell stage in IVF mouse embryos (Dunning et al. 2010a). For cattle embryos, supplementing complete culture medium (containing 0.5mM glucose, 0.35mM pyruvate and 10.5mM lactate) with 5mM L-carnitine improved development to the morula stage (Sutton-McDowall et al. 2012). Porcine embryos contain a larger complement of endogenous lipids compared with embryos of other domestic livestock species, making them an ideal model for lipid studies. Supplementation with 3.10mM L-carnitine during culture of porcine parthenotes decreased ROS levels and reduced apoptosis in blastocysts (Wu et al. 2011). Given the changing needs of the developing embryo, it was hypothesised that embryos may preferentially utilise lipids for energy production at specific stages of development.

The aim of this study was to examine the role of lipid metabolism during porcine embryo culture. The effect of temporal upregulation of lipid metabolism pathways through the addition of L-carnitine on developmental rates and cryosurvival was assessed.

5.3. Materials and methods

5.3.1. In vitro culture

Oocytes were in vitro matured and in vitro fertilised as described in sections 2.1 and 2.2, respectively. Embryos were cultured in vitro as described in section 2.3, with modifications. Embryo cleavage was assessed on Day 3, and embryos were transferred to different medium formulations as per experimental design. Uncleaved embryos were removed from culture. Media dishes were equilibrated for a minimum of 3 h prior to use. On Day 4, 10% FCS was added to each culture droplet.

5.3.2. Experimental design

Experiment 1: L-carnitine dose-response in standard PZM-3
Presumptive zygotes were cultured in standard PZM-3 (PZM-PL; 0mM glucose, 0.2mM pyruvate, 2mM lactate) with 0, 1.5, 3, 6 or 12mM L-carnitine for 7 d to determine the optimal L-carnitine dose. Embryos were assessed for cleavage and blastocyst development rates, and blastocyst cell number. Four replicate experiments were performed with 35 to 45 presumptive zygotes used per treatment group within each replicate.

Experiment 2: L-carnitine dose-response in carbohydrate deficient medium
Presumptive zygotes were cultured in modified PZM-3 containing no glucose, lactose or pyruvate, and with either 0, 1.5, 3, 6, or 12mM L-carnitine, or in PZM-PL (control medium), for 7 d. Embryos were assessed for cleavage and blastocyst development rates. Three replicate experiments were performed with 30 to 50 presumptive zygotes used per treatment group within each replicate.

Experiment 3: Temporal effects of L-carnitine in culture
Using PZM-PL as the base medium, presumptive zygotes were cultured for 7 d either with or without 3mM L-carnitine for the entire period; with or without 3mM L-carnitine for the entire period with a change to fresh medium after 3 d; without L-

carnitine for the first 3 d, with a change to fresh medium containing 3mM L-carnitine; or with 3mM L-carnitine for the first 3 d, with a change to fresh medium without L-carnitine. Embryos were assessed for cleavage and blastocyst development rates, and blastocyst cell number. Three replicate experiments were performed with 30 to 45 presumptive zygotes used per treatment group within each replicate.

Experiment 4: Effect of L-carnitine in a sequential media system

PZM-3 was used as standard (PZM-PL) or modified (PZM-GLU; 5.55mM glucose, 0mM pyruvate, 0mM lactate). The glucose concentration was chosen based on that used in NCSU-23 medium (Petters and Wells 1993), a commonly used porcine embryo culture medium, and the concentration used in sequential porcine embryo culture media (Kikuchi et al. 2002b; Kim et al. 2004; Beebe et al. 2007). Presumptive zygotes were cultured for 7 d in either PZM-PL with or without 3mM L-carnitine for the first 3 d, then in PZM-PL without L-carnitine; PZM-PL with or without 3mM L-carnitine for the first 3 d then in PZM-GLU without L-carnitine; or PZM-GLU with or without 3mM L-carnitine for the first 3 d then in PZM-GLU without L-carnitine. Embryos were assessed for cleavage and blastocyst development rates, and blastocyst cell number. Three replicate experiments were performed with 30 to 50 presumptive zygotes used per treatment group within each replicate.

Experiment 5: Effect of L-carnitine on cryosurvival

Embryos were cultured for 7 d in either PZM-PL with 3mM L-carnitine (no media change; n=29 vitrified); PZM-PL without L-carnitine (no media change; n=71 vitrified); or PZM-PL with 3mM L-carnitine for the first 3 d, then in PZM-GLU without L-carnitine (n=77 vitrified). These treatments were selected to examine the effects of L-carnitine on cryosurvival in the standard single medium system (+ or – L-carnitine in PZM-PL with no media change) compared to the cryosurvival of embryos treated with L-carnitine at an optimal stage in a sequential media system (PZM-GLU with L-carnitine for days 0-3 only). Blastocysts were vitrified on Day 7 and assessed for survival at 24 h post-thaw. Six replicate experiments were performed.

5.3.3. Statistical analysis

All analyses were conducted using GenStat (v. 16, VSN International Ltd). Cleavage rate, blastocyst development and embryo cell number data were analysed by one-way analysis of variance (ANOVA). Fisher's protected LSD post-hoc test was used when significant differences between treatments were detected. Post-thaw survival data was analysed by Generalised Linear Mixed Model (GLMM). For all analyses, $P<0.05$ was considered significant.

5.4. Results

Experiment 1: L-carnitine dose-response in standard PZM-3

The addition of 3mM L-carnitine increased cleavage rates relative to the control group (81% vs 66% respectively; $P<0.05$, Figure 5.1). Blastocyst development rate was reduced with the addition of 6mM L-carnitine during culture (8% vs 25% control; $P<0.05$). There was no difference in the mean cell number per blastocyst between treatments (14-26 average cells per blastocyst; $P>0.05$).

Experiment 2: L-carnitine dose-response in carbohydrate deficient media

In the absence of carbohydrate substrates, all doses of L-carnitine improved cleavage rates (27-42%) relative to the control (3%; $P<0.05$, Figure 5.2). Cleavage rate was significantly higher for embryos cultured in standard PZM-3 (78%). Blastocyst development was inhibited in the absence of carbohydrates regardless of L-carnitine supplementation, and was higher in the standard PZM-3 group (10%; $P<0.05$, Figure 5.2).

