The metabolism of the ruminant cumulus-oocyte complex revisited

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Summary

The progress in understanding the metabolism of the ruminant cumulus-oocyte complex (COC) from large antral follicles has progressed significantly in the past decade. In particular, new insights in the importance of lipid metabolism, β-oxidation and its relationship to oxidative phosphorylation within oocytes have emerged. This provides opportunities for tapping into the potential yield of ATP from lipid metabolism, as ATP content is a major determinant of oocyte competence. However, this new appreciation of lipid metabolism also includes the damaging influence of some free-fatty acids, the non-esterified fatty acids, which are particularly high in the follicular fluid of dairy cows during peak lactation. Historically, studies have focussed on carbohydrate metabolism, which occupies a central significance, especially for cumulus cell metabolism. Glucose has multiple roles; one still largely unexplored is its requirement for extracellular matrix production, via the hexosamine biosynthesis pathway. The availability of oxygen for oocytes in large antral follicles remains to be resolved, and requires new tools to measure intra-follicular O_2 levels to determine its significance. For *in vitro* oocyte maturation, new strategies for improving oocyte competence includes: cAMP management, oocyte secreted factor supplementation, protection against reactive oxygen species with glutathione, and utilizing the endogenous maturation signalling molecules, the EGF-like peptides, during maturation - all of which influence metabolism. The introduction of existing platforms, such as metabolomics, to ruminant oocyte metabolism will broaden our understanding. Ultimately, to appreciate the dynamic nature of metabolism in oocytes of all species requires the development of new nano-scale sensing platforms that will allow us to measure activity in single oocytes and early embryos *in situ* in real time.

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Introduction

In 2006, in chilly Wellington New Zealand, the Ruminant Reproduction Symposium VII was held within that country's spectacular national museum. One of the symposia talks at that meeting was on the same subject reviewed here. In the intervening 8 years there have been notable new insights into the metabolism of the ruminant cumulus-oocyte complex (COC), and so we have attempted to update our understanding in this review, with a strong focus on the bovine cumulus-oocyte complex.

Metabolomics allows comprehensive metabolic profiles to be created from relatively small amounts of sample (Koek, *et al*. 2010, Kraly, *et al*. 2009). This is particularly useful for the study of detailed amino acid or fatty acid profiles. Metabolomics involves the separation and then detection of metabolites; using technologies such as gas chromatography and high performance liquid chromatography for separation and mass spectrometry (MS), nuclear magnetic resonance (NMR) and Raman spectrometry for detection (Issaq, *et al*. 2009, Nagy, *et al*. 2009). Depending on the quality of sample, metabolomics can both identify and quantify (absolute concentration) or semi-quantify (relative changes between treatments) levels of substrates. Such metabolomic techniques have been restricted to human oocytes and embryos, confined to analysing either the embryo's secretome, or sampling the cumulus cells that are normally discarded, with the aim of selecting the best embryo for transfer (Nagy, *et al*. 2009, Nel-Themaat and Nagy 2011). Even though these techniques are capable of providing a comprehensive view of cellular metabolism, sampling over the time-course of oocyte maturation or embryo development is one major limitation in most metabolic studies, generally restricted to a single point in time. It remains that much of what we have learnt of ruminant COC metabolism in the ensuing eight years has still relied on the techniques of micro-sampling "spent" media and either direct analysis of media composition using fluorescence assays, or the application of fluorescence probes for oocytes. Some techniques, such as scanning electrode technology, has had limited application in ruminant oocytes (Sugimura, *et al*. 2012).

Interest in ruminant COC metabolism is driven by questions of how oocyte competence is regulated by the environment encountered during maturation. For example, there are concerns about oocyte quality within the dairy industry during peak lactation; interest grows in understanding the impact of environmental toxins on oocyte quality; and the emergence of a strong *in vitro* embryo production (IVP) market for cattle, and increasingly for sheep, goats, horses and pigs, has driven research to improve IVP efficiencies, with much of the focus on improving oocyte competence, as this is recognized as a bottleneck to IVP performance (Lonergan and Fair 2008, 2014). Such questions have strong links with mechanisms of cumulus cell and oocyte energy production and how metabolic activity is a determinant of oocyte competence.

Oocyte developmental competency – what constitutes metabolic competence?

Oocyte developmental competence is acquired *in vivo* as a result of complete follicular maturation achieved when the dominant follicle responds to the ovulatory surge of LH, initiating a complex signalling cascade that impacts all follicular cells, including the oocyte. There are several transcriptomic studies comparing immature (hence not developmentally competent) to fully grown and ovulated oocytes, revealing major changes in the RNA processing and cell cycle control (e.g. Labrecque and Sirard 2014, Leoni, *et al*. 2007). Several studies have focussed on the cumulus cell transcriptome, in particular comparing *in vivo* matured vs. *in vitro* matured (IVM), in the attempt to understand why IVM-derived oocytes have poorer competence. Many of these studies reveal that metabolism of the COC is a key element in determining oocyte competence, with genes regulating both lipid and carbohydrate metabolism within cumulus cells being identified as associated with competence, (e.g. Peddinti, *et al*. 2010, Salhab, *et al*. 2013).

