# Differential Effects of Specific Phosphodiesterase Isoenzyme Inhibitors On Bovine Oocyte Meiotic Maturation, Gap Junctional Communication, and Developmental Competence

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# Table of Contents

## Abstract

Induction of oocyte maturation *in vivo* is associated with increased follicular cAMP levels, however intra-oocyte cAMP decreases prior to germinal vesicle breakdown (GVBD). The aim of this study was to examine whether the differential regulation of cAMP levels within the oocyte and somatic (cumulus) cell compartments of the follicle regulates bovine oocyte meiotic maturation as a result of the specific cell-type localisation of phosphodiesterase (PDE) isoenzymes. Selective PDE inhibitors were used to modulate cAMP levels in each of the two follicular compartments and to examine their effects on oocyte meiotic maturation.

The type 3 PDE inhibitor, but not the type 4, prevented spontaneous meiotic maturation and elevated intra-oocyte cAMP in cultured oocytes in a dose dependant manner. While the type 4 PDE inhibitor had no effect on the oocyte, it dose dependently elevated mural granulosa and cumulus cell cAMP production. These results indicate that specific PDE isoenzymes are differentially localised within the two compartments of the bovine follicle – the type 3 PDE in the oocyte and the type 4 PDE in the granulosa cells. In addition, results showed oocyte cAMP levels to be primarily regulated in bovine oocytes by its degradation by PDE, whereas granulosa cell cAMP levels are controlled mainly by active adenylate cyclase - with both sources able to participate in oocyte meiotic regulation. This study also demonstrated that FSH, but not forskolin, was able to override the meiotic arrest at the immature GV stage caused by milrinone treatment – suggesting the existence of a form of induced oocyte maturation in the bovine species.

In the growing follicle, communication between the oocyte and its surrounding follicular cells is essential for normal oocyte and follicular development. Gap junction channels metabolically couple the oocyte and the follicular cells to each other, allowing inter-cellular communication and transfer of low molecular weight (<1000 Mr) substrates such as ions, nucleotides, amino acids, metabolites and regulatory molecules between the cells that are important for oocyte growth. Maturation of the fully-grown oocyte in vivo is associated with loss of cumulus cell-oocyte gap junctional communication, preventing entry of meiotic-modulating factors such as cAMP into the oocyte. An assay designed to measure gap junctional communication between the oocyte and its surrounding cumulus cell vestment using the fluorescent dye calcein-AM was developed and validated. In control cumulus-oocyte complexes (COCs), dye transfer from cumulus cells to the oocyte fell progressively from 0 to 9 h of maturation, after which oocyte-CC GJC was completely lost. Loss of gap junctional communication was significantly attenuated ($P<0.05$) by treatment with the oocyte type 3 PDE inhibitor, and also to a lesser extent by the cumulus cell type 4 PDE inhibitor. Importantly, all treatments that prolonged GJC also delayed meiotic resumption, with meiosis generally resuming when fluorescence had fallen to ~40% of initial levels. These results demonstrate that treatments which maintain/elevate cumulus cell and/or oocyte cAMP levels result in prolonged oocyte-cumulus cell communication and delayed meiotic resumption. It was hypothesised that this may have a positive effect on the capacity of an oocyte to undergo cytoplasmic maturation and therefore may improve oocyte developmental potential. Inclusion of isoenzyme-specific PDE inhibitors to bovine oocyte IVM media (containing FSH) was shown to cause a significant delay in the progression of oocyte meiosis to the metaphase II stage, and resulted in a 15% increase in the proportion of cleaved embryos that proceeded to the blastocyst stage of development compared to controls.

Keywords: oocyte, meiotic maturation, meiotic inhibition, cAMP, phosphodiesterase, granulosa cell, gap junction, developmental competence, embryo development.

## Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text. The experiments reported in this thesis were performed by myself and any assistance received from others is specifically pointed out and acknowledged.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Rebecca E. Thomas

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## Publications and Conference Proceedings

### *Publications*

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Rebecca E Thomas, David T Armstrong, Robert B Gilchrist. 'Differential Effects of Specific Phosphodiesterase Isoenzyme Inhibitors On Bovine Oocyte Meiotic Maturation'; Developmental Biology; 2002; 244; 215-225.

II Rebecca E Thomas, David T Armstrong, Robert B Gilchrist. 'Bovine Cumulus Cell-Oocyte Gap Junctional Communication During In Vitro Maturation In Response To Manipulation of Cell-Specific cAMP Levels'; 2003 (Biology of Reproduction – published 20 October, 2003; 10.1095/biolreprod.103.021204).

III Rebecca E Thomas, Jeremy G Thompson, David T Armstrong, Robert B Gilchrist. 'Effect of Specific Phosphodiesterase Isoenzyme Inhibitors During In Vitro Maturation of Bovine Oocytes on Meiotic and Developmental Capacity'; 2004 (Biology of Reproduction - submitted).

### *Conference Proceedings*

Thomas RE, Armstrong DT, Gilchrist RB. 'Bovine Cumulus Cell-Oocyte Gap Junctional Communication During In Vitro Maturation In Response To Manipulation of Cell-Specific cAMP Levels'. ESHRE Oogenesis Workshop 2003, Frascati, Italy.

Rebecca E Thomas, Jeremy G Thompson, David T Armstrong, Robert B Gilchrist. 'Bovine Oocyte-Cumulus Cell Gap Junctional Communication During In Vitro Maturation In Response to Cell-Specific Phosphodiesterase Inhibitors'. Proceedings of the 29th annual conference of the International Embryo Transfer Society; Auckland, New Zealand, 11-15th of January, 2003; Theriogenology; 59(1), Abstract 502.

RE Thomas, JG Thompson, DT Armstrong, RB Gilchrist. 'Manipulation of Bovine Oocyte-Cumulus Cell Gap Junctional Communication During In Vitro Maturation by Modulation Cell-Specific cAMP Levels'; Proceedings of the 33rd annual conference of the Society for Reproductive Biology; Reproduction, Fertility and Development; 14 (Suppl); Abstract 73; 2002; Adelaide, SA.

Rebecca E Thomas, David T Armstrong, Robert B Gilchrist. 'Cell-Specific Phosphodiesterase Inhibitors on Bovine Oocyte-Cumulus Cell Gap Junctional Communication'. North Western Adelaide Health Service Research Day; 2002; Adelaide.

Rebecca E Thomas, David T Armstrong, Robert B Gilchrist. 'Differential Effects of Specific Phosphodiesterase Isoenzyme Inhibitors On Bovine Oocyte Meiotic Maturation'. The 32nd Annual Conference of the Society for Reproductive Biology; 2001; Gold Coast, QLD (Abstract 41).

Armstrong DT, Thomas RE, Gilchrist RB. Bovine oocyte meiotic maturation and granulosa cell cAMP levels are differentially regulated by type-specific phosphodiesterase inhibitors.

The 34th annual meeting of the Society for the Study of Reproduction; Biology of Reproduction; 64 (Suppl 1): 138 [Abstract]; 2001; Ottawa, ON, Canada.

Rebecca E Thomas, David T Armstrong, Robert B Gilchrist. 'Differential Effects of Specific Phosphodiesterase Inhibitors On Bovine Oocyte Meiotic Maturation and Granulosa Cells'. North Western Adelaide Health Service Research Day; 2001; Adelaide (Abstract 36).

## Awards

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| *Australian Postgraduate Award* (Scholarship) | 2000-03 |
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| *North Western Adelaide Health Service* Research Day Prize Finalist | 2002 |
| *Society for Reproductive Biology* Travel Scholarship | 2001 |
| *North Western Adelaide Health Service* Research Day Prize Finalist | 2001 |

## Glossary/Abbreviations

| | |
|---|---|
| AC | adenylate cyclase |
| AM | acetoxy-methyl ester |
| 5'-AMP | adenosine 5'-monophosphate |
| ATP | adenosine triphosphate |
| BSA | bovine serum albumin |
| B-TCM | bicarbonate-buffered tissue culture medium |
| CAM | calcein acetoxy-methyl ester |
| CAM-BTCM | CAM-bicarbonate buffered tissue culture medium |
| cAMP | cyclic adenosine monophosphate |
| cAMP-PKA | cAMP-dependant protein kinase A |
| CDO | cumulus-oocyte complex derived oocyte |
| cGMP | cyclic guanosine monophosphate |
| CM | cilostamide |
| COC | cumulus-oocyte complex |
| dbcAMP | dibutyryl cyclic adenosine monophosphate |
| DMSO | dimethyl-sulphoxide |
| DO | denuded oocyte |
| FAF | fatty acid-free |
| FF-MAS | follicular fluid meiosis activating sterol |
| FK | forskolin |
| FSH | follicle stimulating hormone |
| G2 | growth phase two of the cell cycle |
| GC | granulosa cell |
| GJC | gap junctional communication |
| GV | germinal vesicle |
| GVBD | germinal vesicle breakdown |
| hCG | human chorionic gonadotrophin |
| H-TCM | hepes-buffered tissue culture medium |
| iAC | invasive adenylate cyclase |
| IBMX | 3-isobutyl-1-methylxanthine |
| ICM | inner cell mass |
| IVF | *in vitro* fertilisation |
| IVM | *in vitro* maturation |
| IVP | *in vitro* production (of embryos) |
| LH | luteinising hormone |
| M | meiosis phase of the cell cycle |
| MGC | mural granulosa cell |
| MI | metaphase I |
| MII | metaphase II |
| MR | milrinone |
| PDE | phosphodiesterase |
| PDE3 | phosphodiesterase subtype 3 |
| PDE4 | phosphodiesterase subtype 4 |
| PVA | polyvinyl alcohol |
| rhFSH | recombinant human FSH |
| RIA | radioimmunoassay |
| RP | rolipram |

# Literature Review

# 1. Literature Review

## 1.1 Introduction

### 1.1.1 Oocyte Formation and Growth

Oocytes are terminally differentiated germ cells formed in mammals during early development from oogonia, mitotic cells of the germ line located in the ovary. During oogenesis, developing oocytes accumulate proteins, mRNA, and molecular precursors required for fertilisation and embryonic development. Oocytes enter the initial stages of meiosis during early life, only to become arrested at the dictyate stage of prophase I - also known as the germinal vesicle (GV) stage. This arrest occurs during the first meiotic division at about the time of birth and is maintained until each oocyte is committed to either atresia or ovulation [1].

Surrounded by a single layer of flattened epithelial cells, oocytes within primordial non-growing follicles in the ovary, from which oocytes are recruited throughout reproductive life. An unknown signal triggers a cohort of these primordial follicles to develop into primary, secondary, and eventually pre-ovulatory antral follicles (Figure L1). During this process of follicular development, the immature germinal vesicle stage-arrested oocyte undergoes a growth phase where it acquires the capacity to complete meiosis and support early embryo development (i.e. acquires developmental competence) [1].

Immature germinal vesicle stage oocytes resume meiosis in response to gonadotrophic hormonal stimulation. The majority of vertebrate oocytes are then again arrested at the

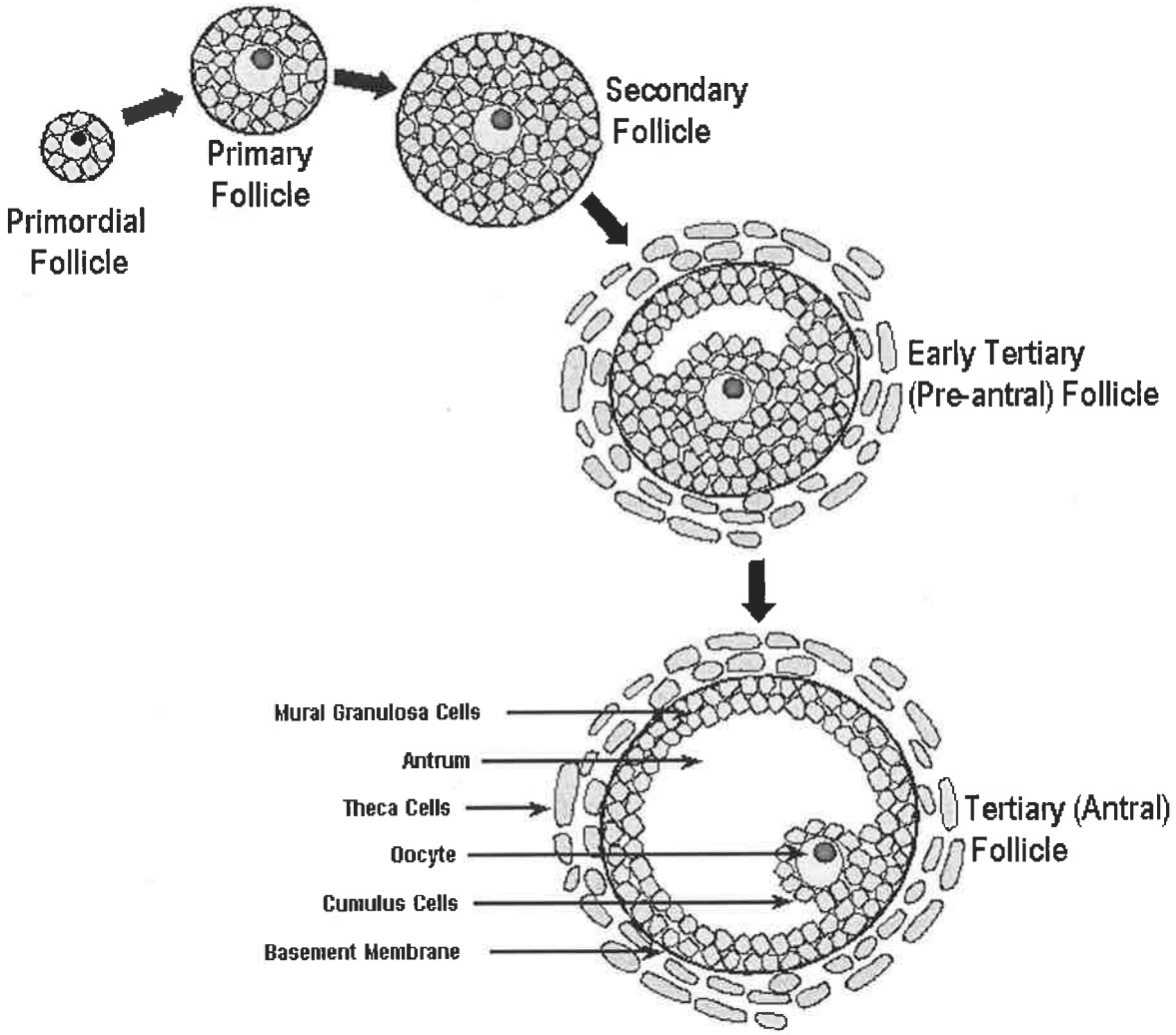


**Figure L1.** Preovulatory follicles consist of a central fluid-filled cavity called the antrum. Layers of cumulus cells surround the oocyte inside the follicle, while the mural granulosa cells surround the central antrum.

metaphase II stage of meiosis. This second meiotic arrest is relieved by fertilisation of the oocyte or by artificial parthenogenetic activation under experimental situations.