Experiment 3: Temporal effects of L-carnitine in culture

There was no difference in cleavage or blastocyst development rates between treatments ($P>0.05$, Figure 5.3). Blastocyst cell numbers were similar among treatment groups, although there was a strong trend for higher cell numbers in blastocysts obtained from embryos cultured with 3mM L-carnitine for the first 3 d only compared with blastocysts exposed to L-carnitine for the duration of culture ($P=0.06$).

Experiment 4: Effect of L-carnitine in a sequential media system

Cleavage rates were decreased in groups cultured in PZM-G for the first three days compared with those in PZM-PL, regardless of L-carnitine supplementation (44% vs 68-77% respectively; $P<0.05$, Figure 5.4). Culture in PZM-G for the entire period also reduced blastocyst development rate (0-3%), compared with groups cultured in PZM-PL for Day 0-3 (18-20%; $P<0.05$). There was no difference in cleavage or blastocyst development rates between groups cultured in PZM-PL for the first three days, regardless of L-carnitine supplementation or subsequent medium formulation. Embryos cultured in PZM-PL medium without L-carnitine for the entire culture period had a lower mean cell number per blastocyst compared with those cultured in PZM-PL with 3mM L-carnitine for the first three days only, or PZM-PL for the first three days either with or without L-carnitine followed by PZM-G for the remainder of culture ($P<0.05$, Table 5.1).

Experiment 5: Effect of L-carnitine on cryosurvival

Embryos cultured in PZM-PL without L-carnitine for the duration of culture with a change to fresh medium on Day 3 had a lower post-thaw survival rate (31%) compared with those cultured in PZM-PL with 3mM L-carnitine for the duration of culture with a change to fresh medium on Day 3 (48%) and those cultured in PZM-PL with 3mM L-carnitine for the first three days and PZM-G without L-carnitine for the remainder of the culture period (48%; $P<0.05$, Table 5.2).

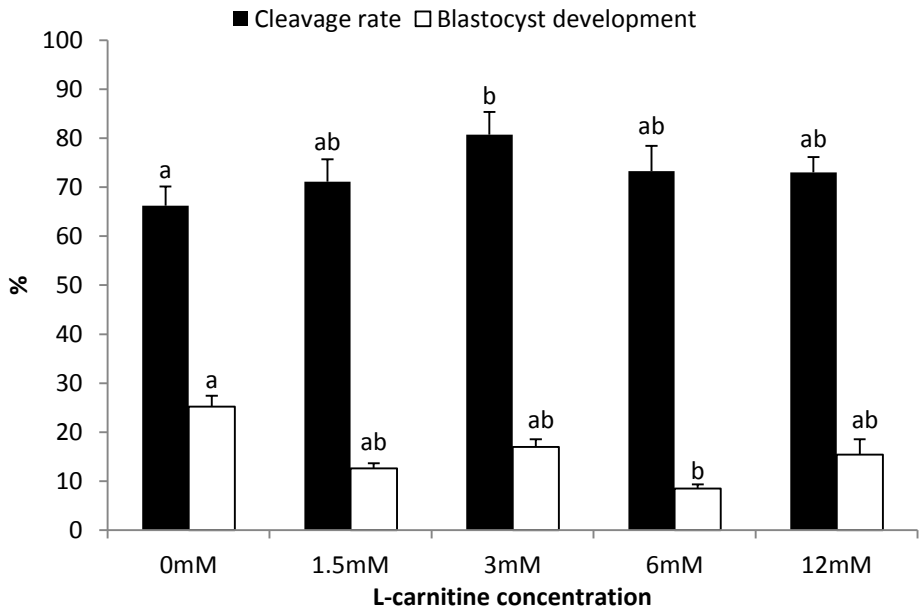

Figure 5.1. Mean ± SEM cleavage and blastocyst development rates in porcine embryos cultured in standard PZM-3 with 0, 1.5, 3, 6 or 12mM L-carnitine for the duration of culture. Cleavage data is presented as a percentage of total oocytes inseminated; blastocyst development is presented as a percentage of cleaved embryos. Within cleavage rate and blastocyst development, groups represented by bars without a common letter differ significantly ($P<0.05$).

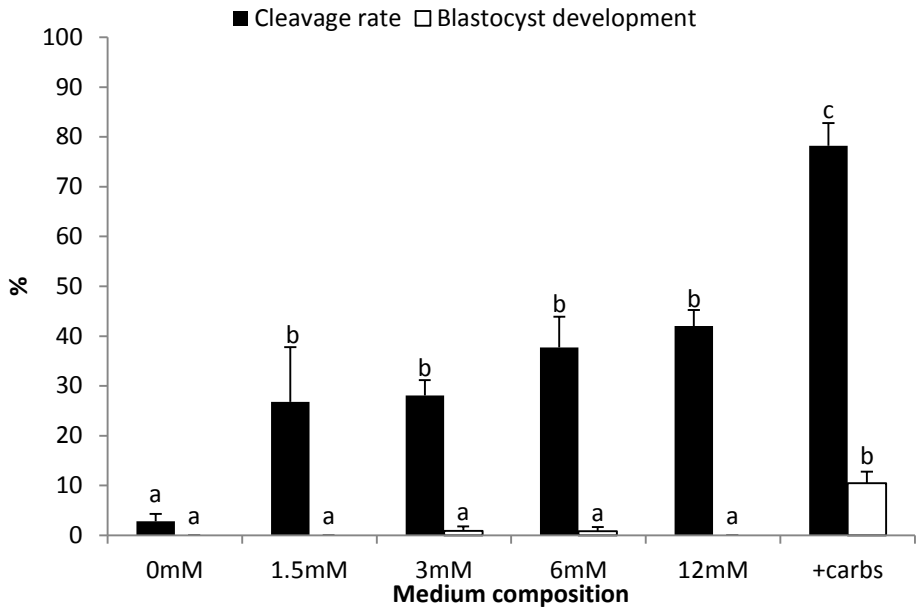

Figure 5.2. Cleavage rates and blastocyst development of embryos cultured without L-carnitine in complete media, or without glucose, pyruvate and lactate and supplemented with 0, 1.5, 3, 6 or 12mM L-carnitine for the duration of culture. Mean ± SEM cleavage and blastocyst development rates of porcine embryos cultured in standard PZM-3 without L-carnitine (+carbs) or in modified PZM-3 (0mM glucose, 0mM pyruvate, 0mM lactate) with 0, 1.5, 3, 6 or 12mM L-carnitine for the duration of culture. Cleavage data is presented as a percentage of total oocytes inseminated; blastocyst development is presented as a percentage of cleaved embryos. Within cleavage rate and blastocyst development, groups represented by bars without a common letter differ significantly ($P<0.05$).

