**INVESTIGATION OF EXPOSURE TO LIFESTYLE AND
ENVIRONMENTAL FACTORS ON CUMULUS-OOCYTE
COMPLEX FUNCTION**

KELLY ANNE CAMPEN

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ABSTRACT

Pathways involved in bi-directional communication within the cumulus-oocyte complex (COC) include gap junction (GJ) communication, oocyte growth factor production, and glucose metabolism and are essential for oocyte health. Perturbation of these pathways may result in reduced oocyte quality due to altered COC function. Using rats as a model, *in vitro* effects of exposure to bisphenol A (BPA), caffeine, nicotine, ethanol, methylenedioxymethamphetamine (MDMA), or Δ^9 -tetrahydrocannabinol (THC) on COC function were investigated. Furthermore, MDMA was administered to rats to compare *in vitro* with *in vivo* effects.

The transfer of a fluorescent dye (calcein) from cumulus cells (CC) to the oocyte was used as a measure of GJ communication. Expression of CC-derived (*Atr*, *Cx43*, *Cycs*, *Gfpt1*, *Pfkp*) and oocyte-derived (*Atr*, *Bmp15*, *Cx37*, *Gdf9*) genes were measured using multiplex TaqMan quantitative PCR. Levels of CX43 and GDF9 proteins were quantified using Western blotting.

Optimisation of the GJ bioassay included the addition of phosphodiesterase inhibitors (rolipram and dipyridamole), and a 1 hour post-calcein incubation period to allow dye transfer. Quantification of gene expression in calcein-treated CC and oocytes was validated, enabling direct comparisons between GJ communication and gene expression.

To determine the *in vitro* effects, COC were incubated with test factors at high physiological concentrations over 25 hours. GJ communication decreased over time in control COC. This reduction was attenuated after exposure to BPA and nicotine, and partially by caffeine. Furthermore, exposure to ethanol maintained oocyte meiotic arrest, whereas MDMA and THC promoted meiotic resumption.

Oocyte-derived gene expression was mostly unaffected by *in vitro* exposure to the lifestyle and environmental factors, although a treatment x time interaction for *Cx37* levels following nicotine exposure was observed. Of the CC-derived genes, *Cx43* was the most sensitive where BPA, MDMA, and THC increased, and caffeine and ethanol decreased, expression. In CC, exposure to MDMA and THC increased *Gfpt1* levels and exposure to MDMA resulted in a treatment x time interaction in *Cycs* and *Pfkp* expression.

In COC, caffeine increased CX43 protein levels after 1 hour. Nicotine initially reduced, but with time increased CX43 levels. Furthermore, CX43 levels decreased and increased after 25 hour exposures to ethanol and MDMA, respectively. GDF9 protein levels in COC exhibited wide within-treatment variation. Overall, BPA and caffeine reduced GDF9 levels after 1 hour whereas GDF9 levels were increased following exposure to BPA, caffeine, MDMA, and THC for 25 hours.

To determine *in vivo* effects, female rats were administered saline or 5 mg/kg/day MDMA for 3 days. COC from MDMA-treated rats had higher levels of CX43 protein but gene expression and meiotic reactivation were unaffected.

In conclusion, COC function was altered by *in vitro* exposure to BPA, caffeine, ethanol, nicotine, MDMA, and THC. Furthermore, *in vivo* exposure to MDMA elicits similar, albeit reduced, effects on COC function. A role for CC in protecting the oocyte from harmful contaminants is proposed. Perturbation of the bi-directional communication pathway is likely to influence oocyte quality due to alterations in nutrient availability and timing of follicular events, although these may not be associated with negative outcomes. This study provides evidence that exposure to lifestyle factors and environmental contaminants affect COC function.

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LIST OF COMMONLY USED ABBREVIATIONS

AM	Acetoxymethyl
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
BCA	Bicinchoninic assay
BMP15	Bone morphogenetic protein 15
BPA	Bisphenol A
cAMP	Cyclic adenosine monophosphate
CC	Cumulus cell
cDNA	Complimentary deoxyribonucleic acid
CEEF	Cumulus cell expansion enabling factor
cGMP	Cyclic guanosine monophosphate
COC	Cumulus-oocyte complex
C _T	Cycle threshold
CX	Connexin
CYCS	Cytochrome c, somatic
CYP	Cytochrome P450
dH ₂ O	Distilled water
DMSO	Dimethyl sulfoxide
DO	Denuded oocyte
ECL	Electrochemiluminescence
FSH	Follicle-stimulating hormone
GC	Granulosa cell
GDF9	Growth differentiation factor 9
GFPT1	Glutamine-fructose-6-phosphate transaminase 1
GJ	Gap junction
GLUT	Glucose transport
GnRH	Gonadotrophin releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown

hCG	Human chorionic gonadotrophin
HHMA	3,4-dihydroxymethamphetamine
ip	Intraperitoneal
IVF	<i>In vitro</i> fertilisation
IVM	<i>In vitro</i> maturation
KO	Knockout
LH	Luteinising hormone
MAPK	Mitogen activated protein kinase
MDA	3,4-methylenedioxyamphetamine
MDMA	Methylenedioxymethamphetamine
MII	Meiosis II
mRNA	Messenger ribonucleic acid
OOX	Oocyctectomised
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PFKP	Phosphofructokinase, platelet
PKA	Protein kinase A
PPP	Pentose phosphate pathway
qPCR	Quantitative polymerase chain reaction
RPL19	60S ribosomal protein L19
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMAD	Similar to mothers against decapentaplegic
TBS	Tris buffered saline
TCA	Tricarboxylic acid
TGF β	Transforming growth factor β
THC	Δ^9 -tetrahydrocannabinol
XOD	Xanthine oxidoreductase

1 INTRODUCTION

1.1 Female Reproduction

1.1.1 *The ovary*

Female mammals have two ovaries located in the abdominal cavity on either side of the uterus. The ovary consists of a medulla and cortex enclosed by an epithelial layer called the ovarian surface epithelium. The cortical region contains the ovarian follicles, which are composed of an egg (oocyte) surrounded by a layer or layers of somatic cells and a basal lamina. The medulla region contains the main blood and lymphatic vessels.

1.1.2 *Ovarian follicular formation and growth*

Germ cells are first identified in the yolk sac during early embryonic life in mammals. They migrate from here to the gonadal ridge and differentiate into oogonia. These proliferating oogonia continue to migrate towards the ovarian cortex and, together with adjacent mesenchymal cells (pregranulosa cells), eventually coalesce into a convoluted array of ovigerous cords. Enclosed within these cords, the oogonia continue to proliferate but now are limited to recruiting pregranulosa cells from the proliferating ovarian surface epithelium. During this process, the oogonia enter meiosis and arrest at the diplotene stage of prophase I (see Sawyer et al., 2002). As the oogonia enter meiosis, many undergo apoptosis and their associated pregranulosa cells dissociate from these degenerating oogonia and re-attach to surviving oocytes. Prior to birth, individual meiotically-arrested oocytes surrounded by a single layer of squamous pregranulosa cells and enclosed by a basement membrane emerge from the base of the ovigerous cords as primordial follicles (Aerts and Bols, 2010). The assembly of primordial follicles is completed prior to birth in primates and domestic species, and during neonatal life in rodents (Rajah et al., 1992; Van den Hurk et al., 2000; Sawyer et al., 2002). It is generally accepted that this pool of non-growing primordial follicles is non-renewable although this view has been contested (Johnson et al., 2004).

Follicles initiate growth one at a time from the moment they are formed. Once growth has been initiated, the follicle grows without rest until it either becomes atretic and dies, or undergoes ovulation. Initiation of follicular growth takes place continuously regardless of the stage of reproductive life or time of the oestrous or menstrual cycle. This continuum is only

terminated at death or when the pool of follicles is depleted such as at menopause in humans. The mechanism by which individual primordial follicles are recruited into the growing pool is largely unknown. Activation of growth of primordial follicles is likely to occur as a result of both stimulating factors and removal of inhibitory factors. Several pathways have been identified to be involved in primordial follicle activation including KIT ligand signalling, NOBOX and SOHLH1 transcription factor activation, and anti-Mullerian hormone (Edson et al., 2009). The transition from the primordial to primary follicle stage is characterised by a change in granulosa cell (GC) shape, from flattened to cuboidal (Figure 1-1). All growing follicles that reach the developmental stage that requires support from pituitary hormones will undergo atresia if these hormone concentrations are inappropriate such as any time before puberty (McNatty, 2000), during anoestrus or during certain stages of the oestrous cycle (Hirshfield, 1991).

Progression from the primary stage to the secondary stage involves enlargement of the oocyte and proliferation of the GC to form multiple concentric layers. Theca cells, derived from the interstitial stroma, surround the basal lamina sometime after the primary stage of growth (Edson et al., 2009). Two distinct layers, the theca interna and externa, become evident by the late preantral (i.e. secondary) stages of development: the theca externa is a less-well differentiated layer while the more differentiated and vascularised theca interna region is localised adjacent to the follicular basal lamina. The theca interna is the major source of follicular androgens, notably androstenedione. Androstenedione is a primary substrate for follicular oestradiol production. During antral follicular growth, androstenedione is metabolised to oestradiol by the P450 aromatase enzyme present in the GC (Magoffin, 2005). After initiation of growth, a glycoprotein matrix forms, termed the zona pellucida, separating the oocyte from the GC. The zona pellucida is essential for normal fertilization (Edson et al., 2009). Throughout follicular growth, the GC secrete fluid which gradually coalesces to form an antral cavity during the late secondary stage of growth (Edwards, 1974). This fluid-filled antrum leads to the differentiation of two distinct sub-populations of GC: the cumulus cells (CC) and the mural GC (Figure 1-1 and Figure 1-2).

Figure 1-1: The structure of the ovarian follicle during different growth stages. Following initiation of follicle growth, the GC become cuboidal and proliferate to form multiple concentric layers. Secretions from the GC and exudates from the plasma coalesce to form a fluid filled antrum. Concurrently, the GC differentiate into two populations, the mural GC and the CC. The theca interna and theca externa form outside the basal lamina of the follicle. Modified from Johnston, L (VUW BBMedSci Honours thesis, 2010).

The CC directly surround the oocyte, and serve to nurture it (Su et al., 2009). The mural GC line the outer regions of the follicle, perform steroidogenesis and express receptors for pituitary gland-derived gonadotrophins, follicle-stimulating hormone (FSH) and in later follicular stages, luteinising hormone (LH). The differentiation of the mural GC and CC is proposed to be mediated by opposing gradients of FSH from the blood, and factors secreted by the oocyte (Diaz et al., 2007). Following the formation of the antrum and differentiation of the GC, the follicle is termed an antral or tertiary follicle.

During the primary and secondary phases, follicular growth is not reliant on gonadotrophin signalling; conversely tertiary follicles require a functional LH receptor signalling system in theca interna cells and a functional FSH receptor signalling system in GC for continued GC proliferation and synthesis of steroids such as oestradiol (Kumar et al., 1997). Those developing follicles that acquire functional LH receptors on GC are considered to be the 'dominant' follicles which will go on to be ovulated. The number of follicles that are ovulated during each reproductive cycle depends on the species. Species such as primates (e.g. humans) and ruminants typically ovulate one oocyte per cycle whereas species such as

rodents and pigs will ovulate multiple oocytes per cycle. These few dominant follicles are the major source of oestrogens and inhibins, which rise cyclically during the mid- to late-follicular stage of the oestrous cycle. Oestrogens and inhibins secreted from the dominant follicle(s) suppress FSH, but not LH, release from the pituitary, thus preventing any follicles that do not have LH receptors expressed on GC from sustaining oestradiol production and further maturation. As FSH is an essential factor for antral follicular development, it is thought a key mechanism to reduce the number of dominant follicles is lowering the plasma concentrations of FSH while increasing the GC responsiveness to LH. This allows maturation of the dominant follicle(s) to continue in increasingly low concentrations of FSH but rising concentrations of LH (Edson et al., 2009).

Figure 1-2: Sheep ovarian follicles at various stages of development. A pre-antral follicle with multiple concentric layers of GC may be seen at the bottom of the image. The follicle above this is beginning to form an antrum, while the next follicle has a more developed antrum. The top-most follicle has a large antral space, and the differentiated CC-oocyte complex (COC) is visible in the middle of the follicle. Image supplied by Ken McNatty.

The final stages of follicular growth involve the maturation of the oocyte nucleus and cytoplasm in preparation for ovulation (Eppig, 1996). Nuclear maturation includes the resumption of meiosis and will be discussed in Section 1.1.3. Cytoplasmic maturation prepares the oocyte for activation and preimplantation development and involves the storage of messenger ribonucleic acid (mRNA) transcripts and proteins for use after fertilisation. These mRNA stores are essential as the embryonic genome is not activated until a few days after fertilisation (Gosden and Lee, 2010). During cytoplasmic maturation, ultra-structural changes also occur including redistribution of the mitochondria, endoplasmic reticulum, and cortical granules (Marteil et al., 2009).

1.1.3 Oocyte meiotic arrest

From the time of follicular formation until the pre-ovulatory LH surge, the oocyte is arrested at the diplotene stage of the first meiotic prophase. During this time the DNA is contained within the nuclear envelope, termed the germinal vesicle (GV). The oocyte gains the ability to progress through meiosis around the same time as the antrum forms, although meiotic arrest is maintained until the preovulatory LH surge (Mehlmann, 2005). Resumption of oocyte meiosis can be recognised morphologically by the breakdown of the GV (GVBD). Meiotic progression results in release of the first polar body, but arrests again at metaphase II and only resumes at fertilization. Meiosis is completed following fertilization and the release of the second polar body (Edson et al., 2009)

Meiotic arrest has long been known to be regulated by high levels of cyclic adenosine monophosphate (cAMP) in the oocyte (Cho et al., 1974). Removal of the CC-oocyte complex (COC) from the follicle, or inhibition of gap junction (GJ) transfer, causes cAMP levels within the oocyte to decline, and the oocyte to resume meiosis (Pincus and Enzmann, 1935; Racowsky et al., 1989; Tornell et al., 1990). Within the oocyte, cAMP is synthesised by adenylyl cyclase which is stimulated by a constitutively activated G protein coupled receptor (Norris et al., 2009). Despite the presence of adenylyl cyclase (Conti et al., 2012), it has been shown that the oocyte receives much of its cAMP from the somatic cells via GJs (Thomas et al., 2002; Webb et al., 2002). Thus, it is understood that intra-oocyte cAMP levels are the net result of a supply of this second messenger from CC via GJ, synthesis within the oocyte itself and the rate of enzymatic degradation. The degradation (hydrolysis) of cAMP occurs mainly by phosphodiesterases (PDEs). The major PDE isoform reportedly expressed in mammalian

oocytes is PDE3A, and this has been shown to prevent oocyte nuclear maturation both *in vitro* and *in vivo* (Conti et al., 2002). While PDE3A preferentially and rapidly hydrolyses cAMP, it also hydrolyses another secondary messenger (cyclic guanosine monophosphate; cGMP), but at a much slower rate. Thus the presence of cGMP within the oocyte slows the hydrolysis of cAMP and is described as an inhibitor of cAMP degradation by PDE3A (Beavo, 1995). During meiotic arrest, cGMP is transferred to the oocyte from the CC via GJs, resulting in a reduction of cAMP hydrolysis by PDE3A (Norris et al., 2009).

One way through which FSH contributes to the maintenance of meiotic arrest is stimulation of the cGMP pathway. Studies in the rat using equine chorionic gonadotrophin reveal that activation of the FSH receptor stimulates mRNA expression of GC-derived natriuretic peptide precursor type C (*Nppc*), and its receptor on CC (*Npr2*). The action of NPPC on NPR2 results in an increase in cGMP production by the GC and CC (Zhang et al., 2010). In addition, oestradiol increases the levels of *Npr2* mRNA and maintains the ability of NPPC to stimulate cGMP production *in vitro* (Zhang et al., 2011). Conversely, treatment of mouse follicles with LH caused a rapid reduction in NPR2 guanylyl cyclase activity, without reducing NPR2 protein levels (Robinson et al., 2012). The preovulatory LH surge also triggers closure of the GJs as a result of mitogen activated kinase (MAPK)-dependent phosphorylation of the GJ protein connexin 43 (CX43) (Norris et al., 2008). The net result of NPR2 inhibition and closure of the GJs is that both cAMP and cGMP concentrations within the oocyte decrease and meiosis resumes.

As discussed earlier, oocyte maturation in response to the preovulatory LH surge is a cAMP-mediated process. However, cAMP has paradoxical actions in the CC and oocyte. Following the LH surge, a transient rise in cAMP levels occurs in the GC. In the rat, elevated levels of cAMP levels in the follicular cells as a result of inhibition of PDE4 promotes oocyte maturation in the absence of LH (Tsafriri et al., 1996). PDE4D is the major PDE isoform expressed in the CC and GC in rat follicles and is cAMP specific (Tsafriri et al., 1996). PDE4D regulation of LH receptor signalling is essential, as demonstrated by a PDE4D knockout (KO) mouse model. Following human chorionic gonadotrophin (hCG) stimulation, follicles from KO mice showed an abnormal pattern of cAMP accumulation and failed to rupture, resulting in a decreased ovulation rate (Park et al., 2003).

In addition to PDE3 and 4, PDE1, 5, 7, 8, 10, and 11 have also been detected in follicles from various species (Sasseville et al., 2009a; Conti, 2011). However, the role of these PDE families in oocyte maturation is less well known and is thought to be secondary to the actions of PDE3/PDE4 in most species. The exception to this is PDE8, which accounts for around 20 % and 60 % of PDE activity in the bovine oocyte and CC, respectively (Sasseville et al., 2009a).

Detection of DNA damage and replication errors during meiosis is critical for maintaining genetic integrity. Two protein kinases that detect DNA damage and regulate progression of meiosis have been identified. The ataxia telangiectasia mutated (*Atm*) gene is activated by ionizing radiation and double-strand breaks. The ataxia telangiectasia and Rad3-related (*Atr*) gene is primarily activated by UV light, hydroxyurea, stalled replication forks and single-stranded DNA, but may also be activated by ATM in response to double-strand breaks (Rhind, 2009). Activation of the ATR protein causes the phosphorylation of multiple proteins including CHK1, BRCA1, and NBS1 and results in the formation of check point complexes and G₂/M arrest (Abraham, 2001; Miles et al., 2010). ATR is localised at unsynapsed chromosomes during the pachytene stage of prophase in human oocytes (Garcia-Cruz et al., 2009). Pachytene is a stage of prophase during which crossing over between homologous chromosomes occurs. The pachytene checkpoint monitors DNA damage, repair of recombination intermediates, and chromosomal synapsis prior to chiasma formation (Kogo et al., 2012). In addition, phosphorylation of ATR/ATM substrates has been detected in bovine oocytes during the second stage of meiosis (MII) (Siemer et al., 2009). Moreover, *Chk1* mRNA expression is present in germ cells of female mice at 14.5 days post conception (Hasthorpe et al., 2007) and is essential for maintaining meiotic arrest in *Xenopus laevis* oocytes (Nakajo et al., 1999).

1.1.4 The pre-ovulatory LH surge and ovulation

As follicles grow and synthesise inhibin subunits and oestradiol, levels of inhibins and oestradiol within both the follicular fluid and circulation increase. The high levels of inhibins act on the pituitary gland to inhibit FSH production. Oestradiol production in dominant follicles is driven by the increasing release of LH acting on LH receptors expressed in mural GC of dominant follicles (Edson et al., 2009). The positive feedback effect of oestradiol leads to a preovulatory LH surge. As discussed previously (Section 1.1.3) the LH surge triggers

oocyte nuclear maturation and resumption of meiosis. In addition, the LH surge initiates morphological changes of the follicle, which are required for ovulation.

The rise in LH levels stimulates the CC to secrete a specialised extracellular matrix, composed of a backbone of hyaluronan in a process termed CC expansion. CC expansion is mediated by MAPK signalling (Su et al., 2002) and oocyte paracrine factors (Buccione et al., 1990b) and results in the CC transforming from a tightly packed mass to an expanded mucified complex. CC expansion is essential for extrusion of the oocyte from the follicle (Kidder and Vanderhyden, 2010). The follicle wall is also remodelled through capillary haemorrhage, proteolytic degradation and cell death at the apical site and luteinisation of the GC. These events culminate in follicular rupture thereby allowing the expanded COC to be released from the follicle for entry into the reproductive tract (Russell and Robker, 2007).

The preovulatory LH surge also stimulates luteinisation of the mural GC. Following ovulation and release of the expanded COC, the luteinised GC and, in some species, the theca cells differentiate from the remnants of the haemorrhagic follicle to form the corpus luteum (CL). The CL is a specialised structure that in all species produces progesterone (and in some species oestradiol and inhibin) to stimulate the uterus to prepare for pregnancy (Edson et al., 2009).

1.2 The Role of GJs in Folliculogenesis

1.2.1 General GJ structure and function

GJs are intercellular channels that allow direct passage of small molecules (<1 kDa) between the cytoplasm of two adjacent cells. Cells share ions, small metabolites, and second messengers through GJs as a means of intercellular signalling (Bruzzone et al., 1996). GJs are composed of integral membrane proteins (connexins; CX), which form hexameric pore structures termed hemichannels or connexons (Figure 1-3). The docking of two hemichannels results in formation of a GJ channel.

There are around 20 different connexin proteins encoded in the mammalian genome. Most cells express multiple connexin isoforms, and each connexin isoform has a characteristic tissue expression pattern (Goodenough and Paul, 2009). Connexins are named according to their size; the smallest connexin is CX26 (26 kDa) while the largest connexin is CX57 (57 kDa).

Figure 1-3: Basic structure of GJ channels. CX proteins are transmembrane proteins that form hexameric pore structures (hemichannels). The docking of two hemichannels from adjacent cells forms a GJ channel through which small molecules can pass between the cells.

All connexins have the same structure consisting of a cytoplasmic C terminus, four membrane spanning domains, two extracellular loops, an intracellular loop, and a cytoplasmic N terminus (Figure 1-4).

Oligomerization of six connexins to form a hemichannel occurs in the endoplasmic reticulum and Golgi body (Musil and Goodenough, 1993). The hemichannels are transported in vesicles which move along microtubules to the plasma membrane. The vesicles fuse with the plasma membrane, thus delivering the hemichannels to the cell surface (Gaietta et al., 2002; Segretain and Falk, 2004). Analysis of the crystal structure of human CX26 GJs has shown that the hemichannels possess a positively charged cytoplasmic opening, a narrow funnel, a negatively charged transmembrane region and an extracellular exit (Maeda et al., 2009). A large amount of variation in GJs occurs through the different composition of hemichannels. Hemichannels may contain only one connexin isoform or multiple connexin isoforms; these

Figure 1-4: Structure of connexin proteins. Connexins are transmembrane proteins which have four transmembrane domains, two extracellular loops, one cytoplasmic loop, and cytoplasmic C- and N-termini. Figure adapted from Kidder and Mhawi, 2002; Maeda et al., 2009.

are termed homomeric and heteromeric hemichannels respectively. It is thought homomeric and heteromeric hemichannels have different permeability to small molecules. Although unpaired hemichannels were previously thought to be non-functional, they have recently been found to play a role in extracellular release of adenosine triphosphate (ATP), Nicotinamide adenine dinucleotide (NAD^+), prostaglandins, and glutamate, and transduction of Ca^{2+} waves (Tong et al., 2007).

Two hemichannels from adjacent cells dock through interactions between cysteine residues in the extracellular loops (Dahl et al., 1991). The two hemichannels that make up a GJ channel may be identical or non-identical, forming homotypic or heterotypic GJs respectively (Goodenough and Paul, 2009). The permeability of homotypic GJ channels to small molecules varies greatly. For example, ethidium bromide does not pass through GJs composed of CX31 or CX32, but will pass through GJs composed of CX26, CX37, CX40, CX43,

or CX45. Moreover, total conductance of GJ channels in cells transfected with CX31 or CX45 is only around 50 % of that observed in cells transfected with other isoforms (Elfgang et al., 1995). Less information is available about the permeability of heterotypic GJ channels due to their complexity, but it is likely that different combinations of connexins give rise to GJ channels with different physiological properties, in a similar manner as that described for homotypic channels (Goodenough and Paul, 2009).

GJs are further assembled into large GJ plaques, which may contain several to thousands of GJ channels. Formation of GJ plaques may be essential for GJ function, as CX43 hemichannels that are not part of a plaque are unlikely to form GJs, and those GJs that are formed are not functional. However, within a GJ plaque composed of CX43 GJs only 2-20 % of the GJs are functional, depending on the size of the plaque (Bukauskas et al., 2000).

GJ permeability is also modified in response to cytoplasm acidification and changes in Ca^{2+} concentration however these modifications are slower than voltage gating. Increases in H^+ and Ca^{2+} ion concentrations have been demonstrated to reduce the permeability of almost all GJs (Peracchia, 2004). A number of other compounds that specifically inhibit GJs have been identified. These compounds are termed GJ uncouplers and include glyccerrhetinic acid derivatives, long-chain alkanols, and acidifiers (Bukauskas and Verselis, 2004). Inhibition of GJs by GJ uncouplers is reversible and is thought to reduce conductance to the residual state in a similar manner to voltage gating.

GJs are regulated by phosphorylation. All connexins, with the exception of CX26, are phosphoproteins (Ahmad and Evans, 2002; Moreno, 2005). Multiple phosphorylation steps regulate GJ assembly. Connexins are phosphorylated on specific residues during transport through the Golgi, and these phosphorylation sites appear to be essential for trafficking (Solan and Lampe, 2007). Phosphorylation can also inhibit or stimulate connexin degradation (Goodenough and Paul, 2009). Phosphorylation of CX43 at serine (S)368 is thought to prevent CX43 from being assembled into GJs, and results in a 50 % decrease in unitary conductance (Lampe et al., 2000). CX43, the best studied of the connexin isoforms, contains two serines within its N-terminal region and 21 serines within the C-terminal cytoplasmic region (Lampe and Lau, 2000).

GJs are regulated by rapid turnover: the half-life of CX43 in most cell types is <3 hours. Indeed, most connexin isoforms have a half-life of less than 10 hours (Hervé et al., 2007). Turnover of GJs occurs by removal of the channels at the centre of a plaque, and additionally at the edges (Gaietta et al., 2002). Moreover, GJs may be removed from the cell surface by internalisation of the entire GJ plaque (Jordan et al., 2001).

1.2.2 The Role of GJs in Folliculogenesis

The follicle functions as a syncytium through folliculogenesis, with the oocyte coupled to the surrounding somatic cells by GJ channels. In a tertiary follicle, the oocyte is coupled to the CC, that are then coupled to the mural GC by GJ channels (Gilula et al., 1978). GJs allow intercellular signalling between the oocyte and somatic cells and provide a pathway for the CC to supply the oocyte with essential nutrients. Glucose metabolites, amino acids, cyclic nucleotides, ions, choline, and inositol are all passed to the oocyte through GJ channels (Kidder and Vanderhyden, 2010). In addition, the GC regulate the pH of the growing oocyte by transferring ions to the oocyte via GJs (FitzHarris et al., 2007). The GJ channels between the oocyte and CC occur at the end of CC trans-zonal projections (Kidder and Mhawji, 2002).

The major connexin isoforms expressed in rat ovarian follicles are CX37 (expressed in the oocyte) and CX43 (expressed in CC and GC) (Teilmann, 2005). In addition, CX30.3, CX32 and CX45 are expressed in lower proportions in rat GC. Whilst CX57 has been detected in mouse CC, it has not been measured in rat CC (Kidder and Mhawji, 2002).

The essential role of CX37 in follicular growth has been demonstrated by targeted gene KO studies. As CX37 is the major connexin expressed in the oocyte, oocyte-GC coupling is abolished in mice that have a KO of the *Cx37* (alternatively named *Gja4*) gene that encodes CX37. Growth of follicles initially appears to proceed normally in *Cx37*-KO mice however halts at the preantral stage (Simon et al., 1997). The size of oocytes from these mice reached only 74 % of that of fully grown oocytes from wild-type mice and did not gain meiotic competence (Carabatsos et al., 2000). The ovaries of *Cx37*-KO mice eventually become filled with CL-like structures, indicating premature differentiation of the GC into luteal cells (Simon et al., 1997). Expression of *Cx37* mRNA in oocytes, but not GC, is essential for follicle growth as demonstrated by the use of chimeric ovaries containing wild-type or *Cx37*-KO oocytes and wild-type or *Cx37*-KO GC in all combinations (Gittens and Kidder, 2005). Chimeric ovaries

were created *in vitro* and implanted in the kidney capsule of ovariectomized female wild-type mice. Ovaries containing wild-type oocytes and Cx37-KO GC showed similar proportions of follicles at each stage of development compared to ovaries containing wild-type oocytes and wild-type GC. However, ovaries containing Cx37-KO oocytes and wild-type GC had significantly reduced proportions of late secondary and tertiary follicles compared to completely wild-type ovaries.

Due to the expression of CX43 in many tissues, mice which have a targeted KO of *Cx43* (alternatively named *Gja1*) gene are aborted *in utero*. In order to study the role of CX43 in follicular growth, *Cx43*-KO ovaries from neonatal mice were cultured *in vitro* before being grafted into the kidney capsule of ovariectomized wild-type adult mice (Ackert et al., 2001). GC from *Cx43*-KO mice were unable to proliferate causing follicular growth to arrest at the primary stage. Oocytes from *Cx43*-KO ovaries were morphologically abnormal, did not gain meiotic competence and could not be fertilised. Dye transfer experiments indicated that some GJ channels were retained between the GC, consistent with expression of other connexin isoforms. The essential role of GC, but not oocyte, expression of CX43 was demonstrated using chimeric ovaries containing wild-type or *Cx43*-KO oocytes, and wild-type or *Cx43*-KO GC in all combinations (Gittens and Kidder, 2005). Ovaries with *Cx43*-KO oocytes and wild-type GC contained follicles at all developmental stages in similar proportions to those seen in ovaries containing wild-type oocytes and wild-type GC. Ovaries with wild-type oocytes and *Cx43*-KO GC had significantly reduced proportions of early secondary, late secondary and tertiary follicles compared with the wild-type comparison, consistent with a block at the primary stage of follicular development.

As *Cx37* mRNA is expressed solely by the oocyte and *Cx43* mRNA is expressed in the CC and GC, the GJs that form between the oocyte and CC must be heterotypic. Conductance studies of cell lines transfected with rat *Cx43* and/or human *Cx37* genes showed that homotypic, heterotypic, and heteromeric GJs are able to be formed *in vitro* (Brink et al., 1997). However, to my knowledge this has not been demonstrated *in vivo*.

Within the follicle, *Cx43* mRNA and protein levels are dynamically regulated throughout the oestrus cycle by gonadotrophins. In antral follicles of rats that express FSH receptors, rising levels of FSH stimulate an increase in *Cx43* mRNA and protein levels (Granot and Dekel,

1997). As discussed previously (Section 1.1.3), cAMP and cGMP pass through the GJ to the oocyte to maintain the threshold concentration of cAMP that inhibits meiotic resumption. The FSH-mediated increase in oocyte cAMP is blocked by treatment with the GJ uncoupler carbenoxolone (Webb et al., 2002) confirming that cAMP is transferred to the oocyte from the CC via GJ. Following the LH surge in mice, the decrease in GJ permeability between the oocyte and CC is mediated by the phosphorylation of CX43 at S255, S262, and S279/S282 (Norris et al., 2008). Subsequently, *Cx43* mRNA expression is abolished (Granot and Dekel, 1997). To my knowledge, *Cx37* gene expression has not been reported to be regulated by gonadotrophins.

The CC transfer a range of nutrients to the oocyte via GJs. Compared with denuded oocytes (DO), CC-enclosed mouse oocytes had a significantly higher uptake of [³H]glycine and [³H]choline, however this was abolished following treatment with the GJ uncoupler 18- α -glycyrrhetinic acid (Haghighat and Van Winkle, 1990). Glucose and glucose metabolites are transferred into the oocyte from the CC via GJs. The amount of a fluorescently-labelled glucose derivative in the mouse oocyte was significantly reduced when the GJs were blocked with the GJ uncoupler carbenoxolone (Wang et al., 2012). CC-enclosed mouse oocytes also contain higher amounts of amino acids glycine, alanine, proline, serine, tyrosine, glutamic acid, and lysine compared with DO (Colonna and Mangia, 1983). Moreover, Ca²⁺ and Na⁺ ionic currents between mouse CC and oocytes were abolished following treatment with the GJ uncoupler octanol (Arellano et al., 2002).

In addition to fully-formed GJ channels, undocked hemichannels may play a role in ovarian folliculogenesis. Dye uptake and ATP release, indicative of hemichannel opening, was observed in mouse GC following application of stimuli known to cause hemichannel opening (Tong et al., 2007). However hemichannel function in the absence of GJs is not sufficient for follicle growth. Reaggregated ovaries containing GC with a mutant *Cx43* gene that lacked the conserved cysteines in the extracellular loops required for docking were created. Follicle growth was arrested at the primary stage, similar to *Cx43*-KO mutants.

Figure 1-5: Schematic of a GJ bioassay utilising calcein to measure GJ communication between the CC and oocyte. Calcein-AM enters the CC by permeating the cell membrane (1). Inside the CC, the AM group is cleaved by non-specific esterases (2). The resultant calcein molecule is lipid insoluble and fluorescent (3). Calcein is transferred to the oocyte through GJ channels (4).

A technique developed to determine GJ activity between the CC and oocyte utilised the transfer of a fluorescent dye, calcein (Thomas et al., 2004). Intact COC were exposed to the acetoxymethyl (AM) derivative of the calcein. Calcein-AM is a non-fluorescent, lipophilic molecule that readily crosses the cell membrane. The AM group is cleaved by non-specific esterases inside the cell. As a consequence of removing the AM moiety, the resultant calcein is now lipid insoluble and hydrophilic. Thus being unable to permeate the cell membrane, calcein can now only be transferred between cells through GJ channels (Figure 1-5).

1.3 Oocyte-secreted factors

1.3.1 Oocyte-secreted factors and follicular development

During follicular growth, co-ordination between the follicular somatic cells and the oocyte is essential. As discussed in the previous section, the CC transfer nutrients to the oocyte via GJs. The oocyte regulates CC function through secretion of growth factors, thus creating a bi-directional communication loop. Aspiration of the COC causes the remaining GC to undergo

premature luteinisation (el-Fouly et al., 1970). Soon after it was found that rat GC co-cultured in close proximity to the oocyte retained their GC phenotype whereas GC further away from the oocyte underwent luteinisation (Nekola and Nalbandov, 1971). Further investigation revealed that oocyte-secreted factors form a concentration gradient in the follicle, and this influences the differentiation of GC into either the CC or the mural GC. Compared to mural GC, CC have a higher proliferation rate and lower steroidogenic activity and expression levels of LH receptor. The CC also possess the ability to secrete hyaluronic acid and undergo expansion, unlike the mural GC (Gilchrist et al., 2004a). The best characterised of the oocyte-secreted factor candidates are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). In most species studied to date, GDF9 and BMP15 are co-expressed in a species-specific manner and the two secreted proteins function synergistically (McNatty et al., 2005a; Crawford and McNatty, 2012). Although the addition of GDF9 and BMP15 to cultured GC in part mimics the addition of oocytes, they are not sufficient to produce the range of GC responses caused by oocytes (Gilchrist et al., 2004a). This indicates that other oocyte-secreted factors exist that may play a role in somatic cell function. Alternatively, the recombinant forms of GDF9 and/or BMP15 tested *in vitro* may have been at inappropriate concentrations or ratios, or different biopotencies to that of native GDF9 and BMP15. Currently, other candidates for oocyte-secreted factors are also members of the TGF β family, including BMP6 and the activins.

Bioassays are commonly used to determine the effects of oocyte-secreted factors on GC function. One common method involves removal of the oocyte from the COC to produce oocyctomised (OOX) CC. The basis of this bioassay is to determine the functions of CC that are regulated by oocyte-secreted factors, on the assumption that OOX CC will be significantly altered compared to CC from intact COC. These OOX CC may then be co-cultured with DO which would rescue the CC function (Gilchrist et al., 2008).

The importance of the oocyte on follicular development was demonstrated by transferring mouse oocytes from secondary follicles into primordial follicles. The rate of follicular growth in the re-aggregated follicles was doubled, producing antral follicles within 9 days as opposed to 20 days in control follicles (Eppig et al., 2002). This indicates that the oocyte strongly regulates follicular growth and development, including GC proliferation. Studies involving the culturing of DO with GC revealed oocyte-secreted factors stimulated the

proliferation rate of GC in many mammalian species including cows (Li et al., 2000; Glister et al., 2003), mice (Vanderhyden, 1992; Gilchrist et al., 2004b) and rats and sheep (McNatty et al., 2005a, 2005b; Lin et al., 2012). In mice, co-culture of mural GC with DO caused an up-regulation of the gene encoding cyclin D2 in the GC (Gilchrist et al., 2006). These actions of DO on GC proliferation are due, at least in part, to both GDF9 and BMP15 as is observed in studies in rodents (Otsuka et al., 2000; Vitt et al., 2000, Lin et al., 2012). Immunoneutralization of GDF9 in an *in vitro* bioassay abolished around half of the mitogenic activity of mouse oocytes, indicating that other oocyte-secreted factors are also involved in stimulation of GC proliferation (Gilchrist et al., 2004b). Importantly, GDF9 and BMP15 act in synergy with one another to stimulate proliferation in rat and ovine GC (McNatty et al., 2006). Addition of recombinant murine GDF9 in combination with ovine BMP15 to rat GC enhanced ³H-thymidine uptake by greater than 3-fold compared to either oocyte-secreted factor alone (McNatty et al., 2005a). Addition of recombinant ovine GDF9 in combination with ovine BMP15 to ovine GC enhanced ³H-thymidine uptake by 1.6-fold compared to GC cultured in the absence of oocyte-secreted factors (McNatty et al., 2005b). It should be noted that there are significant species differences in the potency of oocyte-secreted factor action on GC proliferation that are outside the scope of this literature review. Additionally, whilst some oocyte-secreted factors (e.g. murine) strongly stimulate GC proliferation *in vitro* (Gilchrist et al., 2001), oocyte-secreted factors from other species (e.g. bovine and porcine) require the addition of insulin-like growth factor 1 (Li et al., 2000; Hickey et al., 2005).

In addition to the effects of oocyte-secreted factors on GC proliferation, oocyte-secreted factors have also been reported to reduce luteinisation of CC. Relatively high expression levels of the LH receptor and high steroidogenic activity are observed in mural GC from intact human and bovine follicles compared to CC (Amsterdam et al., 1975; Li et al., 2000). Evidence for the involvement of oocyte-secreted factors in regulating somatic cell luteinisation is further strengthened by the observation that progesterone production and LH receptor expression are increased in mouse OOX CC compared to either intact COC or OOX CC cultured with DO (Eppig et al., 1997). Both BMP15 and GDF9 are inhibitors of CC luteinisation in rats. The action of BMP15 alone does not affect steroidogenesis, but inhibits FSH action thereby abolishing FSH-mediated progesterone production and upregulation of the LH receptor and other key receptors involved in luteinisation (Otsuka et al., 2001). GDF9

inhibits the stimulatory effect of FSH on oestradiol and progesterone production, LH receptor activity, and cAMP production by GC (Vitt et al., 2000). Both GDF9 and BMP15 cooperate to inhibit luteinisation of rat and bovine GC (McNatty et al., 2006). In both of these studies, the combined addition of recombinant ovine GDF9 and BMP15 resulted in reduced progesterone production compared to either GDF9 or BMP15 alone.

As discussed in Section 1.1.4, CC expansion occurs just prior to ovulation in response to unidentified oocyte paracrine factors (termed the CC expansion enabling factor; CEEF) and the preovulatory LH surge. In mice, expansion of CC can be prevented by removal of the oocyte or inhibition of oocyte signalling, and can subsequently be restored by co-culturing CC with DO (Buccione et al., 1990a). Though the identity of the CEEF is currently unknown, GDF9 has long been suspected as the primary candidate. This is supported by the findings that GDF9 stimulates CC expansion *in vitro* (Elvin et al., 1999) and that DO from *GDF9* KO mice were unable to stimulate CC expansion in wild-type CC. However, there is evidence negating that GDF9 alone is sufficient to induce CC expansion. For example, immunoneutralization of GDF9 did not inhibit oocyte stimulated CC expansion (Dragovic et al., 2005). Moreover, meiotically incompetent oocytes from small growing follicles express GDF9 (Juengel and McNatty, 2005) but are not able to stimulate CC expansion (Vanderhyden et al., 1990). OOX CC isolated from *BMP15*^{-/-} *GDF9*^{+/-} double mouse mutants had a reduced ability to undergo expansion compared to either the single mutants (i.e. *BMP15*^{-/-} *GDF9*^{+/+} or *BMP15*^{+/+} *GDF9*^{+/-}) (Su et al., 2004). Taken together, these results suggest that while GDF9 may partially account for the ability of the oocyte to promote CC expansion, BMP15 and other oocyte factors may also play a role.

In vitro studies have also revealed that in a similar manner to DO, GDF9 and BMP15 significantly prevent CC apoptosis in bovine OOX CC (Hussein et al., 2005). Interestingly however, culturing OOX CC with GDF9 alone had no effect on CC apoptosis. The action of GDF9 has also been reported on follicular constituents additional to CC. The addition of GDF9 to cultured rat GC stimulates the production of inhibin A and inhibin B (Roh et al., 2003). It has also been reported to regulate theca cell function, as recombinant GDF9 strongly stimulated basal and forskolin-stimulated androstenedione production in theca cells *in vitro* (Solovyeva et al., 2000).

The regulation of GC function by the oocyte varies during folliculogenesis. The ability of oocytes to stimulate GC proliferation decreases as the oocyte matures. Thymidine uptake in murine GC was greatest when the GC were co-cultured with oocytes that had a visible GV. Although maturing and ovulated oocytes (i.e. those that had initiated GVBD) were also able to stimulate thymidine uptake in GC, the effect was attenuated (Gilchrist et al., 2001). Conversely, the ability of the oocyte to inhibit progesterone production in GC increases throughout maturation. Progesterone production was maximally inhibited when murine OOX CC were co-cultured with fully-grown oocytes rather than growing oocytes. However, CC from ovulated COC produced large amounts of progesterone due to a reduced ability to respond to the oocyte-secreted factors (Vanderhyden and Macdonald, 1998). Both LH receptor mRNA expression and CC-responsiveness to FSH and CEEF are developmentally regulated by oocyte-secreted factors in mice in a similar manner to progesterone production (Vanderhyden et al., 1990; Eppig et al., 1997).

1.3.2 Oocyte-secreted factors and follicular metabolic functions

Fully-grown bovine and rat oocytes have a poor capacity for glucose metabolism, yet are completely reliant on oxidative phosphorylation for ATP production (Zeilmaker and Verhamme, 1974; Cetica et al., 2002). In large antral follicles, CC readily import and metabolise glucose, and transfer glucose metabolites to the oocyte for ATP production (Brower and Shultz, 1982; Cetica et al., 2002). The expression levels of enzymes involved in glucose breakdown, including phosphofructokinase (*Pfkp*), aldolase A (*Aldoa*), triosephosphate isomerase (*Tpi*), α -enolase (*Eno1*), pyruvate kinase (*Pkm2*), and lactate dehydrogenase (*Ldh1*), showed a morphogenic gradient across the mouse follicle with highest levels being measured in the CC and lowest levels in the outer mural GC (Sugiura et al., 2005). Gene expression levels of *Eno1*, *Pkm2*, *Ldh1*, and *Pfkp* were significantly downregulated in OOX CC compared with intact COC in mice. However, expression levels were restored to those seen in intact COC when OOX CC were cultured with fully grown oocytes. Similarly, glycolytic activity and tricarboxylic acid (TCA) cycle activity in mice were equivalent in intact COC and OOX CC cultured with fully grown oocytes but were downregulated in OOX CC.