The cumulus-oocyte complex as a metabolic unit

Bi-directional communication in the COC

The fundamental importance of the bi-directional communication between oocytes and their surrounding cellular vestment, the cumulus cell layer, is now a well-established paradigm in gamete biology. This relationship extends to metabolic co-operation as well and is the reason why, wherever possible, research involving the COC as an intact unit is increasingly favoured over the separation of these two distinct cellular types. Many review articles describe how the function of cumulus cells are significantly influenced by factors released from oocytes, primarily through the paracrine oocyte specific growth factors belonging to the TGF-β family (for a review of ruminant oocyte factors, see Thompson, *et al*. 2012). In turn, the cumulus cells, when "healthily" maintained, provide nutrients, such as pyruvate and ATP to the oocyte (Buccione, *et al*. 1990) (Fig. 1). Immature oocytes at the GV stage of meiosis are connected with cumulus cell via a network of gap-junctions. Through junctional-complexes it is well established that nutrients and small molecular weight molecules pass from the cumulus cells to the oocyte (Anderson and Albertini 1976, Atef, *et al*. 2005, Buccione, *et al*. 1990, Sutovsky, *et al*. 1993) (Fig. 1). Gap-junctional communication is a determinant of oocyte competence, as either prolonging gap-junction communication enhances competence, or gap-junction disruption reduces competence following maturation (Luciano, *et al*. 2011, Luciano, *et al*. 2004, Thomas, *et al*. 2004). New insights into maintaining gap junction communication demonstrates the association with the maintenance of intra-oocyte cAMP levels (Luciano, *et al*. 2004) and impact of oocyte-secreted factors (Sugimura, *et al*. 2014). This most likely explains the positive benefit observed to oocyte competence following a brief pre-maturation period of high cAMP levels (e.g. Albuz, *et al*. 2010).

Regulation of meiosis

Prior to the ovulatory signal, oocytes are arrested at the germinal vesicle stage and this requires the production of cyclic adenosine monophosphate (cAMP) and cyclic guanidine monophosphate (cGMP), from cumulus cells and cAMP specifically in the oocyte. These levels are maintained by respective cyclases and phosphodiesterases (PDE). Initiation of the ovulatory surge leads to granulosa cell production of the EGF-like peptides (amphiregulin, epiregulin and betacellulin) that reduces cGMP levels, which in turn releases the inhibition of PDE type 3 within the oocyte, causing the hydrolysis of cAMP and release of meiosis. Recent new insights have demonstrated, at least in the mouse, how this elegant system of meiotic regulation is achieved (Conti, *et al*. 2012, Robinson, *et al*. 2012). From a metabolic perspective, there is a requirement for fentomole levels of cGMP and cAMP production. Energy is then required for chromatin reconfiguration and spindle formation. The picture in ruminants may differ in detail, but in essence the same or at least similar players are likely to operate. Yet differences do exist, for example, Sasseville, *et al*. (2009) has demonstrated that in cattle COCs, PDE8 is a dominant PDE, not present in the mouse.

Intra-oocyte stores

An area not well explored in mammalian oocyte biology is the storage of energy and mobilisation of stored energy. Somatic cells store energy in two forms: lipid and glycogen. Ruminant and porcine oocytes are distinctive for their high levels of lipid within oocytes. Lipids and their metabolism has been a recent focus of research, particularly in cattle oocytes (discussed

elsewhere in more detail). Oocyte lipid content is highly variable across species. Recently, Krisher and colleagues (Paczkowski, *et al*. 2013) have proposed that the level of lipid reflects the time required from fertilisation to embryonic genome activation, being longer in species having lipid-rich oocytes. This suggests that there is some necessity for energy storage, a hypothesis supported by (Cetica, *et al*. 2003). In contrast, there is very little known about glycogen synthesis or storage in COCs. There is a paucity of literature on this subject, even in the mouse (Pike and Wales 1982, Thompson, *et al*. 1995). This is not the case for amphibian and drosophila oocytes, where glycogen has important functions in meiotic control, (e.g. Dworkin and Dworkin-Rastl 1989, Gutzeit, *et al*. 1994). Whether it is present and what role it plays in ruminant oocytes has yet to be determined.

ATP production is what it's all about?

ATP production is the essential purpose for metabolism, yet many studies (including our own) do not always measure ATP (and ADP) levels. Many studies infer differences in ATP production by defacto measurements such as mitochondrial activity and/or oxygen consumption (e.g. Sugimura, *et al*. 2012). Furthermore, if measured, most studies determine ATP levels at single points during oocyte maturation (e.g. Stojkovic, *et al*. 2001). Furthermore, measurement of the ATP:ADP ratio, a measure of ATP turnover as a consequence of demand, is rarely reported.