The stages of meiosis during spontaneous bovine oocyte maturation were classified according to observations of chromatin spreads (Figure L2) and are similar to observations made by Homa et al. and Motlik et al. [2, 3]. Immature bovine oocytes exhibit a germinal vesicle containing dispersed chromatin (GV stage; Figure L2A). Reinitiation of meiosis is characterised by germinal vesicle breakdown (GVBD), leading to diakinesis, where the chromatin is organised into bivalents. The chromatin begins to contract during late diakinesis to metaphase I (Figure L2B). At metaphase I, the contracted bivalents are closely associated at the spindle equator (Figure L2C). Spindle alignment of homologous chromatid pairs and chromatin separation at anaphase I is shown in Figure L2D. Immediately after separation, the oocyte and polar body chromosomes are similar in appearance, characterising early telophase 1 (Figure L2E). Morphologically, the polar body chromosomes appear to degenerate and are enclosed by the polar body at metaphase II stage (Figure L2F). For simplicity in presenting nuclear maturation data in this study, diakinesis and metaphase I stage oocytes were classed as metaphase I stage, and anaphase, telophase, and metaphase II stage oocytes were classed as metaphase II stage oocytes.

### 1.1.2 The Regulation of Mammalian Oocyte Meiotic Maturation

The exact mechanisms and signals that regulate mammalian oocyte meiotic maturation have not been fully elucidated. In non-atretic follicles in vivo, oocytes remain at the germinal vesicle (GV) stage unless exposed to the pre-ovulatory surge of luteinising hormone (LH)

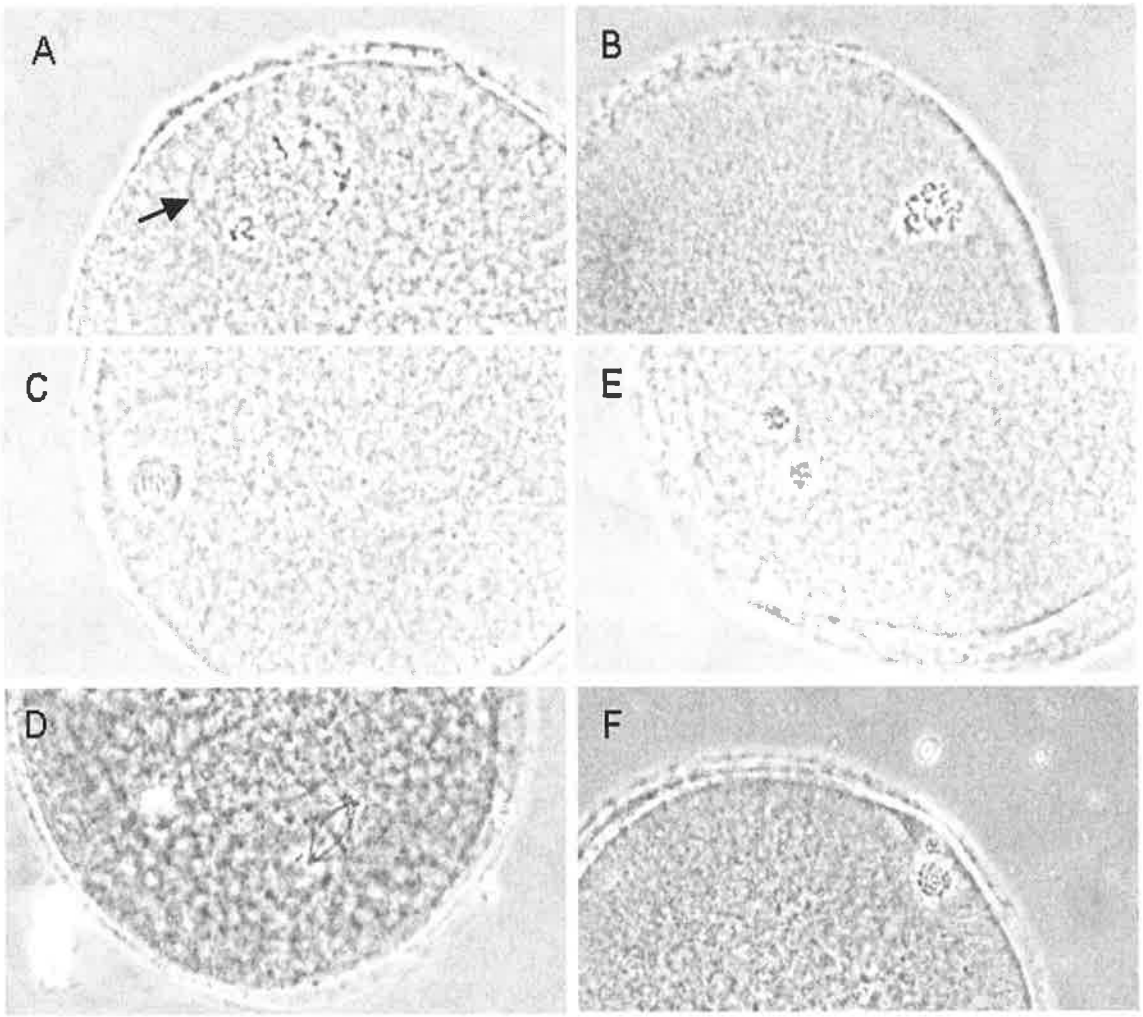


**Figure. L2.** Chronological chromosomal changes during spontaneous maturation of bovine oocytes. All micrographs were originally taken using a 40x objective. a. Germinal vesicle stage, note intact nuclear membrane (arrow). b. Late diakinesis to metaphase I. c. Metaphase I, tetrads are aligned on the spindle. d. anaphase I, some chromatid pairs are at the spindle equator and others at the pole. e. Telophase I, all chromatid pairs are at the spindle pole. f. Metaphase II, the metaphase chromatin is evident as well as a small chromatin-containing polar body.

during each ovarian cycle, however the mechanism by which this stimulation occurs is largely unknown. The oocyte in the dominant follicle(s) then resumes meiosis, undergoing germinal vesicle breakdown (GVBD) and proceeds through metaphase I (MI), only to be subsequently re-arrested at the metaphase II (MII) stage of meiosis (Figure L3). This second arrest is relieved only by fertilisation by a spermatozoon or by artificial parthenogenetic activation of the oocyte.

Alternately, artificial mechanical isolation of the oocyte from an antral follicle induces spontaneous meiotic resumption in vitro without the requirement for gonadotrophic hormonal stimulation, as first observed by Pincus and Enzmann in 1935 [4]. It was this observation, along with experimental results which demonstrated that oocytes cultured inside their own follicle in vitro are maintained in meiotic arrest, that implicate the follicular environment in maintaining the oocyte in meiotic arrest. It is speculated that follicular cells (factors) inhibit oocyte meiosis, probably via the production of inhibitory factors, the suppression of a stimulatory molecule, or by a combination of both [5] - however the mechanism and nature of this inhibition remains undefined.

This review will firstly discuss events occurring and molecules involved in the maintenance of oocyte meiotic arrest. Secondly, the paradoxical actions of cAMP in the control of oocyte meiosis will be discussed and the involvement of phosphodiesterase enzymes in the regulation of mammalian oocyte maturation will be proposed. Finally, the potential involvement of cAMP in cumulus cell-oocyte communication and oocyte developmental potential will be discussed with reference to the use of specific phosphodiesterase isoenzyme inhibitors as tools to reveal a more comprehensive role of cAMP in oocyte meiotic arrest and maturation.

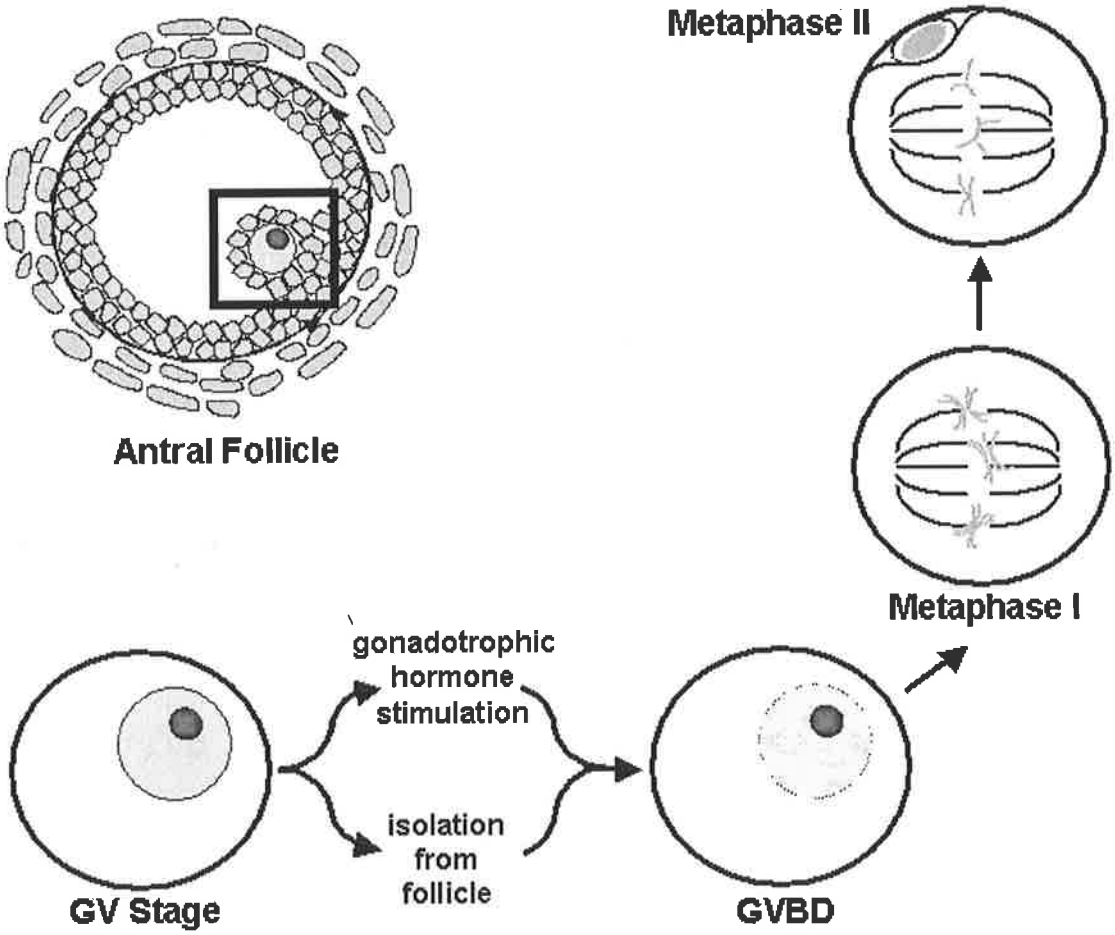


**Figure L3.** Oocytes are arrested at the GV stage and mature in response to gonadotrophic hormone stimulation in vivo, or artificial isolation of the oocyte from its follicle in vitro.

## 1.2 cAMP

### 1.2.1 Introduction

Although the spontaneous resumption of meiosis was observed in mammalian oocytes isolated from follicular cells over sixty years ago [4], research conducted over the past three decades has managed only to propose factors that may be involved in the maintenance of meiotic arrest. The exact means by which follicle-enclosed oocytes are maintained in meiotic arrest has been the subject of much research, however the mechanism and nature of this inhibition remains poorly understood [6].

Intracellular cyclic adenosine monophosphate (cAMP) has been shown to be an important regulator of cell division in a number of cell lines. The importance of cAMP in regulating meiosis during oocyte maturation was first suggested when it was demonstrated that 3-isobutyl-1-methylxanthine (IBMX) and theophylline, which are inhibitors of the cAMP-degrading phosphodiesterase (PDE) enzymes (Figure L4, reaction 5), were able to prevent progesterone-induced maturation in amphibian oocytes [7]. It has been suggested that the arrest of mammalian oocyte meiosis at the germinal vesicle stage *in vivo* may be due to the presence of cAMP in the follicle. The release of the oocyte from this environment (ie. the isolation of the oocyte from the follicle) would therefore result in spontaneous maturation in culture *in vitro* [8, 9]. There is now abundant evidence that cAMP plays an important role in maintaining meiotic arrest in fully-grown mammalian oocytes.