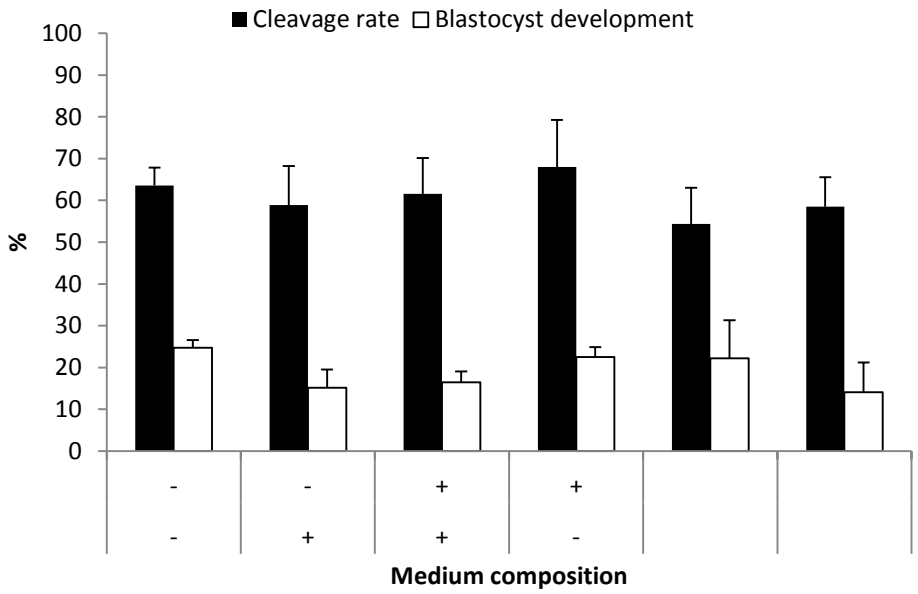

Figure 5.3. Cleavage rates and blastocyst development of embryos cultured with or without L-carnitine for specific periods of culture. Mean ± SEM cleavage and blastocyst development rates in porcine embryos cultured in PZM-3 with (+) or without (-) 3mM L-carnitine for Days 0-3 or Days 4-7, or Days 0-7 with a medium change at Day 3. Procedural controls, without a medium change, were included. Cleavage data is presented as a percentage of total oocytes inseminated; blastocyst development is presented as a percentage of cleaved embryos.

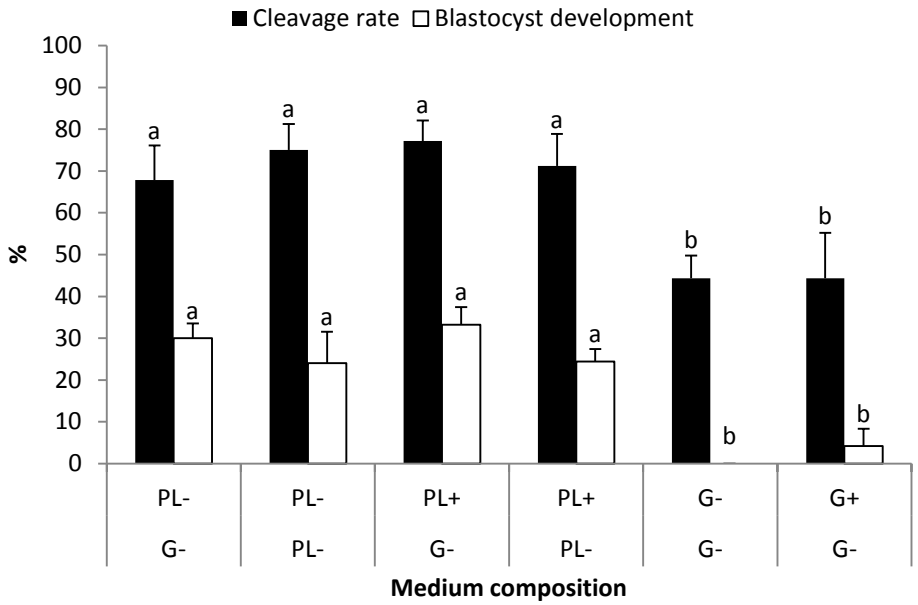

Figure 5.4. Mean ± SEM cleavage and blastocyst development rates in porcine embryos cultured in standard PZM-3 (PL; 0.2mM pyruvate, 2mM lactate, 0mM glucose) or modified PZM-3 (G; 0mM pyruvate, 0mM lactate, 5.55mM glucose), and with (+) or without (-) 3mM L-carnitine, for specific periods of culture. Cleavage data is presented as a percentage of total oocytes inseminated; blastocyst development is presented as a percentage of cleaved embryos. Within cleavage rate and blastocyst development, groups represented by bars without a common letter differ significantly ($P<0.05$).

Table 5.1. Cell number of Day 7 embryos cultured in single or sequential carbohydrate media with or without L-carnitine for specific periods of culture. Mean ± SEM cell number of Day 7 porcine embryos cultured in standard PZM-3 (PL; 0.2mM pyruvate, 2mM lactate, 0mM glucose) or modified PZM-3 (G; 0mM pyruvate, 0mM lactate, 5.55mM glucose), with (+) or without (-) 3mM L-carnitine for specific periods of culture. Values without a common letter differ significantly (P<0.05).