Apart from the provision of energy within the oocyte, ATP is the substrate used by adenylate cyclase for cAMP generation, essential for maintaining meiotic arrest at GV stage in oocytes. New approaches for measuring ATP applied to (mouse) oocyte maturation has revealed that peaks in demand within the oocyte is closely linked with stages of chromatin remodelling during meiotic progression (Dalton, *et al*. 2014). Significantly, oocyte competence is highly correlated to ATP levels (Dalton, *et al*. 2014, Stojkovic, *et al*. 2001). Even more significantly, maintaining gap-junctional communication supports higher levels of ATP within the oocyte, validating this approach for improving oocyte competence (Dalton, *et al*. 2014). Nevertheless, cumulus cell and oocyte metabolism is more than simply the need for oocyte ATP production, for example, the production of cumulus-derived matrix and intra-oocyte glutathione are both examples of alternate metabolic requirements.

Glucose metabolic pathways

Glucose is an essential requirement for the bovine cumulus-oocyte complex for both meiotic and cytoplasmic maturation. A well-founded principle for mammalian species, including cattle, is that the cumulus–oocyte complex consumes high levels of glucose, which is metabolized by the cumulus cells through primarily the glycolytic pathway (Cetica, *et al*. 2002) (Fig. 1). In contrast, the oocyte itself has a low capacity for glucose metabolism, and is reliant on ATP production from oxidative phosphorylation. This low capacity for glucose oxidation in oocytes was recognized in cattle many decades ago (e.g. Rieger and Loskutoff 1994, Rushmer and Brinster 1973). Although attributed to inhibition of phosphofructokinase (Barbehenn, *et al*. 1974), we remain ignorant as to the advantage there is for an oocyte to be reliant on carboxylic acids supplied to it from cumulus cells, rather than having its own capacity to do so.

Glycolysis

Glycolysis is the primary glucose metabolic pathway within the cumulus cells of the COC, and is essential for mammalian (including cattle) maturation. Inhibition of glycolytic activity in cattle COCs impacts negatively on both oocyte meiotic maturation and mitochondrial activity (Gutnisky, *et al*. 2013b). Varying the level of glucose availability also influences meiotic regulation, presumably by a change in glycolytic flux through cumulus cells, impacting oocyte meiotic regulation (Sutton-McDowall, *et al*. 2005). Even though the oocyte has only a limited capacity for intra-oocyte glycolysis, the amount that is metabolised through this pathway has been positively associated with competence (Krisher 2013, Krisher and Bavister 1999). This suggests an as yet un-identified process that requires some degree of oocyte glycolysis.

A concept that first emerged in the 1990's (Rieger 1992) and now revisited is the parallel metabolic profiles of pre-implantation embryos and cancer cells (Krisher and Prather 2012, Smith and Sturmey 2013). Like some cancer cells and other cell types that undergo active proliferation, cumulus cells and post-compaction embryos utilize a large amount of glucose via aerobic glycolysis, resulting in significant levels of lactate production, in preference to a flux of pyruvate through the TCA cycle and oxidative phosphorylation, a phenomenon called the Warburg effect (Warburg 1956). In cancer cells, this occurs despite glycolysis being relatively inefficient at producing ATP compared to oxidative phosphorylation (2 ATP vs. 38 ATP), even though a large demand for ATP to fuel rapid cell division exists. Krisher and Prather (2012) suggest that aerobic glycolysis provide the embryo with substrates for cell division such as nucleic acid synthesis (pentose phosphate pathway, PPP) and reduction-oxidation equilibrium (NADPH and NADH), with other metabolites such as fatty acids and amino acids, providing substrates for oxidative phosphorylation and ATP. This is primarily achieved through the expression of enzymes, such as pyruvate kinase M2 (PKM2) that has low kinase activity, resulting the in the build-up of glycolytic intermediates and preferential metabolism via PPP and the hexosamine biosynthetic pathway (HBP). Confirmation that the Warburg effect applies to cumulus cells within the COC have yet to be established, but it is an attractive framework for understanding cumulus cell metabolism. In 2007 (Thompson, *et al*. 2007), we hypothesized that one function of the bi-directional communication between oocytes and cumulus cells was to establish a metabolic relationship between the oxidative metabolism needs of the oocyte and the glycolytic activity of the cumulus. In the mouse, it is well established that OSFs from the oocyte regulate cumulus cell glycolytic activity (Sugiura and Eppig 2005, Sugiura, *et al*. 2005, Sugiura, *et al*. 2007). Using mathematical modelling, we determined that relative to oocytes, on a per volume of tissue basis, bovine cumulus cells consume 20-fold more glucose than oocytes (Clark, *et al*. 2006, Clark, *et al*. 2011). This led us to examine by mathematical modelling, the relationship between follicular glucose content and the intra-oocyte cortical concentration of glucose, assuming that a glucose concentration gradient across the cumulus cells was established by diffusion (Stokes, *et al*. 2008). Modelling demonstrated a significant glucose gradient across the cumulus layer such that even a modest change (e.g. 1.5 mM) in follicular concentration of glucose made a substantial difference in the concentration at the boundary between the cumulus layer and oocyte. Further work on modelling the differences in glucose uptake between the intact COC and mural granulosa cells revealed a 4- to 6-fold higher uptake by cumulus cells, suggesting bovine COC glucose uptake is probably under the regulation of OSFs (Clark, *et al*. 2011). This differs from our original observation that oocytectomy did not alter glucose uptake (Sutton, *et al*. 2003). The discrepancy is almost certainly due to the impact of supplemental FSH in our original work, as we have seen that FSH has an overriding effect on metabolism in other studies, for example the influence of the OSF, BMP-15 (Sutton-McDowall, *et al*. 2012). A recent finding is that substituting FSH with