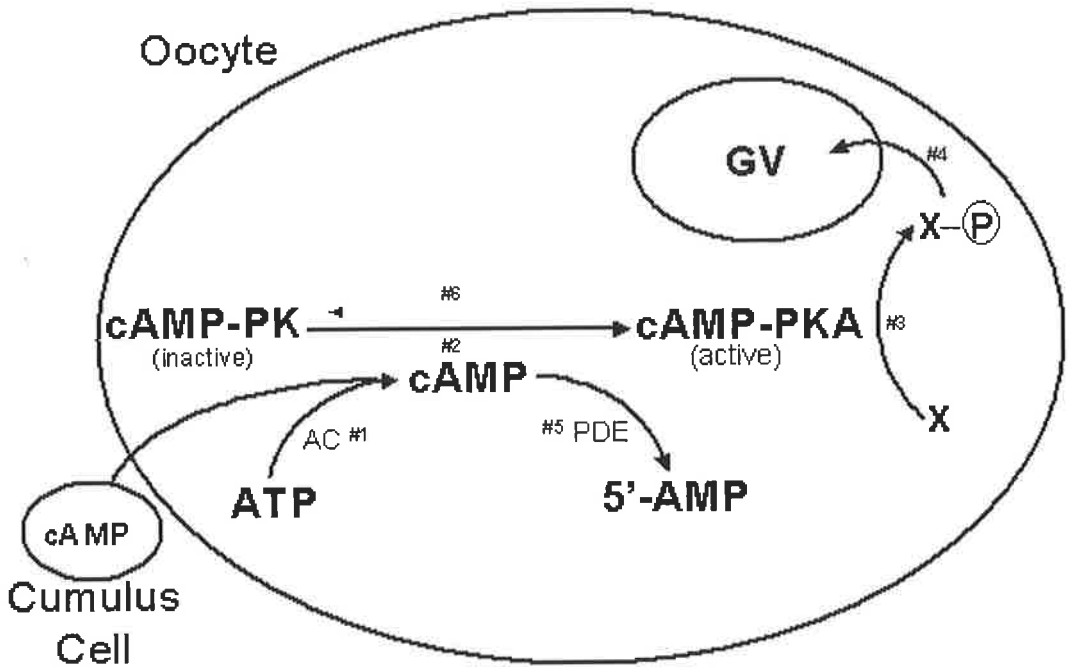


**Figure L4.** Proposed mechanism of cAMP-mediated meiotic arrest. Adapted from [79, 58]. Based on the proposition that high intra-oocyte cAMP maintains meiotic arrest, cAMP is synthesised from adenosine triphosphate (ATP) by the membrane bound adenylate cyclase (AC) enzyme (reaction 1). cAMP then phosphorylates cAMP-dependant protein kinase A (cAMP-PKA), producing its active form (reaction 2). The active cAMP-PKA then causes a cascade of protein kinase A pathway protein phosphorylation (reaction 3). The phosphoprotein(s) (x-P) enable the maintenance of oocyte meiotic arrest (ie. the germinal vesicle stage is maintained) (reaction 4). cAMP-phosphodiesterases (PDEs) cause the degradation of cAMP to adenosine 5'-monophosphate (5'AMP) (reaction 5). The consequent decrease in intra-oocyte cAMP levels due to increased activity of or increased expression of the PDEs leads to a shift in the equilibrium of reaction 2, causing active cAMP-PKA to be de-phosphorylated to its inactive form (reaction 6). This reaction is associated with GVBD and the resumption of oocyte meiosis.

### 1.2.2 Evidence For cAMP: Intra-Oocyte cAMP Measurements

Many studies have been conducted which implicate an elevated level of intra-oocyte cAMP in the maintenance of meiotic arrest. Intra-oocyte cAMP levels in rodent GV-stage oocytes have been shown to be significantly higher when compared to those oocytes which had undergone GVBD both *in vivo* and *in vitro* [10, 11]. Schultz et al. [10] measured a significant drop in cAMP levels that preceded meiotic resumption in the rodent oocyte. Using forskolin, an activator of adenylate cyclase, Yoshimura et al. [12] induced an increase in the level of intra-oocyte cAMP and illustrated a relationship between the abrupt decrease in cAMP levels and frequency of GVBD in rabbit oocytes. Aktas et al. [13] measured cAMP levels in bovine cumulus-oocyte complexes (COCs) in the absence of cAMP-elevators during the course of spontaneous maturation *in vitro*. A subsequent drop in cAMP concentration from 20 fmol to 7.5 fmol was observed fifteen minutes after removal of the complex from the follicle, and this level then remained low for the duration of culture (125 minutes) [13].

### 1.2.3 Artificial Elevation of Intra-Oocyte cAMP

Isolated oocytes from a variety of species have been treated with many chemicals that elevate intracellular levels of cAMP. Cell permeable cAMP analogues such as dibutyryl cAMP (dbcAMP) and 8-bromo-cAMP are able to maintain meiotic arrest in rodent [14-17], porcine [18, 19], bovine [2, 20, 21] and human [22] oocytes [9]. Activators of adenylate cyclase such as cholera toxin, sodium fluoride, prostaglandin E2 and forskolin stimulate the production of cAMP and increase intracellular concentrations [23, 24]. The addition of forskolin to the culture medium of isolated oocytes was found to transiently inhibit the spontaneous maturation of isolated rodent [25-28], rabbit [12], and bovine [2] oocytes by increasing intra-oocyte cAMP as determined by cAMP assay [12, 26]. Aktas et al. [13] showed invasive

adenylate cyclase (iAC) was able to reversibly inhibit the spontaneous maturation of cumulus-enclosed and denuded bovine oocytes due to a subsequent increase in intra-oocyte cAMP. Treatment with all of these cAMP-elevating compounds results in a delay in the spontaneous nuclear maturation of oocytes *in vitro*, indicating cAMP can play a role in the control of meiosis in many species - including the bovine.

### 1.2.4 Follicular Cells

The observation that oocytes isolated from their follicles spontaneously resume meiosis and that explanted follicle-enclosed oocytes are maintained in meiotic arrest led to the understanding that follicular cell factors are capable of inhibiting meiosis (Figure L5a, condition 1 compared with Figure L5b, condition 1). Co-culture of isolated oocytes with monolayers of granulosa or theca cells [29-32] or granulosa cell conditioned medium [33-35] have similarly been shown to have an inhibitory effect on meiosis. The follicular wall is also capable of inhibiting meiosis, with meiotic arrest maintained after follicular hemi-sections or pieces are cultured with or without physical contact with the oocyte [6, 36, 37].

### 1.2.5 Follicular Fluid

Pre-ovulatory follicles contain a large fluid-filled antrum, which has also been implicated to contain components having roles important in maintaining meiotic arrest. Much research has been conducted that examined the ability of follicular fluid, and the components of this fluid to arrest meiosis [30, 33-35]. Preparations of murine and porcine follicular fluid have been found to contain the purines hypoxanthine and adenosine, which are able to maintain mouse

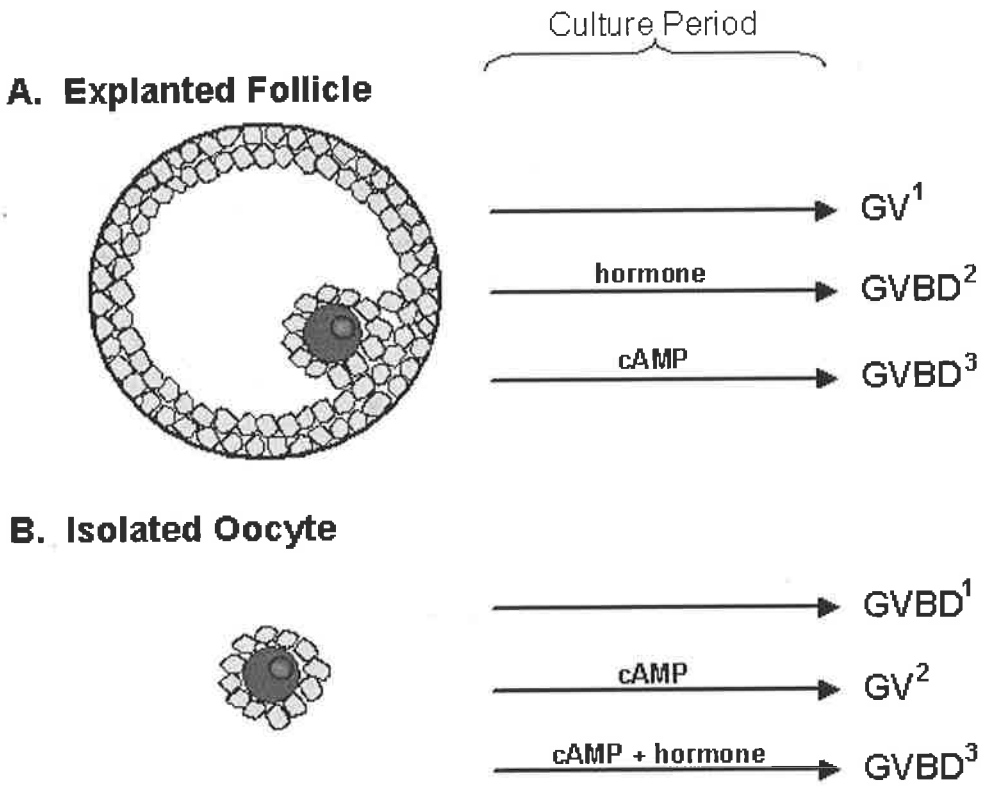


**Figure L5.** Adapted from [42]. *A.* Oocytes contained in explanted preovulatory follicles are maintained in germinal vesicle arrest in the absence of a stimulatory signal, such as gonadotrophic hormones (condition 1). Stimulation with gonadotrophic hormone (condition 2) induces follicle-enclosed oocytes to undergo germinal vesicle breakdown (GVBD). Addition of cAMP to the culture medium induces meiotic resumption (condition 3). *B.* Cumulus cell-enclosed oocytes isolated from their follicles and placed into a standard culture medium undergo spontaneous GVBD without the requirement for hormonal stimulation (condition 1). Addition of cAMP causes the oocyte to be maintained in meiotic arrest (condition 2). This arrest is overcome upon addition of a stimulatory signal, like gonadotrophic hormone, to the medium (condition 3).

oocytes in meiotic arrest [14, 38], even at the relatively low physiological concentrations experienced *in vivo*. It is thought that purines influence oocyte meiotic maturation through the modification of intra-oocyte cAMP levels by their inhibitory action on PDE enzymes [13], however the possibility of effects on other factors can not be ruled out [39]. The inhibitory actions of purines on the oocytes of rat [29], bovine [20, 40] and other species have also been demonstrated. Disruption of specific purine metabolic pathways cause a decrease in intra-oocyte levels of these purines, resulting in the progression of meiosis and oocyte maturation, thus supporting the role for purines in the maintenance of meiotic arrest [41].

### 1.2.6 Phosphodiesterase Inhibitors

The addition of other membrane-permeable PDE inhibitors to the culture medium of isolated mammalian oocytes also prevents spontaneous maturation by increasing intra-oocyte cAMP levels [9]. The non-specific PDE inhibitor IBMX was found to inhibit GVBD of bovine [2, 20, 21] and rodent [14, 17, 28, 39, 42, 43] isolated oocytes in a dose dependent manner. It has been shown that the decrease in intra-COC cAMP concentrations following aspiration from their follicles is prevented by the addition of IBMX or iAC to the collection medium [21, 28]. IBMX is also effective in Xenopus oocytes where it inhibits phosphodiesterase activity and decreases the frequency of oocytes undergoing GVBD in a dose dependent manner [7].

These data support the hypothesis that cAMP-dependent pathways play an important role in controlling meiotic maturation in mammalian oocytes.

## 1.3 The cAMP Paradox

Two major *in vitro* models are used for the investigation of mammalian oocyte meiotic maturation – *in vitro* culture of isolated oocytes (Figure L5b) and *in vitro* culture of follicle-enclosed oocytes (Figure L5a). Both of these methods are now widely employed as tools to investigate oocyte meiotic maturation in most mammalian species, including rabbits, pigs, sheep, cattle and marmoset monkeys.

It was in 1935 that Pincus and Enzmann [4] first demonstrated that rabbit oocytes isolated from their follicles and placed into a standard culture medium underwent spontaneous meiotic maturation from the GV stage to MII, without the requirement for gonadotrophic hormonal stimulation (Figure L5b, condition 1). When cultured in the presence of cAMP or cAMP analogues, these isolated oocytes are maintained at the GV stage and meiosis is arrested (Figure L5b, condition 2). The addition of FSH to the culture medium of these meiotically arrested oocytes overrides the inhibitory effect of cAMP and the oocyte undergoes GVBD and matures (Figure L5b, condition 3).

Follicle-enclosed oocytes *in vivo* remain at the GV stage unless they are exposed to a stimulatory signal, such as the gonadotrophic hormone LH [44]. Tsafriri et al. [44] were the first to explant pre-ovulatory follicles and culture them in a standard medium, where it was discovered the enclosed oocytes were maintained in meiotic arrest (Figure L5a, condition 1). In a similar manner to follicle-enclosed oocytes *in vivo*, supplementation of the culture medium with hormones causes the *in vitro* enclosed oocytes to undergo GVBD and mature (Figure L5a, condition 2) [45]. In contrast to the effect of cAMP on the isolated oocyte, meiotic resumption is induced in follicle-enclosed oocytes following injection of cAMP

analogues in the follicular antrum [44] or exposure of follicles to dbcAMP [46], PDE inhibitors [45] or forskolin [12] (Figure L5a, condition 3).

The results of experiments performed using these two models implicate two conflicting roles for cAMP in oocyte maturation. Results produced using follicle-enclosed oocytes indicate LH-induced oocyte maturation is a cAMP-mediated response, however the continuous presence of cAMP, cAMP analogues, and PDE inhibitors in the culture medium of isolated oocytes prevents their spontaneous maturation *in vitro*. These apparently opposing actions of cAMP present a paradox: a rise in intra-follicular cAMP mediates LH-induced meiotic maturation, however a rise in intra-oocyte cAMP maintains oocyte meiosis in arrest. Several hypotheses to explain this paradox have recently been put forward which propose cAMP is compartmentalised within the follicle and is differentially regulated within the germ cell (oocyte) and the somatic (cumulus) cell compartments.

## 1.4 Phosphodiesterases

### 1.4.1 Introduction

A steady intracellular level of cAMP is maintained by a balance between cAMP synthesis by adenylate cyclase (Figure L4, reaction 1), degradation by phosphodiesterases (Figure L4, reaction 5) [47], and extrusion from the cell [48]. In most systems however, extrusion of cAMP accounts for only a minor proportion of its disposal from cells - Brunton et al. [48] estimated only 20% of all cAMP removal, indicating that phosphodiesterases play a significant role in cAMP degradation.

cAMP, along with cyclic guanosine monophosphate (cGMP), are critical intracellular messengers which mediate a wide variety of biological responses in many different systems and tissues [49]. These second messengers initiate a cascade of intracellular events according to the specific extracellular signals applied to that cell, so it is vital that the intracellular levels of these cyclic nucleotides are tightly controlled by regulating both their synthesis and degradation [49]. 3'5'-cyclic nucleotide phosphodiesterases are responsible for the hydrolysis of these second messengers to their 5'AMP and 5'GMP forms, which are unable to activate the cAMP and cGMP dependent protein kinases. Activation of PDEs serves as a means of rapid feedback regulation of intracellular cAMP concentrations, allowing PDEs to play an important role in the regulation of intracellular concentrations of cAMP, and therefore biological responses to this signal transduction molecule [50-52].