Treatment		Embryos (n)	Cell number (mean ± SEM)
Day 0-3	Day 4-7		
PL-	G-	20	40.25 ± 6.68^{a}
PL+	G-	12	40.08 ± 6.46^{a}
PL-	PL-	20	24.00 ± 3.04^{b}
PL+	PL-	12	43.42 ± 6.28^{a}

Table 5.2. Post-thaw re-expansion rates of embryos vitrified after culture in single or sequential carbohydrate media supplemented with or without L-carnitine for specific periods of culture. Embryo culture was in standard PZM-3 (PL; 0.2mM pyruvate, 2mM lactate, 0mM glucose) or modified PZM-3 (G; 0mM pyruvate, 0mM lactate, 5.55mM glucose), with (+) or without (-) 3mM L-carnitine for specific periods of culture. Values without a common letter differ significantly (P<0.05).

Treatment		Embryos vitrified (n)	24h post-thaw re-expansion (n)	% re-expanded
Day 0-3	Day 4-7			
PL-	PL-	71	22	30.99 ± 3.85^{a}
PL+	PL+	29	14	48.28 ± 4.98^{b}
PL+	G-	77	37	48.05 ± 4.47^{b}

5.5. Discussion

These results demonstrate the importance of tailoring culture media to meet metabolic lipid requirements of pre-implantation embryos. Prior research has predominantly focussed on the use of carbohydrate substrates by embryos, resulting in the design of specific culture media formulations to support these needs.

In the absence of carbohydrate substrates, L-carnitine supplementation increased cleavage rates, although not to the levels achieved when carbohydrate substrates were included in the medium. When pyruvate, lactate and glucose were excluded from the medium, all doses of L-carnitine (1.5-12mM) increased cleavage rates, although only to half the rates achieved in standard medium (0.2mM pyruvate, 2mM lactate) without L-carnitine. Contrary to this, cattle embryos showed similar cleavage rates regardless of carbohydrate or L-carnitine supplementation (Sutton-McDowall et al. 2012). Blastocyst rates were not improved with L-carnitine in the absence of carbohydrates, and were significantly higher in standard medium. Similarly, cattle blastocysts arrested in the early cleavage stages when cultured without carbohydrates (Ferguson and Leese 2006). However, the addition of 1mM L-carnitine to culture medium without carbohydrates supported cattle morula development to the same level as that achieved in complete carbohydrate medium (4.7-5.3%); moreover, the addition of 5mM L-carnitine to carbohydrate-deficient medium promoted a higher rate of morula development (20.4%; Sutton-McDowall et al. 2012). Blastocyst development was not reported in that study. Carbohydrate substrates in culture are not necessarily used for energy generation, with certain metabolic pathways utilising carbohydrate substrates to produce intermediaries necessary for other cellular functions. For example, NADPH is produced by the PPP for biosynthetic reactions and as a reducing agent to protect against toxic ROS accumulation. These alternate purposes may account for why porcine embryos did poorly in the absence of carbohydrates despite stimulation of lipid metabolism. Intermediaries of alternate pathways are also required for full metabolic breakdown of lipid products. Carbohydrates are needed to produce oxaloacetate, which is required to react with acetyl-CoA to prime the tricarboxylic acid (TCA) cycle. The addition of oxaloacetate to

carbohydrate-free IVM medium improved subsequent blastocyst development to levels similar to that of oocytes matured with glucose, and decreased the triglyceride content (Sturmey and Leese 2008). It is possible that in the absence of carbohydrates, the required intermediaries for complete oxidation of lipid substrates are also lacking, preventing lipid from being an effective energy substrate. Further, embryos from ruminant species exhibit a higher level of glycolytic activity than porcine embryos (Thompson et al. 1991; Rieger et al. 1992; Gardner et al. 1993; Swain et al. 2002), which may account for some of the differences seen between species.

When cultured in standard medium, the addition of 3mM L-carnitine for the duration of culture improved cleavage rates relative to no L-carnitine supplementation. However, this result was not replicated across experiments. Morphological assessment of cleavage is not a highly accurate measure of true cleavage, which is accurately assessed by staining the nuclei. This was not possible in this instance as blastocyst data after 7 d culture was also collected. Further, oocyte quality varies weekly and is also subject to seasonal effects, with oocyte quality being significantly lower in summer months. Poorer oocyte quality during certain experiments may have contributed to a greater proportion of oocytes undergoing cytoplasmic fragmentation, which is difficult to morphologically distinguish from normal cleavage, potentially masking the effect of L-carnitine. There is evidence that embryos from rabbits (Khandoker and Tsujii 1998), mice (Hillman and Flynn 1980) and cattle (Ferguson and Leese 1999, 2006) are capable of metabolising fatty acids to produce energy during early cleavage stages. Further, inhibition of β-oxidation during porcine embryo culture blocked development at the zygote stage (Sturmey and Leese 2008), suggesting a requirement for lipid metabolism at cleavage. In contrast, there was no difference detected in the cleavage rates (82-87%) of porcine parthenotes when cultured in PZM-3 supplemented with 0 to 12mM L-carnitine (Wu et al. 2011). Differences in the expression levels of genes relating to metabolism have been shown between bovine IVF embryos and parthenotes, including differences in glucose transporter genes and genes involved in the PPP (Gomez et al. 2009). It is possible that there may be differences in lipid metabolism between porcine

embryos derived from IVF and parthenogenetic activation to explain the inconsistent findings.

Culture in a sequential carbohydrate media system was beneficial to blastocyst quality, supporting results found in earlier studies (Kikuchi et al. 2002b; Beebe et al. 2007). In a single medium system, with embryos cultured in medium containing pyruvate and lactate only for the duration of culture, the inclusion of 3mM L-carnitine for the first 3 d only increased cell number to the same extent as sequential carbohydrate media systems. This result suggests that L-carnitine can increase energy production leading to higher quality embryos in systems where carbohydrate substrates may not be optimum. However, it is unknown whether the benefits of L-carnitine supplementation are attributable to lipolytic or antioxidant effects.