the EGF-like peptide amphiregulin, preserves the impact of BMP-15 on several metabolic measures, and co-operatively enhances developmental competence (Sugimura, *et al*. 2014), therefore acting differently to FSH.

The Hexosamine Biosynthesis Pathway

It is well established that both glucose and glutamine are essential substrates for the hexosamine biosynthesis pathway (HBP), required for the formation of hyaluronic acid and cumulus expansion during IVM (Frank, *et al*. 2014, Furnus, *et al*. 1998, Sutton-McDowall, *et al*. 2010). Prior to a cumulus matrix expansion signal (such the LH surge *in vivo*, or incubation with FSH *in vitro*), the activity of this pathway accounts for little glucose consumed by the COC (Gutnisky, *et al*. 2007, Sutton-McDowall, *et al*. 2004). The activity of this pathway is greatly activated by the cumulus expansion signal (Fig. 1), yet the signalling pathway regulating this is still unexplored in mammalian species to date, although candidates should include the Akt-mTOR pathway (Chen, *et al*. 2013) or ERK (Richani, *et al*. 2013).

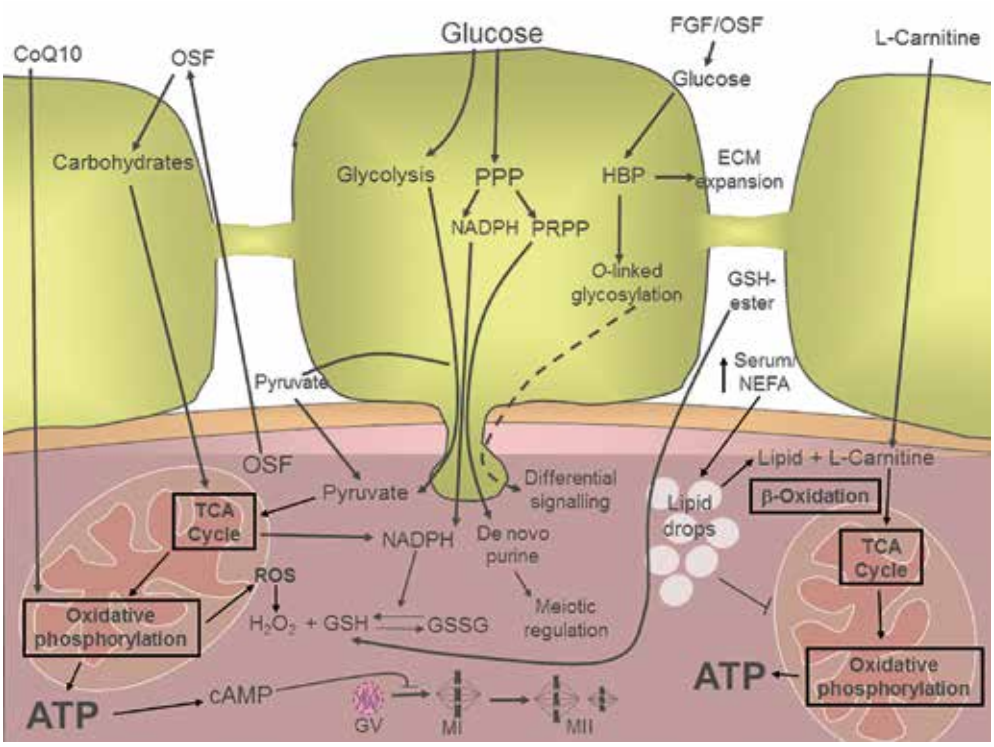

Fig. 1. Metabolism of the intact cumulus oocyte complex (COC). Carbohydrates, in particular glucose, are a primary energy source of energy and substrates for signalling. Glucose is metabolised by the cumulus cells via glycolysis (energy/pyruvate production), pentose phosphate pathway (PPP; NADPH production and meiotic regulation) and the hexosamine biosynthetic pathway (HBP; signalling and extra cellular matrix, ECM, synthesis). Within the oocyte, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation are important for metabolising pyruvate and endogenous lipids following β-oxidation, within the mitochondria. Endogenous anti-oxidants such as glutathione (reduced = GSH, oxidised = GSSG) are extremely important for combating reactive oxygen species (ROS), such as H_2O_2, a product of oxidative phosphorylation. There are numerous emerging factors that influence COC metabolism, such as oocyte secreted factors (OSF), fibroblast growth factor (FGF), co-enzyme Q10 (CoQ10) and L-carnitine.