### 1.4.2 Hormonal Regulation of cAMP PDE Activity

A dramatic surge of LH and FSH occurs during the pre-ovulatory stage of the mammalian ovarian cycle and it is thought that these gonadotrophin surges are the inducers of oocyte maturation. It is widely accepted that FSH regulates granulosa cells by regulation of cAMP-dependent events through its stimulatory action on adenylate cyclase and subsequent cAMP synthesis [53]. These polypeptide hormones induce the resumption of meiosis in follicle-enclosed oocytes *in vitro* [54] and transiently delay the timing of spontaneous maturation of isolated oocytes in association with a temporary rise in cAMP [55, 56]. Salustri et al. [56] demonstrated that FSH-treatment of mouse COCs resulted in both the transient inhibition of spontaneous maturation of the oocyte, and an increase in the intra-oocyte concentration of cAMP, while cAMP levels in DOs remained unaffected by the treatment. Schultz et al. [57] failed to detect significantly enhanced levels of cAMP in mouse oocytes derived from FSH-

treated COCs, however an increase was later observed [58], suggesting the initial failure was attributable to incubation conditions which were not totally inhibitory of endogenous PDE activity, thereby allowing any cAMP which was transferred from the cumulus vestment to be hydrolysed immediately. Evidence that FSH acts via cAMP-dependent events also exists due to the ability of cAMP to mimic the actions of FSH when applied to granulosa cells [53]. In addition, the maintenance of meiotic arrest of isolated rodent oocytes by the presence of cAMP analogues and phosphodiesterase inhibitors *in vitro* is relieved by LH or FSH [39, 42].

Conti et al. [53] studied the *in vitro* effect of gonadotrophins on PDEs in cultured rat granulosa cells. FSH markedly increased cAMP-PDE activity, but not cGMP-PDE activity, in a dose dependent manner when applied to the culture medium, as did treatment with dbcAMP. As anticipated, IBMX + FSH treatment led to a decreased granulosa cell PDE activity compared with that of FSH treatment alone [53]. Hormonal regulation of ovarian PDE was also reported by Schmidtke et al. [59], indicating stimulation of cAMP-PDEs by FSH may represent a mechanism for the regulation of granulosa cell responsiveness to gonadotrophic stimulation.

Therefore, not only are intracellular cAMP levels tightly regulated by phosphodiesterases, the regulation of the phosphodiesterase enzymes themselves is also an important factor determining cAMP levels and the consequent cell processes that follow.

### 1.4.3 Phosphodiesterase Isoenzymes

Following the discovery of cAMP, the diversity between phosphodiesterases became well recognised by the separation and purification of various tissue-distinct types [60]. Since this

time, it has been well demonstrated that mammalian PDEs constitute a large family of isoenzymes, which currently consist of ten distinct gene types, PDE1-10 [47, 61]. All of the phosphodiesterases have a similar structural layout, with a conserved catalytic domain in the C-terminal half and a distinct N-terminal regulatory domain containing the structural elements conferring the specific regulatory characteristics of the different PDE families. For example, the calmodulin-binding domain in PDE1, two cyclic nucleotide binding regions in PDE2, and membrane-targeting domains in PDE4 [47]. Isoenzyme families are defined according to amino acid sequence, physical properties, kinetic characteristics, substrate specificity, and regulation [52]. PDE isoenzymes are differentially expressed and regulated in different cellular and tissue locations, and in addition there are isoforms within a particular PDE isoenzyme family that also localise to different tissues. It must be said that while these variants are currently recognised as 'isoforms' of a particular isoenzyme, further research and clarification may find them to be differently charged isomers or proteolytic by-products of the same enzyme [62]. Studies examining PDE sub-cellular distribution also suggest there are marked differences in the relative phosphodiesterase activity levels and distribution in membrane-bound and cytosolic fractions from different tissues [47].

### 1.4.4 Differential Localisation of Phosphodiesterase Isoenzymes

Of significant relevance to this thesis is the fact that both the type 3 and type 4 PDE isoenzymes have been localised to the rodent ovarian follicle [42, 63]. Although PDE3 and PDE4 isoenzymes have high activity and affinity for cAMP, suggesting both would use a similar range of substrate concentrations, their effects on tissue function are varied. PDE3 isoenzymes characteristically have a high affinity for both cAMP and cGMP [47], although it is usually greater for cAMP than for cGMP [64]. Type 3 PDEs are inhibited by cGMP and it

is proposed that agents responsible for increasing cGMP in certain cells may exert their effects through the resulting intracellular increase in cAMP due to PDE3 inhibition [64]. Two PDE3 isoforms exist, PDE3A and PDE3B, which are products of distinct but related genes and are differentially expressed and regulated in a variety of cells and tissues [47]. *In situ* hybridisation has confirmed the presence of the PDE3A isoform in rat adipose tissue, platelets, and hepatocytes throughout development, while the PDE3B isoform was localised only to heart, bronchial, genitourinary, and gastrointestinal smooth muscle [63]. PDE4 isoforms are a cAMP-specific PDE isoenzyme family and are characterised by their high affinity for cAMP. In contrast to the PDE3 isoenzymes, cGMP has no inhibitory affect on the PDE4 family. Presently, four PDE4 isoforms have been identified (PDE4A-D) in both rats and humans [50].

### 1.4.5 Follicular Localisation of Phosphodiesterase Isoenzymes 3 and 4

The differential roles of specific PDE isoenzymes and the isoforms within these isoenzyme families were confirmed upon their localisation in rodent follicles. Tsafriri et al. [42] used PCR and *in situ* hybridisation to confirm the presence and the precise localisation of the different isoenzymes within the somatic and germ cell compartments of the ovarian follicle (Figure L6).

A PDE3A-specific probe localised only to the oocyte and was not found in the granulosa cells [42, 63], while a PDE3B-specific probe produced no specific signal at all [42]. PDE3A has also been found in human and mouse oocytes by RT-PCR analysis [126]. In contrast, PDE4D-specific probes hybridised only to mural granulosa cells, whereas the PDE4B probes

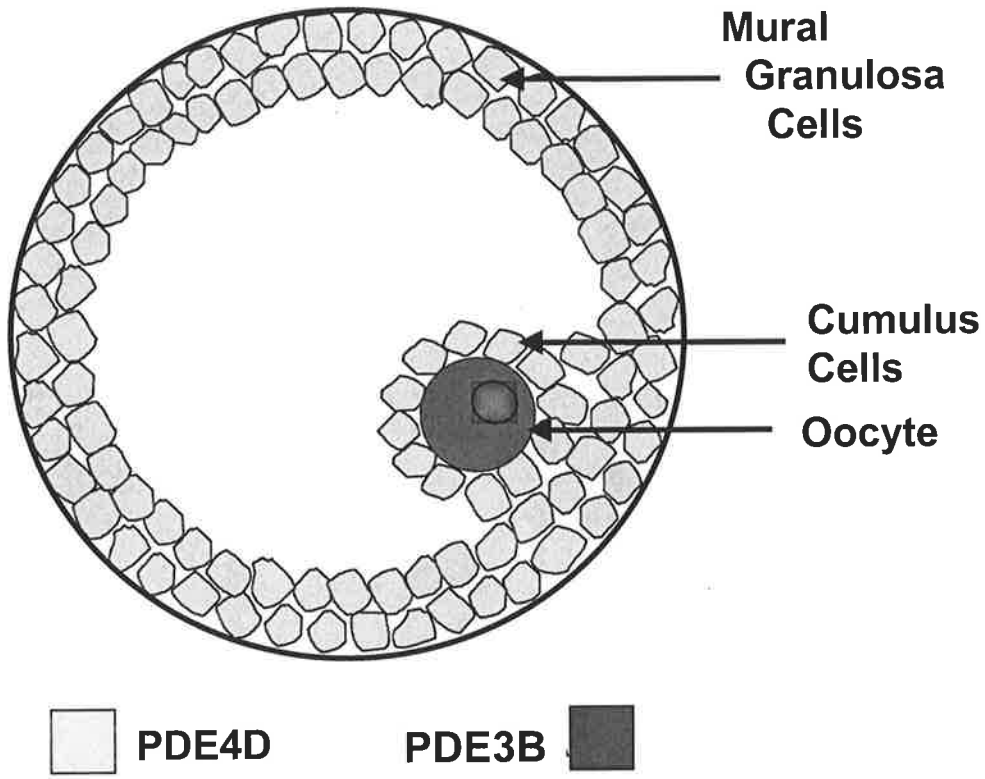


**Figure L6.** *In situ* hybridisation experiments performed in the rat [6, 70] show that the oocyte contains a different PDE isoenzyme compared with that in the cumulus and mural granulosa cells. PDE type 3B were localised inside the oocyte only. PDE type 4D hybridise to the cumulus and mural granulosa cells, with no signal being produced inside the oocyte.

localised only to theca and interstitial tissue [42, 63]. It is of significance to note that the PDE4 probes produced no signal in the oocytes of the rat follicles [42].

## 1.5 Phosphodiesterase Inhibitors

### 1.5.1 Specific Phosphodiesterase Inhibitors

Non-specific PDE inhibitors such as IBMX and hypoxanthine have been used extensively to demonstrate the potency of follicular PDE inhibition in preventing oocyte maturation. Since the initial discovery of the ability of methylxanthines to inhibit phosphodiesterase activity, significant advances have been made in increasing our understanding of the structural requirements of phosphodiesterase inhibitors. The diversity among PDEs has stimulated the development of clinical drugs specifically targeted toward particular PDE isoenzyme families, such as milrinone and cilostamide which specifically inhibit PDE3 isoenzymes [39, 42, 47, 65-69], and rolipram which specifically inhibits those enzymes of the PDE4 family [42, 50, 69, 70]. These isoenzyme-specific inhibitors have and continue to serve as experimental tools to demonstrate the functions of different PDE subtypes in specific cells. For example, the widespread use of the non-specific PDE inhibitor IBMX to examine the functional significance of phosphodiesterase inhibition in the ovary is limited by its pharmacological effects on the many PDE isoenzymes, as well as it's probable side effects on adenosine receptors and $Ca^{2+}$ concentrations [23]. In contrast, the use of isoenzyme-specific inhibitors allows a more precise assessment of the relative importance of different isoenzymes on cyclic nucleotide hydrolysis in the various tissues to which they are localised.

Currently, specific phosphodiesterase inhibitors have allowed the development of specific therapeutic agents that are able to interrupt or change cyclic nucleotide pathways in disease states, without inducing side effects often seen with non-specific PDE inhibitors. PDE3 isoenzymes are the most extensively studied so far, with inhibitors of this family having structural similarity to its natural substrate, cAMP [62]. Milrinone and its analogues are currently used in a clinical situation for the treatment of cardiac failure [71] by improving myocardial contractility due to increased cellular cAMP levels, and therefore $Ca^{2+}$ entry into the cell [65]. Other PDE3 isoenzyme inhibitor effects include smooth muscle relaxation, decreased platelet aggregation, thromboxane synthesis and adherence and phagocytosis of leucocytes, though these have not been therapeutically investigated [47, 65]. Clinical applications of the PDE4 isoenzyme inhibitor rolipram include treatment of atopic and inflammatory diseases, airway smooth muscle relaxation, gastric secretion, antidepressant effects, as well as the suppression of neutrophil degranulation and eosinophil superoxide formation [52].

Though milrinone and rolipram specifically inhibit the PDE3 and PDE4 isoenzyme families respectively, it can be seen that their effects when applied clinically are widespread. This said, the development of inhibitors that act specifically against isoforms *within* a particular phosphodiesterase isoenzyme family (eg. PDE3A vs. PDE3B) may reduce 'side effects', allowing a more accurate picture of the exact role of a particular isoform of isoenzyme family. It must also be noted that many experiments examining the functional activity of PDE inhibitors have shown 'non-selective' doses - where the use of inhibitors selective for PDE3, like other isoenzyme-selective inhibitors, show additional inhibitory activities at higher concentrations which may also contribute to their pharmacological effects (eg. at very high concentrations an inhibitor selective for PDE3 isoenzymes may also inhibit enzymes from

other isoenzyme families, therefore exhibiting pharmacological effects that are not directly attributable to PDE3 isoenzymes).

### 1.5.2 Experimental Use of Phosphodiesterase Inhibitors

PDE inhibitors that inhibit specific PDE isoenzymes are a relatively new phenomenon, with their use so far having been relatively restricted - especially when applied to oocyte and follicular maturation. There are an increasing number of studies using type-specific PDE inhibitors to study the regulation of oocyte and follicular cell function, firstly in Xenopus [72], and recently in rodent [42, 39, 129, 127], bovine [69, 130], macaque [128] and human [126] oocytes.