These results demonstrate the potent effect that oocyte-secreted factors have on CC glucose metabolism. Importantly however, there are significant species differences in the ability of

oocyte-secreted factors to regulate CC glycolytic activity. For example, bovine COC, OOX, and OOX co-cultured with DO showed similar glucose consumption *in vitro* (Sutton et al., 2003). Conversely, mouse OOX have a 10-fold decrease in glycolytic activity and reduced expression of *Pfkp*, and *Ldh1* compared to COC and OOX co-cultured with DO (Sugiura et al., 2005). Oocyte-secreted factors are also implicated in the regulation of cholesterol biosynthesis in CC in mice. The expression of enzymes involved in cholesterol biosynthesis, including mevalonate kinase (*Mvk*), phosphomevalonate kinase (*Pmvk*), farnesyl diphosphate synthase (*Fdps*), squalene epoxidase (*Sqle*), cytochrome p450 51 (*Cyp51*), sterol-C4-methyl oxidase-like (*Sc4mol*), and 3 β -hydroxysteroid- Δ 8, Δ 7-isomerase (*Ebp*) were elevated in CC of COC, compared to OOX CC (Su et al., 2008). Similar to the glycolytic pathway, GDF9 and BMP15 also regulate cholesterol biosynthesis. In *Bmp15* and *Gdf9* double KO mutants, lower levels of enzymes involved in cholesterol biosynthesis, (*Mvk*, *Pmvk*, *Fdps*, *Sqle*, *Cyp51*, *Sc4mol*, and *Ebp*) as well as glycolytic enzymes (*Pfkp* and *Ldh1*), were measured in CC. In addition, reduced cholesterol biosynthesis and glycolytic activity was observed in CC from the double mutants (Su et al., 2008). Treatment of double mutant CC with recombinant BMP15 in combination with fibroblast growth factor 8B (FGF8B) promoted expression of *Pfkp* and *Ldh1*. As GDF9 did not promote expression of *Pfkp* and *Ldh1*, it appears that BMP15 but not GDF9 regulates CC glycolytic activity (Sugiura et al., 2008).

Oocyte-secreted factors have been reported to regulate the uptake of amino acids in CC via the sodium-coupled neutral amino acid transporter *Slc38a3* (Eppig et al., 2005). In mice, the *Slc38a3* gene is expressed in mural GC and CC of early tertiary follicles, but mural GC expression is abolished in late tertiary follicles. Expression levels of *Slc38a3* mRNA and uptake of alanine, histidine, and leucine were similar in intact COC and OOX CC cultured with fully grown oocytes, but were downregulated in OOX CC. Fully-grown, but not growing, oocytes were able to restore *Slc38a3* mRNA levels and uptake of alanine and leucine to those observed in intact COC. Interestingly, *Slc38a3* mRNA was not affected in *Bmp15* or *Bmp15/Gdf9* KO CC, indicating that other oocyte-secreted factors are responsible for regulating amino acid uptake (Su et al., 2009).

1.3.3 GDF9 and BMP15 expression and signalling in the ovary

Both GDF9 and BMP15 are members of the transforming growth factor β (TGF β) superfamily of proteins and are thought to be exclusively expressed in the oocyte of most mammalian

species (Crawford and McNatty, 2012). GDF9 and BMP15 have been reported to be expressed by the somatic cells in pig, goat, and primates (Duffy, 2003; Silva et al., 2005; Paradis et al., 2009) however it is possible that these samples were contaminated with oocyte mRNA. There are significant species differences in the timing and levels of expression. For example, GDF9 is expressed in oocytes from the primordial stage onwards in cows, sheep, and brushtail possums (Bodensteiner et al., 1999; Eckery et al., 2002). BMP15 is also expressed from the primordial stage in the brushtail possum (Eckery et al., 2002), however is expressed from primary stage in sheep (Galloway et al., 2000). GDF9 and BMP15 are expressed only in growing follicles in mice, rats, and humans (Laitinen et al., 1998; Aaltonen et al., 1999; Jaatinen et al., 1999). Recent work indicates that rats and mice produce very little BMP15, compared to GDF9. The ratio of *Gdf9:Bmp15* mRNA was 3.65 and 5.19 in the rat and mouse, respectively (Crawford and McNatty, 2012). Moreover, mature GDF9 protein was detected by Western blotting in rat oocyte lysate and in the oocyte culture medium, indicating secretion of GDF9 (Lin et al., 2012). However, mature BMP15 protein was not detected in rat oocyte lysate or in the culture medium. The promature form of BMP15 was detected in rat oocyte lysate however the biological activity is unknown. Furthermore, immunoneutralization of BMP15 in rat oocytes did not affect the ability of those oocytes to stimulate GC ³H-thymidine uptake indicating BMP15 by rat oocytes does not play a role in regulating GC proliferation.

Unlike most other TGF β proteins, GDF9 and BMP15 lack the conserved cysteine residues that are required for covalent dimer formation. It has been reported that GDF9 and BMP15 may form non-covalent homo- and hetero-dimers *in vitro* (Peng et al., 2013) however the existence of these forms *in vivo* remains a matter of debate (Mottershead et al., 2013). What is known is that GDF9 and BMP15 act synergistically both *in vivo* and *in vitro* to influence somatic cell function (Hanrahan et al., 2004; McNatty et al., 2005a; Mottershead et al., 2012). GDF9 and BMP15 regulate GC function by activating two serine/threonine kinase receptors located on the plasma membrane of GC. Binding of the ligand causes activation of type II receptor kinase activity and phosphorylation of the type I receptor (Figure 1-6). The type I receptor then phosphorylates specific regulatory proteins similar to mothers against decapentaplegic (SMAD) proteins (rSMADs) resulting in the phosphorylated rSMAD binding to the common SMAD4. The rSMAD/SMAD4 complex translocates to the nucleus and

modulates gene expression through interactions with transcription factors, co-activators, and co-repressors (Knight and Glistler, 2006). GDF9 signals through the activin-like receptor kinase (ALK) 5 receptor and BMP receptor type II (BMPRII) and activates the SMAD2/3 pathway (Mazerbourg et al., 2004). BMP15 signals through ALK6 and BMPRII and activates the SMAD1/5/8 pathway (Juengel and McNatty, 2005). The synergistic actions of GDF9 and BMP15 act through the SMAD2/3, but not SMAD1/5/8, pathway (Reader et al., 2011).

Figure 1-6: Schematic overview of the TGFβ family signalling pathway. The ligand dimer binds to the Type II receptor (1) which recruits and phosphorylates the Type I receptor (2). The Type I receptor phosphorylates an rSMAD (3), which binds to the common SMAD (4), and this complex translocates to the nucleus where it modulates gene expression (5).

Whilst GDF9 is required for follicular growth in both sheep and mouse, BMP15 is required for follicular growth in sheep but not mouse. This supports evidence that BMP15 does not play a large role in follicular function of rodents. Follicles from sheep with a naturally-occurring homozygous *Bmp15*^{-/-} or *Gdf9*^{-/-} mutation arrest growth at the primary stage, causing sterility (Hanrahan et al., 2004). Interestingly, sheep heterozygous for mutations in either *Gdf9*^{+/-} or *Bmp15*^{+/-} have a higher ovulation rate than wildtype animals (Galloway et al., 2000; Hanrahan et al., 2004). Conversely, whilst follicular development arrests after the primary follicle stage in *Gdf9* KO mice, mice with a *Bmp15* KO are fertile, although produce smaller litters (Yan et al., 2001). Mice heterozygous for the *Gdf9* KO showed normal fertility (Dong et al., 1996).

1.4 The Role of Glucose Metabolism

1.4.1 Overview of glucose metabolic pathways

Glucose may be metabolised by several different pathways, each playing a different function within the cell. The primary pathways described herein are those concerned with glycolysis and hexosamine biosynthesis, with brief descriptions of the pentose phosphate and the polyol pathways.

Glycolysis functions primarily to generate energy for the cell and involves the conversion of glucose to pyruvate through multiple steps (Figure 1-7). The conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by PFKP is considered to be the rate limiting step (Krisher, 2004). Pyruvate may be converted into lactate by the enzyme LDH1 or into acetyl coenzyme A (CoA) by the enzyme pyruvate dehydrogenase. Lactate can also be recycled back to glucose by gluconeogenesis, or to pyruvate. Acetyl CoA enters into TCA cycle, which produces ATP and nicotinamide adenine dinucleotide (NADH) which, together with ribose, is important for the reduction of the antioxidant glutathione. The NADH produced in the TCA cycle is utilised for oxidative phosphorylation by the mitochondrial electron transport chain. In addition, glycolysis intermediates are used for a number of other cellular pathways including the pentose phosphate pathway (glucose 6-phosphate) and lipid metabolism (dihydroxyacetone phosphate) (Voet and Voet, 2004). During times of rest, or in response to high glucose levels, glucose 6-phosphate may alternatively be converted into the stored form of glucose, glycogen, by the glycogenesis pathway.

Oxidative phosphorylation refers to the coupled processes of ATP synthesis and the electron transport chain within the mitochondria. The electron transport chain removes electrons from NADH and succinate, resulting in conversion of oxygen to water concurrent with the transfer of H^+ across the mitochondrial membrane. This proton-motive force drives ATP synthase resulting in the transfer of adenosine diphosphate (ADP) to ATP. The stepwise removal of electrons from NADH and succinate involves several electron acceptors, including NADH dehydrogenase, succinate dehydrogenase, ubiquinone, cytochrome bc_1 complex, cytochrome c, and cytochrome c oxidase. Cytochrome c is localised in the intermembrane space, and is responsible for transferring one electron to cytochrome c oxidase (Voet and Voet, 2004). Cytochrome c is a common mitochondrial marker, that correlates well to other mitochondrial markers including citrate synthase and respiratory capacity (Fitts et al., 1975). In addition to its role in the electron transport chain, cytochrome c plays an essential role in activation of apoptosis. Mitochondrial activation of the intrinsic apoptotic pathway results in release of cytochrome c into the cytoplasm, where it binds to apoptotic protease activating factor 1 (APAF1) and pro-caspase-9 resulting in activation of effector caspases and initiation of apoptotic processes (Chandra et al., 2002)

Figure 1-7: The glycolysis metabolic pathway. The blue boxes contain the pathway intermediates/products, while the enzymes that catalyse each reaction are beside the arrows. Those enzymes that are outlined in orange are thought to be regulated by oocyte-secreted factors.

The hexosamine biosynthetic pathway converts glucose-6-phosphate to uridine diphosphate (UDP)-*N*-acetyl glucosamine (Figure 1-8). The rate limiting enzyme is glutamine-fructose-6-phosphate transaminase 1 (GFPT1). The hexosamine biosynthesis pathway acts as a fuel sensing pathway. Typically, only a small amount of glucose 6-phosphate (1-3 %) is metabolised through this pathway. Increased flux through the hexosamine pathway causes decreased uptake of glucose, and increased glycogen accumulation (Rossetti, 2000). The UDP-*N*-acetyl glucosamine produced by the hexosamine pathway is used for *O*-linked glycosylation (Sutton-McDowall et al., 2010). The hexosamine pathway plays an additional role in pre-ovulatory follicles, as UDP-*N*-acetyl glucosamine is a precursor for hyaluronic acid. Hyaluronic acid makes up the backbone of the specialised extracellular matrix secreted as a part of CC expansion.

Two other main pathways are involved in glucose metabolism in the COC. The pentose phosphate pathway (PPP) results in production of nicotinamide adenine dinucleotide phosphate (NADPH; involved in cytoplasmic integrity and redox state through glutathione) and phosphoribosylpyrophosphate (a substrate for purine synthesis). The polyol pathway involves the oxidation of glucose to produce sorbitol and fructose. The polyol pathway is utilised in hyperglycaemic conditions, for example it is thought to be involved in the diabetic pathology of many tissues (Gabbay, 1975).

Figure 1-8: The hexosamine biosynthetic pathway. Pathway intermediates/products are contained within the blue boxes. Glc = glucosamine.

1.4.2 Glucose metabolism in the ovarian follicle

The mechanism through which glucose uptake by the COC is regulated is species-specific and occurs primarily via facilitative glucose transporters but may also occur via sodium-coupled glucose transporters (Purcell and Moley, 2009; Wang et al., 2012). For example, bovine and ovine GC express mRNA for the facilitative glucose transporter genes *Glut1*, *Glut3*, and *Glut8* (Nishimoto et al., 2006; Pisani et al., 2008) and mouse and ovine GC additionally express the *Glut4* gene (Williams et al., 2001; Roberts et al., 2004). Furthermore, bovine and ovine oocytes express the genes *Glut1* and *Glut3*, with the bovine oocyte additionally expressing the *Glut8* gene (Augustin et al., 2001; Pisani et al., 2008). Mouse oocytes express the *Glut1*,

and *Glut4* genes (Zhou et al., 2000; Roberts et al., 2004). In addition to glucose transporters in mice, oocytes also express monocarboxylate transporters 1, 2, and 4 indicating that murine oocytes are able to take up lactate and pyruvate directly (Hérubel et al., 2002). Oocytes at both the GV and MII stages of meiosis have been reported to consume pyruvate (Harris et al., 2009). In murine oocytes, the concentration of a glucose analogue, 2-deoxyglucose, was significantly higher in COC than in DO, indicating that the CC facilitate glucose uptake in oocytes. *In vitro* studies of bovine COC revealed that glucose is primarily taken up by the outer CC and gradually moves into the inner CC and oocyte (Sutton-McDowall et al., 2010). In addition, treatment of COC with hCG, which abolishes GJ coupling in a similar manner to LH, reduced the concentration of 2-deoxyglucose in the oocyte to levels similar to those seen in DO (Saito et al., 1994).

It is generally accepted that oocytes metabolise very little glucose compared to CC. Bovine CC consume 23-fold more glucose in comparison to oocytes when assessed per millimetre volume of tissue (Sutton-McDowall et al., 2010). However, both mouse and human oocytes express the enzymes essential for glycolysis (Hardy et al., 1989; Harris et al., 2009) as well as for importing both pyruvate and glucose. Murine oocytes may also metabolise glucose by other pathways as evidenced by expression of hexokinase, glucose-6-phosphate dehydrogenase, GLUTs, and enzymes involved in the PPP (Wang et al., 2012).

The growing oocyte fully depends upon oxidative phosphorylation for ATP production, however has a poor capacity for glucose metabolism (Biggers et al., 1967). The murine oocyte has low PFKP activity, and thus low glycolytic activity. Conversely, murine CC have high activity of enzymes involved in glycolysis such as PFKP and hexokinase (Downs et al., 1996). Thus, the CC produce a significant amount of pyruvate which is able to be transferred to the oocyte (Purcell and Moley, 2009). It is thought that the flux of glucose through the glycolytic pathway in bovine COC remains stable during a 24 hour *in vitro* maturation (IVM) step despite increased glucose consumption, indicating that glucose may be metabolised by other pathways (Sutton et al., 2003). Increased glycolytic activity in bovine oocytes is associated with increased developmental competence, however this may also lead to increased reactive oxygen species, resulting in oxidative damage (Krisher and Bavister, 1999). Oxidative damage leads to impaired mitochondrial function which, in turn, is associated with decreased developmental competence (Krisher, 2004).

Activity through the hexosamine biosynthetic pathway is increased towards the end of oocyte maturation in cows due to the requirement for hyaluronic acid (Gutnisky et al., 2007). However, increased activity through this pathway can also lead to increased *O*-linked glycosylation, leading to activation or inactivation of target proteins. This can have negative effects, and has been reported to be a factor in the pathology of diabetes in somatic cells (Brownlee, 2001). Supplementation of IVM media with glucosamine, a substrate which is exclusively metabolised by the hexosamine pathway, inhibited blastocyst development in bovine embryos. This effect was mediated by increased *O*-linked glycosylation (Sutton-McDowall et al., 2006).

Regulation of the PPP pathway is the primary mechanism by which changes in glucose concentration affect nuclear maturation (Downs, 1996). The PPP is more active within the oocyte than the CC, and influences meiotic resumption and developmental competence in both cattle and mice (Downs et al., 1998; Cetica et al., 2002; Wang et al., 2012). Treatment of murine oocytes with a PPP stimulator caused increased numbers of oocyte to undergo GVBD and stimulated intra-oocyte glucose consumption (Wang et al., 2012). Glucose flux through the PPP is increased in response to FSH in diabetic mice, indicating that this pathway is important for metabolising glucose in hyperglycaemic conditions (Colton et al., 2003). Under normal conditions very little glucose is metabolised via the polyol pathway, with other pathways such as glycolysis or hexosamine biosynthesis being preferred (Brownlee, 2001).

The use of a type I diabetes mouse model has demonstrated the importance of correct glucose levels on oocyte maturation and health (Chang et al., 2005). Pre-ovulatory oocytes from diabetic mice were 30 % smaller than those from control mice. Moreover, oocytes from these mice underwent precocious nuclear maturation. However the proportion of MII oocytes observed was reduced, suggesting a decrease in oocyte competence (Kim et al., 2007). Despite these COC being exposed to hyperglycaemic conditions, the flux through the glycolytic pathway was unaffected as the excess glucose was metabolised through the polyol pathway (Colton et al., 2003; Colton and Downs, 2004). In GC of the type I diabetic mice, GJ communication between the GC and oocyte was decreased by 60 %. In addition, *Cx37* gene and protein expression was decreased by 36 % (Ratchford et al., 2008). Increased *O*-linked glycosylation and decreased concentrations of FSH are thought to primarily modulate the

reduction in oocyte competence caused by high glucose concentrations (Hashimoto et al., 2000). Conversely, it is thought that the major pathways involved in reduced oocyte competence as a result of low glucose concentrations are the glycolytic and PPP pathways (Downs et al., 1998).

1.5 The Influence of Exogenous Factors on Oocyte Quality

Oocyte quality is likely influenced by a mixture of genetic, endocrine and paracrine signals as well as exogenous factors from the environment. The quality of the ovulated oocyte is particularly important for two reasons. First, the embryonic genome, consisting of paternal and maternal genetic information, is only activated after several cell divisions. Thus, the early development of the embryo in the first few days after fertilisation is largely directed by the oocyte (Kidder and Vanderhyden, 2010). Second, most, if not all, of the oocytes that a female will ever have are contained within the ovary by birth. Thus, oocytes that are ovulated later in life may have been formed 1-1.5 (rodents) or 50 years (humans) earlier (McGee and Hsueh, 2000). Not surprisingly, oocyte quality is reported to decrease over time (Navot et al., 1991).

Poor oocyte quality is a major cause of female infertility. For example, the age-related decline in female fertility is attributed to a concurrent decline in both oocyte quality and numbers (Navot et al., 1991). In humans, infertility is defined as the inability to conceive after one year of unprotected intercourse, with one in six couples experiencing this at some stage of their reproductive life (Anderson, 2010). In 2010, there were ~62,000 assisted reproductive technology (ART) cycles performed in Australia and New Zealand. Of these cycles, 3.5 % used donor oocytes (Macaldowie et al., 2012). The large cost of ART, and frequency of adverse effects, means identification of modifiable factors is important for fertility preservation (Chavarro et al., 2007). A person's lifestyle choices may cause an increase in time to pregnancy, and decrease the chance of a healthy, live birth . However, the current data on the effects of lifestyle on fertility are scarce, contradictory or poorly designed (Anderson, 2010). Thus, further research into the effects of lifestyle on fertility may remove myth-based, unnecessary lifestyle restrictions for couples trying to have a baby (Anderson, 2010).

Exogenous factors may affect fertility at a number of levels of the reproductive system (Figure 1-9). The coordination of follicular growth is complex, incorporating signals from the pituitary gland (FSH and LH), along with feedback signals from the ovary (oestrogen and progesterone). In addition, feedback from other organs regulates both ovarian and hypothalamic-pituitary function.

Perturbation of the hypothalamic-pituitary-gonadal axis has been observed following exposure to exogenous factors. For example, both acute and chronic exposure of male rats to methylenedioxymethamphetamine (MDMA; ecstasy) leads to reduced mRNA levels of gonadotrophin releasing hormone (GnRH) in the hypothalamus (Dickerson et al., 2008). MDMA is known to target both serotonergic and dopaminergic pathways, and these also regulate the neurons that produce GnRH. Similarly, GnRH protein levels are reduced as a result of exposure to Δ^9 tetrahydrocannabinol (THC), a compound present in cannabis smoke (Smith et al., 1979). As a result of THC exposure in female rhesus monkeys, inhibition of GnRH signalling causes a reduction in blood gonadotrophin levels (Armstrong, 1986; Park et al., 2004). Additionally, moderate to heavy alcohol consumption in women has been associated with increased plasma oestradiol concentrations (Mendelson et al., 1988). Other known disruptors of the hypothalamic-pituitary-gonadal axis are stress, strenuous exercise, and compounds with oestrogen mimicking properties (Armstrong, 1986).

Exogenous factors may also directly influence the ovary and/or oocytes to reduce fertility. Ovarian follicles, particularly non-growing primordial follicles are sensitive to toxic agents. For example, radiation therapy damages primordial follicles in mice, pigs, monkeys, and humans (Armstrong, 1986). Chemotherapeutic agents also target primordial follicles. Examination of the ovaries of prepubertal girls that had been treated with chemotherapeutic agents found a significantly lower number of oocytes present compared to that in prepubertal girls not exposed to chemotherapy (Himelstein-Braw et al., 1978). Similarly, polycyclic aromatic hydrocarbons, a constituent in automobile exhaust fumes and cigarette smoke, has been shown to destroy primordial follicles in mice (Mattison and Thorgeirsson, 1979). This may also be the case in women, as smokers are reported to undergo menopause significantly earlier than non-smokers (Kaufman et al., 1980).

Figure 1-9: Potential sites of action for disruption of female fertility by exogenous factors. As indicated by the orange arrows. Black arrows indicate interactions between the organs.

A number of exogenous factors decrease oocyte quality without causing toxicity. Bisphenol A (BPA), a compound found in plastics, causes spindle abnormalities including distribution of the centriolar material and chromosome alignment in mouse oocytes (Eichenlaub-Ritter et al., 2008). Mouse follicles treated with BPA *in vitro* had decreased numbers of GC, and a higher proportion of oocytes were unable to resume meiosis (Lenie et al., 2008). BPA exhibits oestrogenic activity, as it binds with both oestrogen receptor α and β (Fujimoto et al., 2010). BPA concentrations in the serum of Asian women were correlated with a decrease in MII oocytes (Fujimoto et al., 2010). Caffeine also directly affects the oocyte through its PDE-inhibitory properties. Superovulation in mice was inhibited by treatment with caffeine, with most of the oocytes collected still in the GV stage (Jagiello et al., 1972). Cigarette smoking and compounds found in cigarette smoke have been reported to affect the quality of oocytes in a range of species. Cigarette smoking (either active or passive) causes an increase in the thickness of the zona pellucida in women undergoing *in vitro* fertilisation (IVF) (Shiloh et al., 2004). Treatment of bovine oocytes with nicotine, the primary addictive agent in cigarettes, inhibited CC expansion, oocyte maturation, and caused meiotic

abnormalities (Liu et al., 2008). When mice were treated with nicotine, the oocytes displayed premature anaphase, and the proportion of oocytes ovulated was halved compared to controls (Mailhes et al., 2000).

Finally, factors may affect other organs causing an indirect reduction in fertility (Armstrong, 1986). In particular, the liver is a target for such actions as steroid hormone metabolism occurs there. For example, rats that were fed fish that had been exposed to high levels of polychlorinated biphenyls (PCBs) showed increases in hepatic expression of cytochrome P450, an enzyme involved in steroid hormone metabolism. Additionally, the rise in oestradiol levels due to moderate to heavy drinking in women as mentioned above is thought to be due to the breakdown of alcohol in the liver, causing inhibition of oestradiol breakdown (Gill, 2000).

1.5.1 Lifestyle factors chosen for this study

Six exogenous lifestyle factors were selected for investigation in this study. These factors were chosen because of their prevalence in our everyday lives (see Table 1-1).

BPA is an endocrine disrupting chemical used in the synthesis of polycarbonate plastics and epoxy resins. BPA accounts for most of the oestrogenic activity that leaches from landfills (Coors et al., 2003) and has been detected in follicular fluid, foetal serum, and amniotic fluid (Ikezuki et al., 2002). Increased BPA concentrations in follicular fluid have been correlated to decreased fertilization rates in both conventional insemination and intracytoplasmic sperm injection (Fujimoto et al., 2010). In Asian women, increased BPA serum concentrations were also linked to reduced oocyte maturation. Urinary BPA concentrations are associated with a reduction in peak plasma oestradiol levels in women during IVF (Mok-Lin et al., 2010), however no relationship was found between urinary BPA and aromatase gene expression levels (Ehrlich et al., 2013). *In vitro* culture of human oocytes that had been discarded during IVF with BPA resulted in reduced proportions of MII oocytes, and increased proportions of oocytes that had degenerated or spontaneously activated (Machtinger et al., 2013).

During IVF, urinary BPA concentrations are associated with reduced oocyte numbers, reduced fertilisation, and reduced oestradiol concentrations (Ehrlich et al., 2012).

Caffeine is the most widely used psychoactive compound and is found in many ingested food products including coffee, tea, and chocolate. In a study of women undergoing IVF, 76% had drunk at least one caffeinated beverage each day in the month prior (Domar et al., 2012). Caffeine has been detected in follicular fluid (Al-Saleh et al., 2010), however the link between caffeine and infertility is contentious (Anderson, 2010). Some studies have found that increased caffeine consumption is associated with a longer time to conception (Hatch and Bracken, 1993; Bolumar et al., 1997) however these failed to adequately take other variables into account, such as smoking. A prospective study showed a significant correlation between not achieving a live birth and consumption of caffeine during IVF (Klonoff-Cohen et al., 2002). A meta-analysis of studies looking at the effect of caffeine and time to conception found that there was no convincing evidence that caffeine increased time to conception (Leviton and Cowan, 2002).

Alcohol (ethanol) is consumed in large amounts throughout most of the world. Approximately 73% of women undergoing IVF reported drinking at least one serving of alcohol in the month prior (Domar et al., 2012). Mild to moderate consumption of alcohol has a significant negative effect on female fertility (Emanuele et al., 2002). For example, alcoholic women are known to have irregular menstruation or even cessation of menses. Alcohol consumption is correlated with increased plasma oestradiol levels (Van Thiel et al., 1978; Mendelson et al., 1988), and has been associated with anovulation (Mendelson and Mello, 1988). Furthermore, alcohol consumption at levels below those that would cause liver damage can cause temporary anovulation and infertility.

Nicotine is the primary addictive agent found in cigarette smoke. Only 2.5% of women undergoing IVF reported that they had smoked at least one cigarette in the previous month (Domar et al., 2012). Cigarette smoke is well documented to adversely affect female fertility (Waylen et al., 2009; Anderson, 2010). *In vitro* or *in vivo* exposure to nicotine in animal models causes disruption to normal oocyte maturation and ovulation (Liu et al., 2008). Nicotine is metabolised to produce nicotine-*N*-oxide and cotinine. Cotinine has been detected in follicular fluid (Weiss and Eckert, 1989; Zenzes et al., 1996). Nicotine has been reported to inhibit CC expansion and oocyte GVBD, and cause chromosomal abnormalities in bovine COC (Liu et al., 2008). Smokers are 1.6 times more likely to experience infertility than

non-smokers (Augood et al., 1998), and need approximately twice as many IVF cycles to achieve pregnancy compared with non-smokers (Feichtinger et al., 1997).

The primary psychoactive compound found in cannabis is THC. Cannabis is the most widely-used illicit drug (U.S. Department of Health and Human Services, 2010) and is reported to suppress LH production in women (Mendelson et al., 1986). Acute exposure to THC causes disruption to the menstrual cycle of rhesus monkeys, however tolerance develops during long-term exposure (Smith et al., 1979). *In vitro* exposure to THC of rat follicles inhibited progesterone, testosterone, and oestradiol production by the follicular cells. In addition, THC stimulated meiotic resumption in rat oocytes (Reich et al., 1982). In a study of couples undergoing IVF, THC was detected in hair samples of 3.7% of the participants (Pichini et al., 2012). A prospective study showed that women who had smoked marijuana within the past year had 25% fewer oocytes collected during IVF (Klonoff-Cohen et al., 2006).

MDMA is a recreational drug that is primarily consumed by young people of reproductive age (U.S. Department of Health and Human Services, 2006). Animal studies have found that MDMA suppresses the production of GnRH and testosterone (Dickerson et al., 2008), delays puberty, and affects sperm quality in male rats (Barenys et al., 2010). There are currently no data on the effects of MDMA on IVF outcome, to the best of my knowledge.

Table 1-1: Measured concentrations of lifestyle factors in biological fluids.

Compound	Species	Concentration	Biological Fluid	Population details	Reference
BPA	Human	2.53 ng/mL (range 0.0-67.4)	Serum	Women undergoing IVF	(Fujimoto et al., 2010)
	Human	2.08 ± 0.8 ng/mL	Serum	Healthy women	(Ikezuki et al., 2002)
	Human	2.4 ± 0.8 ng/mL	Follicular fluid	Women undergoing IVF	(Ikezuki et al., 2002)
	Human	2.2 ± 1.8 ng/mL	Foetal serum	Umbilical cord blood from healthy women	(Ikezuki et al., 2002)
Caffeine	Human	0.913 µg/mL (range 0.0-64.04)	Serum	Women undergoing IVF	(Al-Saleh et al., 2010)
	Human	0.701 µg/mL (range 0.0-6.48)	Follicular fluid	Women undergoing IVF	(Al-Saleh et al., 2010)
	Human	10 µg/mL	Plasma	Males given an oral dose of 7 mg/kg caffeine	(Axelrod and Reichenenthal, 1953)
Ethanol	Human	800 µg/mL	Blood	Legal driving limit for blood alcohol content	New Zealand Transport Agency ¹
Nicotine	Human	30-50 ng/mL	Plasma	Active smokers	(Moyer et al., 2002)
	Rat	40-120 ng/mL	Plasma	Self-administration model of nicotine dependency	(Shoaib and Stolerman, 1999)
	Human	33 ng/mL (range 4-72)	Plasma	Active smokers	(Russell et al., 1980)
MDMA	Human	0.715 µg/mL (range 0.13-2.4)	Serum	Patients who consumed ecstasy at one nightclub and were later treated at two London hospitals	(Greene et al., 2003)
	Human	130.9 ± 38.6 ng/mL	Plasma	Male subjects given 75 mg MDMA	(de la Torre et al., 2000)
THC	Human	67-98 ng/mL	Plasma	Peak plasma THC levels in light and heavy marijuana users respectively who had smoked one marijuana cigarette	(Lindgren et al., 1981)

¹ New Zealand Transport Agency. (Published 26 November 2012). About limits. In *The Official New Zealand Road Code*. Retrieved 30 April 2013. From <http://www.nzta.govt.nz/resources/roadcode/about-limits/alcohol-and-drugs-limits.html>

1.6 Aims

The overall aim of this study was to determine whether the health of mammalian oocytes is directly affected by exposure to exogenous lifestyle and environmental factors. To determine the effects of exogenous factors, a rat COC model was used as the test system. Rats are a commonly used test species for pharmacologically active compounds, enabling investigation into both the *in vitro* and *in vivo* effects of lifestyle and environmental factors on COC function. In addition, the large number of antral follicles present in the prepubescent rat ovary facilitated experiments in which large amounts of biological material was required. The presence of a rat breeding colony at VUW meant that female rats were readily available for this study.

A GJ bioassay was optimised to assess the extent of transfer of a fluorescent dye (calcein) from CC to the oocyte following exposure to targeted reagents. In addition, a multiplex quantitative PCR (qPCR) technique was used to measure gene expression levels in individual rat oocytes and CC masses. Subsequently, both the dye transfer assay and multiplex qPCR were tested in combination and validated, to allow for direct comparison of GJ communication and mRNA levels of candidate genes in the same samples. A Western blot protocol was also optimised to detect CX43 and GDF9 proteins in rat COC.

The effect of *in vitro* exposure to lifestyle factors on rat COC was the major focus of this study utilising the aforementioned assays. The lifestyle factors tested in this study were BPA, caffeine, ethanol, nicotine, MDMA, and THC. Moreover, the effect of *in vivo* exposure to ecstasy (MDMA) on COC health was also determined. MDMA was administered to adolescent female rats and thereafter the COC were extracted, and mRNA and protein expression levels were measured.

The key endpoints of this study were: measurement of GJ communication between the CC and oocyte using an optimised bioassay; qPCR quantification of *Cx43*, *Gfpt1*, *Pfkp*, *Atr*, *Cycs*, and *Glut1* in CC and *Cx37*, *Gdf9*, *Bmp15*, *Atr*, *Cycs*, and *Glut1* in oocytes; and quantification of CX43 and GDF9 protein levels using Western blotting.

2 GENERAL METHODS

2.1 Effect of *in vitro* exposure to lifestyle factors

For *in vitro* studies, ovaries were collected from pre-pubescent Sprague-Dawley female rats as part of routine culling of a breeding colony in the vivarium at the School of Psychology, Victoria University of Wellington (VUW). Euthanasia for tissue collection was included under a general animal ethics approval for rodent research issued by the VUW Animal Ethics Committee (VUW application number 10695).

2.1.1 Recovery of ovaries

Prepubescent (21-25 days old) female Sprague-Dawley rats were euthanized by carbon dioxide (CO₂) asphyxiation. The ovaries were recovered into dissection media (see Appendix 2) supplemented with PDE inhibitors for transportation back to the laboratory. The concentration and type(s) of PDE inhibitors was dependent upon the experiment performed. In the laboratory, the ovaries were transferred into a 35 mm Petri dish containing dissection media supplemented with PDE inhibitors. Subsequently, the surrounding fatty tissue was trimmed whilst viewing under a Zeiss stereo-zoom dissection microscope (Leica Microsystems; Auckland, NZ).

2.1.2 Collection of COC for culture

All of the visible antral follicles (0.5 – 1.0 mM in diameter) were punctured using a 20 gauge needle fitted onto a 1 mL syringe to release the COC into the dissection media supplemented with PDE inhibitors. The COC that had at least two intact layers of CC were transferred into wells of a non-treated, flat bottomed 48-well polystyrene tissue culture plate (Interlab; Wellington, NZ) and washed twice in 200 µL of wash media (see Appendix 2) supplemented with PDE inhibitors. The dimethyl sulfoxide (DMSO) concentration was maintained ~1 %. Typically 15-20 COC were added to each well.

2.1.3 GJ Assay

An acetoxymethyl (AM) ester derivative of the fluorescent dye calcein (Invitrogen; Auckland, NZ) was used to measure GJ transfer from the CC to the oocyte. Calcein fluoresces with excitation and emission wavelengths of 495 and 515 nm, respectively.

Two distinct GJ assay protocols were used in this study. The first was used to optimise the amount of calcein transfer to the oocyte to allow enhanced assay sensitivity (Figure 2-2). The second protocol was used for all subsequent experiments, including those where the COC were exposed to lifestyle factors (Figure 2-4).

In experiments where the aim was to optimise calcein transfer to the oocyte, COC were exposed to 2 μ M calcein-AM (see Appendix 2) in 200 μ L incubation media supplemented with PDE inhibitors, in the presence or absence of treatments, for 10 minutes in incubator conditions of 37 °C, 5 % CO₂ and 96 % humidity. The COC were then transferred into 200 μ L calcein-AM free incubation media with PDE inhibitors, in the presence or absence of treatments, and cultured at 37 °C either transiently or for up to 24 hours. After being washed in 200 μ L of incubation media free of PDE inhibitors and treatment, the CC were removed from the oocyte. This was accomplished by vigorous and repeated pipetting of 200 μ L of incubation media containing the 15-20 COC. The DO were photographed to allow future analysis of the presence or absence of a GV (Figure 2-1). Oocytes were either categorised as containing (see Figure 2-3A) or not containing a visible GV (see Figure 2-3B). In cases where the GV was not clearly identifiable, the oocyte was categorised as not containing a GV for analysis purposes. The degree of fluorescence emitted by the DO was measured using a Leica inverted microscope (CTR4000) with a CRAIC fluorescence detection system (model QDI302; Leica Microsystems; Wetzlar, Germany). The settings for these measurements were 50 milliseconds over the wavelength range of 300 to 700 nm. A dark scan was performed to determine the amount of electronic noise when the excitation light is turned off. Fluorescence was measured near the centre of each oocyte.

Figure 2-1: Photographs depicting rat COC (A, C) and denuded oocytes (B, D) before and after exposure to calcein-AM. Indicated on the photographs are the cumulus cells (CC), oocyte (O), germinal vesicle (GV), and zona pellucida (ZP).

Figure 2-2: Schematic overview of the protocol used to optimise dye transfer in the GJ assay.

Figure 2-3: Photographs depicting an oocyte with a visible germinal vesicle (A) and without a germinal vesicle (B).

Figure 2-4: Schematic overview of the optimised GJ assay protocol.

For all other experiments, COC were cultured at 37 °C in 200 µL incubation media with PDE inhibitors in the presence or absence of treatment for up to 48 hours. Following the incubation period, the COC were exposed to 2 µM calcein-AM for 10 minutes at 37 °C. The COC were then transferred into calcein-AM free media with PDE inhibitors and with or without treatment and incubated at 37 °C for a further 1 hour to allow dye transfer. In those experiments where the COC were not collected for RNA extraction, the COC were washed twice in 200 µL incubation media free of PDE inhibitors and treatment and the 15-20 oocytes within each well were denuded from their CC as described above. The DO were photographed for GV analyses and the fluorescence was measured using the Leica inverted microscope as described previously.

In those experiments where the COC were collected for RNA extraction, individual COC were transferred into wells of a 96-well plate (Thermo Fisher Scientific; Auckland, NZ) where they were washed twice in 100 μ L phosphate buffered saline (PBS). The COC were transferred into 10 μ L PBS and photographed. Thereafter, the oocytes were separated from the CC and the DO were transferred into 100 μ L incubation media free of PDE inhibitors and treatment. The DO were photographed and fluorescence was measured within the oocyte as described previously. Thereafter the DO were washed twice more in 100 μ L PBS before being transferred into 10 μ L fresh PBS. The 96-well plates containing the separated CC masses and DO each in 10 μ L PBS were stored at -80 °C.

2.1.3.1 GJ assay data analysis

Each oocyte was considered to be a discrete variable, so that the number of samples was considered to be the total number of readings across all replicates. For each replicate experiment, the geometric mean value was calculated for the raw fluorescence measurements of the control group (usually the untreated group at 1 hour). Relative fluorescence values were then calculated by dividing the raw fluorescence measurement for each individual oocyte by the geometric mean of the control group. The geometric mean and 95 % confidence interval of the relative fluorescence values were calculated for each experimental group. All statistical analyses of the data were performed using the GraphPad Prism 5 statistics program.

Experiments were analysed using the appropriate statistical test, namely either the Mann-Whitney test (one-way analysis), the Kruskal-Wallis test with Dunn's multiple comparison post-hoc test (one-way analysis), or the two-way analysis of variance (ANOVA; two-way analysis). Where a two-way ANOVA was utilised, the data were first tested for normality using the D'Agostino & Pearson normality test. Data that was not normally-distributed was transformed using an appropriate transformation and tested for normality again. If no transformation method yielded normally-distributed data, the data points were ranked and a two-way ANOVA was performed on the ranks.

2.1.4 COC culture for Western blotting

For the Western blotting experiments, the dissection, wash and incubation media contained the PDE inhibitors, dipyridamole (50 μ M) and rolipram (50 μ M). Following extraction, COC

were transferred into a 48-well plate and washed twice in 200 μ L wash media. Subsequently, the COC were transferred into 200 μ L incubation media containing PDE inhibitors in the absence or presence of treatment. The COC were then incubated at 37 °C with 5 % CO₂ and 96 % humidity for up to 25 hours.

After the culture period, the COC were washed twice in 200 μ L PBS. A known number of COC (100 COC/group) were transferred into a microcentrifuge tube, and the total volume was made up to 500 μ L with PBS. The microcentrifuge tubes were centrifuged at 800 $\times g$ for 10 min, and the supernatant was removed and dispensed into a culture dish. The supernatant was checked under the dissection microscope to ascertain whether any COC were accidentally removed from the pellet. The COC pellet was then resuspended in PBS supplemented with protease inhibitors and stored at -20 °C.

Due to the time taken to extract the COC, only 100 COC per treatment group were collected in each experiment and these were later pooled if necessary.

2.1.5 CC serial dilution

Rat ovaries were collected in PBS, and the COC were extracted into fresh PBS as described previously. The CC were stripped from the oocyte by vigorous and repeated pipetting and the oocytes were removed from the resulting cell suspension and discarded. A 50 μ L aliquot of the CC suspension was mixed with 5 μ L 0.4 % Trypan blue stain, and the cells were counted using a haemocytometer under an inverted microscope at 400x magnification. The total cell count was determined and the CC were serially diluted in triplicate with PBS to produce samples containing between 280 and 18,000 CC. The samples were stored at -80 °C prior to RNA extraction.

2.2 Effect of *in vivo* exposure to methylenedioxymethamphetamine (MDMA)

2.2.1 Treatment regimen

All experiments involving administering MDMA to rats were approved by the VUW Animal Ethics Committee (VUW application number 2011R16).

Pre-pubescent (21-25 day old) female Sprague-Dawley rats were weaned and housed in groups of 4 in a temperature controlled room at 22 °C and 55 % humidity, with a 12 hour

light/dark cycle. Food and water was available *ad libitum*. At the beginning of each experimental week, MDMA (kindly donated by Mr Richard Moore and Prof Susan Schenk, School of Psychology, VUW), was diluted in sterile saline to a concentration of 2.5 mg/mL and stored at 4 °C. The rats were administered either the vehicle (sterile saline; N=12) or 5 mg/kg/ MDMA (N=12) via intraperitoneal (ip) injection. Each rat was administered one injection every 24 hours for 3 days (total of 3 injections). Due to logistics, three replicate experiments were undertaken (i.e. 4 rats/group/replicate experiment) over three consecutive weeks.

To check the efficacy of the MDMA, locomotor activity of all rats following each injection was measured using Activity Monitor Version 5 software (Med Associates Inc.; St Albans, VT, USA). This test was performed by placing each rat in a locomotor activity box (40 x 40 cm) containing 16 infrared beams and corresponding sensors. When the subject stops a beam from reaching the sensor, this event is recorded. As a subject moves, a 'box' is established which is 3 infrared beams in width and length. The subject is considered ambulatory when it breaks more than 3 beams outside the 'box' within 1000 milliseconds. If the subject fails to meet these criteria, it is considered to be in a resting phase until such time as it meets the ambulatory criteria. The software measures ambulatory counts, which are defined as the total number of beams broken while the subject is in an ambulatory episode.

The rats were habituated in the locomotor activity boxes for 30 minutes, with the Activity Monitor software running to measure their activity. The activity boxes were located inside cupboards to create a darkened environment, and a white noise generator was used throughout the measurement of locomotor activity to minimise environmental disturbances. After habituation, the rats were administered their daily injection of either 5 mg/kg MDMA or saline (N=4/treatment group/week) via ip injection. The rats were returned to the locomotor activity boxes where their activity was monitored for a further 60 minutes. The rats were then returned to the temperature-controlled room. Measurement of locomotor activity was repeated following every injection.

Approximately 18 hours after the final injection, the rats were sacrificed by CO₂ asphyxiation. The ovaries were collected into sterile PBS.

2.2.2 Collection of COC

The ovaries from control and MDMA-treated rats were kept separate at all times. The ovaries were transferred into a 35 mm Petri dish containing sterile PBS and the surrounding fatty tissue was trimmed. The follicles were punctured using a 20 gauge needle, releasing the COC into the PBS. The COC were then transferred into a fresh dish containing sterile PBS for either gene or protein expression studies.

The COC that were collected for gene expression were transferred singly into 10 μ L of PBS in a 96-well plate and thereafter DO and CC were isolated separately as previously described. The DO were photographed before being transferred into 10 μ L PBS. The 96-well plates containing the isolated DO and CC were then stored at -80 °C until gene expression studies were performed.

For protein analysis, 200 COC/group were transferred into a microcentrifuge tube. Additional PBS was added to make the volume up to 500 μ L. The microcentrifuge tubes were centrifuged at 800 $\times g$ for 10 min. The supernatant was transferred into a culture dish to check for the accidental removal of any COC. The COC pellet was then resuspended in 15 μ L PBS containing protease inhibitors. The samples were stored at -20 °C until required.