Carnitine is an important co-factor of the carnitine shuttle for entry of free fatty acids into the mitochondrial matrix. Lipids provide a dense energy source, and contribute to ATP production via metabolic breakdown through β-oxidation and oxidative phosphorylation. Supplementation of IVM medium with L-carnitine led to decreased lipid droplet density in porcine oocytes (Somfai et al. 2011), increased levels of β-oxidation in mice oocytes (Dunning et al. 2010a) and a tendency for higher ATP content in bovine oocytes (Chankitisakul et al. 2013). In vitro culture of bovine embryos with L-carnitine decreased lipid droplet density and increased ATP levels in 2-cell embryos (Takahashi et al. 2013). L-carnitine may also protect against oxidative stress, having been shown to alter the redox state of animal somatic cells via mitochondrial pathways (Pillich et al. 2005; Ye et al. 2010). Addition of L-carnitine to embryo culture medium improved development to the blastocyst stage and reduced ROS formation in mice (Abdelrazik et al. 2009) and cattle (Sutton-McDowall et al. 2012) embryos. Similarly, in porcine parthenotes, L-carnitine supplementation to embryo culture medium reduced intracellular ROS and decreased the incidence of apoptosis in cells of the resulting embryos (Wu et al. 2011). It is still unclear if there is a dual effect of lipid metabolism and antioxidant protection, or if one effect is more dominant than the other.

In this study, culture in glucose alone for the entire period was detrimental to cleavage and blastocyst development. This is in contrast to prior studies conducted using NCSU-23 (Petters and Wells 1993), which is formulated with 5.55mM glucose and no pyruvate or lactate for the entire culture period, that have successfully produced blastocysts (Abeydeera et al. 1998c) and piglets following transfer (Rath et al. 1995; Abeydeera et al. 1998b). However, direct comparison of NCSU-23 and PZM-3 showed that the rate of development to the blastocyst stage, as well as blastocyst inner cell mass and total cell numbers, were all higher when cultured in PZM-3 media compared with NCSU-23 (Yoshioka et al. 2002). Porcine embryos are capable of utilising glucose alone for the duration of pre-implantation culture (Swain et al. 2001), although there is limited glucose metabolism prior to compaction (Biggers et al. 1967; Flood and Wiebold 1988) with the majority of ATP produced during embryo development being via oxidative phosphorylation (Sturmey and Leese 2003). Complete oxidation of the products from lipid metabolism occurs via the electron transport chain, which is coupled with oxidative phosphorylation. Sequential media systems are designed to meet the changing nutrient requirements of embryos to more closely mimic in vivo conditions and produce better quality in vitro embryos. Given that the inclusion of L-carnitine for the first three days of culture only in standard PZM-3 increased blastocyst quality to the same extent as a sequential media system, it is likely that lipid metabolism contributes to early embryo development through increased ATP production.

The inclusion of L-carnitine, either for the entirety of the culture period or for the first three days in a sequential media system, significantly improved the cryosurvival of porcine embryos. Lipid content has been correlated to cryosensitivity (Dobrinsky and Johnson 1994; Abe et al. 2002; Sudano et al. 2011), with lipids causing an increase in oxidative and mechanical damage during ice formation (Isachenko et al. 1998). Stimulation of lipolysis in embryos has proven successful in reducing lipid content and increasing cryotolerance. The addition of 10μM forskolin to culture medium for 24 h on Day 5 of culture increased lipolytic activity and increased survival following vitrification of Day 6 porcine embryos (71% vs 37% control; Men et al. 2006). L-carnitine supplementation also improved the cryotolerance of buffalo embryos (Boccia et al.

2013), but did not affect the post-thaw survival of bovine embryos (Miyashita et al. 2012). In the present study, lipid content was not measured. Therefore, it is uncertain whether the L-carnitine treatment reduced the lipid content of the blastocyst cells or, via the antioxidant activity of L-carnitine, provided protection against ROS formed during the freeze-thaw process.

Accumulation of ROS is accelerated when embryos are exposed to environmental stressors, such as extreme temperatures experienced during vitrification. Oxidative stress can lead to mitochondrial damage, ATP depletion, apoptosis and developmental blocks (Guerin et al. 2001; Gupta et al. 2009). Due to increased levels of intracellular lipids, porcine embryos are susceptible to lipid peroxidation, with delipidation of porcine embryos shown to reduce intracellular accumulation of H_2O_2 (Yoneda et al. 2004). The addition of L-carnitine to maturation medium reduced lipid droplet density in porcine oocytes (Somfai et al. 2011) but did not reduce the proportion of apoptotic cells in parthenogenetic porcine blastocysts when added to culture medium, although it did decrease intracellular ROS levels (Wu et al. 2011). Culture of bovine embryos with 1.52 or 3.03mM L-carnitine lowered lipid droplet density and improved survival following slow freezing (Takahashi et al. 2013). Given that there was no reported improvement in cryosurvival of bovine embryos following the addition of alternative antioxidants to culture medium (Hosseini et al. 2009; Rocha et al. 2012), the improved cryosurvival observed in the present study is likely attributable to decreased lipid content.

In conclusion, the addition of L-carnitine, a co-factor of β-oxidation, to porcine embryo culture medium increased cleavage rates, improved blastocyst quality and increased the cryosurvival of Day 7 blastocysts. The beneficial effects of the L-carnitine treatments were most likely due to an increased utilisation of endogenous lipid reserves for energy production. Importantly, in the complete absence of carbohydrate substrates, the ability of fertilised oocytes to undergo the initial cleavage divisions was vastly improved by supplementing the culture medium with L-carnitine. This finding suggests that lipid metabolism plays an important role in energy generation during porcine embryo development, particularly in the interval immediately following fertilisation. Therefore,

supplementing culture medium with lipolytic stimulators, such as L-carnitine, during the early stages of embryo development is an effective strategy to improve the quality of in vitro produced porcine embryos.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

The application of ARTs in pigs for biomedical or agricultural purposes is currently limited by the inefficiency of in vitro embryo production and the quality of embryos produced and maintained in these systems. Typically, porcine blastocyst rates are lower than those seen in other domestic livestock species, and have a low post-thaw survival rate which limits their long-term storage and global movement. Many of these issues are attributable to the altered metabolic processes seen during the in vitro culture of oocytes and embryos, compared with their in vivo counterparts. There is a wealth of research regarding carbohydrate metabolism and many in vitro media formulations reflect this greater understanding. While it is likely that the carbohydrate requirements of embryos are being met, other metabolic needs are potentially not being supported by these media. Oocytes and embryos of different species contain varying amounts of intracellular lipid substrates, known to be a dense source of energy, although the extent to which these cells rely on endogenous substrates for ATP production is poorly understood. Porcine oocytes and embryos have a much greater intracellular lipid fraction compared with those of other domestic livestock species, making them an excellent model for studying lipid metabolism during IVP.