The results of a pioneering study by Tsafriri *et al.* [42] are summarised in Figure L7. This study blocked oocyte GVBD by the addition of PDE3 specific inhibitors milrinone and cilostamide to the culture medium of isolated rat oocytes (Figure L7b, condition 2). In contrast, treatment with a PDE4 specific inhibitor failed to block spontaneous maturation (Figure L7b, condition 3). The addition of PDE3 inhibitors to cultured explanted follicles prevented the induction of oocyte maturation by LH (Figure L7a, condition 3). Addition of the PDE4 inhibitor rolipram to the culture medium failed to block the action of LH, and instead mimicked its actions by promoting oocyte maturation inside the explanted follicle (Figure L7a, condition 4). These results indicate the PDE4 inhibitor acts mainly on the granulosa cells, inhibiting the degradation of cAMP in this compartment, and thereby enhances the LH induction of oocyte maturation in follicle-enclosed oocytes [42]. In contrast, the PDE3 inhibitor probably acts directly on the oocyte, suppressing LH-induction of GVBD

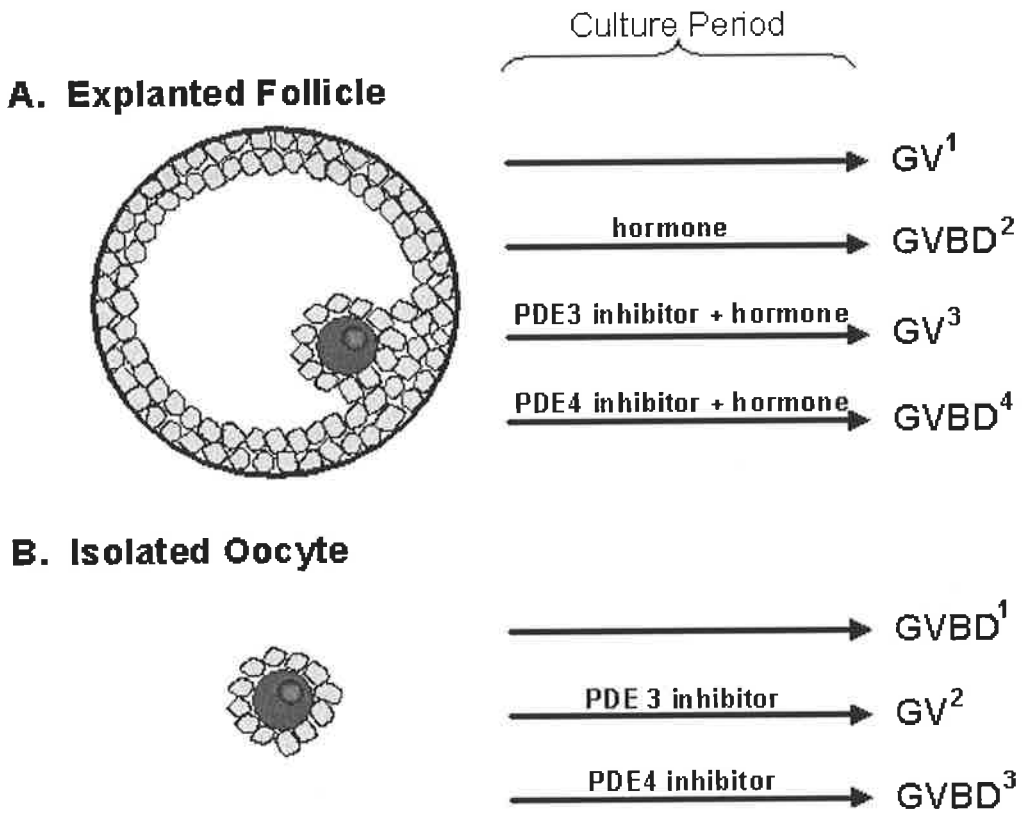


**Figure L7.** Adapted from [42]. **A.** Oocytes contained in explanted pre-ovulatory follicles are maintained in germinal vesicle arrest in the absence of a stimulatory signal, such as gonadotrophic hormones (condition 1). Stimulation with hormone (condition 2) induces follicle-enclosed oocytes to undergo germinal vesicle breakdown (GVBD). Addition of phosphodiesterase (PDE) type 3 inhibitors to the culture medium prevents the action of the hormone, maintaining the oocytes in meiotic arrest (condition 3). Addition of the PDE type 4 inhibitor to the medium does not disrupt the action of the hormone, and instead mimics its action by promoting oocyte maturation (condition 4). **B.** Cumulus cell-enclosed oocytes isolated from their follicles and placed into a standard culture medium undergo spontaneous GVBD without the requirement for hormonal stimulation (condition 1). Addition of PDE type 3 inhibitors to the medium maintains these isolated oocytes in meiotic arrest (condition 2). The addition of PDE type 4 inhibitors to the medium of isolated oocytes has no effect on the oocyte and GVBD occurs (condition 3).

in rat follicle-enclosed oocytes by increasing cAMP concentrations in this compartment [42]. For the first time, this study provided some insight into the paradoxical role of cAMP in mammalian oocyte maturation.

Based on the observation from Tsafriri et al. [42] that specific PDE isoenzymes are compartmentalised in the follicular somatic and germ cells, Wiersma et al [39] used PDE3 inhibitors to block oocyte meiosis in ovulating rodents *in vivo*. Meiotic maturation of ovulated oocytes was blocked by the PDE3 inhibitor at a 100 fold lower dose than that of the non-specific PDE inhibitors IBMX and hypoxanthine. PDE4 inhibitors were found to be ineffective in preventing meiotic resumption, except at doses 500 times above the effective dose of the PDE3 inhibitors - which was attributed to a non-specific action on the PDE3 isoenzymes of the oocyte. Not only did the PDE3 inhibitor block maturation *in vivo*, but ovulation of these oocytes proceeded normally, resulting in the extrusion of immature GV stage oocytes from their follicles.

Prior to this study [39], the complete separation of oocyte maturation from ovulation in cycling rats resulting in the extrusion of un-fertilisable immature oocytes from follicles had not been previously reported. This has significant implications, not only for our understanding of ovarian functions, but also for the development of a novel contraceptive. Wiersma et al. [39] claimed that treatment with the PDE3 inhibitor had no effect on oestrous cyclicity, however it is not made clear whether the rats used to test cyclicity were induced to ovulate or not. If the former case were true, the authors cannot positively state that oestrous cyclicity is maintained, and cycling rats with natural cycles should be used and monitored. Nevertheless, the use of selective PDE inhibitors has allowed investigation of the paradoxical role of cAMP in the somatic and germ cell compartments of the rodent follicle [42]. The differential

regulation of PDE subtypes was implicated in selectively controlling cAMP levels in the two compartments. An extension of the studies conducted by both Wiersma et al. [39] and Tsafriri et al. [42] could be to measure the cAMP content of the PDE inhibitor-treated oocytes - it is this aspect of oocyte phosphodiesterase inhibition which has not yet been investigated.

More recently, the spontaneous maturation of isolated mouse [127], bovine [69], macaque [128], and human [126] COCs was inhibited by the addition of PDE3 inhibitors milrinone, cilostamide, or Org 9935 to the culture medium, suggesting PDE3 isoenzymes do play a role in bovine oocyte meiosis. In agreement with work in the rodent [39, 42], the type 4 PDE inhibitor had no effect on meiotic resumption, implying PDE4s are absent from the oocyte [69, 128]. Additionally, the inhibitory effect of theca cell monolayers on meiotic resumption was significantly enhanced by treatment with PDE3 inhibitors [69].

The exact role of PDE3 isoenzymes in the regulation of oocyte meiotic maturation is as yet unknown, however high expression of PDE3A in rat, mouse and human oocytes [42, 63, 126], and the ability of PDE3 inhibitors to inhibit oocyte maturation in rodent [39, 42, 127], bovine [69], macaque [128] and human [126] oocytes suggest a role of some significance.

## 1.6 cAMP and Inter-Cellular Gap-Junctional Communication

### 1.6.1 Follicular Sources of Intra-Oocyte cAMP

As stated previously, intracellular cAMP concentrations are derived either by increasing cAMP synthesis by the enzyme adenylate cyclase, or inhibiting cAMP degradation by phosphodiesterases.

The spontaneous maturation of isolated cumulus enclosed oocytes (COCs) is transiently inhibited by the addition of forskolin (a stimulator of adenylate cyclase) to the culture medium. Dekel et al. [25] inhibited the resumption of meiosis in rat COCs by treatment with forskolin, but found maturation of oocytes denuded of their surrounding cumulus cells were unaffected (suggesting the absence of adenylate cyclase from the oocyte). This finding was also reported by Racowsky et al. [26], providing further evidence that adenylate cyclase is present in the cumulus cells and contributes to intra-oocyte cAMP levels.

Magnusson et al. [17] demonstrated that forskolin stimulation of rat COCs produces significantly higher levels of intra-oocyte cAMP over that in similarly treated denuded oocytes, implicating the cumulus cells to be a significant source of cAMP for the oocyte. Though transfer of labelled cAMP from the cumulus compartment to the oocyte has not been demonstrated, Bornslaeger et al. [58], Racowsky et al. [24, 26], and Bilodeau et al. [23] have shown that where cAMP concentrations were high in COCs, the concentration was also high in the oocytes derived from these complexes when compared to oocytes cultured without their cumulus vestment.

In some species however, oocytes denuded of their cumulus cells (DOs) do produce cAMP upon stimulation by forskolin [24, 73, 74]. Adenylate cyclase has been localised to the oolemma in bovine oocytes by immuno-cytochemical studies [75], and Bilodeau et al. [23] showed that forskolin was able to stimulate cAMP production and significantly delay the

resumption of meiosis in denuded and zona pellucida-free bovine oocytes. Kuyt *et al.* showed that following stimulation with forskolin, adenylate cyclase levels in the cumulus cell projections connecting with the oocytes increases [75]. These results suggest that the bovine oocyte does possess adenylate cyclase, and that cAMP can be synthesised in sufficient amounts to affect nuclear maturation *in vitro*, although in the absence of forskolin stimulation, the oocyte may not have enough adenylate cyclase enzymatic activity to affect its maturation.

### 1.6.2 Cumulus Cell-Oocyte Gap Junctional Communication

It is well established that direct communication exists between the follicular cells and the oocyte via gap junction transmembrane channels throughout folliculogenesis. Gap junctions provide for communication between cumulus cells, and between the innermost layer of cumulus cells and the oocyte [76-78].

Gap junction channels are made up of two symmetrical units called connexons – each consisting of a hexamer of proteins from the connexin family [79]. Each connexon is a cylindrical organelle inserted into the cell membrane so that when two connexon hemichannels on adjacent cells dock together, a hydrophilic intercellular gap junction channel is formed. Many different connexin types and combinations have been localized in the ovarian follicle [80]. The recent characterization of specific connexin-knockout mice has demonstrated the essential role of gap junction channels in both folliculogenesis and oogenesis. Mice deficient in connexin 37 (Cx37), found on the oocyte surface beneath the zona pellucida, display arrested folliculogenesis and oocyte growth [81], and oocytes do not achieve meiotic competence [82]. In contrast, antral follicles in ovaries from mice deficient

in Cx43 (expressed in granulosa cells) fail to properly develop granulosa cell layers, which is detrimental for the oocyte [83].

### 1.6.3 Loss of GJC and the Resumption of Oocyte Meiosis

Gap junction channels allow passage of low molecular weight metabolites and regulatory molecules that permit oocyte growth, control maturation, and consequently play important roles in oocyte maturation and subsequent embryo development. [38, 76, 84]. Consequently, gap junction-mediated communication constitutes an important part of the process of oocyte cytoplasmic maturation and therefore also the acquisition of developmental competence. In addition, it is highly likely that gap junctional communication is also involved in the regulation of oocyte meiotic maturation.

As previously discussed, oocytes *in vivo* and in intact-explanted follicles *in vitro* are maintained at the immature germinal vesicle (GV) stage of meiosis, but spontaneously resume meiosis and progress through to the metaphase II stage following artificial isolation from the follicle. This suggests follicular factors are responsible for the maintenance of meiotic arrest. Several studies have suggested that a loss of gap junctional communication between the oocyte and its surrounding cumulus cells *in vivo* could trigger the resumption of oocyte meiosis due to a cessation in the transfer of meiotic inhibitory substances from the follicular cells to the oocyte, such as cAMP, purines, and other putative regulatory molecules [85-87]. In agreement with this mode of meiotic inhibition are several lines of evidence that indicate that meiotic resumption occurs as a result of a disruption in intercellular communication. The exact relationship between the loss of this coupling and the resumption of oocyte meiosis has been the subject of much controversy - various studies suggest gap junction disconnection

precedes meiotic resumption, however there are conflicting experiments which suggest the resumption of meiosis precedes the uncoupling of gap junctions.

Both *in vivo* and *in vitro* experiments in cattle have suggested the loss of gap junctions between the cumulus cells and the oocyte occur at a time parallel to that of GVBD. *In vivo*, gap junctions were shown to breakdown 9-12 hours after the LH peak in super-ovulated cattle [88], while the frequency of gap junctions was markedly reduced after only 3 h of in vitro culture [78]. A causal relationship (where the breakdown of gap junctions induces meiotic resumption) has been suggested by some studies in rodent oocytes [45, 58, 89], while other studies in human, porcine [90], and murine [9] oocytes suggest GVBD precedes the decrease in gap junctions. As an aside, some studies indicate that gap junctional connections between the cumulus cells and the oocyte are maintained for a number of hours after the loss of gap junctional coupling within the cumulus vestment itself [77, 84, 90-92]. Although the kinetics and timing of gap-junctional uncoupling with reference to GVBD is different between studies, it is possible that the relationship between gap junctional uncoupling and meiotic resumption is a variation attributable to species-specific differences.

## 1.7 Oocyte Maturation - Nuclear and Cytoplasmic Maturation

### 1.7.1 Oocyte Nuclear and Cytoplasmic Maturation

Oocyte maturation is a complex process involving both nuclear maturation (the progression of the meiotic cycle) and cytoplasmic maturation, with the endpoint of both being the release of a mature (MII stage) oocyte from the follicle that is competent to support normal embryonic

development. Ovulation of the oocyte is followed by sperm penetration, egg activation, the completion of meiosis and entry into mitosis [93]. Proper cytoplasmic maturation of the developing oocyte increases the developmental potential of the oocyte post-fertilisation [94]. During the oocyte growth phase, the oocyte gradually and sequentially acquires the capacity to resume meiosis, complete meiosis and undergo activation. During this phase of oogenesis there is restructuring of the cytoskeleton, and reprogramming of protein synthesis [94] and post-translational modifications [95], while proteins, mRNA, and molecular precursors also accumulate in the cytoplasm. These changes are required to occur prior to activation of the embryonic genome (which occurs between the 8 to 16 cell stage in the bovine, 2-cell in murine, 4-cell in porcine, 8-16-cell in ovine, and 2-cell in caprine) for fertilisation and embryonic development to proceed normally [96].

Cumulus cells surround the mammalian oocyte throughout its development in the ovarian follicle until ovulation, and in almost all species a certain proportion of these cells are still present during fertilisation [97]. Cumulus cells play a very important role during oocyte growth and maturation. They are known to supply nutrients [77] and/or messenger molecules which aid oocyte development [8, 84] and mediate the effects of hormones on the cumulus-oocyte complex [98] - all of which contribute to cytoplasmic maturation of the oocyte. Some studies have demonstrated that the proportion of oocytes capable of maturing to the metaphase II stage *in vitro* is lower when the cumulus cells are removed prior to culture [97, 99-101]. Maturation of most mammalian oocytes is accompanied by a dramatic remodelling of the extra-cellular matrix leading to expansion of the cumulus complex and changes in the ultrastructure of the oocyte surface [102].