2.3 Quantitative PCR (qPCR)

For the optimisation steps for each qPCR gene set, cDNA from pools of 50 COC were used as the template. All subsequent qPCR experiments were performed on individual DO and associated CC masses.

2.3.1 RNA extraction and cDNA synthesis

Total RNA was extracted using the ArrayPure Nanoscale RNA purification kit (Epicentre Biotechnologies; Madison, WI, USA) following the manufacturer's instructions. In detail, 30 μ L of Nanoscale Lysis solution containing 5 μ g of Proteinase K was added to each sample, and mixed thoroughly by pipetting. If the sample was in a 96-well plate, this solution was transferred to a microcentrifuge tube before proceeding. The contents of the microcentrifuge tubes were vortexed for 1 minute before being incubated at 65 °C for 15 minutes. The samples were then placed on ice for 5 minutes, followed by the addition of 18 μ L of MPC Protein Precipitation Reagent and mixing by vortex for 10 seconds. The precipitated protein was pelleted by centrifugation at 12,000 $\times g$ at 4 °C for 7 minutes, and

the supernatant was transferred into a fresh microcentrifuge tube. In order to precipitate the RNA, 50 μ L of isopropanol was added to the supernatant and vortexed for 30 seconds before being pelleted by centrifugation at 12,000 $\times g$ at 4 $^{\circ}$ C for 5 minutes. The isopropanol was discarded, and the RNA pellet was air dried for 5 minutes. The pellet was resuspended in 20 μ L of DNase buffer containing 0.5 U DNase I enzyme by vortexing for 1 minute. To remove any contaminating genomic DNA, this solution was incubated at 37 $^{\circ}$ C for 30 minutes. Following the incubation, 20 μ L of 2x Nanoscale Lysis solution was added and the tubes were vortexed for five seconds. Subsequently 20 μ L of MPC protein precipitation reagent was added, and the tubes were vortexed for a further 10 seconds. To precipitate any remaining protein, samples were then placed on ice for 5 minutes before being centrifuged at 12,000 $\times g$ at 4 $^{\circ}$ C for 5 minutes. The supernatant was transferred to a fresh microcentrifuge tube and RNA was precipitated with the addition of 50 μ L of isopropanol. The tubes were vortexed for 30 seconds and then centrifuged at 12,000 $\times g$ at 4 $^{\circ}$ C for 5 minutes. The isopropanol was discarded, and the RNA pellet was washed with 50 μ L of 70 % ethanol. The samples were centrifuged at 12,000 $\times g$ at 4 $^{\circ}$ C for a further 3 minutes and the ethanol was discarded. The RNA pellet was allowed to air dry for 5 minutes before being resuspended in 10 μ L Ultrapure distilled H₂O (Invitrogen) by vortexing for 2 minutes. To ensure full resuspension of the RNA pellet, the samples were incubated at 55 $^{\circ}$ C for 2 minutes before being either stored at -80 $^{\circ}$ C until required or added directly to the complementary DNA (cDNA) synthesis reaction as described below.

The synthesis of cDNA from total RNA was performed using the SuperScript[®] Vilo[™] cDNA Synthesis Kit (Invitrogen) following manufacturer's instructions. In detail, the entire total RNA sample (10 μ L) was added to 4 μ L of 5x VILO reaction mix, 2 μ L of 10x SuperScript[®] Vilo[™] enzyme and made up to a total volume of 20 μ L with distilled H₂O (dH₂O; 4 μ L). Following mixing by vortex, the samples were incubated in the Corbett Rotor-Gene 6000 machine (model no. 11754-050; Corbett Life Science; Sydney, Australia) under the following conditions: 25 $^{\circ}$ C for 10 minutes, 42 $^{\circ}$ C for 120 minutes, 85 $^{\circ}$ C for 5 minutes. The samples were removed immediately following cDNA synthesis and stored at -20 $^{\circ}$ C until required.

2.3.2 Multiplex qPCR

Gene expression was quantified using multiplex TaqMan qPCR. TaqMan probes contain a fluorophore (FAM, HEX, ROX, or Cy5) at the 3' end that differ in excitation and emission

wavelengths, an oligonucleotide sequence specific to the query gene, and a quencher at the 5' end. The fluorophore does not emit fluorescence due to the close proximity of the quencher. During the annealing steps of the amplification cycle, the primers and TaqMan probe bind to the template sequence. As the DNA polymerase moves along the sequence during the extension step, it displaces the fluorophore from the probe containing the quencher, allowing the emission of fluorescence.

Each reaction contained optimised concentrations of the appropriate forward and reverse primers (Invitrogen) and TaqMan probes (Sigma; Auckland, NZ) a pre-made master mix (Agilent Technologies; Santa Clara, USA), cDNA template, and was made up to a total volume of 52 μ L with UltraPure dH₂O. This reaction was mixed by vortex before being pipetted in duplicate 25 μ L aliquots into 0.1 mL strip tubes. Each reaction run contained a calibrator control and a no template control. The calibrator control contained pooled cDNA that had been isolated from a pool of rat COC, and was included in every reaction to enable normalisation against differences in reaction.

The Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies) was used to optimise the primer concentrations. The Brilliant® Multiplex QPCR Master Mix (Agilent Technologies) was used for all other experiments.

The samples were placed in the Corbett Rotor-Gene 6000 machine for thermal cycling and measurement of fluorescence. The thermal cycle conditions were as follows: 10 minutes at 95 °C, then 40 repeats of 15 seconds at 95 °C and 60 seconds at 60 °C. To check primer specificity, a final melt step was added when SYBR green technology was used, involving heating the samples from 60 °C to 95 °C, with 5 seconds incubation for each 0.5 °C rise in temperature. The qPCR results were analysed using the Corbett Rotor-Gene 600 Series Software 1.7.

2.3.3 Primers and probes

To determine gene expression levels, quadriplex reactions containing three query genes and the reference gene 60S ribosomal protein L19 (*Rpl19*) were prepared and measured using qPCR. Three different quadriplex reactions were included in this study. The first quadriplex reaction contained the oocyte-specific query genes growth differentiation factor 9 (*Gdf9*), bone morphogenetic protein 15 (*Bmp15*), and connexin 37 (*Cx37*). The second multiplex

reaction contained the query genes connexin 43 (*Cx43*), glutamine-fructose-6-phosphate aminotransferase (*Gfpt1*), and phosphofructokinase, platelet (*Pfkp*). The third multiplex reaction contained the query genes ataxia telangiectasia and Rad3 related (*Atr*), cytochrome c (*Cyts*), and glucose transporter 1 (*Glut1*).

For each query gene, a forward oligonucleotide primer, reverse oligonucleotide primer, and TaqMan probe were designed using the Beacon Designer software package (Premier Biosoft International, USA). The same *Rpl19* forward and reverse primers and TaqMan probe were used in each multiplex reaction. The sequences of each forward and reverse primer set and each TaqMan probe are listed in Table 2-1 and Table 2-2, respectively.

Table 2-1: Nucleotide sequences for forward and reverse primers for qPCR, including NCBI accession numbers, for each gene investigated.

Gene	NCBI Accession No	Forward (F) and reverse (R) primers
<i>Gdf9</i>	NM_021672	F- GGAACACTTACTCAAATCGGTCTTG R- GCCTTGCTGGAAGACATGGG
<i>Bmp15</i>	NM_021670	F- AACGAGGTAGCATACCAACTAATCAG R- GTTCGACATTTAGGGACCCAAGG
<i>Cx37</i>	NM_021654	F- GACGGTCGTCTTCGATTTCG R- ACGCCACTGGCCATAGAGG
<i>Cx43</i>	NM_012567	F- CAGCCATTAGCCATCGTG R- CGGTTTAAATCTCCAGGTCATC
<i>Gfpt1</i>	NM_001005879	F- AGCAGCCAGAATCTGTTG R- GTGGTAACTTGTCACACA
<i>Pfkp</i>	NM_206847	F- GGGCATAACCAACCTGTG R- CGATCTCACCATTCTTAGCC
<i>Atr</i>	XM_003754445	F- GGTAAGAATCTCTCCACTCCAGTCA R- AAGCCATTTCTCTGTGATGAGCAA
<i>Cyts</i>	NM_012839	F- CCCGTGCTTCAGTGTGTGTAAA R- CAGTGGTGAAAGGCAGCATCA
<i>Glut1</i>	NM_138827	F- CCTTGCCTGAGACCAGTTGAA R- ACAGCAGGGCAGGAGTGTC
<i>Rpl19</i>	NM_031103	F- CCCAATGAAACCAACGAAA R- GGGAATGGACAGTCACAG

Table 2-2: Nucleotide sequences for TaqMan probe sequences for qPCR for each gene investigated.

Gene	Probe Sequence
<i>Gdf9</i>	[6-FAM]CACTCTGAACAACTCCGCTGCTTCCTCCT[BHQ1]
<i>Bmp15</i>	[HEX]CACTGTGGTCTACCGCCATCAACTTCATCTAGT[BHQ1]
<i>Cx37</i>	[ROX]CCTCCAGCACACTCTTACACAGCACACTC[BHQ2]
<i>Cx43</i>	[6-FAM]CTTCCAGCAGAGCCAGCAGC[BHQ1]
<i>Gfpt1</i>	[HEX]CAGAATCAACCGCCGACACCG[BHQ1]
<i>Pfkp</i>	[ROX]TCCAGAAGACCGCTCCACTCC[BHQ2]
<i>Atr</i>	[6-FAM]ACTCCTTGCCAGACTGCTGAGATGCGAA[BHQ1]
<i>Cycs</i>	[ROX]TGCTAAACACCAGGACGGAAGTATTCTTGACA[BHQ2]
<i>Glut1</i>	[HEX]TGTCTTCAGCAGTAAGTTCTCAGCCTCCGA[BHQ1]
<i>Rpl19</i>	[Cy5]CCAATGCCAACTCTCGTCAACAGATCAGGAAG[BHQ3]

Table 2-3: Optimal final concentrations (nM) of forward and reverse primers and TaqMan probes in qPCR reactions.

Gene	Forward Primer	Reverse Primer	Probe
<i>Gdf9</i>	200	100	100
<i>Bmp15</i>	300	200	100
<i>Cx37</i>	300	100	50
<i>Cx43</i>	50	300	50
<i>Gfpt1</i>	100	200	100
<i>Pfkp</i>	300	300	100
<i>Atr</i>	200	200	50
<i>Cycs</i>	100	100	100
<i>Glut1</i>	100	200	100
<i>Rpl19</i>	300	200	100

2.3.4 Multiplex optimisation

The optimal concentration of the forward and reverse primers for each gene was determined by titration of the primer concentrations between 50 nM and 300 nM. This was performed for each gene individually using SYBR green technology. The combination of primer concentrations that gave the lowest cycle threshold (C_T) value without compromising on endpoint fluorescence was selected. The optimal probe concentration was determined for each gene individually by titration of the probe concentration between 50 nM and 300

nM. As each probe was labelled with a fluorophore, the TaqMan technology was used. The optimal primer and probe concentrations for each gene are shown in Table 2-3.

The reaction efficiency of each gene in each quadruplex reaction was determined after the optimised conditions were determined for each gene. A cDNA dilution series was prepared, with each successive dilution containing half the concentration of cDNA. If the reaction had 100 % efficiency, the C_T value for each successive dilution would differ by 1.0, as C_T values are on a logarithmic scale. The reaction efficiencies for each of the genes are shown in Table 2-4.

Table 2-4: The amplification efficiency for each gene in three different qPCR quadruplex reaction sets.

Gene	Threshold	Efficiency (%)
Quadruplex 1	0.03 (slope correct)	
<i>Bmp15</i>		92
<i>Gdf9</i>		93
<i>Cx37</i>		98
<i>Rpl19</i>		92
Quadruplex 2	0.03 (slope correct)	
<i>Cx43</i>		90
<i>Gfpt1</i>		91
<i>Pfkp</i>		95
<i>Rpl19</i>		98
Quadruplex 3	0.07	
<i>Atr</i>		101
<i>Cybs</i>		96
<i>Glut1</i>		98
<i>Rpl19</i>		99

2.3.5 qPCR Analysis

Each oocyte was considered to be a discrete variable, so that the number of samples was considered to be the total number of readings across all replicates. Relative gene expression, compared to the control group, was calculated as described previously (Livak and Schmittgen, 2001). The CC samples were further corrected for the reference gene *Rpl19*, to give gene expression relative to RNA input. The oocyte samples were corrected for inter-assay reaction differences (i.e. internal calibrator control), to give total gene expression per single oocyte.

For each CC sample, the C_T value for *Rpl19* was subtracted from the C_T value of the query gene, to give the ΔC_T value. For the oocyte samples, the C_T value of the calibrator control for each query gene was subtracted from the C_T value for each oocyte sample for the same query gene. The average ΔC_T of the control group was calculated, and this was subtracted from the ΔC_T values for each sample to give the $\Delta\Delta C_T$. The $\Delta\Delta C_T$ values were converted to linear values by applying the formula $2^{-(\Delta\Delta C_T)}$. This formula gives a positive change in linear values as mRNA levels increase. Any sample that had a C_T value for *Rpl19* of ≥ 30 was excluded from analysis due to insufficient template. Samples that had a C_T value for the query genes that was out of the detection range (>34) were given a C_T value of 40 for analysis purposes.

In order to plot the data, the formula $2^{-(\text{Average}\Delta\Delta C_T)}$ was applied to the average $\Delta\Delta C_T$ for each group, to give the mean relative change in gene expression. The upper and lower errors were calculated using the formula $2^{-(\text{Average}\Delta\Delta C_T \pm \text{SEM})}$, where SEM is the standard error of the mean.

Statistical analysis was performed using the GraphPad Prism 5 software program. In general, either a Kruskal-Wallis test or a two-way ANOVA was employed to analyse the data. Where a two-way ANOVA was used, the data were first tested for a normal distribution using the D'Agostino & Pearson normality test. Where the data were not normally-distributed, the appropriate transformation method was used and the data were tested for a normal distribution again. If no transformation method made the data normally-distributed, the data were ranked and a two-way ANOVA was performed on the ranked data.

2.4 Western Blotting

2.4.1 Protein quantification

For Western blots, the total amount of protein loaded was determined using a bicinchoninic (BCA) based assay (Thermo Scientific; Auckland, NZ). This assay relies on the reduction of Cu^{+2} ions to Cu^{+1} by protein in an alkaline environment. Each Cu^{+1} ion is chelated by two BCA molecules, and this complex has strong absorbance at 592 nm.

The microplate procedure was used as described below. Bovine serum albumin was dissolved in PBS at concentrations of 0, 5, 10, 20, 30, 40, 50, 75, and 100 $\mu\text{g/mL}$. An aliquot (~67 COC; usually in 10 μL) of the crude COC lysate collected for Western blotting was diluted with 80 μL PBS buffer. Each COC sample was serially diluted to produce two sample dilutions, each containing half the amount of protein as the previous sample solution. The assay was performed in a 96-well plate. The assay plate was prepared by dispensing 25 μL of each standard and sample solution into the appropriate wells. The standards were assayed in triplicate and the samples were assayed in duplicate as described below.

The BCA working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. This working reagent (200 μL) was added to each well, and the plate was mixed for 30 seconds using an Eppendorf MixMate plate shaker (Eppendorf; Hamburg, Germany). The plate was sealed and incubated at 37 °C (standard protocol) or 60 °C (optimised protocol) for 2 hours. The plate was removed from the incubator and allowed to cool to room temperature. The absorbance of the wells at 490 nm was measured using a VICTOR3 multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

The data were analysed by subtracting the absorbance of the blank from the standard and sample absorbance values. A standard curve was created using a best-fit linear regression of the absorbance values of the standards from which the protein concentrations of the samples were calculated. The amount of protein in each of the serial dilutions was corrected for the dilution factor, and an average protein concentration over the two serial dilutions was calculated.

2.4.2 SDS-PAGE

Proteins were separated by molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gels, with a 13.5 % separating gel and 4 % stacking gel (Appendix 2), were prepared one day in advance and stored overnight at 4 °C in paper towels soaked in dH₂O.

Sample buffer was added to each crude COC lysate to a final concentration of 2x (Appendix 2). Each blot also contained a pre-stained SDS-PAGE Low-Range standard (BioRad; Auckland, NZ). All the samples were denatured at 95 °C for 10 minutes before being briefly centrifuged. An SDS-PAGE running cassette was set up using the polyacrylamide gel and 1x SDS-PAGE running buffer (Appendix 2). The entire crude COC lysate (minus the aliquot utilised for the BCA assay) was added into the appropriate well. Therefore, each COC sample lane contained protein from ~133 COC and this was kept constant between treatment groups and experimental replicates. If the entire volume of sample would not fit in one well, the gel was electrophoresed at 150 volts for 5 minutes to allow some of the sample to migrate into the stacking gel. Subsequently, the remainder of the sample was loaded into the appropriate well. Electrophoresis of the gel was then continued at 150 volts for a further 90 minutes, or until the blue marker dye had reached the bottom of the gel.

2.4.3 Western Blotting

A transfer sandwich was assembled to transfer the protein contained within the polyacrylamide gel onto a nitrocellulose membrane. All components, except the gel, were pre-soaked in transfer buffer (Appendix 2). The transfer sandwich components, in order from bottom to top, were: black face (negative charge), scotch pad, blotting paper, gel, nitrocellulose membrane, blotting paper, scotch pad, red face (positive charge). A roller was used to gently remove any air bubbles between each layer. The transfer sandwich was placed in the transfer stand along with a magnetic stirrer bar and ice pack, and transfer buffer was added to cover the transfer sandwich components. The transfer was performed at 100 volts for 70 minutes at 4 °C. To check that the transfer was successful, the nitrocellulose membrane was incubated with Ponceau S stain (Appendix 2) for 1 minute at 60 rpm on a Labnet Orbit LS low speed platform shaker (Total Lab Systems Ltd.; Auckland, NZ). The membrane was de-stained with dH₂O, and the position of the lanes was marked. The membrane was washed three times in Tris buffered saline (TBS; Appendix 2) before

being blocked in 5 % low-fat milk powder solution (Appendix 2) for 1 hour on the rotating platform shaker. The membrane was then incubated overnight in the appropriate primary antibody diluted in 5 % low-fat milk powder solution on the rotating platform shaker. The primary antibodies are detailed in Section 2.4.5. The membrane was washed three times in TBS, before being incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) in 5 % low-fat milk powder solution. The secondary antibodies are detailed in Section 2.4.5. The membrane was washed three times in TBS, before being incubated for 1 minute in electrochemiluminescence (ECL) reagents (Appendix 2). The membrane was wrapped in cling film and adhered to an exposure cassette. The x-ray films were exposed to the membrane for between 1 and 10 minutes, depending on the amount of protein signal. Following the development and fixturing of the x-ray film, the ladder and lane positions were marked by comparison with the membrane.

2.4.4 Western blot stripping

The Western blots were stripped to remove the bound primary and secondary antibodies, allowing the membrane to be re-probed with different primary and/or secondary antibodies. The membrane was incubated in stripping buffer (Appendix 2) for 30 minutes at 50 °C. The membrane was washed vigorously in TBS, before undergoing 3 further wash steps in TBS on a rotating platform shaker. Thereafter, the Western blot protocol was carried out from the 1 hour incubation step in low-fat milk powder solution.

2.4.5 Antibodies

A commercial anti-CX43 monoclonal antibody, MAB3067, was purchased from Millipore (Auckland, NZ). The MAB3067 antibody was raised in mice against the peptide sequence RSDPYHATTGPLSPSKDC, which corresponds to residues 252-270 of mouse CX43. This sequence is identical in rat CX43 and contains one potentially modified residue, a phosphoserine at residue 255. The MAB3067 antibody was used at a final concentration of 1 µg/mL in 5 % low-fat milk powder solution. For ECL detection, blots that had been incubated with the MAB3067 antibody were subsequently incubated for 3 hours in 0.33 µg/mL rabbit anti-mouse secondary antibody conjugated to HRP (Jackson ImmunoResearch; Westgrove, PA, USA) in 5 % low-fat milk powder solution. For blocking experiments, MAB3067 was pre-absorbed overnight at 4 °C on a rotating wheel with 5X (w/w) the corresponding control

peptide (Millipore). The blocked antibody was centrifuged at 13,000 x *g* for 10 minutes, and the supernatant was added to 5 % low-fat milk powder solution.

The monoclonal antibody used to detect GDF9 (MAB47B) was produced by Oxford Brookes University for AgResearch. The MAB47B antibody was raised in mice against the peptide sequence SEYFKQFLFPQNEC, which corresponds to residues 122-135 (C-terminal region) of ovine GDF9. This sequence differs by one residue (bolded) in rat GDF9 (SEYF**R**QFLFPQNEC). Although the rat and sheep sequences are only 93 % homologous, MAB47B has previously been shown to recognise rat GDF9 (Lin et al., 2012) and in this study was used at a concentration of 1 µg/mL. For ECL detection, blots that had been incubated with MAB47B were subsequently incubated for 3 hours in 0.33 µg/mL rabbit anti-mouse secondary antibody conjugated to HRP. For blocking experiments, MAB47B was pre-absorbed overnight at 4 °C on a rotating wheel with 5X (w/w) *E. coli* produced recombinant ovine GDF9. The blocked antibody was centrifuged at 13,000 x *g* for 10 minutes, and the supernatant was added to 5 % low-fat milk powder solution.

2.4.6 Western blot analysis

Quantitative analyses of the western blots were performed using ImageJ 1.45s software. After scanning the x-ray film at high resolution, the scanned image was viewed in the ImageJ software package. The migration of the molecular standard was measured and used to estimate the size of the bands in the sample lanes. To measure the density of the bands, the image type was changed to 8-bit (grey-scale), and the subtract background process was used. The rectangular tool was used to select a band, and the measure function was used. The area of the rectangle was kept identical in all the corresponding bands of the same size. The integrated density (IntDen) value corresponds to the area and the mean grey value of the selection. For each band, the IntDen value was divided by the IntDen value of the time zero control. This value was subsequently divided by the relative amount of protein in each sample, as determined in the BCA assay.

3 VALIDATION OF A GJ BIOASSAY TO DETERMINE GJ ACTIVITY IN COC

3.1 Introduction

GJ communication between the CC and oocyte is essential for the transfer of essential nutrients and maintenance of meiotic arrest. A novel bioassay utilising the properties of a fluorescent dye (calcein) for measuring GJ communication between CC and oocytes was first reported in cultured bovine COC *in vitro* (Thomas et al., 2004). This bioassay involves the passive entry of the acetoxymethyl derivate (calcein-AM) into cells via permeation of the cellular membrane. Once inside the cell, the acetoxymethyl group is cleaved by non-specific esterases rendering calcein as a membrane impermeable fluorescent compound. Thus, when calcein-AM is incubated with CC-enclosed oocytes, the majority (~80 %) of the fluorescent calcein measured within the oocyte has been transferred from the CC through GJ (Thomas et al., 2004). Indeed, treatment of bovine COC with carbenoxolone, a GJ blocking agent that disassembles GJ plaques and dephosphorylates GJ proteins (Guan et al., 1996; Cruciani and Mikalsen, 2002), inhibited calcein transfer to the oocyte in a dose dependent manner (Thomas et al., 2004).

Rat COC that have been isolated from their follicular environment resume meiosis within 1-2 hours under *in vitro* conditions (Wert and Larsen, 1989). Meiotic arrest in oocytes is maintained by high levels of cAMP and evidence suggests that COC extraction from follicles results in the breakage of GJ (Wert and Larsen, 1989) and a subsequent decline in intra-oocyte cAMP concentrations. Studies in bovine COC indicate that the primary mechanisms that regulate cAMP levels in the oocyte are the production of cAMP by adenylyl cyclase in the CC followed by transfer to the oocyte, and the degradation of cAMP by PDE expressed in the oocyte (Thomas et al., 2002). Additionally, the degree of intracellular communication facilitated through GJ has been reported to be regulated by cAMP via protein kinase A (PKA)-mediated phosphorylation (Cruciani and Mikalsen, 2002). Thus, maintenance of cAMP levels in the oocyte by the addition of adenylyl cyclase stimulators or PDE inhibitors during *in vitro* culture of COC is critical for maintaining GJ communication and meiotic arrest in oocytes. It has long been thought that the major PDE isoform expressed in the rodent oocyte is PDE3, while the major isoform expressed in rodent CC is PDE4D (Tsafriri et al., 1996; Richard et al., 2001). However, recent research has shown that inhibiting PDE8 activity in bovine COC

increased cAMP levels and delayed oocyte nuclear maturation (Sasseville et al., 2009a). This suggests that species differences in the forms of PDE may exist.

Whilst the GJ bioassay had been modified for measuring GJ communication in rat COC (Patel, 2009; Yadav, 2010), the specificity of the assay and the effectiveness of a range of compounds involved in maintaining meiotic arrest and GJ communication has not been fully elucidated. Therefore, the aims of this chapter were to (1) determine the optimal *in vitro* conditions for maximising the rate of fluorescent dye transfer in rat COC and (2) demonstrate that fluorescent dye transfer from the CC to the oocyte is specific to GJ transfer and can be blocked using the GJ uncoupler carbenoxolone.

3.2 Methodology

3.2.1 Tissue collection

For experiments in sections 3.2.2-3.2.5 described below, ovaries were extracted and follicles were punctured in dissection media supplemented with 100 μ M milrinone. The COC were transferred into 48-well plates containing wash media with 100 μ M milrinone. Due to the results obtained in these aforementioned sections, subsequent experiments utilised a modified collection methodology. Namely for experiments in sections 3.2.6-3.2.8, ovaries were extracted, follicles were punctured and COC were transferred into 48-well plates containing dissection media supplemented with 50 μ M dipyridamole and 50 μ M rolipram (see Sections 2.1.1 and 2.1.2 for detailed tissue collection methodology).

3.2.2 Dose-response effects of DMSO concentrations

To determine the effects of the solvent (DMSO) used to facilitate solubilisation of PDE inhibitors and/or treatments, milrinone was dissolved in differing volumes of DMSO to achieve a final milrinone concentration of 100 μ M and DMSO concentrations of 0.2 %, 0.5 %, 1.0 %, and 2.0 % in media. The COC were washed twice in wash media containing 100 μ M milrinone and the appropriate concentration of DMSO. The COC were then incubated with 2 μ M calcein-AM in incubation media containing 100 μ M milrinone and the appropriate concentration of DMSO for 10 minutes. All incubations were carried out in an incubator at 37 °C under 5 % CO₂ in air with 96 % humidity. Subsequently, the COC were transferred into incubation media containing 100 μ M milrinone and the appropriate concentration of DMSO without calcein-AM and incubated for either 0 or 4 hours. At the end of the incubation

times, each COC was transferred into fresh incubation media (without milrinone or DMSO) where the oocyte was denuded and fluorescence was measured as described previously.

3.2.3 Dose-response effects of forskolin

Following collection, the COC were washed twice in wash media containing 100 μ M milrinone and incubated with 2 μ M calcein-AM in incubation media with 100 μ M milrinone for 10 minutes. To determine the effects of forskolin, the COC were then transferred to incubation media containing 100 μ M milrinone and 0, 10, 20, or 50 μ M forskolin. After 0 or 4 hours incubation, the COC were transferred to fresh incubation media (no milrinone or forskolin) and the oocyte was denuded and fluorescence was measured as described previously (Section 2.1.3).

3.2.4 Dose-response effects of single PDE inhibitors

Three different PDE inhibitors were tested at the concentrations listed in Table 3-1. Milrinone is a specific inhibitor of PDE3 (Tsafiriri et al., 1996), rolipram is a specific inhibitor of PDE4 (Sasseville et al., 2009a) and dipyridamole is reported to inhibit several PDE isoforms including PDE7, 8, 10, and 11 (Sasseville et al., 2009a). All concentrations of PDE inhibitors were dissolved in DMSO and the control media was supplemented with DMSO so that regardless of treatment, the final concentration of DMSO in media was 1 %.

Following collections, COC were washed twice in wash media without (control) or with the appropriate PDE inhibitor at the concentrations described above. The COC were incubated in 2 μ M calcein-AM without or with the appropriate PDE inhibitor for 10 minutes and then transferred into fresh incubation media (no calcein-AM) containing the appropriate PDE inhibitors or media alone (control). Following 0 or 4 hours incubation, the COC were transferred into incubation media alone (no PDE inhibitors) and the oocytes were denuded and fluorescence was measured as described previously (Section 2.1.3).

Table 3-1: Concentrations of PDE inhibitors used for the GJ dose-response experiments

PDE inhibitor	PDE isoforms inhibited	Concentrations tested (μ M)
Milrinone	PDE3	100, 300, 500
Dipyridamole	PDE5, 6, 7, 8, 10, 11	10, 50, 100
Rolipram	PDE4	10, 50, 100

3.2.5 Effects of combined PDE inhibitors

Each PDE inhibitor was tested in combination with each other at the optimised concentration (as determined in section 3.2.4) as follows: 300 μ M milrinone alone, 50 μ M dipyridamole alone, 50 μ M rolipram alone, 300 μ M milrinone + 50 μ M dipyridamole, 300 μ M milrinone + 50 μ M rolipram, 50 μ M dipyridamole + 50 μ M rolipram, and 300 μ M milrinone + 50 μ M dipyridamole + 50 μ M rolipram. The final concentration of the DMSO used to solubilise the PDE inhibitors in the media was ~1 %. Following collection, COC were washed twice in wash media without (control) or with the various PDE inhibitors as listed above. The COC were incubated for 10 minutes in 2 μ M calcein-AM in media alone (control) or with the appropriate PDE inhibitor(s). Thereafter, the COC were transferred to fresh incubation media (without calcein-AM) in the absence (control) or presence of PDE inhibitors. Following 0 or 4 hours incubation, COC were transferred into fresh media (free of PDE inhibitors) and the oocytes were denuded and the fluorescence intensity measured as described previously (Section 2.1.3).

3.2.6 Effects of time on dye transfer to the oocyte

Following collection, the COC were washed twice in wash media containing 50 μ M dipyridamole and 50 μ M rolipram. The COC were incubated in 2 μ M calcein-AM in incubation media containing 50 μ M dipyridamole and 50 μ M rolipram for 10 minutes. To determine the effect of incubation time on oocyte fluorescence, COC were transferred into fresh media (without calcein-AM) containing 50 μ M dipyridamole and 50 μ M rolipram and incubated for 0, 1, 2, 3, 4, 12, or 24 hours. Following incubation, COC were transferred into fresh incubation media containing no PDE inhibitors and the oocytes were denuded and the fluorescence intensity measured as described previously (Section 2.1.3).

3.2.7 Testing the specificity of the GJ bioassay

To determine the specificity of fluorescent dye transfer from the CC to the oocyte by GJs, COC were incubated with varying concentrations of the GJ uncoupler carbenoxolone. Following collection, COC were washed twice in wash media containing 50 μ M dipyridamole and 50 μ M rolipram. The COC were incubated for 10 minutes in 2 μ M calcein-AM in media containing 50 μ M dipyridamole and 50 μ M rolipram. COC were then transferred into fresh incubation media containing 50 μ M dipyridamole and 50 μ M rolipram and either 0, 100, 200, 300, 500, or 1000 μ M carbenoxolone. Following 0 or 4 hours incubation, COC were

transferred into incubation media free of PDE inhibitors and carbenoxolone and the oocytes were denuded and the fluorescence intensity measured as described previously (Section 2.1.3).

3.2.8 *Testing the robustness of the GJ bioassay*

To determine the robustness of the *in vitro* GJ bioassay over time, the proportion of fluorescence that entered the oocyte via a non-GJ pathway over time was assessed. Following collection, COC were washed twice in wash media containing 50 μ M dipyridamole and 50 μ M rolipram. The COC were transferred into incubation media containing 50 μ M dipyridamole and 50 μ M rolipram and incubated for 0, 2, 10, or 22 hours. Those COC that were incubated for 2, 10, or 22 hours were then transferred to incubation media containing 1 mM carbenoxolone, 50 μ M dipyridamole and 50 μ M rolipram and incubated for a further 2 hours, so the total culture times were 4, 12, or 24 hours, respectively. Following incubations, COC were exposed for 10 minutes to 2 μ M calcein-AM in incubation media containing 1 mM carbenoxolone, 50 μ M dipyridamole and 50 μ M rolipram. The COC were incubated for 1 hour in calcein-AM free incubation media containing 1 mM carbenoxolone, 50 μ M dipyridamole and 50 μ M rolipram. The COC were then transferred into incubation media without carbenoxolone or PDE inhibitors, the oocytes were denuded and fluorescence intensity measured as described previously (Section 2.1.3).

3.2.9 *Data Analysis*

The data were analysed as previously described in Section 2.1.3.1. Briefly, the oocyte fluorescence in each individual sample was corrected for the geometric mean of the control group (usually the untreated group at 0 hours). The data were analysed using the Mann-Whitney test, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, or a two-way ANOVA. Where a two-way ANOVA was used, the data were first tested for normality using the D'Agostino & Pearson normality test. Data that was not normally-distributed was transformed using a natural log transformation, tested for normality again, and a two-way ANOVA was performed on the transformed data.

3.3 Results

3.3.1 Dose-response effects of DMSO concentrations

The dose-response effects of DMSO in media on GJ communication in rat COC is depicted in Figure 3-1 where the fluorescent intensity values plotted are those at 4 hours normalised against the mean control value at 0 hours. There were no significant differences in relative oocyte fluorescent intensity at any of the DMSO concentrations tested ($P=0.055$). Nonetheless whilst not significant, there appeared to be a reduction in intra-oocyte fluorescent intensity at the highest dose (2 %) of DMSO, thus the DMSO concentration of 1 % was used in all subsequent assays.

Figure 3-1: Effect of differing concentrations of DMSO on fluorescence transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, relative geometric mean=1.0). The data represent an N of 22-25 readings from individual oocytes in each group generated from 2 replicate experiments. The data were analysed using the Kruskal-Wallis test. No significant effect of DMSO concentration on relative fluorescent intensity was noted. The mean $\pm$ SEM fluorescent reading in the control group at T0 was 104 ± 36 .

3.3.2 Dose-response effects of forskolin

The dose-response effects of forskolin (an adenylyl cyclase stimulator) on GJ communication in rat COC is shown in Figure 3-2, where the fluorescent intensity values plotted are those at 4 hours normalised against the mean control value at 0 hours. Incubation of COC for 4 hours

with the two lowest doses of forskolin (10 μ M or 20 μ M) did not affect oocyte fluorescence. In comparison, incubation of COC for 4 hours with the highest dose of forskolin tested (50 μ M) resulted in a significant reduction ($P<0.001$) in oocyte fluorescence. The incubation time of 4 hours resulted in significantly increased ($P<0.0001$; Mann-Whitney test) fluorescent intensity in the control oocytes (no forskolin), compared to that at 0 hours (i.e. note the ~4-6 fold higher values; Figure 3-2A, B, & C). The proportion of oocytes with a visible GV was similar between experimental treatment groups after 4 hours of incubation (Fisher's exact test; data not shown).

Figure 3-2: Effect of forskolin on fluorescent dye transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, relative geometric mean=1.0). The data represent an N of (A) 46-64 readings from individual oocytes in each group generated from 5 replicate experiments, (B) 30-41 readings from individual oocytes in each group generated from 3 replicate experiments, (C) 8-19 readings from individual oocytes in each group generated from 1 replicate experiment. The data were analysed using the Mann Whitney test. No significant changes in relative fluorescent intensity were noted for A or B. For C, groups that do not share a letter are statistically different ($P<0.05$). The mean $\pm$ SEM raw fluorescent reading in the control group at T0 was (A) 56 ± 18 , (B) 46 ± 16 , and (C) 140 ± 46 .

3.3.3 Dose-response effects of single PDE inhibitors

The dose-response effects of milrinone (a PDE3 inhibitor) on GJ communication in rat COC is shown in Figure 3-3, where the fluorescent intensity values plotted are those at 4 hours normalised against the mean control value at 0 hours. There were no significant differences in oocyte fluorescent intensity following a 4 hour incubation of rat COC with 100 and 300 μM of milrinone. In contrast, the highest dose of milrinone (500 μM) resulted in a significant reduction ($P<0.01$) in fluorescence compared to the other two dosages. The incubation time of 4 hours resulted in significantly increased ($P<0.05$; Mann-Whitney test) fluorescent intensity in the control oocytes (no milrinone), compared to that at 0 hours (i.e. note the ~2 fold higher value; Figure 3-3). The proportion of oocytes with a visible GV, indicating arrested meiosis, was similar between all experimental treatment groups after 4 hours of incubation (Table 3-2).

Figure 3-3: Effect of differing concentrations of milrinone on fluorescent dye transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, relative geometric mean=1.0). The data represent 31-35 readings from individual oocytes in each group generated from 3 replicate experiments. The data were analysed by a Kruskal-Wallis test and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. Groups that do not share a letter are statistically different ($P<0.05$). The mean $\pm$ SEM raw fluorescent reading in the control group at T0 was 206 ± 28 .

Table 3-2: Effect of differing concentrations of milrinone on the proportion of oocytes in which a GV was observed. The table shows the proportion (%) of oocytes with a visible GV, with the actual number of oocytes with a GV observed in brackets. The data from 3 replicate experiments (N=31-35) were analysed by a Fisher's exact test. No significant effect of milrinone concentration on the presence of a GV was noted.

Milrinone concentration (μ M)	GV oocytes (%)
0	78 (25)
100	94 (33)
300	79 (26)
500	74 (23)

The dose-response effects of dipyridamole (a PDE7, 8, 10, and 11 inhibitor) on GJ communication in rat COC is depicted in Figure 3-4, where the fluorescent intensity values plotted are those at 4 hours normalised against the mean control value at 0 hours. The incubation of COC with 50 μ M dipyridamole for 4 hours caused a significant increase in oocyte fluorescence relative to the appropriate control (4 hour incubation with the vehicle alone). No other concentration of dipyridamole tested resulted in a significant change in oocyte fluorescence relative to the control. The incubation time of 4 hours resulted in significantly increased ($P<0.01$; Mann-Whitney test) fluorescent intensity in the control oocytes (no dipyridamole), compared to that at 0 hours (i.e. note the ~2 fold higher value; Figure 3-4). The proportion of oocytes with a visible GV was similar between experimental treatment groups after 4 hours of incubation (Table 3-3).

Figure 3-4: Effect of differing concentrations of dipyridamole on fluorescent dye transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, relative geometric mean=1.0). The data represent 34-38 readings from individual oocytes in each group generated from 4 replicate experiments. The data were analysed by a Kruskal-Wallis test and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. Groups that do not share a letter are statistically different ($P < 0.05$). The mean $\pm$ SEM raw fluorescent reading in the control group at T0 was 207 ± 24 .

Table 3-3: Effect of differing concentrations of dipyridamole on the proportion of oocytes in which a GV was observed. The table shows the proportion (%) of oocytes with a visible GV, with the actual number of oocytes with a GV observed in brackets. The data from 3 replicate experiments (N=34-38) was analysed by a Fisher's exact test. No significant effect of dipyridamole concentration on the presence of a GV was noted.

Dipyridamole concentration (μM)	GV oocytes (%)
0	59 (22)
10	66 (25)
50	81 (29)
100	59 (20)

The effect of incubating rat COC for 4 hours with differing concentrations of rolipram (a PDE4 inhibitor) on GJ communication is shown in Figure 3-5, where the fluorescent intensity values plotted are those at 4 hours normalised against the mean control value at 0 hours. No differences were observed in oocyte fluorescence between any experimental groups. The

incubation time of 4 hours resulted in significantly increased ($P<0.005$; Mann-Whitney test) fluorescent intensity in the control oocytes (no rolipram), compared to that at 0 hours (i.e. note the ~2 fold higher value; Figure 3-5). The proportion of oocytes with a visible GV was similar between experimental treatment groups after 4 hours of incubation (Table 3-4).

Figure 3-5: Effect of differing concentrations of rolipram on fluorescent dye transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, relative geometric mean=1.0). The data represent 26-29 readings from individual oocytes in each group generated from 3 replicate experiments. The data were analysed by a Kruskal-Wallis test and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. No significant changes in relative fluorescent intensity were noted. The mean $\pm$ SEM raw fluorescent reading in the control group at T0 was 212 ± 23 .

Table 3-4: Effect of differing concentrations of rolipram on the proportion of oocytes in which a GV was observed. The table shows the proportion (%) of oocytes with a visible GV, with the actual number of oocytes with a GV observed in brackets. The data from 3 replicate experiments (N=26-29) was analysed by a Fisher's exact test. No significant effect of rolipram concentration on the presence of a GV was noted.

Rolipram concentration (μ M)	GV oocytes (%)
0	32 (12)
10	53 (18)
50	43 (17)
100	50 (18)

3.3.4 Effects of combined PDE inhibitors

The effects of incubating rat COC for 4 hours with combinations of the aforementioned PDE inhibitors on GJ communication is shown in Figure 3-6, where the fluorescent intensity values plotted are those at 4 hours normalised against the mean control value at 0 hours. A combination of dipyridamole and rolipram resulted in a significantly higher ($P<0.05$) fluorescence intensity in oocytes compared to that in the untreated control group. All other treatment groups (PDE inhibitors alone and in combination) had oocyte fluorescent intensities similar to that of the control. The incubation time of 4 hours resulted in significantly increased ($P<0.05$; Mann-Whitney test) fluorescent intensity in the control oocytes (no PDE inhibitors), compared to that at 0 hours (i.e. note the ~2 fold higher value; Figure 3-6). The proportion of oocytes with a visible GV was similar between all experimental treatment groups after 4 hours of incubation (Table 3-5).

Figure 3-6: Effect of single or combinations of PDE inhibitors on fluorescent dye transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, relative geometric mean=1.0). The data represent 22-30 readings from individual oocytes in each group generated from 3 replicate experiments. The data were analysed by a Kruskal-Wallis test and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. Groups that do not share a letter are statistically different ($P<0.05$). The mean $\pm$ SEM raw fluorescent reading in the control group at T0 was 232 ± 46 .

Table 3-5: Effect of single or combinations of PDE inhibitors on the proportion of oocytes in which a GV was observed. The table shows the proportion (%) of oocytes with a visible GV, with the actual number of oocytes with a GV observed in brackets. The data from 3 replicate experiments (N=22-30) was analysed by a Fisher's exact test. No significant effect of PDE inhibitor concentration on the presence of a GV was noted.

PDE inhibitor combination	% GV oocytes
No PDE inhibitors	64 (18)
Milrinone	86 (25)
Dipyridamole	70 (16)
Rolipram	73 (19)
Milrinone + Dipyridamole	83 (25)
Milrinone + Rolipram	81 (22)
Dipyridamole + Rolipram	71 (20)
Milrinone + Dipyridamole + Rolipram	59 (13)

3.3.5 Effects of time on dye transfer to the oocyte

The effect of varying the incubation time following exposure to calcein-AM on the fluorescent intensity of the oocyte is depicted in Figure 3-7. Initially, oocyte fluorescence increased with time to reach maximum levels after 2-3 hours of incubation. As a result of these experiments and to remain within a responsive fluorescent intensity range, COC were incubated for 1 hour after the 10 minute exposure to calcein-AM for all subsequent assays.

Figure 3-7: Effect of time on fluorescent dye transfer from adjacent CC to oocytes. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group. The data represent 23-27 readings from individual oocytes at each time generated from 3 replicate experiments. The data were analysed by a Kruskal-Wallis test and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. Groups that do not share a letter are significantly different ($P < 0.05$). The mean $\pm$ SEM raw fluorescent reading at T0 was 297 ± 38 .

3.3.6 Testing the specificity of the GJ bioassay

To determine the proportion of calcein transfer to the oocyte that is facilitated through GJ, rat COC were exposed to increasing concentrations of the GJ blocking agent carbenoxolone. These results are depicted in Figure 3-8, in which the fluorescent intensity values plotted are those at 4 hours normalised against the mean control value at 0 hours. Carbenoxolone inhibited calcein transfer to the oocyte in a dose-dependent manner. The lowest concentration of carbenoxolone to cause a significant reduction (~ 60 %; $P < 0.05$) in oocyte fluorescence, compared to the control group, was $200 \mu\text{M}$. The lowest dose that caused the maximum inhibition (> 88 %; $P < 0.0001$) of GJ communication within COC was $500 \mu\text{M}$ carbenoxolone. The incubation time of 4 hours resulted in significantly increased ($P < 0.0001$; Mann-Whitney test) fluorescent intensity in the control oocytes (no carbenoxolone), compared to that at 0 hours (i.e. note the ~ 4 -fold higher value; Figure 3-8).