Recently, (Caixeta, *et al*. 2013) demonstrated that mRNA levels for the HBP rate limiting enzymes, glutamine:fructose-6-phosphate transaminase (GFPT) 1 and 2 are up-regulated by either BMP-15 or FGF-10, with both these growth-factors stimulating a 30% increase in glucose uptake without a concomitant rise in lactate production, suggestive of an increased flux through the HBP.

Our previous review (Thompson, *et al*. 2007) pointed to the likely inhibitory consequences for post-compaction embryo development from an up-regulated HBP during oocyte maturation. This involves the participation of an "energy-sensing" pathway, β-*O*-linked glycosylation (O-GlcNAc), and its impact on intracellular signalling (reviewed by Frank, *et al*. 2014). This pathway is intimately involved in glucose sensing and heavily implicated in the pathology of diabetes (Frank, *et al*. 2014). Although our subsequent studies have been conducted in the mouse, it is pertinent to recall that the original observations were made in cattle and pigs (Sutton-McDowall, *et al*. 2006). In dairy cattle, oocyte quality is more likely to be affected by hypoglycaemic conditions. This too can implicate the HBP and O-GlcNAc (Frank, *et al*. 2013), yet there are no studies investigating this in ruminant oocytes.

Pentose Phosphate Pathway

There is increased interest in the Pentose Phosphate Pathway (PPP) as a result of the application of a relatively simple assay (the brilliant cresyl blue, or BCB assay) that measures activity for the pathway's rate-limiting enzyme, glucose-6-phosphate dehydrogenase (G-6-PDH). First described for use in GV-stage pig oocytes (Roca, *et al*. 1998), it has rapidly become a test for immature oocyte competence e.g. (Castaneda, *et al*. 2013, Pujol, *et al*. 2004). On addition of BCB, when G-6-PDH is active, the cytoplasm of GV stage oocytes turn from a deep blue colour to transparent. This is associated with poor cytoplasmic maturity. In contrast, oocytes remaining deep blue are more competent following maturation. Interestingly, this assay has overwhelmingly been used prior to oocyte maturation as an assessment of GV-stage oocyte development. However, PPP activity has been associated with meiotic and developmental competence of mature oocytes in the mouse and cow (Downs, *et al*. 1998, Gutnisky, *et al*. 2013a). Despite this, there are few studies investigating post-maturation use of the BCB assay. The exception is Mirshamsi, *et al*. (2013), who observed that whereas competent GV-oocytes stained blue, competent zygotes had clear cytoplasm, suggesting an increase in PPP activity over the course of maturation and fertilization.

Oxygen supply to the follicle and oocyte oxidative phosphorylation

An intriguing aspect of oocyte energetics is the oocyte's absolute requirement for oxidative phosphorylation (Fig. 1). This has been well established in several model species, including mouse and cow (Rushmer and Brinster 1973, Thomson 1967). Pyruvate is the preferred carboxylic acid, feeding into the tricarboxylic acid cycle. The O_2 consumption of the oocyte (per ml of tissue) is 3–fold higher than cumulus cells (Clark, *et al*. 2006), even though it would appear if cumulus does have a significant oxidative capacity (Cetica, *et al*. 2003). Further modelling, based on actual levels of O_2 consumption by whole COCs (Sutton, *et al*. 2003), suggest that the decrease in O_2 concentration at the cortex of the oocyte is approximately 3-4 mmHg (about 0.5%) less than within the follicular antrum, over a physiological range of antral pO_2 (Clark, *et al*. 2006). Therefore, relatively little O_2 is consumed by the cumulus layer, ensuring that the oocyte avoids hypoxia. The intrigue referred to above is that within large antral follicles, the oocyte lies within a highly avascular environment, many cell layers thick. Despite the publication by Van Blerkom (Van Blerkom, *et al*. 1997) on human follicles remaining a standout in this area, the question of whether follicular pO_2 regulates oocyte competence remains poorly addressed, largely because of the technical difficulties that such measurements present.

Using an alternative mathematical modelling approach, Redding and colleagues (Redding, *et al*. 2007, 2008) rationalised the formation of the follicular antrum as a response to O_2 and nutrient demand. Their conclusion is that formation of an expanding antrum allows cellular proliferation to occur without adding to the number of avascular cell layers surrounding the oocyte. As a consequence, they predicted that antral pO_2 would reflect the thickness of layers of mural granulosa and cumulus cells. In doing so, they predicted the human antral follicle pO_2 was quite variable, around 1.5 – 6.7% O_2 (11-51 mmHg); this appears similar to their data for cattle follicles (Redding, *et al*. 2006). At such concentrations, it is feasible that hypoxic response mechanisms occur, but there has been very little work conducted on mechanisms such as activation of the hypoxia inducible factor family of transcription factors in oocytes and follicles, with much of what is known being focussed on rodent models (Alam, *et al*. 2004, Alam, *et al*. 2009, Kind, *et al*. 2014).