### 1.7.2 Nuclear and Cytoplasmic Maturation – Relation to Follicular Development

Since meiotic maturation was first described by Pincus and Enzmann in 1935 [4], many papers have been published which report that oocytes aspirated from large antral follicles of many species spontaneously progress through to the MII stage *in vitro*, the majority of which readily undergo fertilisation [4]. In bovine oocytes, COCs derived from follicles >1.8mm undergo spontaneous nuclear maturation, requiring ~24hrs to complete the first meiotic division. However, oocytes from follicles <1.6mm have not completed their growth phase and consequently have an impaired ability to complete the first meiotic division [103]. Similarly, it has been demonstrated that oocytes have a size-related ability to undergo meiotic maturation, with rates of cleavage and development to blastocysts/hatched blastocysts increasing with oocyte diameter [104].

Oocyte developmental competence is acquired gradually and increases concomitantly with both follicular development and oocyte growth. Bovine oocytes derived from larger diameter follicles (>2-3 mm in diameter) have a greater developmental potential than those from small follicles [105-107].

### 1.7.3 *In vitro* vs. *In vivo* Oocyte Maturation Outcomes

While it is true that the proportion of oocytes competent to complete nuclear and cytoplasmic maturation increases during follicular development, some oocytes that are able to undergo nuclear maturation to MII are of low developmental potential and fail to develop to the blastocyst stage. The process utilised for the mass harvesting of oocytes from abattoir-derived ovaries for bovine IVF does not discriminate between oocytes retrieved from different follicle sizes or between oocytes retrieved from partially atretic follicles. Khatir et al. [108]

state that 90% of bovine oocytes progress through meiosis to the MII stage under routine *in vitro* culture conditions, however most laboratories can only produce blastocysts from 30-40% of these inseminated oocytes [109]. It is well known that not all GV stage oocytes, or all oocytes which have matured *in vitro* to MII for that matter, possess equivalent developmental competencies [110], and that the nuclear maturity of an oocyte cannot be used as an indicator of its developmental competence. The fact that *in vivo* matured oocytes fertilised and cultured *in vitro* yield twice as many blastocysts as those matured *in vitro* [103], suggest that suboptimal *in vitro* oocyte maturation conditions [93, 103] lead to incomplete oocyte cytoplasmic maturation [111]. It is therefore likely that the low rates of blastocyst production seen in many IVM/IVF programs are not only the result of poor oocyte quality, but are also significantly influenced by the suboptimal culture conditions used to produce them.

## 1.8 cAMP and Embryonic Development

### 1.8.1 Oocyte-Cumulus Cell Gap Junctional Communication & Cytoplasmic Maturation

The premature disconnection of oocyte-cumulus cell gap junctions observed *in vitro* during spontaneous nuclear maturation of isolated oocytes or following FSH treatment of follicle-enclosed oocytes may be detrimental to the final developmental competence of the oocyte. The metabolic coupling and communication between the oocyte and its surrounding cumulus and follicular cells is almost certainly necessary for complete cytoplasmic maturation of the oocyte. Prolonging gap junctional connection in the cumulus-oocyte complex – either during GVBD or concomitant with GVBD inhibition, may therefore result in a longer period of

cytoplasmic maturation and produce oocytes with a greater capacity to support early development.

Salustri et al. [91] demonstrated a significant reduction in gap junctional coupling in isolated murine COCs (to 65% of the initial value) at 3 hours of *in vitro* culture in control medium. In contrast, treatment of COCs with FSH prevented this reduction and delayed meiotic resumption for at least 3 hours of culture. Total gap junctional uncoupling occurred after 3 hours in both the control and FSH treated conditions. Interestingly, Salustri et al. [91] prevented the loss of cumulus-oocyte communication for at least 6 hours by the addition of 2 mM dbcAMP to the culture medium, although cumulus expansion, uncoupling, and meiotic resumption was observed after 12 hours of culture. The continual correlation between meiotic resumption and uncoupling suggests that the two events may be regulated by a common factor – with a good candidate for this factor being cAMP.

### 1.8.2 Low Levels of cAMP and the Development of a Defined Chemical Media

In addition to the intrinsic quality of the oocyte, culture conditions using basic oocyte maturation medium such as serum, hormones, and growth factors have a major effect on subsequent maturation and developmental potential [112-114]. It is interesting to note that almost all of these substances act either directly or indirectly on the cAMP-synthesising adenylate cyclase pathway which is paradoxical to the use of cAMP-elevating compounds for the inhibition of spontaneous meiotic resumption of bovine oocytes [2, 20, 21, 23, 40].

Luciano et al. [115] demonstrated that while high concentrations of cAMP transiently inhibited the spontaneous maturation of bovine oocytes, low concentrations stimulated

nuclear maturation. Media supplemented with low concentrations of invasive adenylate cyclase (0.01 μg/ml iAC) resulted in increased rates of development to the blastocyst stage (by ~15%) compared to those matured and fertilised in medium containing serum and gonadotrophins. The addition of 0.1 μg/ml iAC and 0.5 mM IBMX in the collection medium also improved the blastocyst rate by 20% when IVM was performed in control medium or by 25% in medium supplemented with iAC [115]. These results suggest there may be an optimal level of intracellular cAMP during IVM that ensures isolated oocytes maintain or obtain a high level of developmental competence. The use of iAC may offer a means of developing a completely defined chemical media for the collection and maturation of oocytes, however its high cost suggests a need for an alternative compound.

### 1.8.3 High cAMP Levels and Sustained Meiotic Arrest

The maintenance of meiotic arrest in bovine oocytes has been widely reported using physiological and pharmacological methods [13, 21, 31, 36, 37, 40, 116], however very few authors have examined the developmental competence of these oocytes following the inhibition of meiotic resumption. It has been hypothesised that oocytes cultured *in vitro* under conditions which maintain meiotic arrest at the germinal vesicle stage for an extended period of time, may be able to obtain greater developmental competence [117] - possibly as a result of increasing the time over which cytoplasmic maturation occurs.

Fouladi Nashta et al. [117] demonstrated that *in vitro* culture of intact bovine antral follicles was most effective in preventing GVBD (97% GV after 24 hours culture) compared with 63 and 25% GV after culture in hemi-sections or attached to part of the follicle wall, respectively. When compared with other agents which are able only to transiently inhibit

meiosis, antral follicle culture seems to be the most effective in maintaining arrest over a relatively long period of time, although this form of culture still resulted in the eventual resumption of oocyte meiosis - probably due to degeneration of the follicle [117].

Spontaneous oocyte maturation has also been prevented using various agents, such as inhibitors of protein synthesis or phosphorylation (eg. cycloheximide and 6-dimethylaminopurine [109], or inhibitors of specific cyclin-dependant kinases [131] (eg. butyrolactone I [132-134] and roscovitine [135-137])), after which the inhibitor is removed and maturation allowed to continue. However, these studies generally show that manipulation of spontaneous meiotic progression in this manner fails to improve oocyte quality, often adversely affecting the developmental potential of these oocytes.

It is well known that the second messenger cAMP plays a role in the regulation of mammalian oocyte maturation, and it is suggested that concomitant inhibition of precocious nuclear maturation and stimulation of intracellular cAMP levels may prove the production of oocytes of high developmental capacity to be achievable. Nogueira et al. [127] recently demonstrated that rodent oocytes arrested with a type 3 PDE inhibitor had significantly improved fertilisation rates, and comparable rates of pre-implantation development and production of live offspring, compared to those oocytes collected following *in vivo* maturation (although levels of GJC were not examined). The effectiveness of various modulators of cAMP are not constant when applied across species - while purines, cAMP, and follicular cells have significant effects on many laboratory animals, the oocytes of cattle are not as sensitive [30]. Several studies demonstrate that the treatment of ungulate oocytes with chemicals which maintain high levels of cAMP exert only a transitory inhibitory effect on germinal vesicle breakdown in the bovine [2, 20, 40]. Despite this, a study conducted by Funahashi et al. [118]

showed the frequency of IVM-IVF porcine embryos that developed to the blastocyst stage was doubled when COCs were exposed to a high inhibitory level of dbcAMP for the first 20 hours of IVM compared to controls. Results such as these show that cAMP has a key role in the regulation of meiotic and cytoplasmic maturation, and the addition of agents that modulate intracellular cAMP levels during IVM clearly have a role in the improvement of these processes *in vitro*, with the outcome of enhanced embryo development.

## 1.9 Summary

Research investigating oocyte maturation, fertilisation, and embryonic development is necessary for improved assisted-reproduction technologies in animals and humans and to reveal causes of inferior or abnormal *in vitro* embryonic development.

cAMP is known to directly inhibit oocyte maturation and the fact that this nucleotide can apparently move from the cumulus cells to the oocyte suggests it could possibly serve as the physiological factor preventing oocyte maturation *in vivo*. Consistent with this idea is the observation that the resumption of meiosis is associated with a reduction in intra-oocyte cAMP levels.

This review presents strong evidence that supports the differential actions of specific PDE isoenzymes in the somatic and germ cell compartments of the ovarian follicle. Selective regulation of the activities of these subtypes may account for the paradoxical roles of cAMP seen in the experimental situation and may provide an explanation for the mechanism of oocyte meiotic control.

The exploitation of specific PDE isoenzyme inhibitors would have significant implications as experimental tools to study the oocyte and surrounding cumulus cells in separation, and to demonstrate the functions of the different PDE isoenzymes - and thereby cAMP, in the two follicular compartments.

FSH induces cumulus expansion, which is associated with gap junction disconnection in the cumulus-oocyte complex and a transitory inhibition of meiosis, followed by the nuclear maturation of isolated oocytes *in vitro* and follicle-enclosed oocytes *in vivo* and *in vitro*. cAMP acts in a similar manner *in vivo* and in explanted follicles *in vitro*, thus mimicking the effects of gonadotrophins, while *in vitro* cAMP prolongs gap junctional connection. Several studies have discovered or suggest a causal relationship between the loss of intercellular coupling and the resumption of meiosis and suggest a role for cAMP in this process. The specific actions of subtype-specific phosphodiesterase inhibitors on the somatic and germ cell compartments of the follicle implicate them as a useful and powerful tool to modulate gap-junctional communication and possibly alter the level of cytoplasmic maturation of the *in vitro*-matured oocyte.

The effective use of 'high' levels of cAMP for the total inhibition of spontaneous oocyte maturation over an extended period of time using PDE3 inhibitors may increase developmental competence, sustaining cytoplasmic maturation during this extended period of arrest. Use of the PDE3 specific inhibitors has an advantage over non-specific PDE inhibitors as intra-cellular cAMP concentrations in the somatic compartment of the follicle will remain relatively unaffected. If meiotic arrest at the GV stage could be maintained in vitro, it would

be possible to influence both the cytoplasm and the nucleus with hormones or granulosa cells before nuclear maturation.

There seems to exist two levels of intracellular cAMP that exert different effects on oocytes *in vivo* and *in vitro*. In contrast to the above use where high levels of cAMP maintain isolated oocytes in meiotic arrest, a lower non-inhibitory level may also exist which is optimal to promote high developmental competence. This approach is more likely to have an application in non-rodent IVM/IVF systems than in rodent systems. Again, the specificity of a type 3 PDE inhibitor would stimulate this particular level of cAMP elevation in the oocyte, but not the somatic cells, thereby eliminating side-effects and allowing the development of a defined culture system capable of supporting oocyte growth and producing blastocysts at a rate above that of existing culture systems. These approaches will allow for studies aimed at defining the precise roles of hormonal and growth factor modulators in supporting early development.

The successful use of specific isoenzyme inhibitors during oocyte maturation will prove important for a more defined understanding of the fundamental mechanisms surrounding the regulation of mammalian oocyte maturation.

## 1.10 Hypotheses and Aims of the Present Study

### 1.10.1 Hypotheses

1. Phosphodiesterase isoenzymes types 3 and 4 are differentially localised within the bovine ovarian follicle, where the type 3 is localised to the oocyte and the type 4 to the granulosa cells.
2. Regulation of bovine oocyte maturation is influenced by the differential regulation of cAMP levels in the oocyte and somatic cells.
3. Modulation of intra-oocyte cAMP levels affects oocyte maturation - including meiotic capacity and cumulus cell-oocyte gap junctional communication - and as a result, subsequent developmental capacity.

### 1.10.2 Aims

At the onset of this study, the exact role of cAMP in the regulation of meiotic maturation in the bovine oocyte was comparatively undefined when compared to that known about the regulation in rodent oocytes. The aims of this thesis are to examine whether the differential localisation of specific phosphodiesterase isoenzymes in the two compartments of the follicle:

1. Differentially regulates cAMP levels in the germ cell (oocyte) and somatic (mural and cumulus granulosa cells) compartments of the bovine follicle, and the effect this has on the spontaneous meiotic maturation of bovine oocytes *in vitro*.
2. Differentially controls cumulus cell-oocyte gap junctional communication.
3. When inhibited by isoenzyme-specific agents included in standard *in vitro* maturation culture media, improves the developmental capacity of oocytes to progress through to the blastocyst stage following in vitro fertilisation.

# Experimental Studies

## 2. Paper I - Differential Effects of Specific Phosphodiesterase Isoenzyme Inhibitors On Bovine Oocyte Meiotic Maturation

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Thomas, R.E., Armstrong, D.T., and Gilchrist, R.B., (2002) Differential effects of specific phosphodiesterase isoenzyme inhibitors on bovine oocyte meiotic maturation. *Developmental Biology, v. 244 (2), pp. 215-225.*

# 3. Paper II – Bovine Cumulus Cell-Oocyte Gap Junctional Communication During In Vitro Maturation In ResponseTo Manipulation of Cell-Specific cAMP Levels

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Thomas, R.E., Armstrong, D.T., and Gilchrist, R.B., (2004) Bovine cumulus cell-oocyte gap junctional communication during in vitro maturation in response to manipulation of cell-specific cyclic adenosine 3',5'-monophosphate levels. *Biology of Reproduction, v. 70 (3), pp. 548-556.*

# 4. Paper III – Effect of Specific Phosphodiesterase Isoenzymes During In Vitro Maturation of Bovine Oocytes on Meiotic and Developmental Capacity

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## Thesis Discussion

The crucial role of the second messenger cAMP in the maturation of mammalian oocytes has long been recognized, however its exact role in the regulation of bovine oocyte maturation remains unclear. As one of the primary means for determining intracellular cAMP levels, original experiments beginning in the late 1980s utilized non-specific phosphodiesterase inhibitors (such as IBMX and theophylline) to modulate intracellular cAMP levels. These initial studies suggested PDE involvement in the regulation of oocyte maturation, however it was the lack of specificity toward specific PDE subtypes, in addition to the toxic side effects of these inhibitors, which restricted their usefulness. From 1995, several studies identified and demonstrated the differential localization of different phosphodiesterase subtypes within the two compartments of the rodent follicle - the germ cell (oocyte; type 3 PDE) and the somatic cell (cumulus and mural granulosa cells; type 4 PDE). Subsequently, newly developed subtype-specific PDE inhibitors were used against the type 3 and 4 PDE subtypes of the rodent ovarian follicle – the results of which were compiled in two key publications by Tsafriri et al.[42], and Wiersma et al. [39], both of which constituted the foundations for the current study in the bovine model: 'Differential Effects of Specific Phosphodiesterase Isoenzyme Inhibitors On Bovine Oocyte Meiotic Maturation, Gap Junctional Communication, and Developmental Competence'.