The findings reported in this thesis contribute to an overall understanding of the metabolic substrates utilised by porcine oocytes and embryos during IVM, IVF and IVC, and the impact of lipid metabolism during these times. The results provide evidence to support plasticity of oocyte and embryo metabolism, with these cells showing some level of adaptability to the substrates available that support growth. This study identified reasons for the conflicting findings of prior studies, with the carbohydrate composition of media impacting on the differing responses of oocytes to upregulation or inhibition of lipid metabolism, highlighting the importance of specific media additives to cell growth. Other results show that by upregulating lipid metabolism during IVF or IVC, the cryosurvival of Day 7 embryos can be improved. Collectively, these results support the hypothesis that

oocytes and embryos utilise endogenous lipid substrates during in vitro maturation and in vitro culture respectively, although not to the extent that is thought to occur in vivo.

Varied results from prior studies examining the upregulation and inhibition of lipid metabolism are likely attributable to differences in the carbohydrate composition of the medium used. Beneficial effects of L-carnitine on nuclear maturation were seen in prior studies using NCSU-23 and NCSU-37 (no pyruvate) as the IVM media (Somfai et al. 2011; Wu et al. 2011) but not when TCM-199 (0.91mM pyruvate) was used (You et al. 2012). Similarly, this study demonstrated that while there was a lesser effect of upregulating or inhibiting nuclear maturation in complete POM media, there was a greater impact of these treatments seen in media with insufficient carbohydrates. When maturation media provides proper carbohydrate support, lipid metabolism appears to play a minor role during oocyte maturation in vitro. In the absence of pyruvate and lactate, the nuclear maturation rate was halved, and when all carbohydrate substrates were removed it was reduced nine-fold. This highlights the importance of providing an optimum media formulation, and demonstrates the requirement for carbohydrates to support nuclear maturation. These results show that oocyte metabolism is adaptable, and different metabolic pathways can be utilised to compensate for deficiencies in energy substrates to a degree.

There is evidence that in vitro matured oocytes lack the necessary gene transcripts and enzymes involved in lipid metabolism. In mouse COCs, in vitro maturation led to dysregulation of gene transcripts that are related to lipid metabolism and reduced the rate of fatty acid oxidation by 2.8-fold compared to in vivo maturation (Dunning et al. 2014). Expression of genes relating to long-chain fatty acid synthesis, conversion of acetoacetyl-CoA to acetyl-CoA, and regulation of cholesterol levels were upregulated in bovine blastocysts derived from IVM oocytes compared with in vivo matured oocytes (Gonzalez-Serrano et al. 2013). The fatty acid oxidation genes acyl-CoA synthetase long-chain family member 3 (ACSL3) and long-chain acyl-CoA dehydrogenase (ACADL) were differentially expressed between IVM and in vivo matured oocytes; further, these genes were dysregulated in IVM oocytes of poor developmental competence (Yuan et al.

2011). Inhibition of lipid metabolism during IVM decreased the abundance of gene transcripts involved in fatty acid oxidation, glycolysis and oxidative stress in porcine oocytes (Paczkowski et al. 2013). Further, the morphology of lipid droplets was found to differ between in vitro- and in vivo-derived porcine embryos (Kikuchi et al. 2002a). This suggests that lipid metabolism is altered in oocytes and embryos cultured in vitro, and that in vitro matured oocytes are less able to effectively utilise lipids for ATP production. This could also explain why supplementation of maturation medium with L-carnitine had little effect on meiotic progression and ATP production. Further studies are required to understand these deficiencies and to determine whether expression of relevant genes and enzymes can be improved prior to or during in vitro maturation.

There are dual effects of L-carnitine that can complicate the interpretation of results. Supplementation of culture medium with L-carnitine may be beneficial due to its antioxidant activity or its stimulatory effect on lipid metabolism. In vitro assays have shown L-carnitine to be an effective antioxidant, with greater reducing power, DPPH radical and superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities than other commonly used antioxidant compounds (Gulcin 2006). L-carnitine is also an important co-factor involved in the entry of free fatty acid into the mitochondrial matrix, the site of β-oxidation. However, living systems must be maintained in a balance between metabolic rate and ROS production, to ensure the redox system can continue to effectively neutralise harmful radicals. At high L-carnitine concentrations, lipid metabolism may be increased to an extent that results in excess accumulation of ROS, leading to increased oxidative stress and an overall detrimental effect on cells. Where possible, these results have been compared with prior studies utilising alternative antioxidants in similar circumstances to indicate if the effects of L-carnitine are more likely attributable to its antioxidant activity or its role in lipid metabolism. Future studies would benefit from quantification of ROS levels following L-carnitine treatment to determine the primary effect within an IVP system.

Very few prior studies have examined the role of lipid substrates during insemination. This period is marked by an increased ATP requirement to support

gamete fusion, calcium oscillations, the block to polyspermy, protein synthesis, completion of meiosis, pronuclear formation and other post-fertilisation cellular events. In this study, incubation of oocytes in 3mM L-carnitine for an hour prior to insemination increased cleavage rates; at higher doses, cleavage and blastocyst development were both inhibited, possibly due to the antioxidant properties of L-carnitine. Low levels of ROS are required at fertilisation for normal sperm function and capacitation, sperm-oocyte interactions, cell signalling, homeostasis and growth (Miesel et al. 1993; de Lamirande et al. 1998; de Lamirande and O'Flaherty 2008; Finkel 2011; Fujii and Tsunoda 2011). This is consistent with a prior finding that high concentrations of antioxidants in IVF media decrease cleavage and blastocyst rates in cattle embryos (Ali et al. 2003). Conversely, addition of L-carnitine during sperm preparation had a beneficial effect on subsequent embryo development. Previous reports have shown improved sperm motility with the addition of L-carnitine during centrifugation (Banihani et al. 2012), and improved motility and chromatin quality with supplementation of L-carnitine during room temperature incubation (Aliabadi et al. 2012). L-carnitine has a dual role in providing beneficial effects for spermatozoa. The antioxidant activity has a protective role for sperm membranes against ROS (Agarwal and Said 2004; Gulcin 2006), while also promoting lipid metabolism and thereby decreasing lipid peroxidation.