Within the oocyte, mitochondrial activity and localisation reflect oocyte competence (Hashimoto 2009, Sugimura, *et al*. 2012). Highly competent ovine oocytes are associated with a peri-cortical and peri-nuclear mitochondrial distribution (Martino, *et al*. 2012). Generation of reactive oxygen species (ROS) is a normal consequence of oxidative phosphorylation within functioning mitochondria (Martino, *et al*. 2012, Morado, *et al*. 2009). Oxidative bursts within fertilizing mouse and cow oocytes are associated with chromatin reconfiguration (Dumollard, *et al*. 2004, Morado, *et al*. 2013). Morado and colleagues (Morado, *et al*. 2009) measured the changes in ROS patterns over the time-course of IVM and observed that ROS levels decrease between 6-18 hr maturation, coinciding with meiotic reconfiguration, usually at their lowest by 12 h (MI) in cattle oocytes. Against current dogma, these authors saw little association between ROS levels and inhibition or stimulation of oxidative phosphorylation, but observed a significant reduction with the addition of cysteine, an essential amino acid required for glutathione (GSH) synthesis. Much has been written about the requirement for antioxidant therapy, both during IVM of oocytes and subsequent embryo culture, yet most therapies applied *in vitro* yield disappointing results. Reduced GSH is a well characterised antioxidant and increasing intra-oocyte concentrations is known to be associated with improved oocyte competence in several ruminant species (Curnow, *et al*. 2010, de Matos, *et al*. 1995, de Matos, *et al*. 2002) (Fig. 1). Strategies to increase intra-oocyte GSH levels have involved the supplementation to medium of cysteine, usually in the presence of another reduced thiol compound, such as cysteamine or β-mercaptoethanol (de Matos and Furnus 2000). Cumulus cell GSH production from thiols such as cysteine and cysteamine appears to be important for the oocyte (de Matos, *et al*. 1997). An alternative that has yet to be fully exploited is the use of a GSH-ester, which has been demonstrated to increase intra-oocyte GSH levels in both cumulus-enclosed and denuded oocytes (Curnow, *et al*. 2010). Another powerful antioxidant that is generating interest is co-enzyme Q10 (CoQ10). CoQ10 is an essential component of the respiratory chain, and is widely used as an antioxidant dietary supplement. Supplementation of CoQ10 to bovine oocytes during *in vitro* maturation has been associated with increased ATP production (Stojkovic, *et al*. 1999) and can impact mitochondrial localisation and membrane potential, as well as gene expression and overall competence in a compromised oocyte model (Gendelman and Roth 2012).

The new insight in ruminant COC metabolism – lipid metabolism

At the time of writing the 2007 review (Thompson, *et al*. 2007), there was very little known regarding lipid metabolism in mammalian oocytes. In the intervening 8 years, this is where the greatest new insights have been obtained in the field, with wide implications for the fertility of cattle and the development of ruminant *in vitro* media systems.

Ruminant oocytes and cumulus cells are particularly lipid rich, containing considerably higher levels than human and mouse. For example, sheep and cow oocytes contain 21 ng/ml

and 15 ng/ml w/w lipids, respectively, compared with 6.3 ng/ml in mouse oocytes (reviewed by Sturmey, *et al*. 2009). The "time to attachment" hypothesis (Paczkowski, *et al*. 2013, Sturmey, *et al*. 2009), suggests the period between ovulation and implantation dictates lipid content; there is 4-6 days between ovulation and implantation in the mouse verses 15-30 days in sheep and cattle.

Triglycerides are the predominant lipid found within the oocyte, with palmitic acid and oleic acid accounting for 32% and 25% w/w of total lipids within cow oocytes and 25% and 26% w/w within sheep oocytes (McEvoy, *et al*. 2000a). While lipids are important substrates for steroid hormone, prostaglandins and membrane biosynthesis, lipids are also a valuable energy source. Lipid-derived fatty acids are metabolised to acetyl CoA via β-oxidation and when completely metabolised, 1 M of lipid (such as palmitic acid) will yield ~106 M ATP, compared to 27-31 M ATP from 1 M of oxidised glucose. Indeed, in the absence of carbohydrates, the addition of L-carnitine (a co-factor of carnitine palmitoyl transferase-I, the rate-limiting enzyme in β-oxidation (Dunning and Robker 2012)) assists in the mobilisation of endogenous lipid stores, capable of supporting pre-compaction embryo development similar to rates achieved in the presence of carbohydrates (Sutton-McDowall, *et al*. 2012) (Fig. 2). Conversely, inhibiting β-oxidation during maturation and early embryo development significantly perturbs oxygen consumption and blastocyst development (Ferguson and Leese 2006).