Figure 3-8: Effect of increasing concentrations of carbenoxolone on fluorescent dye transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, relative geometric mean = 1.0). The data represent 25-37 readings from individual oocytes in each group generated from 4 replicate experiments. The data were analysed by a Kruskal-Wallis test and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. Groups that do not share a letter are significantly different ($P < 0.05$). The mean $\pm$ SEM raw fluorescent reading in the control group at T0 was 195 ± 35 .

3.3.7 Testing the robustness of the GJ bioassay

The robustness of the *in vitro* GJ bioassay over time was assessed by determining the proportion of fluorescent dye that enters the oocyte via a means other than GJs over time. These results are depicted in Figure 3-9, in which the fluorescent intensity values plotted are normalised against the mean control value at 0 hours. As carbenoxolone takes at least 2 hours to effectively block GJ (see Appendix 1), the specificity of calcein transfer to the oocyte in the COC incubated for 1 hour was not able to be determined. A 2 hour incubation of COC with carbenoxolone, regardless of a prior 2, 10, or 22 hour incubation without carbenoxolone, caused a marked decrease (91 %, 85 %, and 92 %, respectively) in dye transfer to the oocyte by the end of the total incubation period. All of the incubation times tested resulted in significantly increased ($P < 0.0001$; Mann-Whitney test) fluorescent intensities in the control oocytes, compared to those at 1 hours (i.e. note the ~3.5-fold higher values, Figure 3-9).

Figure 3-9: Effect of 1 mM carbenoxolone on fluorescent dye transfer from adjacent CC to oocytes after 5, 13, and 25 hours incubation in rat COC. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control (i.e. untreated) group is represented by the unfilled bars (□), while the carbenoxolone-treated group is represented by the filled bars (■). Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the control group at 1 hour (not shown, relative geometric mean = 1.0). The data represents 23-44 readings from individual oocytes in each group generated from 3 replicate experiments. The natural log-transformed data were analysed by a two-way ANOVA. The mean $\pm$ SEM raw fluorescent reading in the control group at 1 hour was 112 ± 14 .

3.4 Discussion

These validation studies reveal for the first time in the rat that, of all the cAMP-modulating compounds tested the combination of dipyridamole and rolipram was arguably the most effective in maintaining GJ communication between the CC and oocyte. Interestingly, the inhibitors milrinone and rolipram that block the PDE isoforms reported to be primarily expressed in rat oocytes (i.e. PDE3; Richard et al., 2001) and CC (i.e. PDE4; Tsafiriri et al., 1996), respectively had no effect on GJ communication in this study either singly or in combination with one another. Dipyridamole is reported to inhibit the actions of PDE7, 8, 10, and 11 (Sasseville et al., 2009a). Whilst the expression of PDE8 has previously been detected in the rat ovary (Kobayashi et al., 2003), PDE8 activity on regulating cAMP concentrations or oocyte meiotic resumption in this species has not been reported. Additionally, to the best of my knowledge, the expression of PDE7, 10, and 11 in the rat COC has not been previously detected. Therefore, these data indicate for the first time that the levels of PDE7, 8, 10, and/or 11 are active in rat COC. In the cow, PDE8 has been reported to account for up to 20 % and 60 % of PDE activity in oocytes and CC, respectively. If a similar relationship exists in

the rat COC, these results suggest that inhibition of PDE activity in the CC, rather than the oocyte, is more important for maintaining GJ communication in this species. These results also suggest that prevention of cAMP breakdown (tested by the addition of PDE inhibitors), rather than stimulation of cAMP production (tested by the addition of forskolin) is more important for maintaining GJ communication in the rat.

The ability of the GJ inhibitor carbenoxolone to inhibit calcein transfer to the oocyte up to 25 hours in culture indicates that these culturing conditions allow the COC to retain its structural integrity *in vitro*. Furthermore, calcein transfer from the CC to the oocyte was reduced in a dose-dependent manner with increasing concentrations of carbenoxolone. Maximum inhibition (~90 %) of fluorescence transfer from the CC to the oocyte was achieved at a dose of 500 μ M carbenoxolone. Thus, approximately 10 % of fluorescence measured within DO using the bioassay was the result of transfer facilitated by a means other than GJ. Indeed, other transfer channels exist in oocytes including aquaporins and calcium channels (Ford et al., 2000; Tosti et al., 2000). Aquaporins are involved in the transfer of water and neutral solutes such as polyols, purines, pyrimidines, and carbamides, and are thought to mediate oocyte volume control (Tsukaguchi et al., 1998; Ford et al., 2000). Calcium channels are thought to play a role in oocyte meiotic resumption (Tosti, 2006).

The optimal parameters for measuring GJ communication in rat COC using the bioassay herein include the addition of 50 μ M dipyridamole and 50 μ M rolipram in media. The inclusion of these PDE inhibitors in media should be used for ovary collections and dissections, throughout all *in vitro* incubation periods and the 1 hour incubation time following calcein-AM exposure to allow for appropriate dye transfer. Furthermore, the final concentration of the solvent DMSO should remain at <1 %.

In summary, we report a successful bioassay for measuring GJ communication in rat COC that utilises a novel combination of PDE inhibitors (dipyridamole and rolipram). This study infers that PDE7, 8, 10 and 11 are active in the rat COC, and may play an important role in the maintenance of meiotic arrest in the oocyte in this species. Moreover this study verifies that GJ communication within the rat COC is a dynamic process that the bioassay described herein can accurately and repeatedly measure.

4 VALIDATING THE USE OF COC PREVIOUSLY UTILISED IN GJ ASSAYS FOR GENE EXPRESSION ANALYSES

4.1 Introduction

Ovarian follicles are activated to grow one at a time such that each one is at a different stage of development at any one time. Even when a large number of pre-ovulatory follicles are produced in sheep through hormonal superovulation protocols, it has been shown that GC isolated from these similarly-sized follicles showed significant differences in their responses to FSH, and hCG (McNatty et al., 2010). Thus, superovulation protocols are unable to overcome the hierarchical growth of ovarian follicles. Moreover, large differences in oocyte quality have also been reported following superovulation in humans (Krisher, 2004; Lasien et al., 2009) confirming that similar-sized follicles are unlikely to be at an identical stage of development, or to respond identically to external stimuli. These observations highlight the importance of assessing both the degree of GJ communication and the expression levels of candidate genes in individual COC. Moreover, the ability to directly correlate the degree of GJ communication and candidate gene expression levels would be invaluable for assessing the regulatory pathway(s) that are involved in CC-oocyte interactions.

In order to directly correlate CC and oocyte gene expression with GJ activity, the aim of this study was to determine whether (1) the fluorescent properties of the dye (calcein) used in the GJ assays interfered with the fluorescence of the TaqMan probes used in the qPCR technique and (2) the exposure of COC to calcein affected mRNA levels of the candidate genes.

4.2 Methods

For all experiments described below, the fluorescence was measured in a Corbett Rotor-Gene 6000 machine following a thermal cycle programme of: 95 °C for 10 minutes; and then 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. The Corbett was set to acquire fluorescence on the green, orange, red, and yellow channels. The excitation and emission wavelengths for these channels (Table 4-1) determine the four fluorescence dyes chosen for the tags on the TaqMan probes, namely FAM, ROX, CY5 and HEX, respectively. Calcein has excitation and emission wavelength of 495 and 515 nm, respectively.

Table 4-1: Excitation and emission wavelengths for the green, orange, red, and yellow channels of the Corbett Rotor Gene 6000

Channel	Excitation (nm)	Emission (nm)
Green (FAM)	470	510
Orange (ROX)	585	610
Red (CY5)	625	660
Yellow (HEX)	530	555

4.2.1 Detection of calcein fluorescence in qPCR reactions

To determine whether the fluorescence emitted by calcein in a diluted solution was detected by the Corbett apparatus, a 1 mM calcein-AM stock was diluted to a concentration of 40 μ M in dH₂O. Fluorescence was subsequently measured using the Corbett under the thermal conditions described above.

To determine whether fluorescence in DO subjected to a GJ assay (i.e. exposed to calcein-AM) was detectable by the Corbett apparatus, COC were extracted and included in a routine GJ assay. In brief, COC were incubated with incubation media supplemented with 50 μ M dipyridamole and 50 μ M rolipram and in the absence (control) or presence (treated) of 2 μ M calcein-AM for 10 minutes. The COC were then transferred into calcein-AM free incubation media containing 50 μ M dipyridamole and 50 μ M rolipram and incubated for a further 4 hours. The CC were removed from the oocyte and fluorescence was measured within each individual oocyte as described previously (see Section 2.1.3). The oocytes were stored at -80 °C until further use. To determine whether fluorescence from the calcein is detected in cDNA of oocytes that were previously subjected to the GJ assay procedure, RNA was extracted from some of the oocytes collected in the previous experiment and cDNA synthesised. Fluorescence was detected using the Corbett under the thermal conditions described previously.

4.2.2 Correlation of Rpl19 C_T value to CC number and oocyte diameter

To test the relationship between CC number and *Rpl19* expression levels, COC were extracted from ovaries into PBS as described previously. The oocytes were stripped of the CC by vigorous pipetting and the oocytes were discarded from the resulting cell suspension. The number of CC/mL was determined by counting the CC under an inverted microscope using a

haemocytometer and Trypan blue stain (Section 2.1.5). The CC were serially diluted in triplicate, and stored at -80 °C prior to extraction of total RNA and synthesis into cDNA. The level of *Rpl19* mRNA was ascertained in each sample in duplicate using TaqMan qPCR.

To test the relationship between oocyte diameter and *Rpl19* expression levels, oocytes with no CC contamination were selected from the experiment in Section 4.2.3. The diameter of each oocyte was measured, and this was compared to the level of *Rpl19* mRNA measured using multiplex TaqMan qPCR.

4.2.3 Effect of calcein/calcein-AM on expression of candidate genes

To test whether exposure to calcein-AM during the GJ assay affects the expression levels of the candidate genes, a GJ assay was performed in which the COC were, or were not, exposed to calcein-AM. The COC were extracted from ovaries as described previously, and incubated either for 0, 4, 12, or 24 hours in incubation media containing 50 µM dipyrindamole and 50 µM rolipram. The COC were further incubated for 10 minutes in the aforementioned incubation media in the absence (control) or presence (treated) of 2 µM calcein-AM. The COC were transferred into fresh incubation media (same as above) but without calcein-AM and incubated for a further 1 hour. The oocyte was denuded of CC, and oocyte fluorescence was measured as described previously. Individual CC masses and DO were collected separately in PBS and stored at -80 °C until further use.

Total RNA was extracted from the thawed CC and oocyte samples, and cDNA was synthesised as previously described (see Section 2.3.1). Multiplex TaqMan qPCR was performed to determine the mRNA levels of the following sets of genes: *Atr*, *Cygs*, *Glut1* and *Rpl19* in DO and CC separately; *Cx43*, *Gfpt1*, *Pfkip* and *Rpl19* in CC; and *Gdf9*, *Bmp15*, *Cx37* and *Rpl19* in DO. The data were analysed using the $2^{-(\Delta\Delta C_T)}$ method (Section 2.3.5; Livak and Schmittgen, 2001). As discussed herein, CC-derived genes were corrected for the reference gene *Rpl19* to give gene expression relative to input, while oocyte-derived genes were corrected to an internal calibrator control to give total gene expression in a single oocyte. The relative linear mRNA levels were analysed for normality using the D'Agostino & Pearson normality test. Data that were not normally-distributed were transformed (natural log, $1/(y+1)$, or square root($y+0.5$) transformations) and reanalysed using the D'Agostino & Pearson normality test to confirm a normal distribution. Normally-distributed data were

analysed by a two-way ANOVA. In cases where data were not normally-distributed after all transformation options were exhausted, the data were ranked and a two-way ANOVA was performed on the ranked values.

4.3 Results

4.3.1 Detection of calcein fluorescence

The fluorescence levels detected in a 40 μ M calcein-AM solution is shown in Figure 4-1. Fluorescence was detected at very high levels on the green channel but was not detected above baseline levels on the orange, red, or yellow channels.

Figure 4-1: Detection of fluorescence in a 40 μ M calcein-AM solution over 40 cycles. The red lines show, in duplicate, the fluorescence measured on each channel in the calcein-AM solution.

The fluorescence levels detected in the lysate of individual oocytes that had been incubated in the absence or presence of calcein-AM is shown in Figure 4-2. Fluorescence was not detected above the baseline levels on any channel in either the control or treated oocytes.

Figure 4-2: Detection of fluorescence in DO that had been incubated with and without calcein over 40 cycles. The blue lines show, in duplicate, a pool of DO that had not been treated with calcein (N=1). The green lines show, in duplicate, the DO that had been treated with calcein (N=8).

The fluorescence levels detected in cDNA of DO that had been incubated in the absence or presence of calcein-AM is shown in Figure 4-3. Fluorescence was not detected above baseline levels on any channel for cDNA from either the control or treated oocytes.

Figure 4-3: Detection of fluorescence in cDNA samples of oocytes that had been incubated with and without calcein over 40 cycles. The purple lines show, in duplicate, cDNA from oocytes that were not treated with calcein (N=3). The blue lines show, in duplicate, cDNA from oocytes that were treated with calcein (N=5).

4.3.2 Correlation of *Rpl19* C_T value to CC number and oocyte diameter

CC were collected, counted, and serially diluted to determine the relationship between CC number and *Rpl19* expression levels as denoted by C_T values. This is depicted in Figure 4-4A. Expression levels of *Rpl19* were highly correlated to the number of CC. The minimum number of cells required to measure *Rpl19* (i.e. the C_T value at the upper sensitivity limit = 30) was 900.

The relationship between oocyte diameter and *Rpl19* expression levels as denoted by C_T values is shown in Figure 4-4B. There was no relationship between oocyte diameter and *Rpl19* expression levels. The average oocyte diameter was 74 μM (range 58–95 μM).

Figure 4-4: Correlation between *Rpl19* C_T value and CC number (A) or oocyte diameter (B). CC were serially diluted and the *Rpl19* levels were measured using TaqMan qPCR. The *Rpl19* mRNA levels in single oocytes were measured using TaqMan qPCR and compared to the diameter of each oocyte.

4.3.3 Effect of calcein on gene expression

The effect of calcein-AM treatment with time on expression of the reference gene *Rp19* in DO and CC masses is shown in Figure 4-5. The average number of CC collected from each COC was 1495 (range 988–2397). Levels of *Rp19* mRNA in the oocyte and CC decreased over time in both the untreated and calcein-AM treated groups. No effect of calcein-AM treatment was measured for *Rp19* mRNA levels in the oocyte or the CC. A treatment x time interaction effect for *Rp19* mRNA levels in CC, but not oocytes, approached significance ($P=0.058$).

Some of the oocyte samples were contaminated with CC due to improper denuding technique. When those oocytes that had >5 CC attached were excluded from the analysis, the effect of time on *Rp19* levels remained ($P<0.05$), but no treatment or treatment x time interaction effects were noted.

For future gene expression studies only the 1 and 25 hour incubation times were used. When the 5 and 13 hour times were excluded from the analysis of *Rp19* levels in the oocyte, the effect of time remained ($P<0.0001$) and no effects due to treatment or a treatment x time interaction were observed. When the 5 and 13 hour times were excluded from the analysis of *Rp19* levels in the CC, a treatment x time interaction effect was measured ($P<0.01$) but no effects of treatment or time were observed.

Figure 4-5: Effect of calcein-AM with time on *Rpl19* expression levels in oocytes (A) and CC (B). The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control (i.e. not exposed to calcein-AM) is represented by the unfilled bars (□), while the calcein-AM treated group is represented by the hatched bars (▨). As the reference gene is present in all gene sets, the mRNA value of *Rpl19* for each oocyte sample was an average from the two oocyte gene quadruplex sets (*Atr/ Cycs/Glut1/Rpl19* and *Gdf9/Bmp1/Cx37/Rpl19*). The mRNA value of *Rpl19* for each CC sample was an average from the two CC gene quadruplex sets (*Atr/ Cycs/Glut1/Rpl19* and *Cx43/ Gfpt1/Pfkp/Rpl19*). Values are shown as means $\pm$ SEM analysed from linearised gene expression values normalised against the mean of the control group at 1 hour, which had a mean of 1. The data represent 6-15 individual samples in each group generated from 3 replicate experiments. A two-way ANOVA was performed on natural log-transformed data.

The effects of calcein-AM treatment on the expression levels of candidate genes of interest for the oocyte (i.e. *Atr*, *Gdf9*, *Bmp15*, and *Cx37*) are shown in Figure 4-6. Oocyte-derived expression levels of *Cycs* and *Glut1* were also tested, but were below the detectable limit. A significant effect of time was observed for *Atr* mRNA levels, with *Atr* levels decreasing over time in both the control and calcein-AM treated oocytes. No effect of calcein-AM treatment was observed for *Atr* mRNA levels. A significant effect of time was observed for *Bmp15* levels

in rat oocytes. Overall *Bmp15* mRNA levels decreased over time. *Bmp15* levels were similar between the control and calcein-AM treated groups at all time points except at 13 hours. At 13 hours, the level of *Bmp15* was reduced in the control oocytes but was unchanged in the calcein-AM treated oocytes. However, no effect of calcein-AM treatment on *Bmp15* levels in rat oocytes was observed. *Cx37* mRNA levels in rat oocytes were significantly affected by both treatment and time. *Cx37* mRNA was reduced in the control oocytes after 13 and 25 hours, however this decrease was not observed in the calcein-AM treated oocytes. Significant time and calcein-AM treatment effects were observed for *Gdf9* mRNA levels in rat oocytes. *Gdf9* mRNA was decreased after 13 and 25 hours in the control group, however this decrease was not observed in the calcein-AM treated group.

For most future gene expression studies, only the 1 and 25 hour incubation times were included. Therefore, the data were reanalysed with the 5 and 13 hour time points excluded. When only the 1 and 25 hour time points were included from the *Atr* analysis, both treatment ($P<0.05$) and time effects ($P<0.0001$) were observed. Expression levels of *Atr* mRNA were decreased at 25 hours, compared to 1 hour, in both the control and calcein-AM treated oocytes. In addition, *Atr* mRNA was lower in the calcein-AM treated oocytes compared to the control oocytes at both time points. No treatment, time, or treatment x time interaction effects were observed for *Bmp15* levels. Time effects ($P<0.01$) were noted for *Cx37* and *Gdf9* levels ($P<0.01$ and 0.05 respectively), but no treatment or treatment x time interaction effects were measured. *Cx37* and *Gdf9* mRNA levels were decreased over time in the control oocytes and, to a lesser extent, in the calcein-AM treated oocytes. These results are summarised in Table 4-2.

Figure 4-6: Effect of calcein-AM with time on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D) in rat oocytes. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control is represented by the unfilled bars (□), while the calcein-AM treated group is represented by the hatched bars (▨). Values are shown as means ± SEM analysed from linearised gene expression values normalised against the mean of the control group at 1 hour, which has a value of 1. The data represent 7-16 readings from individual oocytes in each group generated from 3 replicate experiments. B, C, and D were transformed (1/y+1) and A was not transformed prior to analysis by a two-way ANOVA.

Table 4-2: Summary of calcein-AM treatment, time, and interaction effects on mRNA levels of candidate genes in rat oocytes. Genes are ordered from most abundant to least abundant.

Gene	Abundance relative to <i>Rpl19</i>	All time points			1 and 25 hours only		
		Treatment effect	Time effect	Treatment x Time effect	Treatment effect	Time effect	Treatment x Time effect
<i>Gdf9</i>	7.93 ± 0.36	P<0.05	P<0.01	-	-	P<0.05	-
<i>Cx37</i>	7.80 ± 0.61	P<0.01	P<0.05	-	-	P<0.01	-
<i>Bmp15</i>	1.62 ± 0.08	-	P<0.05	-	-	-	-
<i>Atr</i>	0.35 ± 0.02	-	P<0.05	-	P<0.05	P<0.0001	-

The effects of calcein-AM treatment on the expression levels of candidate genes of interest for the CC (i.e. *Atr*, *Cygs*, *Cx43*, *Gfpt1*, and *Pfkp*) are shown in Figure 4-7. Significant treatment x time interaction and time effects were observed for *Atr* mRNA levels in rat CC. The mean *Atr* mRNA levels were more variable in the calcein-AM group as evidenced from the SEM. In the control group, *Atr* mRNA in CC declined progressively after the 5 hour incubation whereas this decline wasn't evident in the calcein-AM treated CC until the 25 hour time point. A significant effect of time was observed for *Cx43* levels in rat CC. *Cx43* mRNA levels were markedly reduced from 5 hours onwards in both the control and calcein-AM treated CC. No effect of calcein-AM treatment was observed for *Cx43* expression. Significant treatment, time and treatment x time interaction effects were observed for *Cygs* levels in rat CC. *Cygs* mRNA levels had decreased from 5 hours onwards in the control CC, however levels in the calcein-AM treated CC did not decrease until 25 hours. The mean *Cygs* mRNA levels were consistently higher in the calcein-AM treated CC, compared to the control CC, at 5, 13, and 25 hours. Significant treatment, time and treatment x time interaction effects were observed for *Gfpt1* mRNA levels in rat CC. The mean *Gfpt1* levels had declined from 5 hours and remained low thereafter in the control CC, However, in the calcein-AM treated CC, the mean mRNA levels appeared to be different with a decrease noted at 5 hours but not at 13 or 25 hours. A significant effect of time was observed for *Pfkp* mRNA levels in rat CC. *Pfkp* mRNA was markedly decreased from 5 hours onwards in both the control and the calcein-AM treated CC. No effect of calcein-AM treatment on *Pfkp* levels was observed.

A further re-evaluation of these data for CC gene expression was undertaken using only the 1 and 25 hour data sets as it seemed that the effects of calcein-AM had their major interactive effects at the 5 and 13 hour time points. When this restricted data set was analysed for *Atr*, a significant effect of time ($P<0.05$) but no treatment or time x treatment interaction effects were observed. For *Cx43*, *Cygs*, and *Pfkp*, significant effects of time (all genes, $P<0.0001$) but no treatment or time x treatment interaction effects were found. However, for *Gfpt1*, there were significant effects of time ($P<0.0001$), treatment ($P<0.05$) and a treatment x time interaction ($P<0.01$). These results are summarised in Table 4-3.

Figure 4-7: Effect of calcein-AM with time on mRNA levels of *Atr* (A), *Cx43* (B), *Cycs* (C), *Gfpt1* (D), and *Pfkfb3* (E) in rat CC. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control is represented by the unfilled bars (□), while the calcein-AM treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ SEM analysed from linearised gene expression values normalised against the mean of the control group at 1 hour, which has a value of 1. The data represent 9-16 readings from individual oocytes in each group generated from 3 replicate experiments. C was transformed (square root($y+0.5$)) and E was transformed (natural log) prior to analysis by a two-way ANOVA. A, B and D were ranked and a two-way ANOVA was performed on the ranked data.

Table 4-3: Summary of calcein-AM treatment, time, and interaction effects on mRNA levels of candidate genes in rat CC. Genes are ordered from most abundant to least abundant.

Gene	Abundance relative to <i>Rpl19</i>	All time points			1 and 25 hours only		
		Treatment effect	Time effect	Treatment x Time effect	Treatment effect	Time effect	Treatment x Time effect
<i>Cx43</i>	2.231 ± 0.31	-	P<0.0001	-	-	P<0.0001	-
<i>Pfklp</i>	0.178 ± 0.02	-	P<0.0001	-	-	P<0.0001	-
<i>Cyts</i>	0.068 ± 0.004	P<0.0001	P<0.0001	P<0.0001	-	P<0.0001	-
<i>Gfpt1</i>	0.056 ± 0.01	P<0.01	P<0.0001	P<0.01	P<0.05	P<0.0001	P<0.01
<i>Atr</i>	0.047 ± 0.005	-	P<0.01	P<0.05	-	P<0.05	-

4.4 Discussion

Fluorescence was detected on the green channel in the sample of diluted calcein-AM solution, indicating that the thermal cycling caused the removal of the acetoxymethyl group resulting in activation of fluorescence. However, fluorescence was not detected in the lysate of oocytes from COC that had been treated with calcein-AM suggesting that residual calcein contained within the oocytes is at undetectable concentrations. Similarly, no fluorescence was detected in cDNA from oocytes that had been treated with calcein-AM. These results demonstrate that residual calcein fluorescence in oocytes is unlikely to add to or interfere with the fluorescence emitted from any of the TaqMan probes during qPCR reactions.

In this study, the incubation times of 1, 5, 13, and 25 hours differed in the lengths of time COC are incubated before exposure to calcein-AM (i.e. 0, 4, 12, or 24 hours respectively). However, thereafter the time that COC are exposed to calcein-AM (i.e. 10 minutes) and the subsequent calcein-AM free interval (i.e. 1 hour) were identical for all samples. While it might be expected that exposure to calcein-AM would affect gene expression in a similar manner across all time points, it is also possible that since the functions of individual COC after isolation are so variable, they are much more sensitive to calcein-AM effects over the first 13 hours of incubation. As a consequence of this variability, most future gene expression studies after calcein-AM treatment were limited to the 1 and 25 hour time points.

The reference gene (*Rpl19*) used in this study is a component of the ribosome 60S subunit, and is a commonly used reference gene in CC and GC (Dragovic et al., 2006; Feuerstein et al., 2007; Su et al., 2008). Selection of a stable reference gene that is expressed at similar levels to the candidate genes and is not affected by experimental conditions is essential. Two other reference genes commonly used for COC are *Gadph* and *18s* RNA. These were not considered for the current study as *Gapdh* levels are affected by oestrogens (Diel et al., 2000), and therefore would not be suitable when testing compounds with oestrogenic activity such as BPA, while *18s* RNA is very highly expressed and therefore would not provide a good comparison for the candidate genes selected for this study. This current study revealed that *Rpl19* is strongly correlated to CC number ($P < 0.0001$). All of the CC-derived candidate genes were corrected for *Rpl19* to remove potentially large amounts of variability in CC-derived candidate gene expression as a result of differences in CC number. The exposure of COC to the fluorescent dye calcein-AM used in the GJ assay did not directly affect expression of *Rpl19* in rat CC, when all incubation times were taken into account. While, *Rpl19* levels declined with time this was likely due to reduced CC number as COC diameter was significantly reduced at 13 and 25 hours, compared to at 1 hour ($P < 0.01$; Kruskal-Wallis test). Therefore, *Rpl19* is a suitable reference gene for rat CC.

Interestingly, *Rpl19* gene expression levels were not correlated to oocyte size. None of the oocyte genes were corrected for *Rpl19*, but were instead corrected for a calibrator control sample. Thus for the oocyte genes, mRNA levels were calculated as expression levels in each single DO. Although oocyte-derived candidate genes were not corrected for a reference gene, *Rpl19* was measured in all future DO samples, as levels below the detectable level suggested that the RNA extraction was unsuccessful. DO samples that had an *Rpl19* C_T value > 30 were excluded for this reason.

The exposure of COC to the fluorescent dye calcein-AM used in the GJ assay did not directly affect expression of *Rpl19* in rat CC or DO, when all incubation times were taken into account. Thus, we conclude that treatment with calcein-AM does not inhibit or interfere with the RNA extraction and cDNA synthesis procedures.

When analysing only the 1 and 25 hour time points, there was no effect of calcein-AM treatment on levels of *Rpl19* mRNA in the oocyte. However, a treatment x time interaction

was noted for *Rpl19* levels in CC. These levels were increased in the calcein-AM treated CC at 1 hour, but were at similar levels to the control CC at 25 hours. It is unlikely that this difference in *Rpl19* was a direct result of calcein-AM exposure but rather was due to a difference in the number of CC collected. Indeed, when reviewing the pictures that were taken of each individual COC, those that were collected in the calcein-AM treated group at 1 hour had noticeably more CC than those in the untreated group.

When all of the time points were included in the analyses of the effects of calcein-AM on oocyte-derived genes, a treatment effect was observed for two genes (*Cx37* and *Gdf9*). Interestingly, *Cx37* and *Gdf9* were the most abundant genes measured in the oocyte. When only the 1 and 25 hour time points were included in the analysis, no treatment effects on *Cx37* and *Gdf9* were observed. In fact, including the 1, 5, and 25 hour time points (i.e. excluding the 13 hour time point) also removes the treatment effect. These results indicate that the difference in the levels of these genes between the calcein-AM treated and control groups at 13 hours contribute disproportionately to the treatment effect observed when all the time points were analysed. After 13 hours of incubation, the expression of *Cx37* and *Gdf9* may be particularly sensitive to perturbations. In the control oocytes, both *Cx37* and *Gdf9* were reduced at 13 hours however, calcein-AM may influence this reduction in gene expression.

When only the 1 and 25 hour time points were included in the analyses of the effects of calcein-AM on oocyte-derived genes, a treatment effect was observed for *Atr*. The gene *Atr* was the least abundant of all the oocyte genes measured in this study. Oocyte-derived *Atr* was reduced in the calcein-AM treated group compared to the control group. The effect of calcein-AM treatment on oocyte *Atr* levels was the same at both time points. In addition, the expression levels of *Atr* were closely related to the levels of *Rpl19*, especially at 1 hour, indicating this effect may have been due to differences in RNA input. The effect of calcein-AM on *Atr* mRNA levels in the oocyte may be related to the function of ATR as a DNA damage sensor and regulator of meiosis. The down-regulation of *Atr* levels in calcein-AM treated oocytes indicate that calcein-AM may protect against DNA damage, resulting in a reduced requirement for ATR, or that calcein-AM prevents the oocyte from responding to DNA damage by reducing *Atr* expression. Either way, this effect of calcein-AM may mask any potential effects of lifestyle factors on *Atr* levels and the DNA damage pathway. It is possible

that calcein-AM exposure simply reduces the baseline level of *Atr* in oocytes, however any interpretations of *Atr* levels will be treated with caution in future experiments.

When all of the time points were included in the analyses of the effects of calcein-AM on CC-derived genes, a treatment effect was observed for two CC-derived genes (*Cycs* and *Gfpt1*) while a treatment x time interaction was noted for three of the genes (*Atr*, *Cycs* and *Gfpt1*). The genes *Atr*, *Cycs* and *Gfpt1* were the least abundant of all the genes measured in the CC. When only the 1 and 25 hour time points were analysed, the treatment effect was not observed for *Cycs* and no treatment x time interaction effects were noted for *Atr* or *Cycs*. The *Atr* mRNA levels reduced over time, and this reduction began after 13 hours. However, calcein-AM exposure appeared to delay the reduction in *Atr* expression, resulting in a large difference in levels between the calcein-AM treated and control groups at 13 hours. The *Cycs* and *Gfpt1* levels were also reduced over time in the control CC from 5 hours onwards. However, calcein-AM treatment appeared to delay the reduction in *Cycs* levels until 25 hours. This resulted in large differences in the levels of *Cycs* mRNA between the calcein-AM treated and control CC at 5 and 13 hours. The *Gfpt1* levels in the calcein-AM treated CC were similar to the control at 5 hours, but were significantly upregulated at 13 and 25 hours.

When only the 1 and 25 hour time points were included in the analyses of calcein-AM on CC gene expression, treatment and treatment x time interaction effects were only observed for one gene (*Gfpt1*). CC-derived *Gfpt1* was increased in the calcein-AM treated group at 25 hours, but not at 1 hour. This treatment x time interaction effect is of particular concern, as it indicates that the effect of calcein-AM on *Gfpt1* expression is inconsistent over time. These results suggest that flux through the hexosamine biosynthetic pathway is upregulated at 25 hours in calcein-AM treated CC. Thus, the interpretation of CC-derived *Gfpt1* expression levels over time in COC subjected to the GJ assay will be treated with caution.

The ability to compare directly the degree of the GJ communication (as determined by the GJ assay) and the expression levels of genes thought to be involved in maintenance of oocyte health is a very powerful tool. This study concludes that despite a few small and transitory differences in expression levels of a couple of genes following the exposure of COC to calcein-AM, it would be acceptable to measure gene expression levels by multiplex qPCR in CC and oocytes from COC that were subjected to the GJ assay. However, when gene

expression is measured at 1, 5, 13, and 25 hours, caution must be taken when interpreting the changes in mRNA levels of oocyte-derived *Cx37* and *Gdf9* and CC-derived *Atr*, *Cycs*, and *Gfpt1* due to the effects of calcein-AM exposure on expression of these genes. In addition, when gene expression is measured at 1 and 25 hours, the levels of oocyte-derived *Atr* and CC-derived *Gfpt1* must also be interpreted carefully.

5 EFFECT OF TIME IN CULTURE ON COC HEALTH

5.1 Introduction

Removal of the COC from the follicle, and subsequent *in vitro* culture can have a significant impact on COC health. As described previously, aspiration of the COC from the follicular environment results in the progression of meiosis within the oocyte (Wert and Larsen, 1989). Additionally, the communication network comprised of GJs appears to undergo dynamic changes following the removal of COC from the follicle. For example, GJ communication in bovine COC decreased progressively over 21 hours *in vitro* (Thomas et al., 2004). However, in porcine COC, GJ communication increased over the first 18 hours of culture, before declining at 24 hours and then remaining unchanged for 24 hours thereafter (Sasseville et al., 2009a). Thus, species differences exist in the impact of removing the COC from its follicular environment.

Alterations in GJ communication within the COC would be expected to be associated with molecular and metabolic changes in both the CC and oocyte. Expression of *Gdf9* and *Bmp15* mRNA transcripts were reduced in rat DO that had been co-cultured with GC for 24 hours (Lin et al., 2012). Utilization of radioactively labelled glucose ([5-³H] glucose) was observed to increase during IVM of bovine COC over 24 hours (Steeves and Gardner, 1999). Moreover, bovine CC that were isolated from COC that underwent IVM had a two-fold increase in cell death, along with increased expression levels of genes involved in apoptosis, compared with COC that were matured *in vivo*. Furthermore, COC that underwent IVM compared with those matured *in vivo* had augmented activation of the TGF β and MAPK signalling pathways and increased mRNA and protein levels of hyaluronan synthase 2, which is responsible for the synthesis of hyaluronic acid for CC expansion (Salhab et al., 2012). Therefore, while there is some evidence that the removal of the COC from the follicle and its subsequent incubation *in vitro* can have marked changes in cell-to-cell communication, gene expression levels and metabolic activity in COC of some species, there is little information regarding these changes in the rat.

The aim of this chapter was to determine the impact of incubation time on the health of rat COC in this study. Specifically, the changes in GJ communication, candidate gene expression (oocyte-derived *Atr*, *Bmp15*, *Cx37*, and *Gdf9* and CC-derived *Atr*, *Cx43*, *Cyts*, *Gfpt1*, and *Pfkp*)

and protein expression levels (oocyte-derived GDF9 and CC-derived CX43) in COC that had been incubated for varying amounts of time were investigated.

5.2 Methods

5.2.1 Effect of time on GJ communication

Ovaries were extracted into dissection media supplemented with 50 μ M dipyridamole and 50 μ M rolipram, and the COC were recovered and transferred into a 48-well plate as described previously (section 2.1.1 and 2.1.2). The COC were washed twice in wash media containing 50 μ M dipyridamole and 50 μ M rolipram. The COC were then incubated for 0, 4, 12, or 24 hours in incubation media containing 50 μ M dipyridamole and 50 μ M rolipram. The COC were exposed to 2 μ M calcein-AM for 10 minutes before being incubated for a further hour in fresh incubation media (calcein-AM free) containing 50 μ M dipyridamole and 50 μ M rolipram. The COC were transferred into incubation media free of PDE inhibitors where the oocyte was denuded and then fluorescence was measured. The oocytes and separate CC masses were then stored at -80 °C until further processing for RNA extraction. The oocyte fluorescence for each individual sample was corrected for the geometric mean of that at 1 hour. The relative fluorescent levels were analysed by a Kruskal-Wallis test, and where a significant difference was observed, a Dunn's multiple comparison post hoc test was performed.

5.2.2 Effect of time on mRNA levels

Total RNA was extracted from the DO and CC samples from the GJ bioassay and synthesised into cDNA as previously described (Section 2.3.1). Three quadriplex qPCR reactions were used to measure expression of candidate gene in CC and/or DO. The candidate genes were as follows: CC expression of *Cx43*, *Gfpt1* and *Pfkip*; oocyte expression of *Bmp15*, *Cx37* and *Gdf9*; and both oocyte and CC expression of *Atr*, *Cycs* and *Glut1*. The reference gene *Rpl19* was included in each of qPCR multiplexes. The expression of each gene in each sample was measured in duplicate. Relative levels of mRNA were calculated as described previously (Section 2.3.5). Expression levels of CC genes were corrected for *Rpl19*, enabling candidate gene expression to be standardized relative to CC number. Expression levels of oocyte genes were corrected for an internal calibrator control, giving total candidate gene expression per

oocyte. These data were analysed by a Kruskal-Wallis test. Where significant differences were measured, a Dunn's multiple comparison post-hoc test was performed.

5.2.3 Effect of time on protein expression

Ovaries were extracted into dissection media supplemented with 50 μ M dipyridamole and 50 μ M rolipram, and the COC were recovered and transferred into a 48-well plate as described previously. The COC were transferred into incubation media containing 50 μ M dipyridamole and 50 μ M rolipram and incubated for 1, 5, 13 or 25 hours. These incubation times were chosen to reflect the total incubation period during the GJ assay (i.e. 4 hours incubation plus 1 hour post-calcein incubation). The COC were washed twice in PBS and were collected into a microcentrifuge tube (100 COC/tube). The COC were pelleted by centrifugation and were resuspended in 15 μ L PBS containing proteinase inhibitors. A total of 200 COC were collected per group, comprising of pooled samples from two collection days.

A 10 μ L aliquot (the equivalent of ~67 COC) of each pool of COC was taken and analysed for protein concentration using the BCA assay as previously described (Section 2.4.1). Briefly, BCA reagent was added to each sample in a 96-well plate, the plate was incubated at 60 °C for 2 hours, and the absorbance at 490 nm was measured. The total protein content was calculated by comparing against the 490 nm absorbance values of a bovine serum albumin standard curve. The remaining 20 μ L aliquot (the equivalent of ~133 COC) was analysed for the expression of CX43 and GDF9 proteins using Western blotting as described in Section 2.4. To determine band specificity, the blot was also incubated with either the CX43 or GDF9 specific antibodies pre-absorbed with the appropriate control peptide or recombinant protein.

Band densities were analysed using the ImageJ software. For the CX43 blots, the density of the 43 kDa band was measured. For the GDF9 blots, the densities of the 49 kDa promature and, when present, the 20 kDa mature bands were measured. When multiple bands were unresolved (e.g. when doublet or triplet bands were present for CX43) the density of all bands was measured. The band densities for CX43 protein were corrected for total protein amount, as determined by the BCA assay. The band densities for GDF9 protein were uncorrected to give total GDF9 protein expressed by a known number of oocytes.

5.3 Results

5.3.1 Effect of time on GJ communication

The effect of increasing incubation time on GJ communication in rat COC is depicted in Figure 5-1. Oocyte fluorescence was increased at 5, 19, or 25 hours, compared to that at 1 hour. The proportion of oocytes with a visible GV progressively and significantly decreased over time, with all oocytes containing a GV at 1 hour, but only 44 % of oocytes containing a GV after 25 hours (Table 5-1).

Figure 5-1: Effect of incubation time on fluorescent dye transfer from adjacent CC to oocytes. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the 1 control. The data represent 16-17 readings from individual oocytes at each time generated from 3 replicate experiments. The data were analysed using the Kruskal-Wallis test, and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. Groups that do not share a letter are significantly different ($P < 0.05$). The mean $\pm$ SEM fluorescent reading at 1 hour was 357 ± 28 .

Table 5-1: Effect of selected incubation times on the proportion of oocytes with a visible GV. The table shows the proportion (%) of oocytes with a visible GV, with the actual number of oocytes with a GV in brackets. The data from 3 replicate experiments ($N=16-17$) was analysed by a Fisher's exact test. Groups that do not share a letter are significantly different from one another ($P < 0.05$).

Time (hours)	% GV oocytes
1	100 ^a (16)
5	88 ^{ab} (14)
13	56 ^{bc} (9)
25	44 ^c (7)

5.3.2 Effect of time on mRNA levels in rat oocytes and CC

The effect of increasing incubation time on mRNA levels in cultured rat oocytes is depicted in Figure 5-2. It appeared that levels of *Atr* mRNA in oocytes declined progressively after 1 hour, and were significantly lower ($P<0.05$) at 25 hours. Conversely, the expression levels of *Bmp15*, *Cx37*, and *Gdf9* mRNA in oocytes did not change over time. Oocyte-derived *Rpl19* mRNA levels remained unchanged at 5 hours, compared to 1 hour, but appeared to decline at 13 and 25 hours, however this was not significant. Oocyte-derived *Cycs* and *Glut1* mRNA levels were below the detection limit.

The effect of increasing incubation time on mRNA levels in cultured rat CC is depicted in Figure 5-3. The levels of *Atr* mRNA in CC had increased by 13 hours, compared to 1 hour ($P<0.05$), in culture but this effect had dissipated by 25 hours. The levels of *Cx43* mRNA in CC were clearly reduced ($P<0.0001$) at 5 and 13 hours, compared to that at 1 hour, and remained low after 25 hours. Two samples at 25 hours contained relative *Cx43* levels of 1.29 and 1.19 (compared to relative levels of 0.013-0.678 in the other CC samples) and these samples masked the clear reduction in *Cx43* mRNA levels at this time point during the statistical analysis. However, these samples were not excluded from the analysis, as the *Rpl19* C_T values were well within the sensitive range. The levels of *Cycs* mRNA remained constant until 25 hours, at which time they had significantly declined ($P<0.05$). The levels of *Gfpt1* mRNA in CC increased between 5 and 13 hours ($P<0.01$), but this effect was attenuated by 25 hours. The levels of *Pfkp* mRNA were markedly reduced ($P<0.01$) at all time points, compared to 1 hour. Similar to that observed in the oocyte, *Rpl19* mRNA levels declined with time, with a significant difference at 13 and 25 hours compared to 1 hour ($P<0.05$ for both time points). CC-derived *Glut1* mRNA levels were below the detection limit.

Figure 5-2: Effect of incubation time on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D), and *Rpl19* (E) in rat oocytes. Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of that at 1 hour. The data represent 7-16 readings from individual oocytes in each group generated from 3 replicate experiments. The data were analysed using the Kruskal-Wallis test, and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. For *Atr* levels groups that do not share a letter are significantly different ($P < 0.05$). No significant effects of time on *Bmp15*, *Cx37*, *Gdf9*, or *Rpl19* levels were noted.

Figure 5-3: Effect of incubation time on mRNA levels of *Atr* (A), *Cx43* (B), *Cyts* (C), *Gfpt1* (D), *Pfkf* (E), and *Rpl19* (F) in rat CC masses. Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of that at 1 hour. The data represent 7-16 readings from individual CC masses in each group generated from 3 replicate experiments. The data were analysed using the Kruskal-Wallis test, and where significant differences were observed, a Dunn's multiple comparison post-hoc test was performed. In all graphs, groups that do not share a letter are significantly different ($P < 0.05$).

The relationship between oocyte fluorescence and expression levels of *Cx43* and *Cx37* mRNA, as denoted by C_T values, in each individual sample is shown in Figure 5-4. There was a very weak relationship between oocyte fluorescence and *Cx43* expression levels. Oocytes with higher fluorescence readings tended to be associated with CC with lower *Cx43* mRNA levels (high C_T value). No relationship was observed between oocyte fluorescence and expression levels of *Cx37* mRNA (denoted by C_T values).

Figure 5-4: Correlation between oocyte fluorescence values and C_T values for *Cx43* (A) and *Cx37* (B).