The first major aim of this study was therefore to examine whether there was differential regulation of cAMP levels within the oocyte and somatic (cumulus) cell compartments of the bovine follicle (made possible by exploiting the cell type-specific localisation of PDE isoenzymes), and the effect of this on the regulation of bovine oocyte meiotic maturation. In particular, the study investigated whether subtypes of PDEs are differentially localised within the two compartments of the bovine follicle – inferred by experiments demonstrating that

inhibition of specific isoenzymes caused modulation of cAMP levels in the corresponding compartment of the bovine follicle. The effect of altering cAMP levels on the progression of spontaneous bovine oocyte meiotic maturation was also examined. These investigations showed PDE isoenzymes to have differing activities within the two compartments – where the type 3 PDE was present in the oocyte, and the type 4 in the cumulus and mural granulosa cells (paper I). The type 3, but not the type 4 PDE inhibitor, elevated cAMP levels in the oocyte and prevented the spontaneous resumption of meiosis in vitro. In contrast, the type 4 PDE inhibitor had no effect on oocyte cAMP levels or on the progression of meiosis, but was able to elevate both cumulus and mural granulosa cell cAMP production (paper I). This study was the first in any species to measure intracellular cAMP levels after subtype-specific PDE regulation. This study demonstrates that distinct PDE subtypes do selectively control cAMP levels in the granulosa and germ cell compartments of the bovine follicle, with PDE3 being active in the oocyte, and PDE4 in the cumulus cells. These results supported findings in the rodent [63, 42, 39] and preliminary observations in bovine [69] studies, and confirmed that PDE subtypes are compartmentalised in the bovine follicle, providing a mechanism for the differential regulation of cAMP in the non-rodent follicle.

Because cAMP is a known regulator of GJC between cells of various types and in different tissues, the present study also aimed to investigate the effects of cAMP level modulation in the somatic or germ cell compartments of the bovine follicle on cumulus cell-oocyte GJC using subtype-specific PDE inhibitors. In order to do this, a novel assay was developed and validated for the quantitative measurement of cumulus cell-oocyte GJC during in vitro maturation (paper II). In particular, experiments were designed to investigate the temporal relationship of the time of onset of bovine oocyte germinal vesicle breakdown (GVBD) with the change in cumulus cell-oocyte GJC. In addition, the effect of differentially modulating

cAMP levels in the somatic or germ cell compartment of the bovine follicle on cumulus cell-oocyte communication was also examined. Results from the present study have indicated that the level of GJC between the oocyte and its surrounding cumulus cells dramatically decreases upon release of COCs from their follicular environment. Communication continued to drop until 9 h of *in vitro* culture, after which levels were effectively zero and did not decrease further (paper II). Results suggest that the presence of cAMP during in vitro maturation enhances cumulus cell-oocyte GJC, where the dramatic loss seen under control conditions was attenuated in response to treatment with the type 3 PDE inhibitor milrinone, and also to a lesser extent by the type 4 PDE inhibitor rolipram. The results suggested the inhibitory effect of the oocyte type 3 PDE inhibitor on spontaneous meiotic resumption is either a direct result of the attenuation in GJC loss (paper II), or is a separate phenomenon attributable to the elevation of oocyte cAMP (paper I). When stimulated, cumulus cell cAMP production (as induced by a combination of adenylate cyclase stimulation and type 4 PDE inhibition) was found to be the major contributor to the total cAMP content of the oocyte (paper I), and in these experiments was observed to alter levels of GJC accordingly (paper II).

GJC between the oocyte and its surrounding follicular cells permits transfer of many low molecular weight substrates such as ions, nucleotides, amino acids, metabolites and regulatory molecules between the cell types. Because these molecules are important for oocyte growth, it is hypothesised that GJC constitutes an important component of oocyte cytoplasmic maturation, and therefore also in the acquisition of oocyte developmental competence. In conjunction with the delay in meiotic maturation (paper I), this study proposes that prolonged communication between the two cell types (paper II) may enable increased maturation of the oocyte cytoplasm, thereby increasing the developmental potential of *in vitro* matured oocytes for embryo development. Having established the positive effects of cAMP modulation on

levels of communication in the *in vitro* matured COC (paper II), this study aimed to investigate the effects of prolonging GJC on oocyte acquisition of developmental capacity (paper III). *In vitro* maturation of oocytes for the production of embryos requires the presence of FSH in the culture medium – and as such FSH (paper III) was substituted for forskolin (paper II) as the agent used to stimulate cAMP production, along with PDE inhibition. Although there was a less significant attenuation of GJC loss between the oocyte and the cumulus cells than that observed with forskolin (paper II), inclusion of isoenzyme 3 and 4-specific PDE inhibitors with FSH in the culture media during IVM nonetheless increased rates of development of cleaved embryos to the blastocyst stage following *in vitro* fertilisation and embryo culture by approximately 10% (paper III). This study is the first in a non-rodent species to demonstrate improved oocyte acquisition of developmental competence during *in vitro* maturation via this methodological approach (inhibition of precocious spontaneous maturation, with enhancement of oocyte-cumulus cell GJC following treatment with subtype-specific PDE inhibitors). Most recently, Noguiera *et al.* [127] demonstrated that rodent oocytes arrested with a type 3 PDE inhibitor, followed by removal of the inhibitor and completion of oocyte maturation, had significantly improved fertilisation rates, and comparable rates of pre-implantation development and production of live offspring, compared to those oocytes collected following *in vivo* maturation – however GJC involvement or modulation was not considered.

The work presented in this thesis demonstrates that the exploitation of subtype-specific PDE inhibitors is a powerful experimental approach to study the oocyte and surrounding cumulus cells in separation, and to investigate the functions of cAMP in the two follicular compartments. The successful use of specific PDE isoenzyme inhibitors will prove important

in the development of a clearer and more defined understanding of the fundamental mechanisms regulating mammalian oocyte maturation.

Ovaries collected post-mortem from abattoirs are an enormous source of economical and freely available immature COCs – and as such the successful maturation *in vitro* of these oocytes constitutes an economical platform technology for the production of high yield, quality embryos. Mechanisms regulating oocyte maturation and the acquisition of developmental competence need to be researched and fully understood - not only in terms of the economic and agricultural importance of successful livestock IVM – but also in order to enable significant advancements in assisted-reproduction technologies in both animals and humans, and to reveal causes of inferior or abnormal *in vitro* embryonic development. Results from the present study demonstrate a means of improving oocyte cytoplasmic maturation during IVM – where inhibitors of specific PDE isoenzymes may prove to be a beneficial component of oocyte IVM culture systems, increasing in vitro embryo production (in terms of both quantity and quality) for assisted reproduction applications in laboratory animals, livestock, endangered species and humans.

Future directions of this study include: Investigation of the developmental capacity acquired by oocytes matured *in vitro* in the presence of PDE inhibitors combined with forskolin initially (as opposed to FSH); investigation of gap junction channel regulation in the bovine follicle (using gap junction blockers, such as carbenoxolone, and enhancers (such as all-trans-retinoic acid); characterisation of bovine follicular gap junction composition (connexin subtypes); investigation of connexin regulation in the bovine follicle; characterisation and

investigation of the process of oocyte chromatin condensation (in terms of sub-stages of the immature germinal vesicle stage) configuration following PDE inhibitor treatment.

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# Appendices

# Appendix 1 - Work-Up Experiments

***Use of IBMX in the Collection Media***

From bovine literature, Homa et al [2] demonstrated the ability of an alternative non-specific PDE inhibitor IBMX (5mM) to almost totally inhibit meiotic resumption of bovine DOs after 24hrs of culture. 5 and 2mM IBMX treatment groups were included in this experiment to evaluate the effectiveness of IBMX in inhibiting oocyte maturation in bovine COCs in our system (Figure A1). After 16 h of culture, 2 mM IBMX maintained approximately 10% of oocytes at the immature GV stage, and all other oocytes were arrested at the MI stage of meiosis, preventing progression through to the MII stage (~90% MI; 0% MII). In response to treatment with 5 mM IBMX, approximately 98% of oocytes were arrested at the immature GV stage of meiosis, while only ~2% progressed through to the MI stage after 16 h of culture. To prevent premature commitment of oocytes isolated from their follicles to commence spontaneous meiotic maturation based on these results, follicular fluid freshly aspirated from ovarian follicles was immediately supplemented with IBMX (5 mM) for all subsequent experiments.

***The Effect of Milrinone on the In Vitro Nuclear Maturation of Murine COCs***

The spontaneous maturation of murine COCs was blocked in a dose dependent manner by the type 3 PDE inhibitor milrinone after 16 h of culture (Figure A2). A response to milrinone was observed at a concentration of 1 μM, where 13 fold more oocytes were maintained at the GV stage compared to control oocytes. Using 10 μM milrinone, almost all of the oocytes were maintained in meiotic arrest, while a maximal response of 100% of oocytes being maintained at the immature GV stage was seen at the 100 μM concentration. A fifth treatment group included as a positive control is 5mM IBMX, the non-specific PDE inhibitor (IBMX was used in the collection media to prevent premature commitment to the resumption of meiosis during the aspiration, collection, and sorting procedures). Compared to controls, 5mM IBMX maintained all oocytes in meiotic arrest (100% GV), this response being comparable to that of 10μM milrinone (94% GV; Figure A2).

***Effect of the Type 3 PDE Inhibitor Milrinone on Bovine COCs***

At the beginning of the current study, pure milrinone compound was diluted to 2.45 mM with 1part MQ H2O and 2parts dimethyl-sulphoxide (DMSO). DMSO was used as the diluent because milrinone is insoluble in water. A dose response of milrinone was conducted using 0, 1, 10, 100, 250, and 500 μM concentrations, meaning that a 500μM treatment well would contain 66 μl of DMSO (13.2% of the entire well volume; Figure A3). 250 μM milrinone significantly inhibited bovine oocyte meiotic resumption (40% GVBD) compared to that in the control group (85% GVBD), and 500 μM milrinone completely inhibited meiotic resumption (0% GVBD). It was concluded from this experiment that high concentrations of the type 3 PDE inhibitor milrinone were effective in preventing meiotic resumption of oocytes over a 16 h incubation period. However when oocytes were cultured in the presence of an equivalent amount of DMSO to that used to dilute MR to a concentration of 500 μM, a significant inhibitory effect on spontaneous meiotic resumption was seen (100% GV; Figure A3). Consequently, the observed inhibitory effect of milrinone was assumed to be completely

attributable to that of the DMSO diluent. A new stock of milrinone compound was obtained and stocks were prepared again in DMSO, but to a concentration of 250 mM.

This new stock of milrinone failed to maintain bovine oocyte meiotic arrest (Figure A4), even at concentrations fifty times higher (500μM: 90% GVBD) than that required to maintain 100% of murine oocytes in the GV stage (Figure A2: 10μM: 6% GVBD). No significant difference can be seen between MI and MII frequencies with increasing concentrations of milrinone, demonstrating that not only was the inhibitor unable to maintain oocytes in GV, but also the inhibitor was not able to transiently inhibit bovine oocyte meiosis. Preliminary experiments in this study indicated that high concentrations of DMSO have a significant inhibitory effect on bovine oocyte maturation (Figure A3). Due to the fact that milrinone stocks were all dissolved in DMSO, a DMSO control was included to account for the percentage of DMSO diluent in each milrinone treatment group. It can be seen that the DMSO control containing 0.2% DMSO (equivalent to that of the 500 μM milrinone treatment) induced no significant increase in the frequency of GV stage, nor MI and MII stage oocytes compared to control-treated oocytes.

When added to the in vitro maturation medium of bovine oocytes, rolipram failed to inhibit their spontaneous maturation (90% GVBD) which proceeded undisturbed, even at the high concentration of 500μM (89% GVBD; Figure A5). The frequencies of MI and MII were also not significantly different from that of control oocytes, indicating rolipram was unable to transiently inhibit meiosis over the 16hr incubation period (control: 36%MI, 55% MII; 500μM: 34%MI, 55%MII).

An experiment done culturing the oocytes aspirated from only four ovaries in the presence of milrinone finally demonstrated an inhibitory effect on spontaneous meiotic resumption (Figure A6). The time required for aspiration of follicular fluid, isolation of COCs, and transfer to culture was only 10-20 minutes. As a result, experiments were performed to investigate the effects of COC processing time on the ability of milrinone to prevent spontaneous meiotic resumption.

***Effect of Time in Follicular Fluid Supplemented IBMX on the Ability of Milrinone to Prevent Spontaneous Oocyte Meiotic Maturation***

Tubes of follicular aspirate were supplemented with H-TCM containing IBMX so that the final concentration of IBMX was 5 mM. COCs from each tube of aspirate were isolated and put into culture after 20 min, or 1, 1.5, 2, or 2.5 h. After 16 h of culture in the presence of milrinone, COCs were assessed for their meiotic status. As the time spent in supplemented follicular fluid increased, the ability of milrinone to arrest oocytes at the immature GV stage of meiosis decreased (Figure A7). Approximately 40% of oocytes were arrested at the GV stage following a processing time of only 20 minutes, with this proportion decreasing with time until all oocytes had undergone GVBD after a processing time of 2.5 h (Figure A7).

In the attempt to try and increase the ability of MR to arrest oocyte meiosis at the GV stage, follicular fluid was supplemented with MR instead of IBMX, however the improvement seen was not great enough to justify the cost of substituting IBMX (Figure A8). We also tried increasing the concentration of IBMX in the collection media to 7 mM, but with no improvement seen in the proportion of oocytes maintained at the immature GV stage by milrinone (data not shown).