Incubating mature oocytes in fertilisation media supplemented with L-carnitine for 1 h prior to co-incubation with sperm increased cleavage rates of the resulting embryos. These results suggest that there is a limited use of lipid substrates during fertilisation, and that the beneficial effects of L-carnitine are likely attributable to the antioxidant action. However, the addition of L-carnitine for the 5.5 h duration of gamete co-culture did not improve developmental outcomes. In cattle, the inclusion of cysteine, N-acetyl-L-cysteine, catalase or super oxide dismutase for the duration of the IVF period resulted in a significant reduction in the subsequent morula and blastocyst development rates (Ali et al. 2003). Inclusion of β-mercaptoethanol in porcine IVF medium following a transient co-culture without, increased blastocyst cell number, while inclusion for the entire co-culture period decreased cleavage and blastocyst development rates (Funahashi 2005). Together these findings indicate that there may be temporal effects of

adding antioxidants during IVF, and differential effects due to the types of antioxidants used. Further studies should focus on the impact of L-carnitine on ROS-induced oocyte damage and ATP production throughout fertilisation, to determine the optimum time of addition during IVF.

When lipid metabolism was upregulated during embryo culture, cleavage rates were improved relative to the control. In in vivo derived cattle blastocysts, expression of CTP-1 was not detected, while transcripts of fatty acid synthesis genes were increased at this stage (Algriany et al. 2007). When added to media without any carbohydrate substrates, 1.5-12mM L-carnitine increased cleavage rates, although not blastocyst development rates. This implies a temporal role for lipid metabolism, with lipid usage greater during the early cleavage divisions than later in development. Given that embryo nutrient requirements change with the stage of development, it was hypothesised that the addition of L-carnitine during the early cleavage stages only would further improve development. While this targeted L-carnitine exposure did not improve the blastocyst development rate, blastocyst cell numbers were increased, indicating better quality embryos. Several studies have shown the beneficial effects of altering the carbohydrate composition of media on porcine embryo development (Kikuchi et al. 2002b; Karja et al. 2004; Beebe et al. 2007). The addition of L-carnitine to such a sequential media system for the first three days of culture did not improve developmental outcomes, indicating that carbohydrate substrates are preferentially utilised by porcine embryos in vitro. Conversely, temporal addition of L-carnitine to a single medium system (containing pyruvate and lactate, but no glucose) for the first 3 d only improved blastocyst cell numbers to the same extent as a sequential media system (containing glucose only at Day 3-7) without L-carnitine. This again shows that the changing requirements for energy substrates can be fulfilled in a number of ways to produce higher quality embryos. Assessment of ATP content and β-oxidation rate at critical developmental stages throughout culture may further reveal links between the timing of lipid utilisation and improved developmental outcomes.

Supplementation of medium at specific times during IVP was shown to improve the cryotolerance of the resulting embryos. Post-thaw survival was increased by

incubation in the presence of L-carnitine prior to co-incubation with sperm, and when embryo culture medium was supplemented with L-carnitine. Although not confirmed in this study, the increased cryosurvival following L-carnitine treatment is most likely attributable to a decrease in lipid content, as cryotolerance is associated with lipid content (Nagashima et al. 1995; Abe et al. 2002). Further, studies using other lipolytic stimulants have shown positive results regarding the cryosurvival of porcine embryos (Men et al. 2006; Gajda et al. 2011; Gomis et al. 2013). This result is also consistent with those of previous studies in cattle, whereby L-carnitine was shown to reduce intracellular lipid and improve embryo cryosurvival (Sutton-McDowall et al. 2012; Ruiz et al. 2013; Takahashi et al. 2013). Although there was no associated increase in ATP content of oocytes incubated in L-carnitine prior to insemination, blastocyst cell numbers were also similar to those not exposed to L-carnitine, indicating the embryos were of equivalent quality. This is an important finding as the ability to successfully cryopreserve embryos promotes the long-term storage and global movement of valuable genetic lines. Frozen genetic material is an important factor in the application of herd genetic improvement programs in the cattle and sheep industries, but is yet to be realised in the pig industry. Further improvements to the cryostorage of porcine embryos are essential for embryo transfer to become an integral component of advanced breeding programs.

There was a consistent pattern of increased embryo cleavage rates found when L-carnitine was added during IVF and IVC. This suggests that lipid stores were primarily utilised during the early cleavage divisions. This is interesting given that NCSU-23 medium, a culture medium commonly used for porcine embryos, only provides 5.55 mM glucose as the sole carbohydrate substrate (Petters and Reed 1991; Petters and Wells 1993). However, exposure to glucose during the early cleavage stages was found to reduce embryo quality in the medium used in the present study, PZM-3, consistent with poor development of embryos seen when they were cultured in unmodified NCSU-23 medium (Kikuchi et al. 1999). Blastocyst development rates and embryo quality were improved by replacing glucose with pyruvate and lactate for the first two days of culture in NCSU-37, and NCSU-37 medium conditioned with oviduct cells supported the production of piglets following transfer (Kikuchi et al. 2002b). The PZM media formulations were

developed based on substrate concentrations of oviductal fluid (Yoshioka et al. 2002). Both PZM-3 and PZM-4 (with PVA replacing BSA for a fully defined medium) produced embryos with higher ICM and total cell numbers than those cultured in NCSU-23 medium (Yoshioka et al. 2002). Further, embryos cultured in vitro in PZM-3 and PZM-4 had similar ICM to total cell ratios as that of in vivo produced blastocysts, and the transfer of PZM-4 cultured embryos to recipient females resulted in farrowing and live birth rates similar to those achieved following the transfer of in vivo produced embryos (Yoshioka et al. 2002). Higher blastocyst development rates and embryo cell numbers were obtained for both parthenotes and reconstructed nuclear transfer embryos when cultured in PZM-3 compared with NCSU-23 medium (Im et al. 2004).