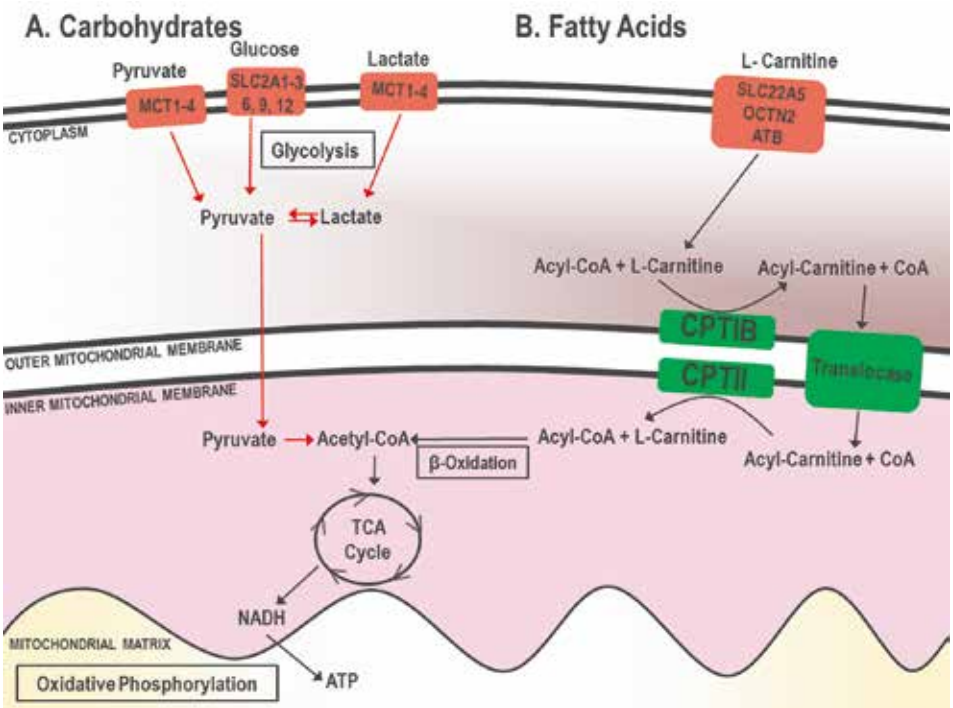

Fig. 2. Both A) carbohydrates and B) endogenous fatty acids can be utilised for energy production within the mitochondria, via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. MCT 1-4 = monocarboxylate transporters 1-4 (pyruvate/lactate); SLC2A 1-3, 6, 9, 12 = Solute carrier family 2, facilitated glucose transporter members (glucose); SLC22A5/OCTN2 = Solute carrier family 22 (organic cation/ carnitine transporter), member 5 (L-carnitine); ATB = amino acid/carnitine transporter B (L-carnitine); CPTIB/CPTIIB = carnitine palmitoyltransferase. Adapted from (Sutton-McDowall, *et al*. 2012).

Despite the importance of lipids as an endogenous energy source, increased lipid accumulation within COCs is associated with compromised developmental competence and fetal outcomes, such as large calf syndrome (Leese, *et al*. 1998, McEvoy, *et al*. 2000b). *In vitro* serum supplementation during cow and sheep IVP was identified as a major cause (reviewed by Young, *et al*. 1998). Increased lipid content is associated with poor cryo-tolerance of embryos, possibly due to poor lipid metabolism *in vitro* and the absence of L-carnitine in culture systems. When added during IVM, L-carnitine improved the cryo-tolerance of cattle COCs, resulting in similar on-time embryo development as un-vitrified oocytes following IVF and IVC (Chankitisakul, *et al*. 2013, Phongnimitr, *et al*. 2013). Both these studies used serum as a protein source, indicating that decreasing the serum-induced accumulation of lipid maybe the greatest benefit of L-carnitine under these conditions. It has yet to be determined if L-carnitine supplementation of medium containing either BSA or no protein results in increased developmental competence or cryo-tolerance.

Non-esterified fatty acids and dairy cow fertility

The increasing rate of sub-fertility in high performance, milk-yielding cows has been partially attributed to increased accumulation of fatty acids by the COC, in particular non-esterified fatty acids (NEFAs). NEFAs are long-chain (greater than 14 carbons) free fatty acids that are distinguished from triacylglycerides (esterified fatty acids). Many farming systems involve a 12-month calving interval, requiring cows to be pregnant by 60-80 days post-partum, coinciding with the peak lactation period (0-70 days post-partum). During peak lactation, metabolites (for example, up to 80% of glucose) are partitioned for lactation and away from other organs. As a consequence, body condition scores decrease and cows enter negative energy balance (NEB) (Bell, *et al*. 1995, Hocquette and Bauchart 1999, Walsh, *et al*. 2011). A consequence of NEB is the mobilisation of fats, in particular NEFAs, with the severity of NEB directly related to increases in circulating NEFA levels (Schneider 2004). Considering the final stages of oocyte development and maturation commence 90 days prior to ovulation (Fair 2010), oocyte developmental competence could be compromised, resulting in poor fertilisation, embryo development and early pregnancy losses. This led MacMillan (2007) to describe these cows as suffering from "Phantom Cow Syndrome", where cows fail to return to oestrus by the second round of artificial insemination, indicative of successful fertilisation but subsequent embryonic loss.