5.3.3 *Effect of time on protein levels*

The effect of increasing incubation time on CX43 protein expression in rat COC in culture is depicted in Figure 5-5. Multiple bands were detected in the positive control of both replicates, while no bands were observed in the negative control. A band corresponding to the size of CX43 protein (i.e. 43 kDa) was detected in the positive control of the first replicate (Figure 5-5A), along with unidentified bands around 65 and 22 kDa in size. In the second replicate, a 43 kDa band was not detected in the positive control (Figure 5-5B). In contrast to the COC lysates from the second replicate where only a 43 kDa band was observed, an additional strong unidentified 22 kDa band was observed in the COC lysate from the first replicate. Following stripping of the blot and re-probing with the CX43 antibody that had been pre-absorbed with the control peptide, the bands that had previously been visualised were not apparent (Figure 5-5C).

In comparison with that in COC after 1 hour in culture, the corrected CX43 protein levels appeared to be slightly reduced at 5 hours (Figure 5-6), markedly increased at 13 hours (~3 fold higher) and slightly increased at 25 hours (1-2.5 fold higher). There was a large variability in CX43 protein levels between replicates at 25 hours.

Figure 5-5: The *in vitro* effect of incubation time on CX43 protein levels in rat COC. Western blots were performed in duplicate (A and B) to determine the level of CX43 protein in COC lysate. COC were either incubated for 1, 5, 13, or 25 hours. Rat ovary lysate (A) or rat aorta lysate (B) were included as positive controls (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also run where the membrane (B) was stripped and re-probed with the CX43-specific antibody that had been pre-absorbed with its control peptide. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μg) in each sample was determined using the BCA assay. The relative band densities were corrected for total protein amount, as calculated by the BCA assay.

Figure 5-6: Effect of incubation time on relative CX43 levels in rat COC. The bars depict the mean relative CX43 band densities corrected for protein amount at each time point, with each individual band density represented by the dashes.

The effect of increasing the incubation time of rat COC on GDF9 protein levels is depicted in Figure 5-7. Multiple bands between 20 and 114 kDa were detected in the positive control (Figure 5-7A and C) and the COC lysates (Figure 5-7A, B & C) of both replicates. These included bands of 49 and 20 kDa in size, corresponding to the promature and mature forms of GDF9, respectively. Additional prominent bands of 93, 70, and 40 kDa were also observed. To better visualise the mature 20 kDa band in the COC lysate, the first replicate was subjected to an increased exposure time (Figure 5-7B) and revealed the absence of this band at 5 hours in culture. No bands were observed in the negative control (Figure 5-7A & C). Following stripping of the blot and re-probing with the GDF9 antibody that had been pre-absorbed with recombinant GDF9, the bands that had been visualised were not apparent, with the exception of one band that was markedly reduced in intensity (Figure 5-7D).

Overall, the relative levels of the promature and mature GDF9 proteins did not change over time (Figure 5-8A and B, respectively). There was large variation in the relative levels of both forms of GDF9 at all time points, and especially at 25 hours (i.e. the promature form ranged from 0.8-2 fold; the mature form ranged from 0.5-3.4 fold).

Figure 5-7: The *in vitro* effects of incubation time on GDF9 protein levels in rat COC. Western blots were performed in duplicate (A and C) to determine the level of GDF9 protein in COC lysate. COC were either incubated for 1, 5, 13, or 25 hours. Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A longer exposure time was performed for blot (A) to better visualise the mature band (B). A control experiment (D) was also run where the membrane (B) was stripped and re-probed with the GDF9-specific antibody that had been pre-absorbed with *E. coli* produced recombinant ovine GDF9. Molecular sizes (kDa) are indicated to the left of each blot. The densities of the bands of interest (indicated by arrowheads) were measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay.

Figure 5-8: Effect of incubation time on promature (A) and mature (B) GDF9 protein levels in rat COC. The bars depict the mean relative GDF9 band densities at each time point, with each individual band density represented by the dashes.

5.4 Discussion

The purpose of this chapter was to determine the effects of incubating rat COC *in vitro* over an extended time period on GJ communication and the expression of oocyte- and CC-derived candidate genes and proteins. The candidate genes and proteins were selected due to their key roles in COC health. These results demonstrate that a number of cellular processes were altered over time when rat COC were cultured *in vitro*.

GJ communication in COC showed a biphasic pattern over time in culture where levels increased from 2-5 hours, declined to low levels between 9-13 hours, and then increased again from 19-25 hours. This pattern of GJ activity is markedly different to that seen in

cultured porcine and bovine COC (Thomas et al., 2004; Sasseville et al., 2009b). Interestingly in the current study, the pattern of GJ communication over time was not strongly correlated with gene or protein expression levels of the connexins. This indicates that the regulation of GJ communication in rat COC is more complex than connexin expression levels alone, and may be further regulated by post-translational modifications. In contrast to the current study, GJ communication increased for up to 18 hours in culture in porcine COC, before declining after 24 hours. Moreover, an elegant study by Sasseville et al. (2009b) showed that mechanical rupture of a porcine COC from the follicle results in a transcriptional-dependent upregulation of CX43 protein after 4 hours in culture, which was concomitant with an increase in GJ communication. However, the following decrease in GJ communication by 22 hours in culture in the Sasseville et al. study was not associated to CX43 protein levels, but to GVBD and compartmentalisation of CX43 into lipid rafts in a gonadotrophin-dependent manner. Taking into account that the timings may be different, it appears this pattern of events does not occur in the rat. The increase in GJ communication in rat COC observed after 5 hours in culture in the current study was concomitant with a marked decline in both mRNA and proteins levels of CX43, and no change in Cx37 mRNA levels. Furthermore, the decrease in GJ communication in rat COC after 13 hours in culture was associated with a large increase in CX43 protein levels. However it is not known whether this decline in GJ communication was associated with post-translational or cellular localisation changes of CX43 within the COC. One similarity between this study and that of Sasseville et al. was that this decline in GJ communication after 13 hours was associated with an increased proportion of oocytes undergoing GVBD. However in the current study, GJ communication increased thereafter despite the increased proportion of oocytes resuming meiosis.

Interestingly, after 25 hours in our culture system, ~50 % of rat oocytes still contained a visible GV indicating that half of the oocytes were able to maintain meiotic arrest for longer than 25 hours. There was no apparent correlation with time between the level of GJ communication and the proportion of oocytes that had resumed meiosis. It is well established that oocytes require a connection with the CC to maintain meiotic arrest (Sela-Abramovich, 2006; Norris et al., 2009; Conti et al., 2012). Whilst the exact molecule supplied by the CC for this purpose is currently being debated, it is clear that the critical end-point is high intra-oocyte cAMP levels. In the present study, PDE inhibitors were added to maintain

GJ communication and oocyte meiotic arrest over a long incubation period. This was likely to have decreased the need for the transfer of the meiotic maintenance molecule through the GJs, thus confounding any correlation between GJ communication and nuclear maturation that exists in the absence of PDE inhibitors.

The amount of RNA from the individual oocytes, as indicated by *Rpl19* mRNA levels, was similar across all time points. Therefore, despite not correcting oocyte-derived candidate gene expression levels against *Rpl19* mRNA levels, differences in gene levels are likely to be due to changes in expression, rather than differences in RNA input. The level of *Atr* mRNA in the oocyte progressively decreased with the lowest levels measured after 25 hours in culture. The reduced level of *Atr* expression indicates that the DNA damage checkpoint is less active after 25 hours incubation. Both *Atr* mRNA levels and the proportion of oocytes with a GV decreased over time which is consistent with the role of *Atr* in the regulation of meiosis. ATR detects DNA damage in the oocyte at the pachytene stage of meiotic prophase (Garcia-Cruz et al., 2009). Activated ATR phosphorylates downstream targets, including CHK1, BRCA1, and NBS1, resulting in G₂/M arrest (Abraham, 2001). However, the level of *Atr* mRNA was not significantly associated with the presence or absence of a GV (data not shown). The mRNA levels of *Bmp15*, *Cx37*, and *Gdf9* remained steady over time. Previously (Chapter 4), it was found that treatment with calcein-AM prevented a time-dependent decrease in *Cx37* and *Gdf9* levels. Thus, the finding that *Cx37* and *Gdf9* did not change over time in this study may be attributable to the treatment of all samples with calcein-AM. Interestingly, levels of the promature GDF9 protein also remained steady over time, correlating with *Gdf9* mRNA levels. Taken together, these results suggest that the oocyte health is generally unaffected by a 25 hour *in vitro* incubation period. However, a reduction in the activity of the DNA damage sensing pathway may occur at 13 hours.

The mRNA levels of all of the CC genes tested showed a change over time. The *Rpl19* mRNA levels were reduced at the 13 and 25 hour time points, suggesting that fewer CC were attached to the oocyte at later times. Since each candidate gene was corrected for *Rpl19*, this discrepancy in the number of CC collected at each time point was taken into account. The *Atr* mRNA levels in CC were increased at 13 hours, compared to 1 hour, indicating that the DNA damage checkpoint is more active at 13 hours, and may correspond to increased DNA damage in those CC that are still attached to the oocyte. In pigs, DNA damage in the

oocyte as a result of oxidative stress generated by the xanthine oxidoreductase (XOD) enzyme system was reduced when the oocytes were cultured as COC, rather than as DO (Tatemoto et al., 2000). Given the finding that oocyte *Atr* levels were relatively constant until 13 hours in culture, and then reduced at 25 hours, this supports the hypothesis that the CC protect the oocyte from DNA damage. As discussed previously, *Cx43* mRNA levels were reduced in CC from 5 hours onwards but this change did not correlate with GJ communication in the COC. The levels of *Pfkp* mRNA in CC were reduced after 5 hours, suggesting a significant reduction in glycolytic activity. Interestingly, mRNA levels of *Cycs* were also reduced in the CC although this did not occur until 25 hours. Levels of *Gfpt1* mRNA were upregulated in the CC at 13 hours, compared to 5 hours. Given the concurrent reduction in *Cycs* and *Pfkp* expression, these results suggest that glucose is directed to the hexosamine pathway at 13 hours, rather than the glycolytic pathway. The hexosamine biosynthetic pathway is involved in the production of substrates for *O*-linked glycosylation and in the synthesis of hyaluronic acid for CC expansion, while the glycolytic pathway is involved in energy production. Therefore, the results presented here suggest that CC energy production is reduced at 13 hours and that glucose is instead used for *O*-linked glycosylation and CC expansion, although CC expansion was not observed at any time point in this study (data not shown). Previously (Chapter 4), it was established that calcein-AM treatment prevented a time-dependent decrease in *Cycs* levels. It was also found that calcein-AM treatment resulted in increased *Gfpt1* levels at 13 and 25 hours, and an increase in *Atr* mRNA at 13 hours. Therefore, the time dependent changes in *Atr*, *Cycs*, and *Gfpt1* mRNA levels may be influenced by calcein-AM. However, the results presented here strongly support the notion that glucose metabolism is altered over time under *in vitro* conditions.

Western blotting was used to analyse changes in expression levels of CX43 and GDF9 in rat COC over time in culture. The Western blot protocol yielded variable results probably due to the limited number of replicate samples (N=2) able to be collected (i.e. each replicate sample consisted of 200 COC). Therefore, the results presented here are preliminary and further replicates need to be carried out in the future. Nevertheless, multiple bands were detected using the CX43-specific antibody, including a 43 kDa band assumed to be CX43, and two unidentified bands around 63 and 22 kDa in size. Interestingly, the 43 kDa band and both of the unidentified bands were absent after the blot had been stripped and the CX43

antibody added had been pre-absorbed with the control peptide. However, pre-absorption of the antibody with an antigen proves that the antibody binds to that antigen but does not prove the specificity of single bands (Swaab et al., 1977; Willingham, 1999). Thus, it is possible that the 63 and 22 kDa bands were visualised as a result of nonspecific binding by the CX43 antibody. These bands may also represent differentially processed forms of CX43 protein however this has not been previously reported to the best of my knowledge. A 43 kDa band was notably absent in the rat aorta lysate positive control of the second replicate experiment. The phosphorylation pattern of CX43 is tissue specific (Kadle et al., 1991) thus CX43 from rat aorta may have a different migration pattern to that from the CC.

Multiple bands were also detected using the GDF9-specific antibody, including a 49 kDa band assumed to be the promature protein, a 20 kDa band assumed to be the mature protein (Lin et al., 2012), and unidentified bands of 90, 70, and 40 kDa sizes. All of these bands were absent following stripping of the blot and re-probing with the GDF9-specific antibody that had been pre-absorbed with a recombinant ovine GDF9 protein. It is likely that some of these bands were differentially processed forms of GDF9. Indeed, a preproprotein also exists within the oocyte and is a non-secreted form. Furthermore, GDF9 contains five *N*-linked glycosylation sites (Jaatinen et al., 1999) and is a target for phosphorylation (McMahon et al., 2008).

Analysis of the band densities was undertaken using the ImageJ software. The 43 kDa band was analysed exclusively for the CX43 Western blots. For the GDF9 blots, the 49 kDa and 20 kDa bands were analysed separately. The total protein content was measured in each sample using the BCA assay. The band densities for CX43 were corrected for total protein amount to reduce the variation caused by differences in CC number. The band densities for GDF9 were not corrected for total protein, to provide an indication of the total amount of protein produced by a known number of oocytes.

This study revealed that expression of CX43 protein in COC was increased at 13 hours in culture, compared to 1 and 5 hours, and the levels of CX43 at 25 hours were variable. As discussed previously, CX43 protein levels were not correlated to the pattern of GJ communication over time. This may be due to various GJ regulatory mechanisms, such as

voltage gating, phosphorylation, and incorporation into GJ plaques, playing a major role in up- or down-regulating GJ communication.

Overall levels of promature GDF9 protein did not change over time. However, relatively large variability was observed in the levels of promature GDF9 measured in cultured COC at 5, 13, and 25 hours. The promature band was much denser than the mature band, indicating that the promature form is much more abundant in the cell than the mature form. A previous study found that the mature form of GDF9 was the predominant form detected in rat oocytes, and that promature protein was undetectable (Lin *et al.*, 2012). However, there were significant experimental differences between the Lin *et al.* (2012) study and the experiments presented here. Most notably, the Lin *et al.* study measured GDF9 expression following 24 hours of culturing DO, as opposed to COC in this present study. Therefore, the presence of intact CC layers around the oocyte may alter the processing of uncleaved promature GDF9. The band representing the mature GDF9 protein was faint and highly variable in density between replicates. Although the mature form of the protein is the bioactive region, immunization against the proregion of GDF9 in mice resulted in ovaries with more corpora lutea and smaller litter sizes. This indicates that the GDF9 proregion may also play an important role in regulating fertility (McIntosh *et al.*, 2012). While this influence is likely to be through interactions between the cleaved proregion and the mature region (McIntosh *et al.*, 2008), this may also be as a result of undetermined biological activity of the uncleaved promature protein.

In summary, these results demonstrate that the communication pathways between the oocyte and CC remain active in rat COC throughout 49 hours of *in vitro* culture. In contrast to other species, levels of connexin mRNA (i.e. *Cx43* and *Cx37*) or CX43 protein were not good indicators of functional GJ communication in rat COC. The levels of oocyte-derived mRNAs (with the exception of *Atr*) and GDF9 protein remained stable for up to 25 hours in culture. In the CC, expression levels of genes involved in the glycolytic pathway (i.e. *Pfkfb* and *Cycs*) were reduced after 25 hours. However, *Gfpt1* expression was similar at 25 hours compared to that at 1 hour. These results indicate that culturing rat COC *in vitro* may favour the hexosamine biosynthetic pathway, rather than the glycolytic pathway, for glucose utilisation. Moreover, these results validate the use of the GJ bioassay, qPCR and Western blotting

methodologies to detect changes in key regulatory pathways that are involved in maintaining COC health.

6 EFFECTS OF *IN VITRO* EXPOSURE OF COC TO LIFESTYLE FACTORS ON CC-OOCYTE GJ COMMUNICATION

6.1 Introduction

Maintenance of GJ communication within the COC is essential for oocyte development and maturation. The function of GJ are regulated at multiple levels including transcription, translation, post-translational modifications, incorporation into GJ plaques, voltage gating, and other slow gating processes (Bukauskas et al., 2000; Harris, 2002; Goodenough and Paul, 2009). Disruptions to any of these mechanisms have the potential to modify GJ communication. Moreover, suppression of GJ communication may result in the oocyte not receiving adequate nutrients for development and/or maturation (Kidder and Mhawji, 2002). In this context, blocking GJs between the CC and oocyte, using carbenoxolone, inhibited the transfer of a fluorescent glucose analogue (6-NBDG) and cAMP (Webb et al., 2002; Wang et al., 2012). Moreover, blocking GJs using octanol and heptanol resulted in a decreased proportion of bovine oocytes reaching the blastocyst stage of development (Atef et al., 2005). On the other hand, increasing GJ communication is likely to enhance the transfer of nutrients to the oocyte. This may be advantageous, due to more substrates entering the oocyte for energy production and maturation processes. Conversely, excessive GJ communication may also have a negative impact on oocyte quality by transferring more nutrients than necessary for oocyte growth, or may be indicative of a stressed oocyte requiring an increased need for energy-consuming RNA surveillance mechanisms (Baumann et al., 2007).

The closure of GJs between the oocyte and CC is necessary for normal nuclear maturation (Shimada et al., 2001; Norris et al., 2008). For example, suppression of inhibitory phosphatidylinositol 3 kinase mediated phosphorylation of CX43 in porcine COC resulted in a decreased proportion of MII oocytes following *in vitro* culture (Shimada and Terada, 2001). The timing of meiotic resumption is critical. Blastocyst formation was reduced in bovine oocytes pre-incubated with cyclohexamide (meiosis inhibitor) for 24 or 48 hours prior to IVM and fertilised under inhibitor-free conditions, compared to oocytes not exposed to meiotic inhibitors (Saeki et al., 1997). The effects of increased GJ communication on nutrient transfer to the oocyte have not been examined to the best of my knowledge. However, it is reasonable to assume that increased GJ communication results in more glucose and glucose

metabolites being transferred to the oocyte, and indeed, this occurs under hyperglycaemic conditions (Wang et al., 2012). Hyperglycaemic conditions result in increased O-linked glycosylation (Brownlee, 2001; Schelbach et al., 2010), increased production of reactive oxygen species, and decreased glutathione concentrations in the oocyte (Hashimoto et al., 2000). Hyperglycaemic conditions also lead to an increased rate of oocyte meiotic maturation (Downs et al., 1998; Sutton-McDowall et al., 2005). Increased glucose concentrations are also correlated with increased FSH-induced CC expansion in bovine COC (Sutton-McDowall, 2004).

A number of environmental contaminants and lifestyle factors, including BPA, caffeine, and nicotine metabolites, have been found at measureable concentrations in follicular fluid as well as in serum of women (Zenzes et al., 1996; Ikezuki et al., 2002; Al-Saleh et al., 2010). Some of these compounds have also been reported to negatively affect female fertility (Emanuele et al., 2002; Klonoff-Cohen, 2005; Anderson, 2010). The environmental contaminant BPA and the lifestyle factors caffeine, ethanol and nicotine have all previously been reported to affect GJ communication in various tissue types (Flagg-Newton et al., 1981; Tsai et al., 2004; Mustonen et al., 2005; Lee and Rhee, 2007). However, the effects of physiological concentrations of these and other highly addictive and potentially detrimental compounds (e.g. MDMA and THC) on GJ communication within the COC have not been tested. Moreover, the large number of connexin isoforms, their combinations in differing tissue types and the unique regulatory properties of each isoform suggest that alterations in GJ communication in response to regulatory factors between tissue types will be unique.

The aims of this chapter were to determine the *in vitro* effects of high physiological concentrations of BPA, caffeine, ethanol, nicotine, MDMA, or THC with time on cultured COC in regards to (1) the degree of GJ communication between the oocyte and CC and (2) the timing of meiotic resumption of the oocyte.

6.2 Methods

6.2.1 COC collection

A full description of the COC collection procedure for the *in vitro* culture experiments is described in Section 2.1.2. In brief, ovaries were extracted, follicles were punctured with a 20 gauge needle, and morphologically healthy COC were transferred into 48-well plates

containing dissection media containing 50 μ M dipyridamole and 50 μ M rolipram. All COC were washed twice in wash media containing 50 μ M dipyridamole and 50 μ M rolipram.

6.2.2 *Exposure to lifestyle factors*

Stock solutions of each of the treatment reagents are listed in Table 6-1. The treatments were generally purchased from Sigma with the exception of BPA (donated by Phil Conveny; Nuplex Industries, NZ), MDMA (donated by Richard Moore; VUW), and THC (donated by Ryan Steel, VUW). The final concentrations chosen were based on levels that had been measured in biological fluids (see Table 1.1). Following the wash steps described above, COC were transferred into incubation media containing 50 μ M dipyridamole and 50 μ M rolipram without (control) or with (treated) the appropriate treatment. After 0, 4, 12, or 24 hours incubation at 37 °C in 5 % CO₂, and 96 % humidity, the COC were transferred into incubation media containing 2 μ M calcein-AM, 50 μ M dipyridamole and 50 μ M rolipram as well as the appropriate treatment for 10 minutes. The COC were then transferred into fresh incubation media (calcein-AM free) containing 50 μ M dipyridamole and 50 μ M rolipram and the appropriate treatment, and incubated for a further hour. The COC were washed twice in PBS and the oocyte was denuded of CC and measured for fluorescence as described previously. Thereafter, the oocyte was washed twice more in PBS. These individual DO and separated CC masses were suspended in 10 μ L PBS in the 96-well plates and stored at -80 °C until further analyses (see Chapter 2.1.3).

To reduce the number of samples, the experiments involving the treatments with caffeine and nicotine were combined, and the experiments involving the treatments with MDMA and THC were combined such that the treatments in each experiment were compared to one control group.

As described previously (Section 2.1.3.1), the raw fluorescence measurement for each individual oocyte was corrected for the geometric mean of the control group at 1 hour (i.e. those COC pre-incubated for 0 hours followed by a 1 hour post-calcein incubation). The data were tested for normality using the D'Agostino & Pearson normality test and data that were not normally-distributed were transformed (square root or natural log transformations) and were assessed again for normality. The data were then analysed using a two-way ANOVA.

Table 6-1: Stock and final concentrations of treatment reagents in media. With the exception of ethanol, which was stored in dH₂O, all other reagents were stored in DMSO.

Treatment reagents	Stock concentration	Final Concentration
BPA	4 µg/mL	20 ng/mL
Caffeine	20 mg/mL	100 µg/mL
Ethanol	750 mg/mL	7.5 mg/mL (0.95 % w/v)
Nicotine	10 µg/mL	500 ng/mL
MDMA	400 µg/mL	2 µg/mL
THC	10 µg/mL	500 ng/mL

6.3 Results

6.3.1 Effect of BPA on GJ communication in rat COC

The effect of exposure of rat COC to 20 ng/mL BPA *in vitro* on GJ communication over time is depicted in Figure 6-1. A significant effect of BPA treatment on oocyte fluorescence was noted, but no effects of time or treatment x time interaction were observed. Although no significant effect of time was measured, oocyte fluorescence appeared marginally increased in the control COC at 13 hours, compared to 1 hour, but was decreased at 25 hours. This reduction in oocyte fluorescence in the control COC at 25 hours was not mirrored in the BPA-treated COC. Instead, mean oocyte fluorescence in the BPA-treated COC increased progressively at each consecutive time point, although no significant effect of time was demonstrated. The proportion of oocytes with a visible GV declined over time in both the control and BPA-treated groups (Table 6-2). There was no difference in the proportion of oocytes with a visible GV between the control and BPA-treated groups.

Figure 6-1: Effect of BPA on fluorescent dye transfer from adjacent CC to oocytes over time *in vitro*. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control (i.e. untreated) group is represented by the unfilled bars (□), while the BPA-treated group is represented by the hatched bars (▨). Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the control group at 1 hour. The data represent 11-12 readings from individual oocytes in each group generated from 3 replicate experiments. The data were transformed (square root) prior to being analysed by a two-way ANOVA. The mean $\pm$ SEM fluorescent reading in the control group at 1 hour was 167 ± 39 .

Table 6-2: Effect of BPA on the proportion of oocytes with a visible GV. The table shows the proportion of oocytes with a visible GV (%), with the actual number of oocytes with a GV in brackets. The data from 3 replicate experiments (N=11-12/group) were analysed by Fisher's exact test. No effect of BPA on the presence of a GV was noted.

Time	Control	BPA
1	100 (12)	75 (9)
5	42 (5)	75 (9)
13	67 (8)	58 (7)
25	42 (5)	50 (6)

6.3.2 Effect of caffeine on GJ communication in rat COC

The effect of exposure of rat COC to 100 $\mu\text{g/mL}$ caffeine *in vitro* on GJ communication over time is depicted in Figure 6-2. Overall, there were no significant effects of treatment, time or treatment x time interaction. It is worth noting that the treatment effect almost reached

significance ($P=0.052$). Mean oocyte fluorescence in the control COC remained essentially unchanged for the first 13 hours but by 25 hours, a lower (albeit not significant) mean level of fluorescence was evident. This reduction in oocyte fluorescence at 25 hours was not observed in the caffeine-treated COC. Instead, the mean oocyte fluorescence levels in the caffeine-treated COC were essentially unchanged over 25 hours. The proportion of oocytes with a visible GV declined over time in both the control and caffeine-treated groups (Table 6-3). There was no difference in the proportion of oocytes with a visible GV between the control and caffeine-treated groups.

Figure 6-2: Effect of caffeine on fluorescent dye transfer from adjacent CC to oocytes over time. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control (i.e. untreated) group is represented by the unfilled bars (□), while the caffeine-treated group is represented by the hatched bars (▨). Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the control group at 1 hour. The data represent 10-12 readings from individual oocytes in each group generated from 3 replicate experiments. The data were transformed (square root) and then analysed by a two-way ANOVA. The mean $\pm$ SEM fluorescent reading in the control group at 1 hour was 224 ± 24 .

Table 6-3: Effect of caffeine on the proportion of oocytes with a visible GV. The table shows the proportion of oocytes with a visible GV (%), with the actual number of oocytes with a GV in brackets. The data from 3 replicate experiments (N=10-12/group) were analysed by a Fisher's exact test. No effect of caffeine on the presence of a GV was noted.

Time	Control	Caffeine
1	83 (10)	83 (10)
5	75 (9)	75 (9)
13	42 (5)	67 (8)
25	42 (5)	58 (7)

6.3.3 Effect of ethanol on GJ communication in rat COC

The effect of exposure of rat COC to 7.5 mg/mL ethanol *in vitro* on GJ communication over time is depicted in Figure 6-3. No effects due to treatment or time, or treatment x time interaction were observed. The proportion of oocytes with a visible GV decreased over time in both the control and ethanol-treated groups (Table 6-4). When the data from all time points were pooled, there was a greater proportion of oocytes with a visible GV in the ethanol-treated group compared to the control group ($P < 0.05$).

Figure 6-3: Effect of ethanol on fluorescent dye transfer from adjacent CC to oocytes over time *in vitro*. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control (i.e. untreated) group is represented by the unfilled bars ( ), while the ethanol-treated group is represented by the hatched bars ( ). Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the control group at 1 hour. The data represent 9-12 readings from individual oocytes in each group generated from 3 replicate experiments. The data were transformed (square root) and analysed by a two-way ANOVA. The mean $\pm$ SEM fluorescent reading in the control group at 1 hour was 313 ± 71 .

Table 6-4: Effect of ethanol on the proportion of oocytes with a visible GV. The table shows the proportion of oocytes with a visible GV, with the actual number of oocytes with a GV in brackets. The data from 3 replicate experiments (N=9-12/group) were analysed by a Fisher's exact test.

Time	Control	Ethanol
1	62 (7)	83 (10)
5	50 (6)	75 (9)
13	29 (4)	75 (9)
25	33 (4)	42 (5)

6.3.4 Effect of nicotine on GJ communication in rat COC

The effect of exposure of rat COC to 500 ng/mL nicotine *in vitro* on GJ communication over time is depicted in Figure 6-4. No significant effects of treatment or time were detected but a significant treatment x time interaction was noted. The mean oocyte fluorescence in the

control COC remained essentially unchanged over the first 13 hours but appeared to be lower at 25 hours. This lower (albeit not significant) level at 25 hours in the control group was not evident in the nicotine-treated group where the mean fluorescence levels remained essentially unchanged over the 25 hour period. There was no difference in the proportion of oocytes with a visible GV between the control and nicotine-treated groups (Table 6-5).

Figure 6-4: Effect of nicotine on fluorescent dye transfer from adjacent CC to oocytes over time *in vitro*. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control (i.e. untreated) group is represented by the unfilled bars (□), while the nicotine-treated group is represented by the hatched bars (▨). Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the control group at 1 hour. The data represent 12 readings from individual oocytes in each group generated from 3 replicate experiments. The data were transformed (square root) and analysed by a two-way ANOVA. The mean $\pm$ SEM fluorescence reading in the control group at 1 hour was 224 ± 24 .

Table 6-5: Effect of nicotine on the proportion of oocytes with a visible GV. The table shows the proportion of oocytes with a visible GV (%), with the actual number of oocytes with a GV in brackets. The data from 3 replicate experiments (N=12/group) were analysed by a Fisher's exact test. No effect of nicotine on the presence of a GV was noted.

Time	Control	Nicotine
1	83 (10)	67 (8)
5	75 (9)	67 (8)
13	42 (5)	42 (5)
25	42 (5)	67 (8)

6.3.5 Effect of MDMA on GJ communication in rat COC

The effect of exposure of rat COC to 2 µg/mL MDMA *in vitro* on GJ communication over time is shown in Figure 6-5. A significant effect of time but not treatment was noted. Moreover, there was no significant treatment x time interaction. Overall, mean oocyte fluorescence was lower at 13 and 25 but not 5 hours in both the control and MDMA-treated groups. The proportion of oocytes with a visible GV decreased over time in both the control and MDMA-treated groups (Table 6-6). When observations from all time points were pooled, there was a lower proportion of oocytes with a visible GV in the MDMA-treated group compared to the control group ($P < 0.001$).

Figure 6-5: Effect of MDMA on fluorescent dye transfer from adjacent CC to oocytes over time. The incubation time indicates the pre-calcein incubation + 1 hour the post-calcein incubation. The control (i.e. untreated) group is represented by the unfilled bars (□), while the MDMA-treated group is represented by the hatched bars (▨). Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the control group at 1 hour. The data represent 11-12 readings from individual oocytes in each group generated from 3 replicate experiments. The data were transformed (square root) and analysed by a two-way ANOVA. The mean $\pm$ SEM fluorescent reading in the control group at 1 hour was 139 ± 31 .

Table 6-6: Effect of MDMA on the proportion of oocytes with a visible GV. The table shows the proportion of oocytes with a visible GV (%), with the actual number of oocytes with a GV in brackets. The data from 3 replicate experiments (N=11-12/group) were analysed by a Fisher's exact test.

Time	Control	MDMA
1	92 (11)	92 (11)
5	83 (10)	25 (3)
13	67 (8)	25 (3)
25	58 (7)	17 (2)

6.3.6 Effect of THC on GJ communication in rat COC

The effect of exposure of rat COC to 500 ng/mL THC *in vitro* on GJ communication over time is depicted in Figure 6-6. Overall, there was a significant effect of incubation time but no effect of treatment and no treatment x time interaction. Mean oocyte fluorescence was lower at 13 and 25 hours in both the control and THC-treated groups. The proportion of oocytes with a visible GV declined over time in both the control and THC-treated groups (Table 6-7). When observations from all time points were pooled, the difference in the proportion of oocytes with a visible GV between the control and THC-treated groups almost reached significance ($P=0.054$). When observations from the 5, 13, and 25 hour time points were pooled, the proportion of oocytes with a visible GV was significantly reduced in the THC-treated group compared to the control ($P<0.05$).

Figure 6-6: Effect of THC on fluorescent dye transfer from adjacent CC to oocytes over time *in vitro*. The incubation time indicates the pre-calcein incubation + the 1 hour post-calcein incubation. The control (i.e. untreated) group is represented by the unfilled bars (□), while the THC-treated group is represented by the hatched bars (▨). Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the control group at 1 hour. The data represent 12 readings from individual oocytes in each group generated from 3 replicate experiments. The data were transformed (natural log) and analysed by a two-way ANOVA. The mean $\pm$ SEM fluorescent reading in the control group at 1 hour was 139 ± 31 .

Table 6-7: Effect of THC on proportion of oocytes with a visible GV. The table shows the proportion (%) of oocytes with a visible GV, with the actual number of oocytes with a GV in brackets. The data from 3 replicate experiments (N=12/group) were analysed by a Fisher's exact test.

Time	Control	THC
1	92 (11)	92 (11)
5	83 (10)	42 (5)
13	67 (8)	50 (6)
25	58 (7)	33 (4)

6.4 Discussion

The focus of this study was to investigate the effects of several lifestyle factors and environmental contaminants on the COC communication system and hence their influence oocyte health. The results reveal that there was no treatment effect noted after *in vitro*

exposure of rat COC to high physiological concentrations of caffeine, ethanol, MDMA or THC on fluorescent dye transfer over 25 hours relative to the controls. It is possible that there is an effect of caffeine as this treatment approached significance. However, further studies are needed to confirm whether this is of biological significance. In contrast, exposure to nicotine resulted in a treatment x time interaction, as a result of a dramatic decline in dye transfer that was noted in the control group after 13 hours whereas no change in dye transfer over 25 hours was measured in the nicotine-treated group. The only treatment effect independent of time *in vitro* was noted for BPA where the pattern of fluorescent dye transfer was similar to that for nicotine: again no change was noted for the treated group over 25 hours whereas a dramatic decline after 13 hours was noted for the control. Thus, exposure of COC to BPA and nicotine may require oocytes to demand a higher level of CC-derived factors to mitigate the possible negative effects of these reagents. With respect to measures of oocyte maturation status (i.e. the presence of a GV) after exposure to lifestyle factors, it was noted that *in vitro* exposure to ethanol suppressed oocyte meiotic maturation, whereas *in vitro* exposure to MDMA or THC enhanced the proportion of oocytes undergoing GVBD.

In general, the compounds tested in the present study were at concentrations 10-fold higher than that measured previously in biological fluids. These concentrations were chosen as they were above the normal physiological range, but were not so high to induce a false positive result. The concentrations of those compounds that had not been measured previously in follicular fluid were determined based on the concentration in blood. In general, the concentrations of small molecules are comparable between plasma and follicular fluid (Edwards, 1974).

The exposure of rat COC to BPA caused an overall increase in GJ communication. The largest difference in oocyte fluorescence between the treated and control COC occurred after 25 hours in culture. The reduction in oocyte fluorescence that occurred in control COC after a 25 hour culture period was not observed in those COC exposed to BPA. This observation suggests that BPA prevents closure of the GJs. Interestingly, previous *in vitro* studies using a higher dose of BPA found that BPA suppressed GJ communication in an epithelium-derived rat breast cancer cell line *in vitro* (Lee and Rhee, 2007). Similarly, BPA has also been reported to suppress spontaneous GJ-dependent Ca^{2+} oscillations in mouse oocytes (Mohri and

Yoshida, 2005). However, it is important to note that the concentrations of BPA used in these studies were between 4- and 1000-fold higher than that used in the present study. Whilst the concentration used in this study was lower, it was nevertheless above the normal physiological range. In future studies it would be of interest to test BPA concentrations within the expected physiological range. As discussed previously, the consequence of increased GJ communication may include increased transfer of cAMP, glucose metabolites, and other nutrients to the oocyte. BPA has previously been reported to reduce oocyte quality in both mice and humans (Eichenlaub-Ritter et al., 2008; Fujimoto et al., 2010). Furthermore, *in vitro* exposure to BPA did not affect oocyte nuclear maturation, as the proportion of oocytes undergoing GVBD was unchanged following exposure to BPA for up to 25 hours. It should be noted here that the number of samples used in this study was small resulting in the inability to detect small differences between groups. While the Fisher's exact test is more sensitive than the traditional chi-square test, it still lacks power with small sample sizes (Upton, 1992). Previous reports indicate that BPA does promote meiotic arrest in oocytes. For example, a greater proportion of mouse oocytes underwent GVBD following exposure to 10 µg/mL, but not lower concentrations, of BPA (Eichenlaub-Ritter et al., 2008). Additionally another study revealed that exposure of mouse oocytes to BPA was associated with a decreased ability of oocytes to resume meiosis, and in those that do, an increased proportion arrest after GVBD and fail to extrude a first polar body (Lenie et al., 2008). Again, both of these studies used much higher concentrations of BPA than that used in this study (340-fold and 500-fold higher respectively). Interestingly, the former study found that lower concentrations of BPA did not affect meiotic maturation of the oocyte even after 16 hours of exposure to BPA. Therefore the results from the present study are in agreement with previous publications that determined that high physiological concentrations of BPA do not affect oocyte meiotic maturation.

Overall, caffeine appeared to promote GJ communication in rat COC at some time points although this did not quite reach significance. Similar to BPA-treated oocytes, the largest difference in oocyte fluorescence between the caffeine-treated and control COC occurred after 25 hours in culture. Additionally oocyte fluorescence was highest in the caffeine-treated COC at 5 hours. Again, the reduction in oocyte fluorescence that occurred in control COC after a 25 hour culture period was not observed in those COC exposed to caffeine. This

is consistent with previous reports that caffeine increased GJ communication in rat fibroblasts and epithelial cells *in vitro* (Flagg-Newton et al., 1981). These results suggest that caffeine may affect oocyte quality by preventing closure of the GJs. *In vitro* exposure to caffeine has also been reported to increase the glutathione content of ovine oocytes, and to improve the embryo cleavage rate (Ye et al., 2010). However, evidence regarding the effect of caffeine consumption on female fertility and oocyte quality in humans is inadequate and controversial (Anderson, 2010). Interestingly in the current study, caffeine did not have a significant effect on the proportion of oocytes that underwent GVBD over time. Caffeine is a broad-range PDE inhibitor (Rahimi et al., 2010), that has been shown to maintain meiotic arrest in mouse and porcine oocytes (Jagiello et al., 1972; Kren et al., 2004). However in contrast to the current study, neither of these studies added other specific PDE inhibitors into the culture medium. The use of rolipram and dipyridamole in this study may have inhibited the relevant PDEs to a maximal level, thus potentially masking the PDE inhibitory effects of caffeine.

The exposure of rat COC to ethanol had no overall effects on GJ communication. A study investigating rat gastric mucosal cells showed that ethanol reduced GJ communication, although a higher dose (~8 fold) was used than that in the present study (Mustonen et al., 2005). It is entirely plausible that the incubation conditions may cause ethanol to evaporate out of the media, causing variable or false negative results. However, this has been tested in other studies and 96 ± 2 % of the added ethanol remained in the media following a 25 hour incubation at 37 °C (Messing et al., 1986). Interestingly, ethanol appeared to inhibit oocyte meiotic maturation, as the proportion of oocytes that underwent GVBD was significantly lower over time in the ethanol-treated group compared to the control. A previous study involving the exposure of ovulated mouse oocytes to 7 % ethanol reported a stimulation of parthenogenetic activation (i.e. progression of meiosis from metaphase II to completion), as measured by the number of unfertilized oocytes that had extruded a second polar body (O'Neill et al., 1989). This suggests that ethanol modulates progression of meiosis II in oocytes. However to the best of my knowledge the effect of ethanol on meiosis I (i.e. prior to ovulation) has not been investigated.

Nicotine had no overall effect on GJ communication in rat COC however there was a significant treatment x time interaction. The reduction in oocyte fluorescence that occurred

in control COC after a 25 hour culture period was not observed in those COC exposed to nicotine, as was also the case for BPA and caffeine. These results are in contrast to previous studies that found that *in vitro* exposure to nicotine resulted in decreased GJ communication in human umbilical vein endothelial cells (Tsai et al., 2004). However, the concentration of nicotine used in the study was 195-fold greater than that used in the current study. It is important to note that nicotine is rapidly metabolised into the two major metabolites, cotinine and nicotine-*N*-oxide by the enzymes cytochrome p450 2A6 (CYP2A6) and flavin-containing monooxygenase 3 (FMO3), respectively (Benowitz, 1986; Hukkanen et al., 2005). The enzyme CYP2A6 has been detected in mouse ovaries (Cannady et al., 2003) and cotinine has been measured at concentrations of ~700 ng/mL in follicular fluid and 50-200 ng/mL in plasma of active smokers (Zenzes et al., 1996; Shoaib and Stolerman, 1999). To my knowledge, there is no available data on the activity of FMO3 or the levels of nicotine-*N*-oxide in the ovary. Nicotine did not have a significant effect on the proportion of oocytes that underwent GVBD over time. In contrast to this study, nicotine has previously been reported to promote oocyte nuclear maturation in bovine oocytes (Liu et al., 2008). However, the Liu study used a concentration of nicotine more than 1200-fold greater than was used in the current study.

The effects of the active ingredients in two illicit drugs (i.e. MDMA and THC) on GJ communication in COC was also tested for the first time in this study. Exposure to MDMA did not affect the ability of the oocyte to import dye from the CC. Interestingly though, meiotic maturation of the oocyte was affected by MDMA. Significantly, fewer oocytes with a visible GV were present in the MDMA-treated group compared to the controls. These results suggest that MDMA prematurely advances meiotic progression in rat COC. In the present study, exposure of COC to THC also had no effect on transfer of calcein from the CC to the oocyte compared to the controls. A previous study found that *in vitro* exposure to THC inhibited GJ communication in a rat liver epithelial cell line (Upham et al., 2003). However, the concentrations used were ~8-fold greater than in the present study. The results in this study indicate that exposure to THC may prematurely advance oocyte meiotic maturation, as there were fewer oocytes remaining with a visible GV after exposure to THC compared to the controls. This is in agreement with a previous study that found that exposure of rat follicles to THC *in vitro* induced resumption of meiosis (Reich et al., 1982).

The study design for the GJ bioassay meant that COC were incubated for 0, 4, 12, or 24 hours in the presence or absence of the appropriate treatment followed by incubation with calcein-AM and a subsequent 1 hour post calcein-AM incubation with or without treatment. Incubating COC with the lifestyle factors for up to 24 hours prior to calcein-AM exposure allowed for identification of factors that slowly regulated GJ communication. The 1 hour post calcein-AM incubation was carried out to allow for transfer of the fluorescent dye. Performing the post calcein-AM incubation in the absence of treatment may have released the regulatory action of that treatment, providing a false negative result. Thus, the post calcein-AM incubation was carried out in the presence or absence of treatment, as appropriate.

In summary, this *in vitro* study found that exposure to 20 ng/mL BPA and 500 ng/mL nicotine prevented a time-dependent decrease of GJ communication in rat COC. A reduction in oocyte fluorescence was measured in the control group after 25 hours but not in the BPA or nicotine-treated COC. Moreover, exposure of COC to 100 µg/mL caffeine *in vitro* may promote GJ communication, however this effect was negligible. In contrast, exposure to 7.5 mg/mL ethanol, 2 µg/mL MDMA or 500 ng/mL THC did not affect GJ communication in rat COC. *In vitro* exposure to ethanol inhibited oocyte meiotic maturation, as measured by the proportion of oocytes with a visible GV, while exposure to MDMA or THC promoted oocyte meiotic maturation. None of the other treatments affected the proportion of oocytes with a visible GV.

7 EFFECTS OF *IN VITRO* EXPOSURE OF COC TO LIFESTYLE FACTORS ON GENE EXPRESSION IN OOCYTES

7.1 Introduction

Oocyte competency, defined as the ability of an oocyte to develop into a viable embryo, is dependent upon a two-way communication system between the oocyte and CC that facilitates a co-ordinated maturation process of all follicular constituents (Gilchrist et al., 2008). The oocyte indirectly regulates its own growth and maturation through secretion of oocyte growth factors that act on the CC. The key oocyte growth factors GDF9 and BMP15 regulate important developmental events in GC (e.g. proliferation, steroidogenesis and differentiation, prevention of premature luteinisation and apoptosis, and CC mucification) as well as the synthesis and delivery of essential reagents and substrates from the GC to the oocyte (Gilchrist et al., 2008; Su et al., 2009). Levels of mature GDF9 in follicular fluid have been reported to be positively associated with high quality embryos during IVF (Gode et al., 2011). A number of other processes are also indicative of oocyte competence including glucose uptake and metabolism (Krisher and Bavister, 1999), DNA damage repair (Marangos and Carroll, 2012), and mitochondrial function (Stojkovic et al., 2001).