The studies presented here utilised POM as the IVM medium because it was developed as a completely defined medium capable of supporting oocyte maturation (Yoshioka et al. 2008). Although BSA was used in place of PVA for these studies, this still provides for a more reliable medium compared to the use of undefined factors, such as follicular fluid. When used for IVM, NCSU-23 and -37 media are commonly supplemented with follicular fluid (Petters and Wells 1993), which leads to high variability of media batches due to the addition of undefined factors, and potentially exposes the oocytes to pathogens. The use of POM supplemented with PVA during IVM resulted in higher rates of normal fertilisation compared to POM with follicular fluid, and NCSU-37 medium with either PVA or follicular fluid, while the use of PVA in NCSU-37 medium resulted in lower rates of maturation to the MII stage compared with all other treatments (Yoshioka et al. 2002). Further differences between the two media are in amino acid concentration and composition, with POM containing a total of 22 amino acids and the NCSU media containing a limited number, including glutamine, taurine and hypotaurine. Amino acids act as osmolytes, contribute to intracellular buffering, and can contribute to energy generation. Excess amino acids can be stored, with the carbon skeleton converted to pyruvate, acetyl-CoA and various other intermediaries of the TCA cycle and used for energy production. In the case of ketogenic amino acids, this can also be converted to triglycerides for storage or metabolic use. When non-essential and essential amino acids were added to NCSU-23 supplemented with PVA during porcine oocyte IVM, maturation rates,

sperm penetration and blastocyst formation were similar to that of NCSU-23 medium supplemented with follicular fluid (Hong et al. 2004), highlighting the importance of amino acids in a maturation system. Differences in media compositions all contribute to variable outcomes between studies, and make comparisons of results difficult. Future research should focus on how these other medium components affect oocyte and embryo metabolism, and aim towards standardising the use of a defined medium for scientific research.

In muscle tissue, high glucose availability reduces the mobilisation of fat reserves and favours carbohydrate oxidation (Spriet and Watt 2003). It is proposed that a similar effect is seen within the in vitro oocyte and embryo models presented in these studies. Glucose oxidation produces citrate, which is converted into malonyl-CoA by acetyl-CoA carboxylase, with malonyl-CoA inhibiting entry of fatty acids into the mitochondria at CPT1. Therefore, when glucose oxidation is favoured, high concentrations of malonyl-CoA reduce the rate of fatty acid oxidation. When carbohydrates were present at sufficient concentrations during IVM and IVC, L-carnitine had little to no effect on the rates of nuclear maturation and embryo development. It is likely that with sufficient exogenous energy substrates available, there is a preference to utilise these substrates first while maintaining endogenous energy reserves. In situations where carbohydrates were limiting, there was a greater positive effect of upregulating lipid metabolism, and an increased detrimental effect of inhibiting this pathway. Without the necessary concentrations of exogenous substrates, there is higher mobilisation of endogenous lipid reserves for oxidation, and therefore alteration of the lipid metabolism pathway has greater effects under these circumstances. This is also consistent with a greater effect of L-carnitine shown during IVM with the inclusion of follicular fluid (Somfai et al. 2011), which is rich in exogenous free fatty acids. Increased free fatty acid availability increased muscle acetyl-CoA and citrate, which causes a downregulation of PDH and PFK activities leading to reduced glycolytic rate (Spriet and Watt 2003). Perhaps the key to enhancing lipid metabolism in porcine oocytes and embryos is to increase the rate of hydrolysation of triglycerides, thereby increasing the levels of available free fatty acids for metabolic use.

Future investigations are required to better understand the differences in metabolic processes between IVP and in vivo conditions in more detail. Transcriptomic (Ouandaogo et al. 2012), proteomic ((Kim et al. 2011), metabolomics (Bertoldo et al. 2013; Gu et al. 2015) and lipidomic (Ferreira et al. 2010; Silva-Santos et al. 2014) assessments, and analysis of individual follicles, COCs and embryos using recently developed tools such as novel imaging techniques (Watanabe et al. 2010; Jasensky and Swain 2013), would reveal valuable insights into the development of porcine oocytes and embryos and key differences between good and poor quality cells. Such analyses could also contribute to our understanding of the effects of different environments and media formulations on oocytes and embryos. Continued annotation and analysis of the porcine genome will contribute to studies examining genes involved in metabolism. Advancements in our knowledge of the follicular, oviductal and uterine environments continue to contribute to our understanding of the available substrates during in vivo development. For example, recent characterisation of the metabolomic signatures of porcine follicular fluid showed differences in the concentrations of metabolites, including glucose, lactate and five different amino acids, between good and poor follicular environments (Bertoldo et al. 2013). It is evident that metabolic processes in oocytes and embryos differ between species, making extrapolation from research in other species difficult. Modifications of media should therefore be specific to individual species.

To conclude, the metabolic processes of oocytes and embryos throughout maturation and culture are complex and dynamic. Attempts to examine these processes in vitro are confounded by the ability of oocytes and embryos to utilise different medium substrates, while comparisons between studies are made difficult owing to the different base media formulations used, often leading to contradictory results. This thesis has contributed to further understanding how porcine oocytes and embryos use endogenous lipid substrates throughout in vitro development, and the interaction with carbohydrate substrates. The majority of earlier research focussed solely on the metabolism of carbohydrates, with only recent studies focussing more intently on lipid utilisation. The results of the present study suggest that while lipid metabolism does occur during oocyte maturation and embryo culture, it is at low levels with preferential use of

carbohydrate substrates, namely pyruvate and glucose. However, when these carbohydrates are deficient, lipid metabolism can compensate to some extent to support development. Upregulation of the β-oxidation cycle during embryo culture also enhanced embryo cryosurvival, with porcine embryos being notoriously difficult to cryopreserve due to their high lipid content. Further investigations are required to better understand the complex interactions of the metabolic processes throughout each stage of IVP and thus improve overall IVP efficiency as well as the quality of the resulting embryos. In particular, understanding why porcine oocytes and embryos have such a large intracellular store of lipid and what the fundamental role of this lipid is in embryo formation and development will lead to improved support of these cells in vitro.

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