A cause of sub-fertility in high milk yielding dairy cattle that has recently gained attention is the influence of the peak lactation on the follicular environment. Levels of specific NEFAs, namely the saturated fatty acids palmitic (C16:0) and steric acid (C18:0) and the mono-unsaturated oleic acid (C18:1) are higher in follicular fluid (FF) compared with serum from 16 day post-partum cows (Leroy, *et al*. 2005), but returned to serum levels by 44 days post-partum. In addition, β-hydroxybutyrate levels at Day 16 post-partum mirror the elevated NEFA concentrations, further suggesting mobilisation of body reserves (Matoba, *et al*. 2012). When NEFAs were added to IVM culture of COCs at levels equivalent of Day 16 FF, bovine oocytes had more and larger lipid droplets, a reduced blastocyst yield and resultant blastocysts had less cells, higher rates of apoptosis and altered metabolism (Aardema, *et al*. 2011, Van Hoeck, *et al*. 2011). Gene expression of glucose metabolic enzymes were also influenced, with higher levels of lactate dehydrogenase (LDH1) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) observed in oocytes from COCs exposed to high NEFAs. Furthermore, these genes, in addition to glucose-6-phosphate dehydrogenase (G6PDH) were lower in cumulus cells (Van Hoeck, *et al*. 2013). Given the majority of glucose consumed by the COC is metabolised via glycolysis

within cumulus cells (Sutton-McDowall, *et al*. 2010), this suggests the presence of elevated NEFAs decreases glucose metabolism.

Insights from "metabolomics" studies in ruminants

To date, there are minimal metabolomic studies published investigating ruminant oocytes. Most studies have focused on human clinical oocyte and embryo development (Nagy, *et al*. 2009, Singh and Sinclair 2007). However, Bender and colleagues (Bender, *et al*. 2010) have successfully used gas chromatography-mass spectrometry (GC-MS) to analyse the different fatty acid and amino acid profiles of FF derived from Holstein heifers ("good") vs. lactating cows ("compromised"). Thirty seven fatty acids were detected (25 quantifiable) and 52 aqueous metabolites were detected (20 quantifiable) (Bender, *et al*. 2010). The predominant fatty acids reported here were similar to other reports (Leroy, *et al*. 2005). Matrix-assisted laser desorption ionization (MALDI) mass spectrometry has recently been used to analyse lipid content in oocytes and embryos of several species, including cattle (Ferreira, *et al*. 2010), revealing compositional differences under different O_2 atmospheres and between *in vivo* and *in vitro* derived oocytes. Analysis of amino acids and aqueous metabolites revealed L-alanine was lower and glycine, glutamine and urea were higher in the FF of cows compared to heifers. Metabolomics has also been utilised to determine the amino acid turnover by bovine oocytes during IVM. This study analysed amino acid metabolism of denuded oocytes between 18 – 24 hr IVM, comparing the spent medium profile from oocytes that had undergone cleavage and development to the blastocyst stage against unfertilised oocytes (Hemmings, *et al*. 2012). Oocytes that did not cleave had less glutamine and more alanine in the surrounding medium than successfully cleaving and developing oocytes. The similarity in specific non-essential amino acids that were altered between these two studies may not be a coincidence, but if so, the relationship is not clear.

The future of metabolic profiling of ruminant oocytes

Cattle COC metabolism is now characterised sufficiently to answer questions about differences between the effectiveness of *in vitro* media composition and the impact of maternal environments (such as differences between lactating dairy cattle and heifers). The number of parameters that can be measured is increasing, including reliable measures of the REDOX state, reduced glutathione, ATP/ADP and reactive oxygen species. We are gaining an understanding of how metabolism is facilitated by factors that influence competence, such as the impact of gap-junctional communication, OSFs and EGF-like peptides. What we have learnt is that there is enormous plasticity in metabolic regulation. Simply measuring one metabolic pathway or intermediate metabolite may not reconcile with developmental outcome. Therefore the future direction of metabolic research requires a more holistic and expansive determination of metabolism and this is where true metabolomic studies will be an important element. Nevertheless, endpoints of metabolism, such as ATP and reduced glutathione levels are at least indicative measures of competence.

In many aspects, our tools that have been applied to date to study ruminant oocyte metabolism have reached the limits of their capability. What is really required is the capacity to observe the dynamic nature of metabolism over a developmental period, such as the period of oocyte maturation. There are emerging tools to measure metabolism non-destructively over time, such as time-lapse incubation confocal microscopy. In addition to this, microscopic advances, especially in measuring fluorescence (whether it be autofluorescence or cell compatable

fluorophores) at unprecedented scales of resolution are being rapidly developed. New capabilities in photonics and nano-particle sensing offer opportunities to measure the dynamic nature of early developmental events within single oocytes and embryos, potentially while *in situ*.

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