Within an antral follicle, the CC that surround the oocyte are in direct contact with follicular fluid. In humans, numerous studies have shown that after exposure to certain chemical contaminants such as BPA, caffeine or cotinine, these reagents are able to cross from the blood stream into follicular fluid. In this context, these contaminants have the potential to influence oocyte functions (Zenzes et al., 1996; Ikezuki et al., 2002; Al-Saleh et al., 2010). Moreover, results from Chapter 6 established that *in vitro* exposure of rat COC to BPA or nicotine resulted in increased trafficking from CC to the oocyte via GJ communication. Conversely, these results also showed that *in vitro* exposure to caffeine, ethanol, MDMA, or THC did not affect GJ communication in rat COC. While transfer of nutrients to the oocyte is important for oocyte maturation, this does not provide information about the health of the oocyte itself. Oocyte health may be indirectly measured by quantifying the expression levels of key genes involved in critical pathways that regulate normal oocyte function.

The aim of this chapter was to determine the effects of *in vitro* exposure of rat COC to BPA, caffeine, ethanol, nicotine, MDMA, or THC on *Atr*, *Bmp15*, *Cx37*, and *Gdf9* mRNA expression levels within the oocyte.

7.2 Methods

DO were collected at completion of a GJ bioassay (those samples collected for Chapter 6). The samples were stored at -80 °C prior to extraction of total RNA as described in Section 2.3.1. The mRNA levels of *Atr*, *Cycs*, *Glut1*; and *Gdf9*, *Bmp15* and *Cx37* were measured using multiplex TaqMan qPCR as previously described (Section 2.3.2). Levels of *Cycs* and *Glut1* were below the detection limit, and were not included in the analyses. Expression levels of the reference gene *Rpl19* were also measured in each of these multiplex reactions. Each reaction run included a calibrator sample which contained cDNA from a pool of rat COC and this allowed comparison between reaction runs. The mRNA levels for each candidate gene were corrected for the mRNA levels of the same gene in the calibrator sample. Subsequently, the data were analysed using the $2^{(-\Delta\Delta C_T)}$ method (Livak and Schmittgen, 2001). The relative linear mRNA levels were analysed for normality using the D'Agostino & Pearson normality test. Data that were not normally-distributed were transformed (using natural logarithms, squared, or square root transformations) and reanalysed by the D'Agostino & Pearson normality test to confirm a normal distribution. Normally-distributed data were analysed by a two-way ANOVA.

7.3 Results

7.3.1 Effect of BPA on gene expression in rat oocytes

The effect of *in vitro* exposure of rat COC to 20 ng/mL BPA on mRNA levels of oocytes is depicted in Figure 7-1. For all candidate genes (i.e. *Atr*, *Bmp15*, *Cx37* and *Gdf9*), there were no significant effects of time or treatment although a treatment x time interaction was close to being significant for *Cx37* ($P=0.058$). This appeared to be due to the mean *Cx37* levels decreasing over time in the BPA-treated oocytes, whilst that in the control oocytes appeared to increase. Very similar patterns of gene expression were observed for *Atr*, *Bmp15*, and *Gdf9*. There was a significant effect of time but not treatment on *Rpl19* mRNA levels and no treatment x time interaction was observed. Overall, the levels of *Rpl19* mRNA were lower at 25 hours compared to 1 hour.

Figure 7-1: Effect of BPA on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D), and *Rpl19* (E) in rat oocytes. The control group is represented by the unfilled bars ( ) , while the BPA-treated group is represented by the hatched bars ( ). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 10-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (B, C), natural log transformed data (A, E) or squared data (D).

7.3.2 Effect of caffeine on gene expression in rat oocytes

The effect of *in vitro* exposure of rat COC to 100 $\mu\text{g/mL}$ caffeine on mRNA levels of oocytes is depicted in Figure 7-2. There was no effect of treatment or time, nor any treatment x time

interactions for any of the candidate genes (i.e. *Atr*, *Bmp15*, *Cx37*, and *Gdf9*). *Atr* levels appeared to decrease over time, however this was not significant ($P=0.069$). The *Rpl19* levels were similar across both treatment groups and exposure times.

Figure 7-2: Effect of caffeine on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D), and *Rpl19* (E) in rat oocytes. The control group is represented by the unfilled bars (□), while the caffeine-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 10-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (B, C, D), square-root transformed data (A), or natural log transformed data (E).

7.3.3 Effect of ethanol on gene expression in rat oocytes

The effect of *in vitro* exposure of rat COC to 7.5 mg/mL ethanol on mRNA levels of oocytes is depicted in Figure 7-3. There were no significant effects of treatment, time or treatment x time interactions on *Atr*, *Bmp15*, *Cx37*, *Gdf9*, or *Rpl19* mRNA levels. No significant effects of treatment or time, or treatment x time interactions on *Rpl19* levels were observed. *Rpl19* levels appeared to be higher in the ethanol-treated DO than the control however this was not significant ($P=0.065$).

7.3.4 Effect of nicotine on gene expression in rat oocytes

The effect of *in vitro* exposure of rat COC to 500 ng/mL nicotine on mRNA levels of oocytes is depicted in Figure 7-4. There was no effect of treatment or time, or a treatment x time interaction on *Atr*, *Bmp15* or *Gdf9* levels in rat oocytes. While not significant, *Atr* levels did appear to decline with time ($P=0.068$). For *Cx37*, there was no significant effect of treatment or time however a significant treatment x time interaction was noted. Whilst *Cx37* mRNA levels were similar at both time points in the control oocytes, *Cx37* levels appeared to decrease with time in the nicotine-treated oocytes. There was no effect of treatment or time, or a treatment x time interaction on *Rpl19* levels in rat oocytes.

7.3.5 Effect of MDMA on gene expression in rat oocytes

The effect of *in vitro* exposure of rat COC to 2 µg/mL MDMA on mRNA levels of oocytes is depicted in Figure 7-5. There were no significant effects of treatment or time, or treatment x time interactions with respect to the levels of *Atr*, *Bmp15*, *Cx37*, *Gdf9*, or *Rpl19* mRNA in rat oocytes.

7.3.6 Effect of THC on gene expression in rat oocytes

The effect of *in vitro* exposure of rat COC to 500 ng/mL THC on mRNA levels of oocytes is depicted in Figure 7-6. There were no significant effects of treatment or time, or treatment x time interactions with respect to levels of *Atr* or *Gdf9* mRNA in rat oocytes. For *Bmp15* and *Cx37*, there were significant effects of time but there were no treatment or treatment x time interactions: the levels for both of these genes increased with time, especially in the THC-treated groups. There were no significant effects of treatment, time, or treatment x time interactions on *Rpl19* levels in rat oocytes.

Figure 7-3: Effect of ethanol on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D), and *Rpl19* (E) in rat oocytes. The control group is represented by the unfilled bars (□), while the ethanol-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 10-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (B, C, D), or square root transformed data (A, E).

Figure 7-4: Effect of nicotine on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D), and *Rpl19* (E) in rat oocytes. The control group is represented by the unfilled bars (□), while the nicotine-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 9-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (A, B, C, D) or natural log transformed data (E).

Figure 7-5: Effect of MDMA on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D), and *Rp19* (E) in rat oocytes. The control group is represented by the unfilled bars (□), while the MDMA-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 10-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (B, C, D, E) or square-root transformed data (A).

Figure 7-6: Effect of THC on mRNA levels of *Atr* (A), *Bmp15* (B), *Cx37* (C), *Gdf9* (D), and *Rpl19* (E) in rat oocytes. The control group is represented by the unfilled bars (□), while the THC-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 10-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed (B, C, D, E) or natural log transformed data (A).

7.4 Discussion

These results reveal that the expression of the oocyte-derived genes measured in this study are mostly unchanged following exposure to a range of lifestyle factors and environmental contaminants. There was a treatment x time interaction for Cx37 mRNA expression following nicotine exposure, where nicotine caused a reduction in Cx37 mRNA levels over time in rat oocytes, compared to an increase in control oocytes. Surprisingly, *in vitro* exposure to BPA, caffeine, ethanol, MDMA, or THC alone had no effect on the mRNA levels in rat oocytes of any of the genes tested.

CX37 is the primary component of oocyte-derived GJ channels (Simon et al., 1997; Teilmann, 2005) and is essential for transfer of nutrients to the oocyte. Ratchford et al. (2008) demonstrated an association between the reduced health of oocytes from diabetic mice, reduced Cx37 mRNA levels, and impaired GJ coupling between the oocyte and CC. Therefore, the changes in Cx37 expression levels after exposure to nicotine may be of biological significance.

The oocytes used in this study were collected following a GJ dye transfer assay. As discussed previously (Chapter 6), this GJ assay involved COC being exposed to a treatment for 0, 4, 12, or 24 hours. Thereafter COC were incubated with calcein-AM for 10 minutes and then incubated for a further hour in calcein-free media, but still in the presence of the treatment. The results presented in this chapter suggest that nicotine raises Cx37 expression in the oocyte following a 1 hour exposure, but this effect is attenuated after a 25 hour exposure. The finding that nicotine had different effects depending on exposure time may be related to the metabolism of nicotine over time. It is reasonable to expect that after a 1 hour exposure, most of the nicotine would not have been metabolised. However, after 25 hours *in vitro* it is possible that most of the nicotine had been metabolised. As discussed in Chapter 6, CYP2A6, which catalyses the conversion of nicotine to cotinine, is expressed in the mouse ovary (Cannady et al., 2003). Moreover, cotinine has been detected in follicular fluid (Zenzes et al., 1996; Shoaib and Stolerman, 1999).

Interestingly, exposure to BPA, caffeine, ethanol, MDMA, or THC did not affect mRNA levels of any of the oocyte-derived genes tested. This was unexpected, as BPA, caffeine, and ethanol have all been reported to directly affect oocyte quality following *in vitro* exposure

(Jagiello et al., 1972; O'Neill et al., 1989; Lenie et al., 2008). However, the oocytes in the present study were cultured as intact COC. The CC may have acted as a protective barrier, perhaps preventing at least a proportion of the harmful factors from direct action on the oocyte. In addition, the variability that exists between the maturation stages of individual oocytes may have masked any treatment effects.

The levels of oocyte-derived *Rpl19* were not affected by any of the treatments tested in this study. Similarly, levels of *Rpl19* were not affected by incubation time with the notable exception of the BPA experiment. Levels of *Rpl19* decreased over time in the BPA experiment, indicating that oocytes containing less total RNA were selected at the 25 hour time point. Expression levels of many of the candidate genes showed a similar pattern to that of *Rpl19*. Overall these results reveal that expression levels of oocyte-derived *Atr*, *Bmp15*, *Cx37*, and *Gdf9* are generally stable regardless of time that the COC spent in culture.

In summary, the major finding from this chapter was that exposure of rat COC to potentially detrimental compounds (i.e. BPA, caffeine, ethanol, MDMA, or THC) did not alter the overall expression of oocyte genes measured in this study. The exposure of nicotine did result in increased oocyte-derived *Cx37* at 1 hour, but decreased *Cx37* at 25 hours. The changes in *Cx37* expression levels following exposure to nicotine may influence the availability of nutrients and other molecules necessary for oocyte health.

8 EFFECTS OF *IN VITRO* EXPOSURE OF COC TO LIFESTYLE FACTORS ON GENE EXPRESSION IN CC

8.1 Introduction

During follicular development, the CC provide the oocyte with glucose metabolites, cholesterol metabolites, cAMP, ions and amino acids. Thus, the CC have a nurturing role during oocyte maturation. This is facilitated by the passing of these low molecular weight compounds to the oocyte through GJ (Kidder and Vanderhyden, 2010). However, in addition to important substrates for oocyte maturation, the follicular fluid microenvironment contains exudates from the plasma so that the COC can also be exposed to a number of potentially harmful environmental or lifestyle factors (Weiss and Eckert, 1989; Zenzes et al., 1996; Al-Saleh et al., 2010).

As the CC surround the oocyte within the follicle, they are also considered to have a protective role for the oocyte. For example, the CC protect the oocyte from oxidative stress during follicular development (Tatemoto et al., 2000). Exposure to a reactive oxidative species generating system (XOD) caused meiotic arrest in DO, and this was coincident with the activation of caspase-3 and thus increased activation of the apoptotic pathway. In contrast, COC that were exposed to XOD did not exhibit this activation of caspase-3 and had higher levels of the antioxidant glutathione (Tatemoto et al., 2000).

In Chapter 6 it was determined that *in vitro* exposure of COC to BPA or nicotine resulted in increased GJ transfer from the CC to the oocyte. However, *in vitro* exposure of COC to caffeine, ethanol, MDMA, or THC did not affect GJ communication. In Chapter 7, it was found that an interaction between time and nicotine treatment was observed for the oocyte GJ gene, *Cx37*. The effects of these compounds on gene expression in oocytes are most likely facilitated through regulatory mechanisms within CC. Thus, these compounds would be expected to cause even larger perturbations in gene expression levels of CC.

The aim of this chapter was to determine the effects of *in vitro* exposure of rat COC to BPA, caffeine, ethanol, nicotine, MDMA, or THC on *Atr*, *Cx43*, *Cybs*, *Gfpt1*, and *Pfkp* mRNA expression levels in the CC.

8.2 Methods

The collection of CC masses from individual COC was undertaken following completion of the GJ bioassays described in Chapter 6. The samples were stored at -80 °C prior to total RNA extraction as described in Section 2.3.1. The incubation times referred to henceforth indicate the total time each COC was incubated with the treatment in Chapter 6 (i.e. a 0 or 24 hour pre-calcein incubation followed by a 1 hour post-calcein incubation). The mRNA levels of the candidate genes were measured in quadriplex reaction (*Atr/Cycs/Glut1* and *Cx43/Gfpt1/Pfkp*, respectively) using multiplex TaqMan qPCR as previously described (Section 2.3.2). *Glut1* was excluded from the final analysis, as expression levels were below the detection limit. The final gene in each multiplex reaction was the reference gene *Rpl19*, which was used to correct gene expression for RNA input. Each reaction run included a calibrator sample which contained cDNA from a pool of rat COC; this allowed comparisons to be made between reaction runs. The mRNA levels for each candidate gene were corrected for the *Rpl19* levels of that sample. Subsequently, the data were analysed using the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001).

The relative linear mRNA levels were analysed for normality using the D'Agostino & Pearson normality test. Data that were not normally-distributed were transformed (using natural log, square root, square root($y+0.5$), $y/y+1$, or squared transformations) and reanalysed to confirm a normal distribution. The normally-distributed data were analysed by a two-way ANOVA.

8.3 Results

8.3.1 Effect of BPA on gene expression in rat CC

The effect of *in vitro* exposure of COC to 20 ng/mL BPA on mRNA levels in CC is depicted in Figure 8-1. There were no effects of time or treatment, or a treatment x time interaction on *Atr*, *Gfpt1*, and *Pfkp* mRNA levels: the apparent decline in *Pfkp* levels with time in culture in the control group was not significant ($P=0.068$). However, there was a treatment x time interaction for *Cx43* mRNA levels, along with treatment and time main effects. The expression levels for *Cx43* remained steady over time in the control CC, but had increased markedly after 25 hours in culture in the BPA-treated CC. There was a significant decline in *Cycs* mRNA levels over time in both the control and BPA-treated CC. There were no effects of time, treatment, or a treatment x time interaction on *Rpl19* mRNA levels.

8.3.2 Effect of caffeine on gene expression in rat CC

The effect of *in vitro* exposure of COC to 100 μ g/mL caffeine on mRNA levels in rat CC is depicted in Figure 8-2. There were no significant effects of treatment, time, or a treatment x time interaction on *Atr* mRNA levels although the mean expression levels were ~50 % lower ($P=0.069$) at 25 hours in both the control and caffeine-treated CC. Both treatment and time effects were measured for *Cx43* mRNA where levels were reduced at 25 hours, compared to 1 hour, in both control and caffeine-treated CC. Moreover, *Cx43* mRNA was further reduced in the caffeine-treated CC, compared to control CC at both time points. There was an effect due to time, but not treatment, or a treatment x time interaction for *Cycs*, *Gfpt1*, and *Pfkp* mRNA levels. Expression levels of all of these genes were reduced at 25 hours, compared to 1 hour, in both the control and caffeine-treated groups. There were no effects of time, treatment, or a treatment x time interaction on *Rpl19* mRNA levels.

Figure 8-1: Effect of BPA on mRNA levels of *Atr* (A), *Cx43* (B), *Cxcs* (C), *Gfpt1* (D), *Pfkf* (E), and *Rpl19* (F) in rat CC. The control group is represented by the unfilled bars (□), while the BPA-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 11-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (B, E), natural log transformed data (D, F) or squared-root transformed data (A), square-root($y+0.5$) transformed data (C).

Figure 8-2: Effect of caffeine on mRNA levels of *Atr* (A), *Cx43* (B), *Cycs* (C), *Gfpt1* (D), *Pfkfb* (E), and *Rpl19* (F) in rat CC. The control group is represented by the unfilled bars (□), while the caffeine-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 10-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (A, C, D, E), natural log transformed data (B, F).

8.3.3 Effect of ethanol on gene expression in rat CC

The effect of *in vitro* exposure of COC to 7.5 mg/mL ethanol on mRNA levels in rat CC is depicted in Figure 8-3. There were no effects of time, treatment, or a treatment x time interaction on *Atr* mRNA levels. A treatment x time interaction was observed for *Cx43* expression levels: this was likely due to the mean *Cx43* levels in ethanol-treated CC being higher than the controls at 1 hour, but similar at 25 hours. An effect of time was observed for *Cycs*, *Gfpt1*, and *Pfkp* mRNA levels where expression levels of all these genes were lower at 25 hours relative to 1 hour in both groups. Whilst expression levels of *Gfpt1* appeared to be further reduced in the ethanol-treated, compared to the control, CC, this did not reach significance ($P=0.14$). The mean levels of *Gfpt1* expression in the ethanol-treated groups were skewed by one outlier at 1 hour (relative *Gfpt1* level of 0.012) and two outliers at 25 hours (relative *Gfpt1* levels of 0.004 and 0.008). However, these did not appear to affect the statistical analysis. These samples were not removed from the analysis as the *Rpl19* C_T value was within the sensitive range. There were no effects of time, treatment, or a treatment x time interaction on *Rpl19* mRNA levels.

8.3.4 Effect of nicotine on gene expression in rat CC

The effect of *in vitro* exposure of COC to 500 ng/mL nicotine on mRNA levels in rat CC is depicted in Figure 8-4. There were no effects of time, treatment, or a treatment x time interaction on *Atr* mRNA levels. Although levels of *Atr* mRNA were lower in control CC at 25 hours compared to that at 1 hour this was not significant ($P=0.17$). There were effects of time on *Cx43*, *Cycs*, *Gfpt1* and *Pfkp* mRNA levels. Mean expression levels of each gene were reduced at 25 hours, compared to 1 hour, in both the control and nicotine-treated groups. Moreover, *Cx43* mRNA was further reduced ($P=0.052$) in the nicotine-treated group, compared to control group at 25 hours. Conversely, the levels of *Cycs* mRNA were higher although not significantly so ($P=0.07$), in the nicotine-treated group compared to the control at 25 hours. There were no effects of time, treatment, or a treatment x time interaction on *Rpl19* mRNA levels.

Figure 8-3: Effect of ethanol on mRNA levels of *Atr* (A), *Cx43* (B), *Cxcs* (C), *Gfpt1* (D), *Pfkp* (E), and *Rpl19* (F) in rat CC. The control group is represented by the unfilled bars (□), while the ethanol-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 10-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (B, C, D, E), natural log transformed data (F) and $1/(y+1)$ transformed data (A).

Figure 8-4: Effect of nicotine on mRNA levels of *Atr* (A), *Cx43* (B), *Cxcs* (C), *Gfpt1* (D), *Pfkp* (E), and *Rpl19* (F) in rat CC. The control group is represented by the unfilled bars (□), while the nicotine-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 11-12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (A, C, D), natural log transformed data (B, E, F).

8.3.5 Effect of MDMA on gene expression in rat CC

The effect of *in vitro* exposure of COC to 2 µg/mL MDMA on mRNA levels in rat CC is depicted in Figure 8-5. A time effect was observed for *Atr* mRNA whereby expression levels had increased in both groups at 25 hours, compared to 1 hour. There were treatment effects on expression levels of both *Cx43* and *Gfpt1*. Expression levels of *Cx43* and *Gfpt1* levels were increased in the MDMA-treated group, compared to the control, at both time points. There were effects of time x treatment interactions for *Cycs* and *Pfkp* expression. The mean *Cycs* levels in the MDMA-treated group were similar at 1 hour, but reduced at 25 hours, relative to the control. *Pfkp* levels in the MDMA-treated group were increased at 1 hour, but were reduced at 25 hours, relative to the control group. There were no effects of time or treatment on *Rpl19* mRNA levels.

8.3.6 Effect of THC on gene expression in rat CC

The effect of *in vitro* exposure of COC to 500 ng/mL THC on mRNA levels in rat CC is depicted in Figure 8-6. There was an effect of time on *Atr* expression whereby levels were increased in both THC-treated and control groups at 25 hours, compared to that at 1 hour. The expression levels of *Cx43* were affected by treatment and time. The mean levels of *Cx43* mRNA were similar between the two time points in the control group, but had increased with time in the THC-treated group. An effect of time was observed for both *Cycs* and *Pfkp* genes where expression levels of both *Cycs* and *Pfkp* were decreased in the control and THC-treated groups at 25 hours, compared to 1 hour. Additionally, *Gfpt1* mRNA levels were increased in the THC-treated group at both time points, compared to the control. There were no effects of time or treatment on *Rpl19* mRNA levels.

Figure 8-5: Effect of MDMA on mRNA levels of *Atr* (A), *Cx43* (B), *Cxcs* (C), *Gfpt1* (D), *Pfkfb* (E), and *Rpl19* (F) in rat CC. The control group is represented by the unfilled bars (□), while the MDMA-treated group is represented by the hatched bars (▨). Values are shown as means ± upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (A, B, C, D, F), natural log transformed data (E).

Figure 8-6: Effect of THC on mRNA levels of *Atr* (A), *Cx43* (B), *Cyts* (C), *Gfpt1* (D), *Pfkfb* (E), and *Rpl19* (F) in rat CC. The control group is represented by the unfilled bars (□), while the THC-treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group at 1 hour. The data were generated from a total of 12 readings from individual oocytes in each group. A two-way ANOVA was performed on untransformed data (B, C, D, E), squared data (A), or square-root transformed data (F).

8.4 Discussion

These results reveal that the function of CC within rat COC is influenced by lifestyle or environmental contaminants in the surrounding environment. This study shows that the *in vitro* exposure of COC to BPA, caffeine, ethanol, MDMA and THC, but not nicotine, resulted in modified expression levels of one or more of CC-derived genes.

The finding that *Cx43* mRNA levels increased after a long (i.e. 25 hour) but not a short-term (i.e. 1 hour) exposure to BPA indicates that BPA may act indirectly to modify *Cx43* expression. The increased levels of *Cx43* mRNA suggest that prolonged exposure to BPA may increase the number of GJs and/or hemichannels present in the CC. Interestingly, exposure to BPA did not affect *Atr* expression, a gene that mediates the response to DNA damage by causing G₂/M arrest. BPA inhibits mitosis in murine GC resulting in G₂/M arrest (Xu et al., 2002), however, the Xu study used a dose of BPA that was >1000-fold higher than that used in the current study.

Conversely, the exposure of cultured rat COC to caffeine resulted in an overall reduction in *Cx43* mRNA in CC. This result was surprising as caffeine is a broad-range PDE inhibitor that has been shown to both promote GJ communication and increase intracellular cAMP levels in rat fibroblasts and epithelial cells *in vitro* (Flagg-Newton et al., 1981). Indeed, a previous study found that increases in intracellular cAMP levels that induced GJ communication in a rat hepatoma cell line were coincident with increased *Cx43* mRNA levels (Mehta et al., 1992). Therefore, it was expected that caffeine would increase *Cx43* levels. However as discussed in Chapter 6, the addition of PDE inhibitors for the entire incubation period in the current study may have maximally-inhibited PDE activity therefore masking any similar effects of caffeine. Thus, the mechanism by which caffeine elicits a reduction of *Cx43* mRNA may not be related to the PDE inhibitory properties of caffeine.

Exposing rat COC to ethanol for a short-term period i.e. 1 hour) caused a slight increase in CC-derived *Cx43* mRNA levels, but following a long term exposure, the mean *Cx43* mRNA levels were similar to the control. The results presented here suggest that short exposures to ethanol resulted in an increased number of CX43 GJ channels/hemichannels in rat CC. Thereafter, either receptor desensitization or metabolism of ethanol may result in a reduction of *Cx43* mRNA to levels similar to that of the control. Ethanol is metabolised by

alcohol dehydrogenase (ADH) isoenzymes, the expression of which have been confirmed in murine oocytes (Rout and Armant, 2002). However the major isoenzyme that metabolises ethanol, namely ADH1, was not detected. Desensitization of the cellular response to ethanol over long exposure times has previously been reported in a neuroblastoma cell line (Gordon et al., 1986). Initial exposure of neuroblastoma cells to ethanol resulted in increased production of cAMP, however chronic exposure caused desensitization of the heterologous receptors that are coupled to the GTP-binding protein G_s , resulting in reduced cAMP production (Gordon et al., 1986; Mochly-Rosen et al., 1988). The finding in this present study that *Cx43* mRNA is increased in rat CC following exposure to ethanol for 1 hour is consistent with increased intracellular cAMP (Mehta et al., 1992). In addition, the finding that the ethanol-mediated increase in *Cx43* mRNA was attenuated following a 25 hour exposure is consistent with desensitization of rat CC.

Interestingly, nicotine treatment did not significantly affect mRNA levels of any of the candidate genes tested in CC. However, the results presented in this study suggest that *in vitro* exposure to nicotine may result in a small reduction ($P=0.052$) in *Cx43* mRNA levels in rat CC. This is in contrast with a previous study which found that exposure to nicotine *in vitro* resulted in increased *Cx43* mRNA levels in human umbilical vein endothelial cells (Tsai et al., 2004). However as discussed in Chapter 6, the concentration of nicotine used by the Tsai group was 195 times greater than that used in this study.

The effects of MDMA on gene expression levels in CC were more pronounced than the other aforementioned treatments in that treatment effects or treatment x time effects were observed in four out of the five candidate genes tested. Expression levels for both *Cx43* and *Gfpt1* mRNA were higher at both time points in the MDMA-treated, compared to control CC. In addition, a time x treatment interaction was observed for *Cycs* and *Pfkp* levels where *Pfkp* levels were higher at 1 hour but lower at 25 hours in the MDMA-treated CC, and *Cycs* levels were similar at 1 hour but lower at 25 hours in the MDMA-treated CC, compared to controls. These results indicate that MDMA acts directly on CC (i.e. within 1 hour) to regulate gene expression. To the best of my knowledge this is the first study that has examined the effect of MDMA on *Cycs*, *Gfpt1*, or *Pfkp* mRNA levels in any tissue type. The increase in *Cx43* mRNA suggests that exposure to MDMA results in an increase in the number of GJs and/or hemichannels present between rat CC which is in contrast to neonatal rat ventricular

myocytes where MDMA reduced expression levels of *Cx43* (Zhuo et al., 2013). Interestingly, these results also strongly suggest that MDMA affects expression of genes involved in glucose metabolism. MDMA has been shown to regulate glucose metabolism in several areas of the brain, although the effects are dependent upon the location within the brain (Obrocki et al., 1999). In the present study, MDMA increased the expression patterns of *Gfpt1* and *Pfkp* at 1 hour, indicating that energy production via the hexosamine and glycolytic pathways was increased. The simultaneous increase of both glucose utilisation rate-limiting enzymes in conjunction with increased *Cx43* mRNA is possibly due to increased glucose uptake by CC however the expression levels of the glucose transport *Glut1* mRNA were below detection limits. Enhanced glucose uptake may lead to an increase in all four glucose utilisation pathways in the COC (i.e. glycolysis, hexosamine, pentose phosphate, and polyol pathways) which is linked with an increase in reactive oxygen species and precocious resumption of nuclear maturation (Sutton-McDowell et al., 2010). Interestingly, *Cycs* and *Pfkp* levels were reduced after 25 hours exposure to MDMA. The metabolism of MDMA occurs by two pathways: demethylenation which is catalysed by CYP2D1 and CYP3A2 in rats to produce 3,4-dihydroxymethamphetamine (HHMA); and *N*-demethylation which is catalysed by CYP1A2 and CYP2D1 to produce 3,4-methylenedioxymphetamine (MDA) (Green et al., 2003). The four aforementioned CYP enzymes has been detected in the rat ovary (Hiroi et al., 1998; Lee et al., 2012). However, the expression of these enzymes has not been reported in particular ovarian cell types (i.e. the GC, CC, or oocyte). Therefore, a complex interplay between un-metabolised MDMA, HHMA, and MDA may exist to differentially regulate expression levels of *Cx43*, *Cycs*, *Gfpt1*, and *Pfkp* in rat CC over time.

The exposure of rat COC to THC also resulted in an overall treatment effect for both *Cx43*, and *Gfpt1* mRNA levels. The results presented here suggest that more CX43 GJs/hemichannels may be present in the CC following a 25 hour exposure to THC. The finding that THC takes a longer time to modify *Cx43* mRNA levels, suggests that *Cx43* is less sensitive than *Gfpt1* to THC, or that a THC metabolite is mediating the change in *Cx43* levels. The primary enzymes involved in first pass metabolism of THC are CYP2C and CYP3A (Shimada et al., 1994). Almost 100 metabolites of THC have been identified, however the major metabolite is 11-OH-THC (Lemberger et al., 1973; Grotenhermen, 2003). The finding that *Gfpt1* levels were upregulated at both time points in the THC-treated CC indicates that

the activity of the hexosamine biosynthetic pathway may be preferentially increased over the glycolytic pathways. This is a very interesting result as the major glucose utilisation pathway utilised in the COC is the glycolytic pathway while only a small proportion of glucose is utilised by the hexosamine pathway. The hexosamine pathway is involved in CC expansion, however inappropriate upregulation of this pathway has been linked to compromised developmental competence (Sutton-McDowell et al 2010).

This study reveals that the most sensitive gene of those tested in rat CC was the GJ component *Cx43*. The levels of *Cx43* were affected by all of the treatments included in this study, although the effect of nicotine did not quite reach significance ($P=0.052$). It is perhaps unsurprising that this was the case, as mRNA levels of *Cx43* are rapidly regulated in response to many external stimuli including cAMP (Mehta et al., 1992), prostaglandin E_2 and parathyroid hormone (Civitelli et al., 1998), and epidermal growth factor (Rivedal et al., 1996). The half-life of most connexins is <10 hours (Hervé et al., 2007) suggesting that transcription and translation are important processes by which GJ communication may be regulated. Additionally, transcriptional regulation of the *Cx43* gene in response to external stimuli may be an important mechanism by which the CC protect and nurture the oocyte.

In summary, the main findings of this study were that the gene responsible for GJ transfer potential in CC (*Cx43*) was regulated by most of the compounds tested in this study. Overall, exposure of rat COC to BPA, MDMA, and THC increased CC-derived *Cx43* mRNA levels but exposure of rat COC to caffeine, ethanol, and nicotine either lowered *Cx43* mRNA levels or had little effect. Moreover, exposure of rat COC to MDMA and THC *in vitro* resulted in an overall increase in *Gfpt1* mRNA, whilst a treatment x time interaction for *Cyts* and *Pfkp* was observed following MDMA exposure.

9 EFFECTS OF *IN VITRO* EXPOSURE OF COC TO LIFESTYLE FACTORS ON PROTEIN EXPRESSION

9.1 Introduction

Quantifying the expression level of a gene that encodes a particular protein only gives an indication of how much protein a cell is instructed to synthesise. Subsequent post-transcriptional and post-translational modifications may further enhance or restrict the level of biologically-active protein that is available. Thus, while quantification of gene expression levels are very informative, a true account of the amount of protein being synthesised at any one time is only obtained by measuring protein expression levels. The synthesis of both CX43 and GDF9 proteins are regulated by post-translational events.

GJ communication is regulated in a dynamic manner throughout folliculogenesis by gonadotrophins (Granot and Dekel, 2002), cAMP and PKA (Yogo et al., 2002), and MAPK (Norris et al., 2008). Rats treated with pregnant mare's serum gonadotrophin had increased levels of CX43 protein whereas an additional hCG injection resulted in a marked reduction in CX43 levels (Granot and Dekel, 1997). The *Cx43* gene contains two exons separated by a 30 kilobase intron; the first exon contains a 5'-untranslated region (UTR), while the second exon contains the protein coding region and a 3'-UTR. The first exon is differentially spliced to form at least nine different *Cx43* species in mice, and six different species in rat (Pfeifer et al., 2004). These *Cx43* species had varying translation efficiencies both between different species and in different cell lines. Therefore, as previously stated, levels of *Cx43* gene expression may not correlate with protein levels.

An important mechanism by which GJ communication is regulated is through phosphorylation of connexin proteins. For example, several forms of CX43 are often detected using Western blotting, including a non-phosphorylated form (P0), and two differentially phosphorylated forms (P1 and P2) that migrate at a slower rate (Solan and Lampe, 2009). Phosphorylation on Ser³⁶⁵ is responsible for the altered migration of the P1 form (Solan et al., 2007), while phosphorylation at Ser³²⁵, Ser³²⁸, and Ser³³⁰ is responsible for the altered migration of the P2 form (Lampe et al., 2006). These phosphorylation sites are

thought to be involved in promoting GJ formation and activity (Yogo et al., 2002; Solan and Lampe, 2009).

GDF9 is the primary oocyte-secreted factor involved in regulation of CC/GC function in rodents (Lin et al., 2012). Post-translational modifications are required for GDF9 signalling. As with other members of the TGF β family, GDF9 is initially synthesised as a preproprotein. The signal peptide is removed upon secretion from the oocyte and the promature protein is released into the follicular fluid in humans, mice and sheep (Gilchrist et al., 2004b; McNatty et al., 2006; Gode et al., 2011). The proregions of TGF β proteins are cleaved by proprotein convertase subtilisin/kexin enzymes (Harrison et al., 2011) and evidence exists that this cleavage occurs both intracellularly and extracellularly (Blanchet et al., 2008; Susan-Resiga et al., 2011). Generally, the mature protein has been considered to be the bioactive form. However, a recent study suggests that the proregion also plays an important role in female reproduction. When female mice were immunized with a GDF9 proregion peptide, these mice had more corpora lutea but smaller litter sizes (McIntosh et al., 2012). The exact role of the proregion is unknown, however for other TGF β proteins it mediates dimer formation and targets the protein to the receptor (Sengle et al., 2008; Walton et al., 2009).

Therefore, while transcriptional regulation of gene expression is an important indicator of an effect that a treatment has on a cell, this does not always correspond to what occurs at a protein level (Gygi et al., 1999). In addition, post-translational modifications play an important role in protein bioactivity and function.

The aim of this chapter was to determine the effect of *in vitro* exposure of rat COC to BPA, caffeine, ethanol, nicotine, MDMA, or THC on the expression levels of CC-derived CX43 and oocyte-derived GDF9 proteins.

9.2 Methods

9.2.1 COC collection

Ovaries from prepubescent Sprague-Dawley rats were collected in PBS and the COC were extracted as previously described (Section 2.1.2). The COC were incubated either with treatment or without treatment (control group) for 1 or 25 hours at 37 °C with 5 % CO₂ and 96 % humidity, to mimic the incubation conditions used in the GJ assay (see Chapter 6 and

Table 6-1). Subsequently, 100 COC were washed in PBS before being transferred into a microcentrifuge tube. The tubes were centrifuged at $800 \times g$ for 10 minutes, the supernatant removed, and the COC resuspended in PBS supplemented with protease inhibitors. Each COC collection experiment was performed twice, and the samples were pooled so that the final number of COC in each sample was 200. The samples were stored at -20°C until further processing.

9.2.2 *BCA Assay*

Total protein concentration was measured using the BCA assay (Section 2.4.1). Briefly, a bovine serum albumin standard was serially diluted in PBS and 25 μL was added in triplicate into a 96-well plate. One third of each COC sample (~ 67 COC in $\sim 10 \mu\text{L}$) was serially diluted in PBS and aliquoted in duplicate into a 96-well plate. BCA working reagent (200 μL) was added to each well, and the plate was then incubated at 37°C (original assays) or 60°C (subsequent assays) for 2 hours. The plates were cooled to room temperature and the absorbance at 490 nm was measured using a VICTOR3 multilabel plate reader (PerkinElmer; Surrey Hills, Australia). The concentration of protein in each COC sample was calculated using the equation obtained from the bovine serum albumin standard curve.

9.2.3 *Western blotting*

Two-thirds of each COC sample (~ 133 COC in $\sim 20 \mu\text{L}$) was added to sample buffer, incubated at 95°C in a water bath for 10 minutes, before being loaded onto a polyacrylamide gel. The proteins were separated according to size by SDS-PAGE, transferred onto a nitrocellulose membrane, and CX43 or GDF9 protein expression was detected using Western blotting. In brief, CX43 was identified using the monoclonal antibody MAB3067. To determine MAB3067 specificity, the antibody was pre-absorbed with 5x the corresponding MAB3067 control peptide. GDF9 was detected using a monoclonal antibody (MAB47B) which was generated against the C-terminal region that corresponds to the mature region of ovine GDF9. To determine MAB47B specificity, the antibody was pre-absorbed with 5x *E. coli* produced recombinant ovine GDF9. The membranes were incubated with electrochemiluminescence reagents for 1 minute before being exposed to an X-ray film for between 1 and 10 minutes.

9.2.4 Data analysis

The density of the bands present on the Western blots was measured using ImageJ software. For the CX43 blots, the density of the 43 kDa band was measured. For the GDF9 blots, the densities of the 49 kDa promature and, when present, the 20 kDa mature bands were measured. When multiple bands were present (e.g. when doublet or triplet bands were present for CX43) the density of all bands was measured. The total protein amount in each sample was used to correct for CX43, but not GDF9, protein levels. The reasoning behind this was that samples contained entire COCs which consisted mostly of large numbers of CC and a few (i.e. ~200) oocytes. Thus, differences in total protein levels between samples would predominantly be due to differences in the number of CC, and not oocytes. Therefore, CX43 protein levels are referred to as corrected levels and GDF9 protein levels are referred to as uncorrected levels.

Each experiment comprised of 200 COC for each experimental group at each time point, therefore there were only two replicated experimental samples at each time point. Thus statistical analysis was inappropriate and therefore interpretation of the data is simply based on the evidence of two replicate but independent studies.

9.3 Results

9.3.1 Effect of BPA and caffeine on protein levels in rat COC

The effects of *in vitro* exposure of rat COC to 20 ng/mL BPA or 100 µg/mL caffeine on CX43 protein levels are depicted in Figure 9-1. A 43 kDa band was observed in the positive control in both experimental replicates, while no bands were observed in the negative control in either replicate. There was some non-specific background signal in the first replicate (Figure 9-1A), however this did not obscure the bands of interest. A 43 kDa band was observed in each of the lanes containing COC lysate. When the CX43 antibody was pre-absorbed with the control peptide, no bands were detected (Figure 9-1C).

In the first replicate experiment (Figure 9-1A), a 1.7-fold reduction in CX43 levels with time in culture was noted in the control COC. Following exposure to BPA and caffeine for 1 hour, CX43 levels were 2.0-fold and 1.8-fold greater than the levels in the control at that time. Similar to the control, CX43 levels decreased over time in the BPA-treated and caffeine-

treated COC whereby a 10-fold and 1.8-fold reduction in CX43 levels over time were observed for these treatments so that levels were 3-fold lower and 1.7-fold greater than the levels in the control at 25 hours.

The treatment responses in the second replicate relative to the control differed somewhat from the first replicate but the CX43 levels over time followed the same pattern in all treatment groups (Figure 9-1B). CX43 levels in the control COC declined 10-fold with time in culture. CX43 levels were 1.4-fold lower or 1.4-fold greater after incubation with BPA and caffeine for 1 hour, compared to the control. Similar to the control, 1.8-fold and 7-fold reductions in CX43 levels were observed with time in culture so that levels were 4-fold and 2-fold greater than that in the control following 25 hours incubation with BPA and caffeine, respectively.

Regardless of treatment, there was a consistent decrease in the mean CX43 levels in cultured COC with time (Figure 9-2). CX43 levels following exposure to BPA were not different from that in the control, although there was variability between the BPA treated replicates after 1 hour of culture. Exposure of COC to caffeine after 1 hour resulted in a reproducible increase in CX43 levels compared to controls, however the overall decline in expression over 25 hours was similar to that noted in the control and BPA treated groups.

Figure 9-1: The effects of exposing rat COC to BPA and caffeine *in vitro* on CX43 protein levels over time. Western blots were performed in duplicate (A and B) to determine the level of CX43 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U), BPA (B), or caffeine (C). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also performed where the blot shown in (B) was stripped and re-probed with the CX43-specific antibody that had been pre-absorbed with its control peptide. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay. The relative band densities were corrected for total protein amount, as calculated by the BCA assay.

Figure 9-2: Effect of BPA and caffeine on relative CX43 levels in rat COC. The control group is represented by the unfilled bars (□), the BPA-treated group is represented by the striped bars (▨), while the caffeine-treated group is represented by the dotted bars (▩). The bars represent the means, while the dashes represent each individual band density measurement.

The effects of exposing rat COC to 20 ng/mL BPA or 100 µg/mL caffeine on GDF9 protein levels are depicted in Figure 9-3. Multiple bands between 20 and 114 kDa were detected in the positive control in both replicates. The bands in the positive control of the first replicate (Figure 9-3A) were overexposed, so most individual bands were unable to be distinguished. Bands of 49 and 20 kDa in size, corresponding to the promature and mature forms of GDF9 respectively, were detected in the positive control lanes. Additional prominent bands of 93, 70, and 40 kDa were observed. No bands were observed in the negative control. When the GDF9 antibody was pre-absorbed with recombinant GDF9, no bands were observed (Figure 9-3C). Multiple bands corresponding to the aforementioned molecular weights observed in the positive controls were observed in each of the lanes containing COC lysate.

In the first replicate experiment (Figure 9-3A), promature GDF9 in control COC declined 5-fold over time. Promature GDF9 levels were 2-fold and 3.3-fold lower than the control following incubation with BPA and caffeine for 1 hour. In contrast to the pattern of promature GDF9 levels over time in the control, the amount of promature GDF9 in the BPA-treated and caffeine-treated COC increased 1.6-fold or 2.7-fold with time so that levels were 4-fold greater in both the BPA- and caffeine-treated groups than that in the control at 25 hours. Mature GDF9 protein was only detected in the control COC at 1 hour and in the treated COC at 25 hours.

In the second replicate experiment (Figure 9-3B), promature GDF9 levels in control COC decreased slightly (1.3-fold) with time. Promature GDF9 levels were 3.3-fold and 1.3-fold lower than the control following 1 hour exposure to BPA and caffeine respectively. In contrast to the pattern of promature GDF9 levels with time in the control, levels of promature GDF9 levels increased 6-fold and 2.4-fold with time in the BPA-treated and caffeine-treated COC, so that levels were 2.3-fold and 2.4-fold greater than the control at 25 hours. Similar to the first replicate, mature GDF9 was only detected in the control COC at 1 hours and the treated COC at 25 hours.

Overall in the control COC, the mean promature GDF9 levels declined over time (Figure 9-4), notwithstanding the variability between the replicates after 25 hours in culture. Compared to the control, the mean levels of promature GDF9 for the BPA-treated and caffeine-treated group were lower after 1 hour in culture but both had recovered to levels above that in the controls after 25 hours in culture.

A

	U	B	C	U	B	C	+	-
	1			25				
Uncorrected promature band density	1.0	0.5	0.3	0.2	0.8	0.8	-	-
Uncorrected mature band density	1.0	0.0	0.0	0.0	0.7	0.7	-	-
Total protein content	5.0	4.4	3.7	4.3	4.6	5.2	-	-

B

	U	B	C	U	B	C	+	-
	1			25				
Uncorrected promature band density	1.0	0.3	0.8	0.8	1.8	1.9	-	-
Uncorrected mature band density	1.0	0.0	0.0	0.0	1.2	5.6	-	-
Total protein content	4.4	2.8	4.5	3.1	4.9	5.3	-	-

C

Figure 9-3: The effects of exposing rat COC to BPA and caffeine *in vitro* on GDF9 protein levels over time. Western blots were performed in duplicate (A and B) to determine the level of GDF9 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U), BPA (B), or caffeine (C). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also performed where the blot shown in (B) was stripped and re-probed with the GDF9-specific antibody that had been pre-absorbed with *E. coli* produced recombinant ovine GDF9. Molecular sizes (kDa) are indicated to the left of each blot. The densities of the bands of interest (indicated by arrowheads) were measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay.

Figure 9-4: Effect of BPA and caffeine on relative GDF9 levels in rat COC. The control group is represented by the unfilled bars (□), the BPA-treated group is represented by the striped bars (▨), while the caffeine-treated group is represented by the dotted bars (▩). The bars represent the means, while the dashes represent each individual band density measurement.

9.3.2 Effect of ethanol on protein levels in rat COC

The effect of exposing COC to 7.5 mg/mL ethanol *in vitro* on CX43 protein levels is depicted in Figure 9-5. A 43 kDa band was observed in the positive control of both experimental replicates, while no bands were observed in the negative control of either. A 43 kDa band was observed in each of the lanes containing COC lysate. Multiple smaller bands were also present in all lanes however the identities of these bands are unknown. When the CX43 antibody was pre-absorbed with the control peptide no bands were observed (Figure 9-5C).

In the first replicate experiment (Figure 9-5A), CX43 levels in control COC did not change over time. CX43 levels were similar between the ethanol-treated and control COC at 1 hour. In the ethanol-treated COC, the CX43 levels decreased slightly (1.4-fold) over time and were 1.3-fold lower after 25 hours of culture compared to the controls.

A similar response was observed in the second replicate experiment (Figure 9-5B). Levels of CX43 in the control COC did not change over time. After ethanol exposure CX43 levels were 1.3-fold greater after 1 hour, compared to the control and by 25 hours had decreased 1.6-fold at which time the levels were 1.3-fold lower than that measured in the control.

Overall in the control COC, CX43 levels did not change with time in culture (Figure 9-6). However in the ethanol-treated COC, there was a consistent reduction in CX43 levels after 25 hours in culture, however this was negligible.

Figure 9-5: The effects of exposing rat COC to ethanol *in vitro* on CX43 protein levels over time. Western blots were performed in duplicate (A and B) to determine the level of CX43 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U) or ethanol (E). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also performed where the blot shown in (B) was stripped and re-probed with the CX43-specific antibody that had been pre-absorbed with its control peptide. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μg) in each sample was determined using the BCA assay. The relative band densities were corrected for total protein amount, as calculated by the BCA assay.

Figure 9-6: Effect of ethanol on relative CX43 levels in rat COC. The control group is represented by the unfilled bars (□), while the ethanol-treated group is represented by the striped bars (▨). The bars represent the means, while the dashes represent each individual band density measurement.

The effect of exposing rat COC to 7.5 mg/mL ethanol on GDF9 protein levels are depicted in Figure 9-7. Multiple bands between 20 and 114 kDa were detected in the positive control of both replicates. The bands in the positive control of the first replicate (Figure 9-7A) were overexposed, so most individual bands were unable to be distinguished. Bands of 49 and 20 kDa in size, corresponding to the promature and mature forms of GDF9 respectively were detected in the positive control lanes. Additional prominent bands of 93, 70, and 40 kDa were observed. No bands were observed in the negative control. When the GDF9 antibody was pre-absorbed with recombinant GDF9, no bands were detected (Figure 9-7C). Multiple bands were observed in each of the lanes containing COC lysate, corresponding to the aforementioned molecular weights in the positive control.

In the first replicate (Figure 9-7A), levels of promature GDF9 increased 2.4-fold over time in the controls. Promature GDF9 levels were 1.4-fold lower in ethanol-treated COC at 1 hour, compared to the control. Similar to the control, levels of promature GDF9 in the ethanol-treated COC increased 3.4-fold over time such that levels were similar between the ethanol-treated and control COC at 25 hours.

In the second replicate, levels of promature GDF9 increased 1.6-fold over time in the controls (Figure 9-7B). Promature GDF9 levels were 1.3-fold lower following incubation with ethanol for 1 hour, compared to the control. In the ethanol-treated COC, levels of promature

GDF9 only increased slightly (1.3-fold) with time in culture so that levels were 1.6-fold lower at 25 hours, compared to the control.

Overall, levels of promature GDF9 consistently increased after 25 hours in culture in both the control and ethanol-treated samples (Figure 9-8). However, no difference in the levels of promature GDF9 following incubation treatment were noted.

Figure 9-7: The effects of exposing rat COC to ethanol *in vitro* on GDF9 protein levels over time. Western blots were performed in duplicate (A and B) to determine the level of GDF9 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U) or ethanol (E). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also performed where the blot shown in (B) was stripped and re-probed with the GDF9-specific antibody that had been pre-absorbed with *E. coli* produced recombinant ovine GDF9. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay.

Figure 9-8: Effect of ethanol on relative GDF9 levels in rat COC. The control group is represented by the unfilled bars (□), while the ethanol-treated group is represented by the striped bars (▨). The bars represent the means, while the dashes represent each individual band density measurement.

9.3.3 Effect of nicotine on protein levels in rat COC

The effect of exposing rat COC to 500 ng/mL nicotine on CX43 protein levels is depicted in Figure 9-9. A 43 kDa band was observed in the positive control of both experimental replicates, while no bands were observed in the negative control of either replicate. A 43kDa band was observed in each of the lanes containing COC lysate. Multiple smaller bands were also present in all lanes however the identities of these bands are unknown. When the CX43 antibody was pre-absorbed with the control peptide, the bands that had previously been visualised disappeared (Figure 9-9C).

In the first replicate experiment (Figure 9-9A), CX43 levels decreased 5-fold over time in the control group. Levels of CX43 were 5-fold lower after incubation with nicotine for 1 hour when compared to the control group. After 25 hours of culture, the CX43 levels increased 2.5-fold over time in the nicotine-treated group such that levels were 2.5-fold greater in the nicotine-treated group compared to the control at 25 hours.

A similar response was observed in the second replicate experiment (Figure 9-9B), CX43 levels decreased 2.5-fold over time in the control group. Similar to the first replicate experiment, CX43 levels were 5-fold lower in COC incubated with nicotine for 1 hour compared to the control. CX43 levels increased 7.5-fold over time in the nicotine-treated COC increased, such that levels were 3.8-fold greater in the nicotine-treated group compared to the control at 25 hours.

Overall, there was a consistent decrease in CX43 levels with time in culture in the control group (Figure 9-10). Conversely, CX43 levels were markedly lower at 1 hour incubation in nicotine-treated COC compared to the controls but were higher than the equivalent controls after 25 hours of incubation.

Figure 9-9: The effects of exposing rat COC to nicotine *in vitro* on CX43 protein levels in rat COC over time. Western blots were performed in duplicate (A and B) to determine the level of CX43 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U) or nicotine (N). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also run where the blot shown in (B) was stripped and re-probed with the CX43-specific antibody that had been pre-absorbed with its control peptide. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay. The relative band densities were corrected for total protein amount, as calculated by the BCA assay.

Figure 9-10: Effect of nicotine on relative CX43 levels in rat COC. The control group is represented by the unfilled bars (□), while the nicotine-treated group is represented by the striped bars (▨). The bars represent the means, while the dashes represent each individual band density measurement.

The effects of exposing rat COC to 500 ng/mL nicotine on GDF9 protein levels are depicted in Figure 9-11. Multiple bands between 20 and 114 kDa were detected in the positive control of both replicates. The bands in the positive control of the first replicate (Figure 9-11A) were overexposed, so most individual bands were indistinguishable. Bands of 49 and 20 kDa in size, corresponding to the promature and mature forms of GDF9 respectively were detected in the positive control lanes. Additional prominent bands of 93, 70, and 40 kDa were observed. No bands were observed in the negative control. When the GDF9 antibody was pre-absorbed with recombinant GDF9, no bands were detected (Figure 9-11C). Multiple bands were observed in each of the lanes containing COC lysate, corresponding to the aforementioned molecular weights in the positive control.

In the first replicate experiment (Figure 9-11A), promature GDF9 levels did not change over time in the control group. Incubation of COC for 1 hour with nicotine resulted in promature GDF9 levels 2.5-fold lower than that in the control. However, promature GDF9 levels increased 3.3-fold with time in the nicotine-treated group such that levels were similar to that in control COC at 25 hours. Mature GDF9 was detected in the control COC after 1 hour of incubation, but the levels declined 1.7-fold with time. Mature GDF9 was undetectable in the nicotine-treated COC at 1 hour in culture but had increased by 25 hours in culture to levels nearly twice that of control COC.

Similar to the first replicate, promature GDF9 levels in the second replicate experiment did not change over time in the control group (Figure 9-11B). Levels of promature GDF9 were 1.3-fold greater in the nicotine-treated group at 1 hour compared to the control, however decreased 1.2-fold with time to levels similar to that in the control at 25 hours. Mature GDF9 was undetectable at all time points regardless of treatment.

Overall, due to the large difference between replicates in the promature GDF9 levels in the nicotine-treated group at the 1 hour time point, no effect of time or treatment was observed for promature GDF9 levels (Figure 9-12).

Figure 9-11: The effects of exposing rat COC to nicotine *in vitro* on GDF9 protein levels over time. Western blots were performed in duplicate (A and B) to determine the level of GDF9 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U) or nicotine (N). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also run where the blot shown in (B) was stripped and re-probed with the GDF9-specific antibody that had been pre-absorbed with *E. coli* produced recombinant ovine GDF9. Molecular sizes (kDa) are indicated to the left of each blot. The densities of the bands of interest (indicated by arrowheads) were measured and corrected for the control at 1 hour. Total protein (μg) in each sample was determined using the BCA assay.

Figure 9-12: Effect of nicotine on relative GDF9 levels in rat COC. The control group is represented by the unfilled bars (□), while the nicotine-treated group is represented by the striped bars (▨). The bars represent the means, while the dashes represent each individual band density measurement.

9.3.4 Effect of MDMA and THC on protein levels in rat COC

The effects of exposing rat COC to 2 µg/mL MDMA and 500 ng/mL THC on CX43 protein levels are depicted in Figure 9-13. A 43 kDa band was observed in the positive control of both experimental replicates, while no bands were observed in the negative control of either replicate. A 43 kDa band was observed in each of the lanes containing COC lysate. When the CX43 antibody was pre-absorbed with the control peptide, no bands were detected (Figure 9-13C).

In the first replicate experiment (Figure 9-13A), CX43 levels decreased 2.5-fold with time in the control COC. Similarly, the levels of CX43 also decreased 3.3-fold with time in the THC-treated COC, so that levels were similar to the controls at both times. In contrast, CX43 levels were 1.25-fold lower in the MDMA-treated COC at 1 hour compared to the control, and did not change with time so that the levels were 1.75-fold greater than that in the control COC at 25 hours.

In the second replicate experiment, levels of CX43 decreased 3.3-fold with time in the control COC (Figure 9-13B). CX43 levels were similar between experimental groups after 1 hour of culture time, and a reduction in CX43 levels over time was noted in all groups (Figure 9-13B). Levels of CX43 decreased 2-fold and 1.2-fold with time in the MDMA- and THC-treated COC respectively. The CX43 levels in COC incubated with MDMA and THC for 25 hours were 1.7-fold and 3-fold greater respectively compared to the control.

A consistent trend of a decrease in CX43 levels over time was observed in all experimental groups but this was more evident in the control group (Figure 9-14). The smaller decreases in the MDMA- and THC-treated group resulted in CX43 levels being higher in the treated COC, compared to the control, after 25 hours in culture.

Figure 9-13: The effects of exposing rat COC to MDMA and THC *in vitro* on CX43 protein levels over time. Western blots were performed in duplicate (A and B) to determine the level of CX43 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U), MDMA (M) or THC (T). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also performed where the blot shown in (B) was stripped and re-probed with the CX43-specific antibody that had been pre-absorbed with its control peptide. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay. The relative band densities were corrected for total protein amount, as calculated by the BCA assay.

Figure 9-14: Effect of MDMA and THC on relative CX43 levels in rat COC. The control group is represented by the unfilled bars (□), the MDMA-treated group is represented by the striped bars (▨), while the THC-treated group is represented by the dotted bars (▤). The bars represent the means, while the dashes represent each individual band density measurement.

The effects of exposing of rat COC to 2 µg/mL MDMA and 500 ng/mL THC on GDF9 protein levels are depicted in Figure 9-15. Multiple bands between 20 and 114 kDa were detected in the positive control of both replicates. Bands of 49 and 20 kDa in size, corresponding to the promature and mature forms of GDF9 respectively were detected in the positive control lanes. Additional prominent bands of 93, 70, and 40 kDa were observed. No bands were observed in the negative control. When the GDF9 antibody was pre-absorbed with recombinant GDF9, no bands were detected (Figure 9-11C). Multiple bands were observed in each of the lanes containing COC lysate, corresponding to the aforementioned molecular weights in the positive control.

In the first replicate experiment (Figure 9-15A), levels of promature GDF9 decreased 1.25-fold with time in the control group. Levels of promature GDF9 were similar after 1 hour in culture in all experimental groups. Promature GDF9 levels increased slightly (1.4-fold and 1.2-fold) with time in the MDMA- and THC-treated COC to levels 1.6-fold greater in the MDMA- and THC-treated compared to the control COC at 25 hours. Mature GDF9 was detected at every time point regardless of treatment. Mature GDF9 levels declined 2.5-fold with time in the control COC. Following incubation with MDMA or THC for 1 hour, mature GDF9 levels were 2.5-fold lower than that in the control. Mature GDF9 levels increased 2.75-fold with time in the MDMA-treated COC so that levels were 2.8-fold greater than the

control at 25 hours. Mature GDF9 levels remained constant in the THC-treated COC so that levels were similar to the control at 25 hours.

Similar to the first replicate, levels of promature GDF9 decreased 2-fold with time in the control group (Figure 9-15B). Promature GDF9 levels were 1.7-fold and 5-fold lower in the MDMA- and THC-treated COC compared to the control COC at 1 hour. Levels of promature GDF9 in COC incubated with MDMA or THC increased with time by 1.5-fold and 6-fold respectively. This resulted in the levels of promature GDF9 being 1.8-fold and 2.4-fold higher in the MDMA- and THC-treated COC than the control COC at the 25 hour time point. Mature GDF9 levels were undetectable at any time point regardless of treatment.

The levels of promature GDF9 consistently decreased over time in the control group (Figure 9-16). Conversely, there was high variability in regards to the effect of treatment on the promature GDF9 levels after 1 hour in culture. However with time, both the MDMA and THC-treated COC showed an increase in promature GDF9 levels.

Figure 9-15: The effects of exposing rat COC to MDMA and THC *in vitro* on GDF9 protein levels over time. Western blots were performed in duplicate (A and B) to determine the level of GDF9 protein in COC lysate. COC were either incubated for 1 or 25 hours with the solvent control (U), MDMA (M) or THC (T). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also performed where the blot shown in (B) was stripped and re-probed with the GDF9-specific antibody that had been pre-absorbed with *E. coli* produced recombinant ovine GDF9. Molecular sizes (kDa) are indicated to the left of each blot. The densities of the bands of interest (indicated by arrowheads) were measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay.

Figure 9-16: Effect of MDMA and THC on relative GDF9 levels in rat COC. The control group is represented by the unfilled bars (□), the MDMA-treated group is represented by the striped bars (▨), while the THC-treated group is represented by the dotted bars (▤). The bars represent the means, while the dashes represent each individual band density measurement.

9.4 Discussion

This study revealed a consistent pattern of CX43 protein expression in cultured rat COC with time that enabled the effects of short and long-term exposures to common lifestyle factors and environmental contaminants to be assessed. Overall, the CX43 protein levels were lower after rat COC were cultured for 25 hours, compared to that after 1 hour. However exposure of rat COC to nicotine, MDMA, and THC for 25 hours resulted in CX43 protein levels that were higher than in the appropriate controls. It was interesting to note that the CX43 levels were sometimes altered after a 1 hour exposure of rat COC to some compounds. Specifically, CX43 protein levels were enhanced following a 1 hour exposure to caffeine, and reduced after exposure to nicotine. The effect of time on GDF9 protein expression in cultured rat COC was variable across experiments, thereby confounding interpretations concerning the effects of treatments on protein expression levels. Nevertheless, the evidence did suggest that promature GDF9 levels were reduced following short-term *in vitro* exposures (i.e. 1 hour) to BPA and caffeine, but enhanced after longer exposures (i.e. 25 hours) to BPA, caffeine, MDMA, and THC.

CX43 levels in COC cultured with BPA were not different to the levels in the control group however there was variability between the replicates noted after a 1 hour exposure to BPA. In Chapter 6 it was established that BPA enhanced GJ communication between the CC and

oocyte, particularly after 25 hours exposure. Therefore, it seems that the BPA-mediated upregulation of GJ communication does not occur through changes in CX43 protein expression. The findings from this study are consistent with a previous study which found that BPA did not affect CX43 levels in a rat epithelium derived mammary cell line (Lee and Rhee, 2007). Levels of both promature and mature GDF9 in COC were reduced after a 1 hour exposure to BPA, suggesting a reduction in oocyte growth factor signalling. However, after 25 hours exposure to BPA, promature and mature GDF9 levels were increased, indicating enhanced oocyte growth factor signalling. The finding that short and long term exposures to BPA have opposing effects on GDF9 levels suggest that desensitisation or metabolism of BPA occurs, or that two different regulatory mechanisms are at play in the COC. Given the role of GDF9 in regulating CC functions such as proliferation, metabolism, and apoptosis, further investigation into these pathways would provide insight into how BPA affects COC health.

Rat COC that were exposed to caffeine for 1 hour had increased CX43 levels compared to the control, however this effect was attenuated after a 25 hour exposure. Interestingly, in Chapter 6 it was determined that caffeine may enhance GJ communication ($P=0.052$), and that this effect was most notable after a 25 hour exposure. Therefore, as with BPA, these results suggest that changes in CX43 protein levels are not responsible for the increase in GJ communication that was seen in COC incubated with caffeine for 25 hours. Similar to the effect of BPA on GDF9 protein, levels of promature and mature GDF9 were reduced in COC incubated with caffeine for 1 hour, but were markedly higher than the controls following a 25 hour exposure to caffeine. Again, these findings suggest that metabolism or desensitization to caffeine occurs in the COC, or that two different regulatory mechanisms are at play. The marked effects of caffeine on GDF9 protein levels indicates that CC functions mediated by oocyte-secreted factors may be altered following exposure to caffeine *in vitro*. Specifically, functions such as CC proliferation and metabolism that are enhanced by oocyte-secreted factors may be reduced following a short exposure to caffeine, but increased following a long exposure. Further investigation into oocyte-mediated CC functions will reveal how caffeine affects these pathways.

CX43 levels in COC were not noticeably influenced by ethanol either after a 1 or 25 hour exposure. These results are consistent with those reported in Chapter 6, where it was shown that ethanol did not affect GJ communication in COC. A previous study found markedly

reduced levels of CX43 protein in rat liver epithelial cells that had been exposed to ethanol for 24 hours (Bokkala et al., 2001). Interestingly, in that study CX43 protein levels were also correlated with a reduction in GJ communication. In the current study, a doublet band was observed in both replicates, indicating CX43 was differentially phosphorylated (Solan and Lampe, 2009). However, no consistent pattern of phosphorylation was observed. It should be noted that no effect of culture time on CX43 was noted in the control group of the ethanol experiment. In all other experiments, CX43 levels declined markedly over time in the control group. The reason for this is unknown, however the solvent control used for ethanol, was dH₂O, in contrast to the use of DMSO as the solvent control for all other experiments. Exposure to ethanol did not affect levels of promature GDF9, and the mature protein was not detected in either experiment.

CX43 levels in nicotine-treated COC were markedly reduced at 1 hour, whereas were increased at 25 hours, compared to the control. The variability that was noted in the effect of nicotine over time suggests that metabolism or desensitisation to nicotine occurs in the COC. The effect of nicotine on CX43 protein levels was not dissimilar to the pattern of GJ communication in COC following incubation with nicotine (Chapter 6). GJ communication in COC was unaffected by a 1 hour exposure to nicotine, however it was increased after a 25 hour incubation. These results suggest that the increase in GJ communication in COC following long term exposure to nicotine is mediated by a concomitant increase in CX43 protein levels. However, the reduction in CX43 protein following a 1 hour exposure of COC to nicotine was not observed to influence GJ communication in COC (Chapter 6). Furthermore, the finding that the reduction in CX43 levels over time in the control that was not seen in the nicotine-treated COC indicates that nicotine may, in some manner, prevent the degradation of CX43 protein over time. Interestingly, the results presented in this chapter are in contrast to a previous study which showed that CX43 levels were unaffected by short exposures to nicotine (between 0 and 8 hours) but that long exposures (24 hours) decreased CX43 in human umbilical vein endothelial cells, (Tsai et al., 2004). In the present study, doublet bands were observed in all samples, indicating differentially phosphorylated forms of CX43 are present. The slow migrating form of CX43 likely corresponds to a phosphorylated form (Solan and Lampe, 2009) and generally displayed a reduced intensity compared to the faster migrating isoform. Exposure to nicotine *in vitro* did not affect levels of GDF9 protein in COC.

CX43 and GDF9 levels in COC that were incubated with MDMA for 25 hours were increased slightly compared to the control group. However, a short exposure time (i.e. 1 hour) was without effect. The levels of CX43 protein noted here do not correlate to the pattern of GJ communication in COC following incubation with MDMA (Chapter 6). Indeed, no effect of MDMA on GJ communication was measured. Therefore, while MDMA may increase overall CX43 levels, these connexins may not form functional GJ channels. Furthermore, the finding that MDMA partially prevented the time-dependent decrease in CX43 levels that was seen in the control suggests that MDMA prevents CX43 degradation. The results presented in this chapter are in contrast to a previous study in which MDMA reduced CX43 levels in rat ventricular myocytes following a 1 hour exposure (Zhuo et al., 2013). The effect of MDMA on GDF9 has not been investigated previously, to the best of my knowledge. These results suggest that long-term exposures of COC to MDMA are likely to modulate oocyte-CC interactions and thereby modulate CC functions such as metabolism and proliferation.

Exposure to THC did not affect CX43 levels in rat COC. These results correlate with those from Chapter 6, whereby incubation of COC with THC *in vitro* did not affect GJ communication. Conversely, incubation of COC with THC for 25 hours resulted in increased levels of promature GDF9 compared to the control. Mature GDF9 was only detected in the first replicate experiments however these preliminary results suggest that mature GDF9 was reduced after a 1 hour exposure to THC. Taken together, these results demonstrate that long exposures to THC may influence oocyte-secreted factor production, and thus oocyte-mediated CC functions such as proliferation or metabolism.

In general, these protein expression studies gave less reliable results than the gene expression or GJ communication studies presented in Chapters 6, 7, and 8. Therefore, confirmation of these preliminary results by additional replicate experiments is necessary. The variation observed in the Western blot results is likely attributable to the Western blotting technique used and that only two replicate experiments were carried out due to challenge in undertaking several replicate experiments. Small differences in Western transfer efficiency, primary and/or secondary antibody concentrations, and developing reagents can result in large differences in band density. A commonly used approach to compensate for differences in protein loading is to correct band densities for a reference protein such as GADPH (Ratchford et al., 2008; Wang et al., 2009). In this study the blots

were stripped and re-probed three times (i.e. for CX43, GDF9, and the two pre-absorbed antibody controls). As repeated stripping of the blot can remove protein in addition to the bound antibodies, a reference protein was omitted from this study and instead the total protein content was determined using the BCA assay. Moreover, a constant number of COC (i.e. 200) were included in each group. However, these methods are limited in that they do not account for loading error or differences in protein transfer (Aldridge et al., 2008).

The total protein content of each sample was expected to be influenced mainly by the number of CC. Therefore, the levels of CC-derived CX43 protein was corrected for total protein amount. The amount of protein calculated in each of the samples was extremely variable between experiments, despite using the same number of COC in each. An adapted BCA assay protocol was utilised for some experiments, where the plates were incubated at 60 °C rather than 37 °C. This provided much greater sensitivity and allowed for a more accurate detection of protein content however this may have contributed to the variability seen between experiments. Since the total protein content was used relative to the other samples in each experiment, and was not used to compare samples across experiments, this should not have affected interpretation of the results. In contrast to the protein levels for CX43, those for GDF9 were not corrected for total protein as the relative band densities were produced by a known number of oocytes.

The CX43-specific antibody, MAB3067, used in this study was relatively specific. However, additional, unidentified 24 and 68 kDa bands were seen in some experiments that were not apparent when the antibody was pre-absorbed with the control peptide. In some experiments, differentially phosphorylated forms of CX43 were identified as doublet bands (Solan and Lampe, 2009) however these doublet bands were not always resolved. Preliminary testing of an antibody specific for CX43 phosphorylated on serine residues 279/282 (sc12900; Santa Cruz Biotechnology, USA) was undertaken during these studies. Phosphorylation of these residues is involved in the MAPK-dependent reduction in GJ communication following the LH surge (Norris et al., 2008). Furthermore, an antibody specific to CX37 was also tested for the measurement of CX37 in rat COC (CX37B12-A; Alpha Diagnostic, USA). However, the levels of CX37 and these phosphorylated forms of CX43 were extremely low in rat COC. Hence given the challenge in generating large numbers of COC for

protein measurements it was considered to be impractical to include these proteins in the current study.

The GDF9-specific antibody, MAB47B, used in this study was produced by Oxford Brooks University for AgResearch. A large number of bands were detected using this antibody. It is likely that the band or set of bands around 49 kDa in size represent uncleaved promature GDF9 while the 20 kDa band likely represents mature GDF9 (Lin et al., 2012). It is possible that some of the other bands observed were differentially processed forms of GDF9 such as the preproprotein and glycosylated and/or phosphorylated forms. It is important to note that immunoneutralisation of GDF9 using MAB47B blocked the ability of rat oocytes to stimulate rat GC proliferation (Lin et al., 2012), demonstrating that MAB47B recognises bioactive GDF9. When the monoclonal antibody was pre-absorbed with recombinant ovine GDF9, none of the aforementioned bands were detected. This indicated that the antibody is specific for GDF9 and is capable of recognising multiple forms although this test alone does not confirm complete antibody specificity (Swaab et al., 1977). Thus, there is a possibility that some of the bands detected with MAB47B are due to non-specific binding. On the other hand, the antibody was probing both secreted forms of GDF9 and intracellular protein that were being synthesized and processed. Thus, it is perhaps not surprising that multiple forms were present. In this context, a similar caveat can be applied to the measure of the forms of CX43. In the present study, the promature form of GDF9 was in higher abundance compared to mature GDF9, however at the present time the bioactivity of the promature protein form relative to the mature form of GDF9 is unknown. In addition to the GDF9-specific antibody that was used, several BMP15-specific antibodies were tested for application in this study (H-83 and A-20, Santa Cruz Biotechnology; MAB61a, produced by Oxford Brookes University for AgResearch). However, consistent with a previous study also on rat COC (Lin et al., 2012), the levels of BMP15 protein were very low and therefore, measurement of this protein was not undertaken any further. In summary, this chapter demonstrates that short and long term effects of lifestyle factors on CC-derived CX43 protein levels were detected using Western blotting. Levels of CX43 protein following a short-term exposure to caffeine were increased, but to nicotine were reduced. Furthermore, a long-term exposure to nicotine or MDMA increased CX43 protein in rat COC. The use of Western blotting to determine the effects of lifestyle on oocyte-derived GDF9 protein levels was less definitive due to inconsistencies

between the patterns of GDF9 expression in the control groups. Nevertheless, levels of GDF9 were reduced following short-term exposure to BPA and caffeine, but were increased following long-term exposure to BPA, caffeine, MDMA, or THC. It is important to note that these protein expression studies provide preliminary results, due to the limited number of replicates, and that these results need to be confirmed.

10 EFFECTS OF *IN VIVO* EXPOSURE TO MDMA ON COC FUNCTION

10.1 Introduction

In vitro studies are invaluable for testing the effects of specific compounds on isolated cells however these compounds may elicit different effects on the same cells *in vivo*. This difference in responsiveness is due to many variables such as the route of access to the blood stream, half-life of the compound in the circulation, transit efficiency to the target cells and rate of peripheral metabolism. MDMA (ecstasy) is an amphetamine derivative that is primarily used by young people (Schenk, 2009). Acute physiological effects of MDMA include euphoria, increased energy levels, increased heart rate and blood pressure, chills, sweating, tremor, and muscle tension (Easton and Marsden, 2006). While the use of MDMA is typically infrequent, 35% of subjects interviewed in an Australian study had binged on ecstasy in the past 6 months, with the median length of a binge around 3 days (Topp et al., 1999). In humans and rats, first pass metabolism of MDMA includes *N*-demethylation that produces 3,4-methylenedioxyamphetamine (MDA), and demethylation which produces 3,4-dihydroxymethamphetamine (HHMA) (Green et al., 2003). *In vivo*, these products are further processed into 12 additional metabolites which have varying biological activities.

There is little available information regarding the effect of MDMA on fertility and reproductive health in either males or females. Both acute and chronic exposure to MDMA perturbed the gonadal-hypothalamic axis in male rats resulting in reduced levels of hypothalamic GnRH mRNA expression and serum testosterone (Dickerson et al., 2008). Chronic exposure to MDMA *in utero* in rats was found to disrupt the fertility of male offspring, through elevated levels of DNA damage in sperm, increased interstitial oedema, and decreased sperm counts and motility (Barenys et al., 2010). Similarly, adult male rats that were chronically exposed to MDMA also had increased DNA damage in sperm, tubular degeneration, and interstitial oedema in the testes, however sperm motility, morphology and ability to induce pregnancy were unaffected (Barenys et al., 2009). In the current study it was established that *in vitro* exposure of rat COC to MDMA affected CC function through altered levels of *Cx43*, *Cygs*, *Gfpt1*, and *Pfkp* mRNA (Chapter 8) and CX43 protein (Chapter 9).

The aim of this chapter was to investigate the effects of *in vivo* exposure of female rats to MDMA on COC health. Specifically, the levels of CC-derived *Atr*, *Cx43*, *Cygs*, *Gfpt1* and *Pfkp* mRNA, and oocyte-derived *Atr*, *Bmp15*, *Cx37* and *Gdf9* mRNA were measured. In addition, the effects of *in vivo* exposure to MDMA on protein levels of CX43 and GDF9 in rat COC were determined.

10.2 Methods

Pre-pubescent female rats (N=12/group) were administered 5 mg/kg/day of MDMA (treated) or the vehicle (saline; control) alone via ip injection once per day for three days. The rats were habituated in locomotor activity boxes for 30 minutes prior to receiving the injection. After the injections were performed, the rats were returned to the locomotor activity boxes for 60 minutes thereafter. Whilst in the locomotor activity boxes, the ambulatory counts for each rat were measured at 5 minute intervals as described in Section 2.2.1. Approximately 18 hours after the final injection, the rats were sacrificed and the ovaries were collected in sterile PBS. The COC from rats in the same treatment group were extracted and pooled. The COC were subsequently collected into PBS for either qPCR or Western blotting.

The COC that were collected for qPCR were transferred individually into a 96-well plate containing 10 μ L PBS, where the CC were stripped from the oocyte as described previously. The oocyte was washed twice in PBS, before being transferred into 10 μ L PBS. The 96-well plates containing the DO and CC were stored at -80 °C. Total RNA was extracted from the DO and CC, and was synthesised into cDNA. Multiplex TaqMan qPCR was used to measure the mRNA levels of the following candidate genes: *Gdf9*, *Bmp15*, and *Cx37* in oocytes; *Cx43*, *Gfpt1*, and *Pfkp* in CC; and *Atr*, *Cygs*, *Glut1* in oocytes and CC. The reference gene *Rpl19* was included in each reaction set to correct for cDNA template input. A calibrator sample was included in every run to correct for between-run variation. Those samples which had an *Rpl19* C_T value >30 were excluded from the analysis due to insufficient mRNA levels. For each oocyte gene within each sample, the C_T value was corrected for the C_T value of the calibrator sample from the appropriate run. For each CC gene within each sample, the C_T value was corrected for the *Rpl19* C_T value from the same sample. The data was subsequently analysed

by the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001). The data were examined for normality using the D'Agostino & Pearson test and thereafter subjected to Mann Whitney analyses.

The COC that were collected for Western blotting were transferred into a microcentrifuge tube (200 COC/tube) and additional PBS was added to make the volume up to 500 μ L. The COC were centrifuged at 800 x *g* for 10 minutes, the supernatant was removed, and the COC were resuspended in PBS supplemented with protease inhibitors. The samples were stored at -20 °C until further processing.

One-third of the volume of each sample (~66 COC) was analysed for total protein amount using the BCA assay. The proteins in the remaining two-thirds of each sample were separated by size using SDS-PAGE and transferred onto a membrane. Western blotting procedures were then performed to detect and quantify CX43 and GDF9 protein expression. To determine band specificity, the blot was stripped and incubated with either the CX43 or GDF9 antibodies that had been pre-absorbed with the MAB3067 control peptide, or *E. coli* produced recombinant ovine GDF9 respectively. The density of each protein band was analysed using ImageJ software. For the CX43 blots, the density of the 43 kDa band was measured. For the GDF9 blots, the densities of the 49 kDa promature and, when present, the 20 kDa mature bands were measured. When multiple bands were present (e.g. when doublet or triplet bands were present for CX43) the density of all bands was measured. The band densities for CX43 were corrected for the total protein present in the sample, as determined by the BCA assay. The band densities for GDF9 were uncorrected to give total GDF9 produced by a known number of oocytes.

10.3 Results

The effect of administering three doses of 5 mg/kg MDMA, each 24 hours apart, on ambulatory count before and after each injection in female rats is depicted in Figure 10-1. The mean ambulatory count began relatively high in both the treated and control groups during the habituation period, but this declined to baseline levels before injection of MDMA or saline at Time 0. Following MDMA or saline administration, the mean ambulatory counts were initially raised in both groups, but the counts for the control group declined rapidly to baseline levels. In the MDMA-treated group, the mean ambulatory count rose steadily, peaking at 20-25 minutes post injection. The mean ambulatory count began to

decline thereafter, but did not reach baseline levels before the end of the measurement period.

Figure 10-1: Effects of MDMA on ambulatory counts in female rats. The average ambulatory counts before and after the first injection (A), second injection (B), and third injection (C). Values represent the mean $\pm$ SEM from 12 individuals. The control group is represented by the filled circles (●) while the MDMA-treated group is represented by the unfilled triangles (▲).

The effect of administering MDMA or the vehicle alone to female rats on meiotic arrest in COC is depicted in (Table 10-1). There was no difference in the proportion of oocytes that underwent GVBD between the control and MDMA-treated groups.

Table 10-1: Effect of MDMA on the proportion of oocytes with a visible GV in female rats. The table shows the proportion of oocytes with a visible GV (%), with the actual number of oocytes with a GV in brackets. The data (N=12 oocytes/group from 12 rats) were analysed by a Fisher's exact test.

Control	MDMA
83 (10)	92 (11)

The effect of administering MDMA or the vehicle alone to female rats on candidate gene expression in CC and DO is depicted in Figure 10-2. There were no differences in mRNA levels of any of the genes between the control and MDMA-treated groups.

Figure 10-2: The effect of MDMA on candidate gene expression in CC (A) and oocytes (B) in female rats. The control group is represented by the unfilled bars (□), while the MDMA treated group is represented by the hatched bars (▨). Values are shown as means $\pm$ upper/lower limit of linearised C_T values normalised against the mean of the control group. The data were generated from a total of 12 readings from individual oocytes or CC masses in each group. A Mann-Whitney test was performed for each gene.

The effect of administering MDMA or the vehicle alone to female rats on CX43 protein levels in COC is depicted in Figure 10-3. A 43 kDa band was observed in the positive control of both experimental replicates, along with two smaller bands. No bands were observed in the negative controls of either replicate experiment. A 43 kDa band was observed in both of the lanes containing COC lysate, along with one smaller band. The bands in the COC lanes in the second replicate (Figure 10-3B) were overexposed, so a shorter exposure time was undertaken to visualise the bands better (Figure 10-3C). When the CX43 antibody was pre-absorbed with the control peptide, no bands were observed (Figure 10-3C).

Quantified CX43 protein levels in COC of rats administered MDMA or the vehicle alone are depicted in Figure 10-3. A double band of 43 kDa in size is observed. The protein that migrated faster (i.e. the bottom band) is assumed to be the non-phosphorylated form to

which the antibody is produced against, and the slow-migratory band (i.e. top band) which is assumed to be a phosphorylated form. In the first replicate, CX43 protein levels were 5-fold higher in COC of MDMA-treated, compared to control rats. A similar result was observed in the second replicate where CX43 protein levels were 2.7-fold higher in COC of MDMA-treated, compared to control, rats. Overall, CX43 protein levels were increased in COC from MDMA-treated rats, compared to the control (Figure 10-4).

Figure 10-3: The effect of MDMA on CX43 protein levels in COC from female rats. Western blots were performed in duplicate (A and B) to determine the level of CX43 protein in COC lysate. COC were collected from rats injected with either the vehicle control (U), or 5 mg/kg/day MDMA (M). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). The second replicate (B) was developed for a shorter amount of time to allow visualisation of the individual bands (C). A control experiment (D) was also performed where the blot shown in (B) was stripped and re-probed with the CX43-specific antibody that had been pre-absorbed with its control peptide. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay. The relative band densities were corrected for total protein amount, as calculated by the BCA assay.

Figure 10-4: Effect of *in vivo* exposure to MDMA on relative CX43 levels in rat COC. The bars indicate the means, while the dashes represent each individual relative band density measurement.

The effects of administering MDMA or the vehicle alone to female rats on GDF9 protein levels in COC is depicted in Figure 10-5. Multiple bands between 45 and 90 kDa in size were observed in the positive control of both replicates. These included a band at 49 kDa which corresponds to promature GDF9. No bands were observed in the negative control of either replicate. When the GDF9-specific antibody was pre-absorbed with recombinant GDF9, no bands were detected (Figure 10-5C). Multiple bands between 45 and 70 kDa were detected in the lanes containing COC lysate.

In the first replicate (Figure 10-5A), levels of promature GDF9 were similar in COC of MDMA-treated and control rats. Conversely in the second replicate (Figure 10-5B), levels of promature GDF9 were 4.8-fold higher in COC of MDMA-treated rats compared to that in control rats. Mature GDF9 (20 kDa) was not detected in either replicate. Due to the variable response to MDMA between replicate experiments, the effect of MDMA on GDF9 promature levels in female rats observed were equivocal (Figure 10-6). Despite this, it is evident that GDF9 promature levels do not decline in COC following *in vivo* exposure to MDMA in female rats.

A

Uncorrected promature band density	1.0	1.1	-	-
Total protein content	2.5	1.5	-	-

B

Uncorrected promature band density	1.0	4.8	-	-
Total protein content	5.4	4.3	-	-

C

Figure 10-5: Effect of MDMA on GDF9 protein levels in COC from female rats. Western blots were performed in duplicate (A and B) to determine the level of GDF9 protein in COC lysate. COC were collected from rats injected with either the vehicle control (U), or 5 mg/kg/day MDMA (M). Rat ovary lysate was included as a positive control (+) and *E. coli* produced recombinant ovine BMP15 was included as a negative control (-). A control experiment (C) was also performed where the blot shown in (B) was stripped and re-probed with the GDF9-specific antibody that had been pre-absorbed with *E. coli* produced recombinant ovine GDF9. Molecular sizes (kDa) are indicated to the left of each blot. The density of the band of interest (indicated by an arrowhead) was measured and corrected for the control at 1 hour. Total protein (μ g) in each sample was determined using the BCA assay.

Figure 10-6: Effect of *in vivo* exposure to MDMA on relative promature GDF9 levels in rat COC. The bars indicate the means, while the dashes represent each individual relative band density measurement.

10.4 Discussion

This study indicates there is a marked increase in CX43 protein levels in COC of female rats following the administration of MDMA over three consecutive days. This observation is consistent with that observed in the *in vitro* study (Chapter 9) where a 25 hour incubation of rat COC with MDMA resulted in a 1.7-fold increase in CX43 levels, compared to control COC. However in contrast to the effects of MDMA *in vitro*, administration of MDMA to female rats did not affect oocyte meiotic arrest, candidate gene expression in CC or oocytes, or promature GDF9 protein levels in COC.

Behavioural tests were undertaken to ensure a physiologically effective dose of MDMA was administered. Rats that received a dose of 5 mg/kg MDMA were markedly more active compared to rats that received saline. Locomotor activity, as measured by ambulatory counts, is well-known to increase after exposure to MDMA and the effects are dose dependent (Callaway et al., 1990; Brennan and Schenk, 2006). The results in this present study demonstrate that the dose of MDMA administered to rats in this study was consistent with a behaviourally effective dose.

CX43 protein levels were markedly upregulated in COC from MDMA-treated rats, compared to COC from control rats. The differentially phosphorylated forms of CX43 detected in the second, but not the first, replicate, suggest that MDMA did not affect the phosphorylation of

CX43. To the best of my knowledge, there is only one published study investigating the effect of MDMA on CX43 levels. Male rats were administered 20 mg/kg MDMA via ip injection and sacrificed at 24 hours post-MDMA administration (Zhuo et al., 2013). The levels of CX43 as detected using immunohistochemistry were markedly reduced in heart tissue of MDMA-treated, compared to control, rats.

The results presented herein suggest that MDMA increased the number of GJ channels present in the CC and that this is independent of transcription. The increase in CX43 protein may occur via several post-transcriptional or post-translational mechanisms including increased translation of mRNA stores, alternative splicing of the *Cx43* mRNA to variants with increased translation efficiency (Pfeifer et al., 2004), or inhibition of CX43 degradation. As discussed previously (Chapter 6), the implications of increased GJ communication on COC health are largely unknown. Increased nutrient transfer to the oocyte may improve oocyte quality due to the increased availability of substrates for maturation and development. However excessive nutrient availability to the oocyte can also have negative consequences. For example, excessive transfer of glucose and glucose metabolites to the oocyte results in increased utilization of alternate metabolic pathways such as the hexosamine biosynthetic, pentose phosphate or polyol pathways (Sutton-McDowall et al., 2010). While these pathways all have a role in normal oocyte maturation, abnormal upregulation can result in deleterious effects such as aberrant *O*-linked glycosylation, precocious nuclear maturation and altered intracellular redox state (Downs et al., 1998; Brownlee, 2001). In addition, previous studies have found that under hyperglycaemic conditions, increased reactive oxygen species and reduced glutathione concentrations are detected in bovine oocytes (Hashimoto et al., 2000).

For reasons unknown, the GDF9 blots were far less sensitive than those performed for *the in vitro* studies presented in Chapter 9. It is possible that this prevented accurate determination of the levels of promature GDF9 in these samples. Indeed, levels of GDF9 in COC from MDMA-treated rats were very variable compared to COC from the control rats. However, whilst the results presented here do not allow a definitive conclusion on the effect of *in vivo* exposure to MDMA on GDF9 protein levels, it is unlikely that *in vivo* MDMA exposure results in decreased GDF9 promature levels. This observation is supported by the

in vitro studies herein (Chapter 9) where *in vitro* exposure of rat COC to MDMA for 25 hours resulted in increased GDF9 premature protein compared to controls.

The levels of gene expression in CC and DO from rats that received MDMA were not different between treated and control rats for any of the genes tested herein. Furthermore, the proportion of oocytes that were in meiotic arrest (i.e. had a visible GV) was not different between the treated and control rats. These results contradict those observed following *in vitro* exposure of rat COC to MDMA (Chapter 7 and 8) where GVBD was promoted and levels of *Cx43*, *Gfpt1*, *Cycs*, and *Pfklp* mRNA in rat CC were affected. However, differences may exist in the susceptibility of the COC to MDMA between *in vitro* and *in vivo* systems. For example, the location of the COC in the follicular compartment where it is surrounded by both follicular fluid and GC may offer the COC greater protection from MDMA compared to isolated COC in culture. Additionally, MDMA is processed in the body into metabolites, such as MDA or HHMA that may not affect the parameters tested in this study. Another possibility is that any MDMA-mediated effects were attenuated by the time the rats were sacrificed approximately 18 hours post-injection. Studies have shown that a dose of 5 mg/kg MDMA delivered to adult female rats subcutaneously results in maximum plasma concentrations of MDMA (293 ± 50 ng/mL) after 2 hours, while MDA reached maximum concentrations (46 ± 7 ng/mL) after 3 hours (Fonsart et al., 2009). The half-life of both MDMA and MDA in adult female rats when a dose of 1 mg/kg was administered intravenously was 3.1 hours. This result suggests that plasma concentrations of both MDMA and MDA would be very low at 18 hours after administration. However, effects on CX43 protein levels were detected 18 hours after MDMA administration in the current study, and 24 hours after MDMA administration in a previous study (Zhuo et al., 2013).

The dose of 5 mg/kg/day used in this current study is approximately equivalent to that received in one ecstasy tablet (98 mg or 1.4 mg/kg MDMA in humans; McCann and Ricaurte, 2001). Ecstasy is typically consumed orally in humans, however in this study MDMA was administered via ip injection. A comparison between oral, ip, and subcutaneous routes of administration in rats demonstrated that a peak in plasma levels of MDMA occurred between 1-3 hours post administration, and these administration routes were comparable (Green et al., 2009). Differences in MDMA metabolism between rats and humans may complicate comparisons made between studies. For example in humans, MDMA auto-

inhibits its own metabolism resulting in disproportionately large increases in plasma MDMA concentrations following small increases in the dose of MDMA administered (de la Torre et al., 2000). However, auto-inhibition of MDMA metabolism is not observed in rats (Green et al., 2009). In addition, the major MDMA metabolite produced in humans is 3,4-dihydroxymethamphetamine (HHMA) while MDA is a minor metabolite. Conversely in rats, MDA is the major metabolite produced (Easton and Marsden, 2006) and whilst HHMA is detectable in rat urine, it is quickly methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA) in blood (Fonsart et al., 2009). Moreover, different enzymes are involved in the metabolism of MDMA in humans and rats. For example the enzyme involved in the demethylation of MDMA to produce HHMA is CYP2D6 in humans and CYP2D1 in rats (Easton and Marsden, 2006). Therefore, while the results presented in this study demonstrate that *in vivo* exposure to MDMA results in increased CX43 levels in rat COC, this may not translate in humans.

In summary, these results establish that administering 5 mg/kg/day MDMA to adolescent female rats causes a marked increase in the level of CX43 protein in COC, consistent with that of *in vitro* studies. Whilst the response of GDF9 protein levels to MDMA exposure was variable, it is likely not to cause a reduction GDF9 protein levels. Interestingly, despite a definitive increase in CX43 protein levels in COC after *in vivo* MDMA exposure, the proportion of oocytes in meiotic arrest, gene expression levels of CC-derived *Atr*, *Cx43*, *Cycs*, *Gfpt1* and *Pfkp* mRNA and oocyte-derived *Atr*, *Bmp15*, *Cx37* and *Gdf9* mRNA were unchanged.

11 DISCUSSION

One of the major factors for reproductive success is the production of a healthy oocyte by the female for fertilisation. Oocyte maturation is tightly coordinated with follicular cell processes such as proliferation, metabolism, and steroidogenesis. This coordination is achieved, in part, through oocyte-secreted growth factors which modulate the aforementioned CC functions. Moreover, transfer of nutrients and small molecules from the CC to the oocyte via GJs facilitates cytoplasmic maturation while maintaining meiotic arrest. These oocyte growth factor and GJ communication pathways constitute a communication loop which is essential for follicular development. The major objective of this thesis was to investigate the effects of lifestyle factors on pathways involved in the bi-directional communication system of the COC.

11.1 Method optimisation and validation

A key component of this research was to establish, optimise and validate technologies that would enable the testing of environmental contaminants and lifestyle factors on pathways involved in the maintenance of COC communication. Thus, preliminary experiments were performed to: 1) establish a standard protocol to measure GJ communication in rat COC *in vitro*; 2) validate the use of calcein-treated CC and DO for multiplex qPCR; and 3) determine the health of rat COC over 25 hours in culture.

A GJ bioassay first described by Thomas et al. (2004) was modified and optimised to test the effects of specific compounds on the degree of GJ transfer in rat COC in culture. During *in vitro* culture of COC, the intra-oocyte concentration of cAMP is critical for maintaining GJ communication in the COC and meiotic arrest of the oocyte (Wert and Larsen, 1989; Thomas et al., 2002). Elevated intra-oocyte cAMP levels within cultured COC may be attained by the addition of either cAMP stimulators or PDE inhibitors. An important and unexpected finding in this study was that the addition of forskolin, an adenylyl cyclase stimulator, did not influence GJ communication in rat COC. However, inhibition of cAMP breakdown by the addition of PDE inhibitors, specifically a combination of rolipram and dipyridamole, increased GJ communication in rat COC. This suggests a role for PDE7, PDE8, PDE10, and PDE11 in maintenance of the nutrient transfer system in this rodent species. A dose-dependent inhibition of fluorescence transfer to the oocyte occurred when COC were

treated with the GJ uncoupler carbenoxolone. Moreover, oocyte fluorescence was markedly inhibited when COC were treated with carbenoxolone following up to 22 hours in culture. This provides strong evidence that incorporation of calcein in the oocyte occurs predominantly via GJ transfer from the CC. Therefore, the GJ bioassay described herein is a highly specific and robust indicator of GJ activity within rat COC.

The desire to directly compare GJ activity with candidate gene expression led to the validation of a TaqMan multiplex qPCR methodology using cDNA from calcein-treated COC. Baseline fluorescence was not detected in calcein-treated samples by the Corbett Rotor-Gene 6000 machine, and calcein treatment did not affect global gene expression levels, as measured by *Rpl19* mRNA. However, calcein treatment did affect expression levels of some individual genes. Specifically, oocyte-derived *Gdf9* and *Cx37*, and CC-derived *Cyts*, *Gfpt1*, and *Atr* expression levels were all influenced by calcein treatment. Notably, interaction effects were measured for the aforementioned CC genes, indicating an effect of calcein on gene expression over time. It appeared that gene expression levels in those DO and CC that had been incubated for 5 or 13 hours were more sensitive to calcein-mediated changes as excluding these samples from the analysis removed most of the treatment or interaction effects that were measured. However, a treatment effect remained for oocyte-derived *Atr*, and treatment and interaction effects remained for CC-derived *Gfpt1*.

To the best of my knowledge, this study is the first to measure GJ communication and gene expression levels in the same COC. These results show that the effect of calcein should be tested for each candidate gene, as calcein exposure may up or downregulate levels of some genes. Measurement of both GJ communication and gene expression was particularly desirable in this study as it allowed the effects of lifestyle and environmental factors on these pathways to be determined in individual COC. This is important as follicular development is hierarchical resulting in a population of follicles that are functionally distinct despite being morphologically identical. Furthermore, the correlation between GJ communication and gene expression levels may have potentially large implications for determining the genes and pathways that interact with or are affected by GJ activity, although no such correlation was observed in this study. In all experiments where the effects of lifestyle and environmental factors were being tested, both the control and treated COC were exposed to calcein. Therefore, any changes in gene expression levels between the

treated and control groups were a result of the treatment and not a biological effect of calcein exposure. However, the effects of lifestyle and environmental factors on gene expression in COC that were not treated with calcein will need to be confirmed in future experiments.

In order to test the effects of prolonged exposure of rat COC to various compounds *in vitro*, the effects of culturing rat COC under control conditions, in regards to GJ activity, GVBD, and gene and protein expression levels, were characterised (Chapter 5). It was established that some of these parameters were regulated by time in culture. GJ communication was maintained over 49 hours of culture, and moreover was increased at the time points of 5, 19 and 25 hours. Concurrently, the proportion of meiotically-arrested oocytes (i.e. those with a visible GV) decreased over time. These results demonstrate that the reduction in the proportion of meiotically-arrested oocytes, which reached 44 % by 25 hours, was not correlated to a decline in GJ communication. Compared to that after 1 hour in culture, CC-derived CX43 protein levels were markedly reduced at 5 hours, but were increased at 13 and 25 hours. Interestingly, most of the oocyte genes were stable over time, with the exception of *Atr* mRNA levels, which were reduced after 25 hours in culture. Conversely, expression levels of all the CC-derived genes changed over time. Compared to that after 1 hour in culture, the expression levels of CC-derived *Atr* and *Gfpt1* mRNA were higher at 13 hours, while *Cycs* mRNA was reduced at 25 hours. Expression levels of both *Cx43* and *Pfkp* were markedly reduced from 5 hours onwards. However, given that GJ communication was maintained over time as was expression of *Cycs*, which is involved further down the energy production pathway, the findings here reveal that the health of the COC is not necessarily compromised following 25 hours in culture.

Unfortunately, mRNA expression levels of oocyte-derived *Cycs* and oocyte-derived and CC-derived *Glut1* were below detection limits. Therefore, these were unable to be included in the study.

11.2 *In vitro* effects of lifestyle factors

A summary of the *in vitro* effects of lifestyle factors on COC health is presented in Table 11-1. Each of those factors for which a treatment effect on GJ communication was measured (i.e. BPA, caffeine, and nicotine) had the greatest effect after 25 hours exposure. Therefore only

the 1 and 25 hour samples were tested for gene expression levels. This also allowed exclusion of the 5 and 13 hour time points in which gene expression was found to be more sensitive to calcein-AM perturbation.

Table 11-1: Summary of the *in vitro* effects of lifestyle factors on COC function. Arrows indicate that the parameter was up- or down-regulated by treatment. For GJ communication and gene expression results, overall treatment effects ($P < 0.05$) are indicated by arrows alone, while a treatment x time effect ($P < 0.05$) is indicated by an arrow accompanied by the time point at which an effect was noted. For protein expression, arrows indicate a trend of up- or down-regulation at a specific time point (>1.2 -fold change). Dashes indicate that no effect of treatment was observed for that parameter.

	BPA	Caffeine	Ethanol	Nicotine	MDMA	THC
GJ communication	↑	-	-	↑ 25 hours	-	-
Cx43 mRNA	↑ 25 hours	↓	↑ 1 hour	-	↑	↑
CX43 protein	-	↑ 1 hour	-	↓ 1 hour ↑ 25 hours	↑ 25 hours	-
Cx37 mRNA	-	-	-	↑ 1 hour	-	-
GVBD	-	-	↓	-	↑	↑
GDF9 protein	↓ 1 hour ↑ 25 hours	↓ 1 hour ↑ 25 hours	-	-	↑ 25 hours	↑ 25 hours
Gfpt1 mRNA	-	-	-	-	↑	↑
Cyts mRNA	-	-	-	-	↓ 25 hours	-
Pfkp mRNA	-	-	-	-	↑ 1 hour ↓ 25 hours	-

A notable finding of this study was that in general, CC-derived genes, compared to the oocyte-derived genes, were far more sensitive to all the treatments tested herein. The CC are known to protect the oocyte from reactive oxygen species (Tatemoto et al., 2000). The results from this current study support the evidence that CC have a protective role and extend this role to include protection from potentially harmful exogenous factors. Furthermore, expression of the CC-derived gene *Cx43* was significantly more sensitive to exogenous factors than any other gene tested. Indeed, *Cx43* levels were altered by all of the treatments included in this study, although the effect of nicotine was negligible.

The concentrations of lifestyle factors chosen for this study were based upon measurements in biological fluids. Where available, the concentration of the factor in follicular fluid was used as a reference point (i.e. BPA; Ikezuki et al., 2002). In cases where this information was not available, the serum concentration was used as a reference point. In general, the concentrations used in this study were 10-fold greater than that measured in follicular fluid (i.e. BPA) or blood (i.e. caffeine, ethanol, nicotine, MDMA, and THC). The exception to this is caffeine, for which the concentration was 10-fold greater than that measured in serum (Axelrod and Reichenenthal, 1953), but was 150-fold greater than that measured in follicular fluid (Al-Saleh et al., 2010). The reason for this is that the concentration of caffeine was selected prior to this information being published. Selection of high physiological concentrations of lifestyle and environmental factors for the *in vitro* study reduced the probability of false negative results. However, due to not selecting concentrations well above the physiological range, there was increased difficulty in distinguishing real effects from the background variation. Despite this, these results provide convincing evidence that rat COC are sensitive to high physiological concentrations of lifestyle and environmental factors.

A major limitation of this *in vitro* study was that this model only explores the effects of direct exposure of the COC to lifestyle and environmental factors, and does not take into account other mechanisms by which oocyte quality is regulated (such as the hypothalamic-pituitary axis) or the potential protective effects of the follicular environment. Therefore, follow up investigations into the effects of exposure to lifestyle and environmental factors via an *in vivo* route (such as the *in vivo* MDMA experiments presented in this thesis) are necessary.

11.2.1 GJ communication is promoted by BPA, caffeine, and nicotine

GJ communication and CX43 protein levels decreased over time in culture in the control COC. For reasons unknown, this was in contrast to the results in Chapter 5 where oocyte fluorescence was higher at 25 hours in culture, compared to 1 hour. Nonetheless, exposure to BPA and nicotine prevented the reduction in GJ communication over time, with higher fluorescence levels in the treated oocytes than the control oocytes at 25 hours. A similar effect was observed after exposure to caffeine, however this was just under the level of significance ($P=0.052$). Interestingly, GJ communication was not correlated with mRNA levels of either *Cx43* or *Cx37* in individual samples. Furthermore, the pattern of GJ communication was not necessarily correlated with changes in CX43 protein levels in the treated COC. This is consistent with findings from Wang et al. (2009) where only very weak correlations between CX43 protein expression and GJ conductance were observed.

Post-translational modifications and incorporation into GJ plaques are essential for GJ function. In addition, only 2-20 % of CX43 GJs are active within a GJ plaque (Bukauskas et al., 2000). Therefore, it is perhaps unsurprising that GJ communication does not necessarily correlate with mRNA and protein levels of CX43 in this study. Furthermore, it is possible that the presence of oocyte-derived GJs composed of CX37, rather than CC-derived GJs composed of CX43, are the rate-limiting factor for transfer of nutrients into the oocyte. An effort to measure CX37 protein levels was undertaken for this thesis however expression levels were so low that it was not feasible to include it in this study.

Interestingly, *Cx43* mRNA levels were increased together with GJ communication after a 25 hour incubation with BPA. BPA has oestrogenic activity, allowing it to bind to and activate the oestrogen receptor albeit with a lower potency than endogenous oestrogens (vom Saal et al., 2007). Both *Cx43* mRNA and protein levels have been reported to be upregulated in response to oestrogens (Wiesen and Midgley, 1994), therefore these results are consistent with the oestrogenic properties of BPA. These results suggest that BPA promoted GJ communication independently of the number of CC-derived GJs present, because CX43 protein levels were unaffected. This conclusion is similar to that of a previous study that found that BPA inhibited GJ communication in an epithelial cell line independently of mRNA or protein levels of CX43 (Lee and Rhee, 2007). Further investigation into the phosphorylation states and cellular localisation of CX43 or levels of CX37 protein would

provide further information about how BPA prevents the time-dependent decrease in GJ communication. Moreover, determining the level of CX43 protein and GJ communication at later times would reveal whether given time, the increased *Cx43* mRNA seen at 25 hours were translated into CX43 protein and formed functional GJ channels.

Initial exposure to nicotine reduced CX43 protein levels and increased *Cx37* mRNA levels, however GJ communication was unaffected. Furthermore, a slight reduction in *Cx43* levels was observed in the nicotine-treated COC however this did not reach significance. After 25 hours of exposure to nicotine, GJ communication and CX43 protein levels had increased, while *Cx37* mRNA levels were similar to the control. These results suggest that nicotine may prevent the time-dependent decrease in CX43 protein levels, thereby maintaining GJ communication over 25 hours in culture. Given the initial increase in *Cx37* mRNA, the levels of CX37 protein may also have been increased at 25 hours, however this remains to be determined. The finding that CX43 protein levels were differently affected by short and long term incubations with nicotine suggests that this effect may be mediated by a metabolite. Indeed, cotinine, a stable metabolite of nicotine, has been detected in the follicular fluid of smokers (Zenzes and Reed, 1998). However, whether cotinine alters GJ communication of CX43 levels is unknown.

Caffeine initially reduced *Cx43* mRNA and CX43 protein levels, however had no effect on GJ communication. The effect of caffeine on CX43 protein levels was attenuated by 25 hours whereas *Cx43* mRNA levels remained low and GJ communication was slightly increased ($P=0.052$). Therefore, despite the increase in GJ communication at 25 hours in caffeine-treated COC, CX43 protein levels were similar to the control. As with BPA, further investigation into phosphorylation states and cellular localisation of CX43 and levels of CX37 protein may provide insight into how caffeine regulates GJ communication. Indeed, caffeine is a PDE inhibitor and therefore may promote GJ communication through increased cAMP levels in the COC and promotion of PKA-mediated phosphorylation of CX43 (Mehta et al., 1992).

The implication of enhanced GJ communication on COC health is currently unclear. However increased availability of energy sources and other nutrients is likely to improve oocyte health. This is supported by the finding that increased GJ coupling in human CC cells is

correlated to higher pregnancy rates during IVF (Wang et al., 2009). Moreover, maintenance of GJ communication during IVM of bovine COC is known to promote blastocyst formation (Atef et al., 2005). Additionally, molecules that maintain meiotic arrest in the oocyte such as cAMP and cGMP are well documented to be passed through GJs to the oocyte. Human oocytes that were pre-incubated with a PDE3 inhibitor (i.e. maintained in meiotic arrest) during IVM had higher maturation rates after removal of the inhibitor, compared to controls where the inhibitor was absent (Nogueira et al., 2006). No effect of BPA, caffeine, or nicotine on GVBD was noted in this study. However the addition of PDE inhibitors may have reduced the need for GJ transfer of molecules that maintain meiotic arrest.

Conversely, increased GJ communication may be indicative of increased energy requirements to allow the oocyte to carry out RNA surveillance and DNA damage repair in response to environmental stress (Baumann et al., 2007). The findings from this study strongly indicate that BPA and nicotine, and to a lesser extent caffeine, promote the transfer of small molecules, which may include energy substrates, to the oocyte via GJs. The metabolism of glucose via glycolysis occurs in the CC, and subsequently lactate and pyruvate are transferred to the oocyte and undergo metabolism by the TCA cycle and oxidative phosphorylation to produce ATP. Furthermore, by-products of oxidative phosphorylation include reactive oxygen species (Voet and Voet, 2004). Increased glucose concentrations and oxidative phosphorylation are associated with the production of reactive oxygen species in oocytes (Hashimoto et al., 2000), which may result in a reduction in oocyte quality (Agarwal et al., 2003). Moreover, increased transfer of glucose metabolites to the oocyte may result in utilisation of alternate metabolic pathways such as the PPP. Hyperglycaemic conditions result in the activation of the PPP and subsequent precocious oocyte meiotic maturation (Downs et al., 1998). Therefore, increased transfer of PPP substrates to the oocyte may also result in precocious nuclear maturation, however this was not observed in this study. Further investigation into how upregulation of GJ communication affects the precise coordination of oocyte cytoplasmic and nuclear maturation is necessary. Determining whether BPA, caffeine, and nicotine promote the formation of reactive oxygen species and the activity of the PPP would reveal whether these pathways are activated by increased GJ transfer.

Although incubation of COC with ethanol, MDMA, or THC did not affect GJ communication, each of these treatments did affect *Cx43* mRNA levels and/or CX43 protein expression. Exposure of COC to ethanol initially raised mRNA levels of *Cx43*, but this effect was attenuated after 25 hours of exposure. MDMA increased expression levels of *Cx43* mRNA regardless of exposure time, while CX43 protein levels were also increased after a 25 hour exposure. THC increased *Cx43* mRNA levels after 25 hours. These results demonstrate that mRNA and protein levels of CX43 are not good indicators of GJ communication in rat COC.

11.2.2 GDF9 protein levels are altered by BPA, caffeine, MDMA and THC

Exposure to BPA or caffeine for 1 hour caused a reduction in promature GDF9 levels. However, a 25 hour exposure to BPA or caffeine restored promature GDF9 levels to over and above that in the control. Moreover, exposure to MDMA or THC for 25 hours promoted promature GDF9 levels. Interestingly, GDF9 protein levels were not correlated to *Gdf9* mRNA levels, which were unaffected by time or treatments. Detection of the mature form of GDF9 was inconsistent in this study therefore the major conclusions were drawn from levels of promature GDF9. The biological functions of the proregion and promature GDF9 forms are currently unknown. However, mice that were immunised against the GDF9 proregion had smaller litter sizes suggesting a functional role of the proregion in female reproduction (McIntosh et al., 2012). Expression levels of BMP15 were low in rat COC in the current study, and therefore the measurement of BMP15 over a wide range of treatments was not feasible. However, *Gdf9* and *Bmp15* mRNA expression levels are known to be tightly co-regulated in several species including rat (Crawford and McNatty, 2012). In the rat, *Bmp15* mRNA levels were 3.65-fold lower than *Gdf9* mRNA levels, indicating a reduced requirement for BMP15 in rat follicular growth. Indeed, mature BMP15 is not detectable in the rat until just prior to ovulation, consistent with the finding that *Bmp15* KO mice are fertile (Yan et al., 2001; Yoshino et al., 2006). Therefore, although BMP15 protein levels were not measured in this study, they are likely to be altered proportionately with GDF9.

Reduced GDF9 levels are likely to significantly reduce oocyte health. During follicular growth, the oocyte tightly controls CC functions including proliferation, metabolism, apoptosis, luteinisation, and expansion. It is likely that a reduction in GDF9 levels decreases CC proliferation, metabolism, and expansion but increases CC apoptosis and luteinisation. For example, downregulation of GDF9 expression using RNA interference demonstrated that CC

expansion and expression of genes involved in CC expansion (i.e. *Has2* and *Ptgs2*) were significantly inhibited (Gui and Joyce, 2005). Furthermore, polycystic ovarian syndrome, a syndrome characterised by anovulation, polycystic ovaries, and hyperandrogenism, is associated with reduced *Gdf9* expression levels (Teixeira Filho et al., 2002). A complete lack of GDF9 (i.e. in *Gdf9* KO mice) causes follicular growth to arrest at the primary stage (Yan et al., 2001).

Despite an initial reduction following exposure to BPA and caffeine, GDF9 promature levels were higher in the treated COC than the control after 25 hours. This may have been due to the action of metabolites of BPA and caffeine which stimulated GDF9 protein levels. Alternatively, the oocyte may have produced more GDF9 to stimulate CC metabolism, indicating a greater requirement for CC metabolites.

Increased GDF9 protein levels are likely to have a positive effect on follicle growth and maturation. Addition of recombinant GDF9 to is known to promote preantral mouse follicle growth *in vitro* (Hayashi et al., 1999), and to improve oocyte developmental competence (Hussein et al., 2006). Furthermore, higher concentrations of GDF9 were correlated with higher embryo quality during IVF (Gode et al., 2011). This positive effect of GDF9 on follicular development is likely to be mediated through increased CC proliferation and metabolism, and decreased CC steroidogenesis and apoptosis (Gilchrist et al., 2008). Indeed, GDF9 stimulates CC proliferation (Gilchrist et al., 2004b; McNatty et al., 2006), cholesterol metabolism (Su et al., 2008), and amino acid uptake (Eppig et al., 2005). However, aberrant GDF9 production may stimulate these CC functions inappropriately resulting in follicular dysfunction and thus reducing COC health.

There did not appear to be a correlation between GJ communication, *CX43* mRNA, *CX43* protein levels, and GDF9 protein levels. *CX43* is thought to positively regulate TGF β signalling, through competitive interactions with SMAD2/3 on microtubules (Dai et al., 2007). Thus although protein levels of *CX43* and GDF9 were not correlated, it is possible that GDF9 signalling was correlated to *CX43* protein, however this was not investigated.

11.2.3 GVBD is prevented by ethanol but promoted by MDMA and THC

Long exposures to ethanol (i.e. >5 hours) resulted in reduced proportions of oocytes undergoing GVBD. These results suggest that ethanol prevents oocyte meiotic maturation

without inhibiting the transfer of the meiosis-suppressing molecules to the oocyte as GJ communication was unaffected. Artificial maintenance of meiotic arrest allows for cytoplasmic maturation to complete, and promotes oocyte maturation rates during IVM once the inhibitor is removed (Nogueira et al., 2006). Therefore, further investigations into whether oocytes are subsequently able to undergo GVBD when ethanol is removed are needed. Heavy alcohol consumption is currently known to cause anovulation by inhibiting the LH surge. If ethanol completely prevents GVBD in some oocytes, then the results presented here suggest that ethanol may act at both the pituitary and the follicle to prevent ovulation.

MDMA and THC both promoted oocyte GVBD when COC were incubated with these treatments for >5 hours. Furthermore, no additional oocytes appeared to have undergone GVBD at 13 or 25 hours compared to 5 hours, in either the MDMA- or THC-treated groups. This suggests that MDMA and THC stimulated GVBD by 4 hours. Interestingly, at 4 hours MDMA and THC had promoted GVBD in 75 % and 58 % of COC, compared with the control where 17 % had undergone GVBD. This suggests that MDMA has a stronger influence on GVBD than THC. Moreover, these results highlight that the effects of contaminants are not identical for different COC. For example, 17 % of oocytes retained their GV even in the presence of MDMA for 25 hours. Whether these oocytes were meiotically incompetent, or whether they were resistant to MDMA is unknown.

THC has previously been found to promote GVBD in rat COC *in vitro* (Reich et al., 1982). The results from this study suggest that meiotic maturation in oocytes is more sensitive to THC than previously thought as this study used a concentration of THC that was ~30-fold lower than that in the Reich study. Precocious resumption of meiotic maturation is thought to reduce oocyte quality due to inadequate cytoplasmic maturation. *In vivo*, the oocyte gains meiotic competence around the time of follicular antrum formation (Edson et al., 2009) however meiotic arrest must be maintained to allow cytoplasmic maturation such as organelle and cytoskeleton reorganization and storage of mRNAs to take place (Marteil et al., 2009). Therefore, the current study suggests that MDMA and THC may reduce COC health by promoting precocious nuclear maturation.

No correlation between the effects of treatments on GVBD and GJ communication was noted. The transfer of cAMP and cGMP from the CC to the oocyte through GJs is essential for maintenance of oocyte meiotic arrest. However, after the LH surge CX43 is phosphorylated, resulting in closure of the GJs and initiation of GVBD (Norris et al., 2008). However, it is possible that the addition of PDE inhibitors in the current study reduced the need for CC-derived cAMP and cGMP for maintenance of meiotic arrest. Both BPA and nicotine are reported to cause meiotic abnormalities (Mailhes et al., 2000; Liu et al., 2007; Eichenlaub-Ritter et al., 2008). BPA suppresses oocyte meiotic maturation in mice (Eichenlaub-Ritter et al., 2008), while exposure to nicotine promoted meiotic maturation *in vivo*, but not *in vitro* in mice (Mailhes et al., 2000). Moreover, caffeine is a PDE inhibitor that prevents meiotic resumption in a reversible manner by increasing cAMP levels within the oocyte (Jagiello et al., 1972; Kren et al., 2004). Therefore, measuring the effects of those treatments that affected GJ communication (i.e. BPA, caffeine, and nicotine) in the absence of PDE inhibitors may have demonstrated a link between GJ communication and meiotic arrest.

11.2.4 MDMA and THC alter expression of genes involved in glucose metabolism

Initial exposure to MDMA resulted in increased *Gfpt1* and *Pfkp* mRNA levels. Exposure to MDMA for 25 hours resulted in increased *Gfpt1*, but reduced *Cyts* and *Pfkp* mRNA levels. These results suggest that metabolism of glucose via the glycolytic and hexosamine biosynthetic pathways are promoted after a 1 hour exposure to MDMA. However, after 25 hours exposure to MDMA, glycolysis and oxidative phosphorylation were reduced and hexosamine biosynthesis was increased. Oocytes that are undergoing meiosis (i.e. after GVBD and before metaphase II arrest) consume more pyruvate than GV or MII oocytes (Downs et al., 2002). Therefore, the increase in *Pfkp* levels at 1 hour in the MDMA-treated group may correlate with the induction of GVBD and thus an increased requirement for energy. Although expression levels of *Pfkp* were increased at 1 hour, GJ communication was similar to the controls suggesting that transfer of glucose metabolites to the oocyte remained stable. Perhaps by 25 hours, meiosis had progressed such that the need for energy substrates (and *Pfkp* and *Cyts* expression) had abated. The expression levels of *Pfkp* did not correlate to levels of GDF9 in this study. However, GDF9 has not been implicated in the regulation of *Pfkp* in the mouse, with BMP15 and FGF8 reported to be the main regulators of glycolysis (Sugiura et al., 2008).

The promotion of *Gfpt1* levels at both exposure times by MDMA and THC, suggest that glucose metabolism through the hexosamine biosynthetic pathway is upregulated. Interestingly, GDF9 protein levels were also promoted by incubation with MDMA and THC for 25 hours, correlating to the promotion of *Gfpt1* and consistent with the role of GDF9 in promoting CC expansion (Elvin et al., 1999). However, CC expansion was not observed in either the MDMA or THC-treated COC. This may indicate that glucose was used to produce substrates for O-linked glycosylation, rather than hyaluronic acid. Treating porcine and bovine COC with glucosamine, which enters the hexosamine biosynthetic pathway downstream of GFPT1, inhibited embryo development, and this effect was blocked when O-linked glycosylation was inhibited (Schelbach et al., 2010). Therefore, determining whether MDMA and THC promote O-linked glycosylation or hyaluronic acid synthesis would reveal the consequence of upregulation of the hexosamine biosynthetic pathway.

11.3 *In vivo* effects of MDMA

There is currently very little research on the effects of MDMA on female fertility. Given the finding that *in vitro* exposure to MDMA affects several pathways involved in COC function, we chose to investigate the effects of *in vivo* exposure to MDMA on these pathways. *In vivo* exposure to MDMA resulted in upregulation of CX43 protein in rat COC. GJ communication in rat COC exposed to MDMA *in vivo* was not measured, as *in vitro* exposure to MDMA did not affect GJ communication (Chapter 6). However, given the finding that CX43 protein levels in COC were enhanced in rats administered MDMA, further investigation into whether this affects GJ activity is necessary. Interestingly, the findings from the *in vitro* experiments, i.e. MDMA exposure modulates *Cx43*, *Cycs*, *Gfpt1* and *Pfkip* mRNA expression and GDF9 protein levels in rat COC, were not mirrored in the *in vivo* experiment. Furthermore, MDMA promoted GVBD *in vitro*, but did not affect meiotic resumption in oocyte *in vivo*. These differences between the *in vitro* and *in vivo* effects of MDMA on COC health may be due to the delivery of MDMA to the ovary or metabolic processing of MDMA in the circulation. It remains to be determined whether MDMA or its major metabolites are detectable in the follicular fluid. As discussed in Chapter 10, the major metabolic pathways differ between humans and rats. Therefore, future studies may include investigation of the actions of HHMA and MDA on COC health. In addition, prospective studies investigating MDMA usage and infertility in human populations would provide valuable information regarding the influence

of MDMA on female fertility. However, this study provides evidence that MDMA regulates the COC communication network *in vivo*.

11.4 Future directions

Clear differences between the effects of *in vitro* and *in vivo* exposure to MDMA on COC function were observed. These results demonstrate the need for *in vivo* studies to complement *in vitro* studies. The *in vitro* studies from this thesis demonstrate that all of the treatments investigated (i.e. BPA, caffeine, ethanol, nicotine, MDMA, and THC) affect some facet of oocyte function. Therefore, investigation into the effects of *in vivo* exposure to each of these factors would provide further information about their influences on oocyte health. Subsequent mating studies would provide further information about the effect of these lifestyle factors and environmental contaminants on fertility.

The finding that GJ communication is not correlated to CX43 protein or *Cx43* mRNA levels was surprising, although not unprecedented. These findings suggest that other regulatory mechanisms affecting post-translational modifications and/or formation of GJ plaques may play a critical role in regulating function GJ communication. Further investigations into the effects of BPA, caffeine, and nicotine on localisation of CX43 and the phosphorylation states may provide more insight into how these treatments affect overall GJ communication.

In addition, assays investigating oocyte-secreted growth factor actions and glucose metabolic functions would provide further information regarding the effects of treatment on the activity of these pathways. These studies have shown that *Gdf9* mRNA and GDF9 protein levels were also not correlated, perhaps due to several post-translational processing steps. It is possible that the protein data were confounded by measurement mostly of intracellular content rather than the forms secreted into the media. It is noteworthy too that the bioactivity of the promature protein, the major form detected here, is unknown. Therefore, measurements of secreted protein forms and applying appropriate bioassays to assess the bioactivity of GDF9 (e.g. GC proliferation assays) would shed light on how these treatments affect oocyte growth factor function.

The lifestyle factors were tested in isolation in the current study. However, investigation into effects of, and interactions between, multiple factors is a more realistic model. For example,

correlation between the use of tobacco, alcohol, and cannabis is thought to be around 0.3 suggesting a relatively high incidence of co-exposure (Lynskey et al., 1998).

Although these results clearly suggest a role for lifestyle factors in regulating COC function, this study only examined antral follicles (i.e. fully grown oocytes). GJs are expressed in the follicle from the primordial stage (Kidder and Mhawi, 2002), and are positively regulated by FSH levels (Granot and Dekel, 1997). *Cx43* mRNA is abolished after the LH surge, and *CX43* protein levels are reduced. Furthermore, *GDF9* is expressed from the primary stage onwards in rodent follicles (Laitinen et al., 1998). Follicles at different stages of growth may be more sensitive to certain contaminants. For example, radiation therapy primarily targets primordial, rather than growing follicles (Armstrong, 1986). Therefore, investigation into the effects of these lifestyle factors on all stages of follicles will be necessary.

11.5 Conclusion

The major objective of this study was to determine whether exposure to BPA, caffeine, ethanol, nicotine, MDMA, or THC influences the function of rat COC, and in particular the pathways involved in bi-directional communication between the CC and oocyte, that may adversely affect health.

The results presented in this study implicate the bi-directional communication loop and glucose metabolic pathways as potential targets for lifestyle factors. Exposure of COC *in vitro* to each of the contaminants included in this study affected at least one of the parameters tested. Furthermore, this study has identified that *Cx43* mRNA levels are particularly sensitive to contaminants however the levels of *Cx43* mRNA were not an indicator of functional GJ communication in rat COC. The CC appeared to play a protective role, preserving the oocyte from damage caused by harmful factors in the COC microenvironment. The bi-directional communication loop and glucose metabolic pathways appeared to be less sensitive to perturbation by MDMA after *in vivo* exposure, compared to *in vitro* exposure. Therefore, delivery of lifestyle factors in the blood to the ovary, metabolism of these factors, and the containment of the COC within the follicle may be important contributors to the effects of contaminants on COC function.

It is concluded that GJ communication, GDF9 protein levels, expression levels of genes involved in glucose metabolism, and oocyte meiotic arrest are sensitive processes that may be influenced by contaminants within the COC microenvironment.

APPENDIX 1 CARBENOXOLONE ACTION TIME

An experiment was performed to determine how long carbenoxolone takes to inhibit dye transfer in rat COC. Briefly, COC were isolated as described in Sections 2.1.1 and 2.1.2. All steps were performed in the presence of 50 μ M dipyridamole and 50 μ M rolipram. The incubations conditions used were 37 °C, 5 % CO₂, and 96 % humidity. The 0 hour control group was not exposed to carbenoxolone, and was incubated for 0 hours prior to exposure to calcein-AM and further 1 hour incubation as described below. The other groups were incubated in the absence of carbenoxolone for varying times, such that the total incubation time was equivalent to 240 minutes for all groups, prior to being incubated in 1 mM carbenoxolone for 0, 30, 60, 120, 180, or 240 minutes (Table A1-1). Thereafter, the COC were incubated with 2 μ M calcein-AM and 1 mM carbenoxolone for 10 minutes. The COC were transferred into calcein-AM free incubation media containing 1 mM carbenoxolone and incubated for a further hour. The oocyte was denuded and oocyte fluorescence measured as described previously (Section 2.1.3).

Table A1-1: Incubation conditions for determining time required for maximal inhibition of dye transfer by 1 mM carbenoxolone. Note that the total pre-calcein incubation time is identical between all groups.

Group	Initial incubation (min)	Carbenoxolone incubation (min)	Total pre-calcein incubation (min)
0	240	0	240
30	210	30	240
60	180	60	240
120	120	120	240
180	60	180	240
240	0	240	240

Maximal inhibition occurred following a 2 hour exposure to carbenoxolone prior to incubation with calcein-AM ($P < 0.0001$) (Figure A1-1). An exposure time of 2 hours was therefore used to determine the specificity of the GJ bioassay over time (Section 3.3.7). The incubation time of 4 hours resulted in significantly increased ($P < 0.05$; Mann-Whitney test) fluorescent intensity in the control oocytes (no carbenoxolone), compared to that at 0 hours (i.e. note the ~1.5 fold higher value; Figure A1-1).

Figure A1-1: Effect of exposure time to 1 mM carbenoxolone on fluorescent dye transfer from adjacent CC to oocytes after a 4 hour incubation of rat COC. Values are shown as geometric means $\pm$ 95 % confidence intervals of fluorescent ratios normalised against the geometric mean of the T0 control group (not shown, geometric mean=1.0). The data were generated from 2 replicate experiments with a total of 24-32 readings from individual oocytes in each group. The data were analysed by a Kruskal-Wallis test and where significant differences were observed, a Dunn's multiple comparison post hoc test was performed. Groups that do not share a letter are significantly different ($P < 0.05$). The mean $\pm$ SEM fluorescent reading in the T0 control group was 284 ± 31 .

APPENDIX 2 STOCK SOLUTIONS

A2.1 GJ Assay Solutions

Dissection Media

0.1 % Bovine serum albumin	50 mg
100 IU/mL Penstrep	500 µL
15 mM Hepes Buffer	750 µL
Lebowitz media containing phenol red and L-glutamine	43.7 mL
Adjust pH to 7.4. Store at 4 °C.	

Incubation Media

0.3 mg/mL polyvinyl alcohol	15 mg
M199 media containing L-glutamine, 2.2 g/L sodium bicarbonate, Earle's salt (phenol red free)	45 mL
Adjust pH to 7.4. Store at 4 °C.	

Wash Media

0.1 % Bovine serum albumin	50 mg
100 IU/mL Penstrep	500 µL
M199 media containing L-glutamine, 2.2 g/L sodium bicarbonate, Earle's salt (phenol red free)	44.5 mL
Adjust pH to 7.4. Store at 4 °C.	

1 mM calcein-AM solution

Dissolve a 50 µg aliquot in 50 µL of DMSO. Store at -20 °C. Prior to use in the GJ assay, calcein-AM was diluted 1:5 with incubation media to make a 200 µM intermediate dilution. This 100x calcein-AM solution was then added to incubation media, containing the appropriate treatments, at a final concentration of 2 µM.

200x dipyridamole/rolipram working stock

Dilute 1 mg dipyridamole in 99 μ L DMSO

Dilute 1 mg rolipram in 182 μ L DMSO

Mix these solutions in a ratio of 1:1. Store at -20 °C.

25x Protease Inhibitor stock

Resuspend one tablet in 2 mL of dH₂O. Aliquot into smaller volumes and store at -20 °C

PBS

PBS tablets were purchased from Sigma and dissolved in 200 mL dH₂O. PBS was autoclaved and stored at 4 °C.

100x carbenoxolone stock

Carbenoxolone was purchased from Sigma, was dissolved in dH₂O to a concentration of 100 mM, and stored at -20 °C.

A2.2 SDS-PAGE stocks

0.5 M Tris-HCl pH 6.8

Tris base	6.06 g
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Make up to 100 mL with dH₂O. Adjust pH to 6.8. Store at room temperature.

1.5 M Tris-HCl pH 8.8

Tris base	36.34 g
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Make up to 200 mL with dH₂O. Adjust pH to 8.8. Store at room temperature.

10 % (w/v) ammonium persulphate

Ammonium persulphate	1 g
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Make up to 10 mL with dH₂O. Distribute into 1 mL aliquots. Store at -20 °C or at 4 °C for up to 1 month.

10 % SDS

SDS	2 g
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Make up to 20 mL with dH₂O, store at room temperature.

30 % bis-acrylamide mix

The bis-acrylamide mix was purchased from BioRad (Auckland, NZ) and stored at 4 °C.

TEMED

TEMED was purchased from BioRad and stored at room temperature.

13.5 % separating gel

dH ₂ O	2.85 mL
1.5 M Tris-HCl pH 8.8	2.5 mL
10% SDS	100 µL
30 % bis-acrylamide mix	4.5 mL
10 % ammonium persulphate	50 µL
TEMED	5 µL

Set up small and large glass plates in a vertical gel holder. Distribute the separating gel mix into the plates, layer a small layer of dH₂O over the gel, and allow the gel to polymerise.

4 % stacking gel

dH ₂ O	3.05 mL
0.5 M Tris-HCl pH 6.8	1.25 mL
10% SDS	50 µL
30 % bis-acrylamide mix	650 µL
10 % ammonium persulphate	25 µL
TEMED	5 µL

Remove the layer of water from the separating gel. Distribute the stacking gel over the top of the separating gel. Insert a 10 or 15 well comb, and allow the gel to polymerise.

5x sample buffer

0.5 M Tris-HCl pH 6.8	1.0 mL
Glycerol	0.8 mL
10 % SDS	4.6 mL
DTT	80 mg
1.0 % (w/v) bromophenol blue	0.2 mL
dH ₂ O	0.8 mL
β-mercaptoethanol	1.74 mL

Store at -20 °C.

5x SDS-PAGE Running Buffer

Glycine	72 g
Tris base	15 g
SDS	5 g

Make up to 1 L with dH₂O and adjust pH to 8.3, and store at room temperature.

A2.3 Western blotting solutions

10x Tris-glycine stock

Tris base	30.3 g
Glycine	144 g

Make up to 1 L with dH₂O, store at room temperature.

Transfer buffer

10x Tris-glycine stock	200 mL
Methanol	400 mL

Make up to 2 L with dH₂O and store at 4°C.

Ponceau S Stain

Ponceau S	1.0 g
Acetic Acid	2.0 mL

Make up to 200 mL with dH₂O. Store at room temperature.

1 M Tris pH 7.5

Tris base	30.29 g
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Make up to 250 mL with dH₂O.

pH to 7.5

Make up to 250 mL with sterile water and adjust pH to 7.5 Store at room temperature.

TBS

1 M Tris-HCl pH 7.5	20 mL
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NaCl	8.76 g
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Tween20	1 mL
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Make up to 1 L with dH₂O. Store at room temperature.

5 % low-fat milk powder solution

Low-fat milk powder	2.5 g
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TBS	50 mL
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Make up fresh for each experiment. Store at 4 °C.

A2.4 ECL reagents

1 M Tris-HCl pH 8.5

Tris base	30.29 g
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Make up to 250 mL with dH₂O and adjust pH to 8.5. Store at room temperature.

90 mM p-coumaric acid

p-coumaric acid	0.15 g
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Make up to 10 mL with DMSO. Store at -20 °C.

250 mM luminol

Luminol	0.44 g
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Make up to 10 mL with DMSO. Store at -20 °C.

Solution 1

250 mM luminal	100 μ L
90 mM p-coumaric acid	44 μ L
1 M Tris-HCl pH 8.5	1 mL
dH ₂ O	8.85 mL

Solution 2

30 % hydrogen peroxide	6 μ L
1 M Tris-HCl pH 8.5	1 mL
dH ₂ O	9 mL

Prepare Solutions 1 and 2 fresh for each experiment. Mix together just prior to performing Western development.

A2.5 Stripping solutions

62.5 mM Tris pH 6.7

Tris base	1.89 g
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Make up to 250 mL with dH₂O. Adjust pH to 6.7. Store at room temperature

Membrane stripping buffer

62.5 mM Tris pH 6.7	50 mL
SDS	1 g
β -mercaptoethanol	350 μ L

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