### *Effect of Temperature on the Ability of Milrinone to Inhibit Spontaneous Oocyte Meiotic Maturation*

An observation was made that oocytes arrested by milrinone at the GV stage were in the early GV configuration stages. It was proposed that during longer processing times, the kinetics of spontaneous oocyte meiotic maturation allowed progression of oocytes from early GV configurations through to those occurring just prior to Diakinesis (or GVBD), and this may prevent milrinone from inhibiting meiotic resumption. Therefore, ovaries collected at the abattoir were immediately transferred to and transported in thermos flasks at either 37 or 4°C and the germinal vesicle configurations of the oocytes examined at time = 0, immediately following aspiration from their follicles. The total time from ovary excision until follicle aspiration and oocyte fixation was approximately 3-7 hours. The majority (80%) of oocytes from ovaries collected at 37°C were in GV configuration IV, while those ovaries collected at 4°C were mainly in either GV configuration II (50%) or III (35%; Figure A9). This experiment was repeated, but this time the total time from ovary excision until follicle aspiration and oocyte fixation was reduced to 1.5-2 h (Figure A10). A greater proportion of oocytes from ovaries collected at 37°C were in earlier GV configurations (II: 25%; III: 50%), however an even greater proportion of oocytes from those ovaries transported at 4°C were in earlier GV configurations (II: 50%; III: 35%; Figure A10).

To test whether milrinone has an increased ability to inhibit meiotic resumption of those oocytes in earlier GV stages, oocytes from ovaries collected and transported at 4 and 37°C were cultured for 16 h in the presence of milrinone (Figure A11). Compared to oocytes from ovaries at 37°C (10% GV), a greater proportion of those transported at 4°C were arrested by the type 3 PDE inhibitor at the immature GV stage (Figure A11).

Due to time constraints, the mechanisms and relevance of this to the greater study was not investigated further.

### *Use of Un-Supplemented Centrifuged Follicular Aspirate as Collection Media*

Following a personal communication with M-A Sirard and MA Mayes, it was suggested that the use of neat, un-supplemented follicular aspirate would still permit the inhibition of spontaneous bovine oocyte meiotic resumption, while omitting the need and time required for H-TCM/IBMX use. An experiment was performed examining the efficacy of milrinone in inhibiting meiotic progression under these conditions, ensuring the processing time of oocyte isolation and transfer to culture remained between 20 minutes and 1 h (Figure A12). Under these conditions, milrinone maintained ~25% of oocytes at the immature GV stage, compared to ~3% in control media (Figure A12). As a result, un-supplemented follicular aspirate, coupled with COC processing time minimised to between 20 minutes and 1 hour, was used for all future experiments.

### *Increasing the Sensitivity of the cAMP Radioimmunoassay*

Concentrations of intra-oocyte cAMP were sufficiently low to warrant increasing the sensitivity of the in-house cAMP radioimmunoassay used to measure them. The percentage of bound antibody at dilutions of 1/200, 1/400, 1/600, 1/800, and 1/1000 were determined (Figure A13a), and dilutions of antibody that gave 50, 40, 30, and 20% bound antibody were

then used to create standard curves (Figure A13b). From these results, it was inferred that the curve produced using 1/850 dilution of antibody would produce and assay of the highest sensitivity, and all subsequent assays were carried out using this concentration.

***Development and Validation of the Gap Junctional Communication Assay***

The acetoxymethyl (AM) ester derivative of the fluorescent indicator calcein (calcein-AM; 3',6'-Di(O-acetyl)-2',7'-bis[N,N-bis(carboxymethyl) amino methyl]-fluorescein, tetraacetoxy methyl ester; C-3100; Molecular Probes; Eugene, OR, USA) was used in the assay to assess the level of inter-cellular gap-junctional connection between the oocyte and its cumulus vestment during in vitro culture. The concentration of dye required to produce a detectable (but not overly intense) level of intra-oocyte fluorescence was determined by culturing COCs in decreasing concentrations of the calcein-AM (10, 6, 2, 1, and 0.1 μM) freshly made up in CAM-BTCM + 0.3 mg/ml PVA. Concentrations of calcein-AM greater than 1 μM produced intra-oocyte concentrations of fluorescent calcein way too intense to be detected/differentiated between by the microscope photometer (data not shown). Subsequently, COCs were incubated in the presence of 1, 0.1, or 0.01 μM calcein-AM for 10, 20, 40, or 60 minutes before dye not incorporated into the COC was removed by three washes in calcein-AM-free CAM-BTCM and the surrounding cumulus cells removed. 0.01 μM calcein-AM generated an almost undetectable level of intra-oocyte fluorescence - despite increasing the length of time of COC culture, while a calcein-AM concentration of 0.1 μM required COC culture time to be longer than 20 minutes (data not shown). Ultimately it was decided that the COC dye-loading component of the GJC assay was to utilise a concentration of 1 μM calcein-AM and a culture period of 15 minutes (data not shown).

For the remaining validation experiments, please refer to Paper II (Figures 1 and 2).

***Effect of the Gap Junction Blocker Carbenoxolone on Oocyte-Cumulus Cell GJC***

Bovine COCs were cultured with either the type 3 PDE inhibitor milrinone, or the type 4 PDE inhibitor rolipram with or without the gap junction blocker carbenoxolone (CBX; 30μM). After 6 h of culture, the COCs were pulsed with Calcein-AM, denuded, assessed for intra-oocyte fluorescence intensity and then assessed for meiotic maturation status. Carbenoxolone reduced the level of cumulus cell-oocyte gap junctional communication to approximately 32% of that measured in the control-, milrinone-, and rolipram-treated COCs. In control COCs, the decrease in GJC measured in the presence of carbenoxolone was not significant despite the fact it had fallen to 35.4% of that measured in the absence of carbenoxolone (Figure A14). This lack of a significant drop in GJC, as well as the fact that CBX inhibits 80% of the GJC seen in COCs cultured for 2 h (Paper II, Figure 1), suggests that the majority of cumulus cell-oocyte gap junctions have already been closed during the 6 h culture. GJC levels measured in response to milrinone treatment were increased above that of the control by 55% (P=0.067) and carbenoxolone treatment reduced GJC levels to 31.6% of that measured in milrinone-treated COCs. Rolipram treatment doubled the level of GJC observed in control COCs, but this increase was not significantly different from that seen in milrinone-treated COCs. The addition of carbenoxolone to rolipram-treated COCs reduced GJC levels to 31.4% of that measured in the absence of carbenoxolone – a reduction comparable to that seen in control- and milrinone-treated COCs in the presence of this inhibitor.

Treatment of bovine COCs with forskolin alone induced a 2-fold increase in the levels of GJC between the cumulus cells and the oocyte compared to control-treated oocytes (Figure A15), while the presence of carbenoxolone reduced this level of cumulus cell-oocyte GJC by half. The presence of milrinone in the culture medium did not alter the levels of GJC induced by treatment with forskolin alone, and carbenoxolone retained the ability to block ~50 % of GJC (MR+FK: 200% GJC; MR+FK+CBX: 100%). Treatment of COCs with the type 4 PDE inhibitor rolipram induced significantly higher levels of GJC (compared to FK treatment alone, and in combination with milrinone) and in dramatic contrast to FK or MR+FK treatments, carbenoxolone was unable to block RP+FK-induced GJC (Figure A15).

FSH treatment of COCs alone was unable to induce significantly different levels of GJC compared to control-treated COC, however the presence of the type 3 and 4 PDE inhibitors in combination with FSH increased GJC between the oocyte and the cumulus cells to a much higher level (Figure A16). The gap junction blocker carbenoxolone was observed to reduce these induced levels of GJC by ~65% (in all treatment combinations). This is in contrast to that observed with RP+FK (Figure A15) – where carbenoxolone was unable to block the induced GJC. Due to time constraints, the mechanisms/cause of these observations was not investigated further.

## APPENDIX I - Figures

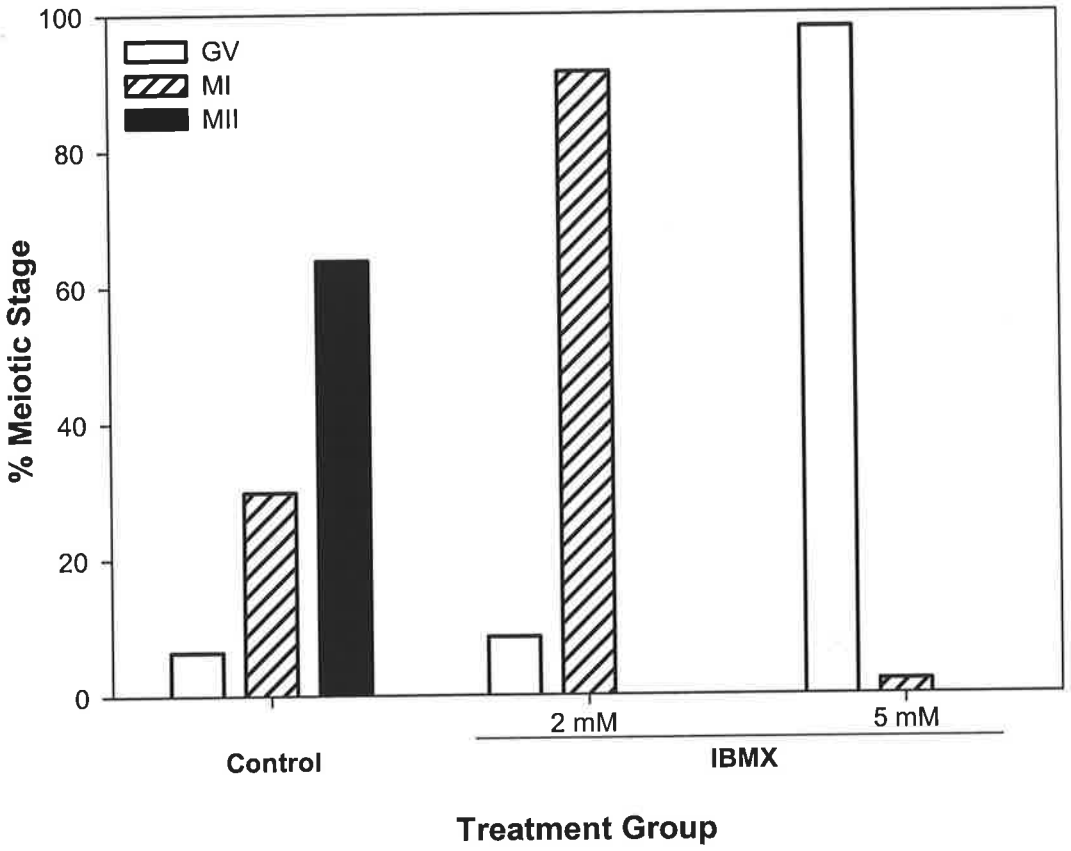


***Figure A1.***
The effects of IBMX on the spontaneous maturation of isolated bovine oocytes following 16h of incubation. Oocytes were assessed and grouped into GV, MI, and MII stages. A mean number of 50 oocytes were used in each treatment. Each column represents a mean of 3 replicates.

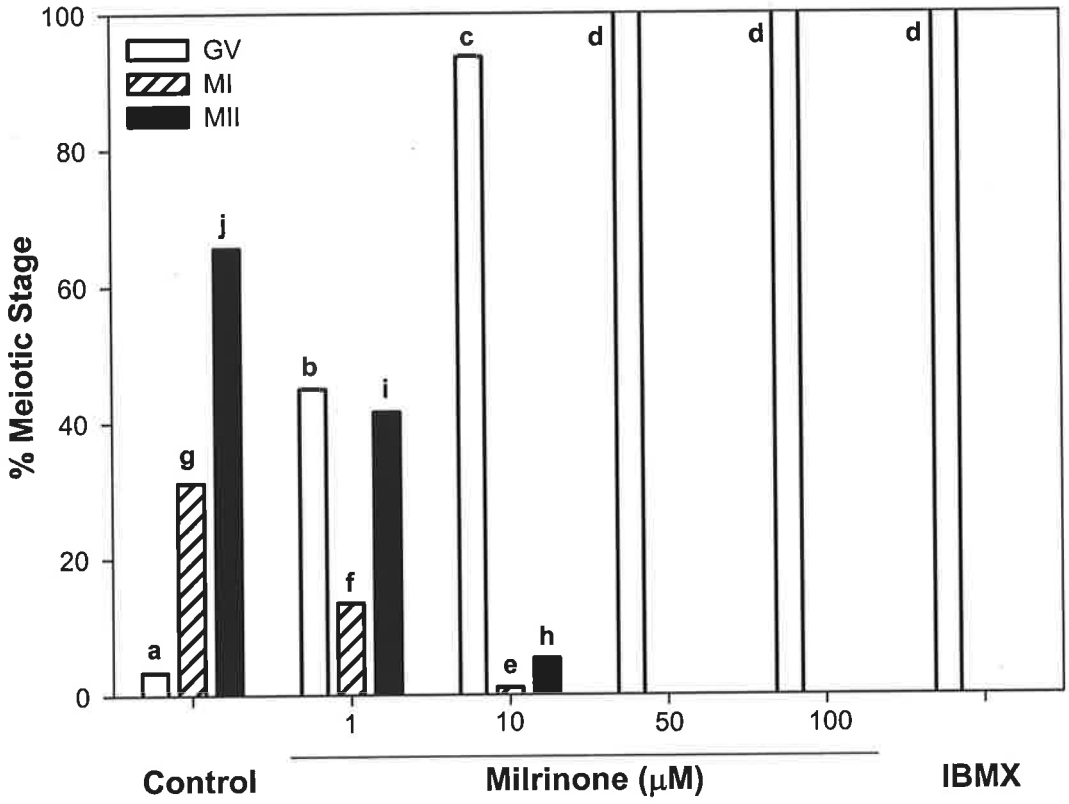


***Figure A2.***
The effects of increasing concentrations of milrinone on the spontaneous maturation of isolated murine oocytes following 16h of incubation. Oocytes were assessed and grouped into GV, MI, and MII stages. A mean number of 30 oocytes were used in each treatment. Each column represents a mean of 3 replicates. Values with different superscript letters indicate significant differences between frequencies of oocytes in the same meiotic stage from different treatment groups (Chi squared analysis, INSTAT package, $P < 0.0001$). [a-d]Significant differences between frequencies of GV stage oocytes; [e-g]Significant differences between frequencies of MI stage oocytes; [h-j]Significant differences between frequencies of MII stage oocytes.

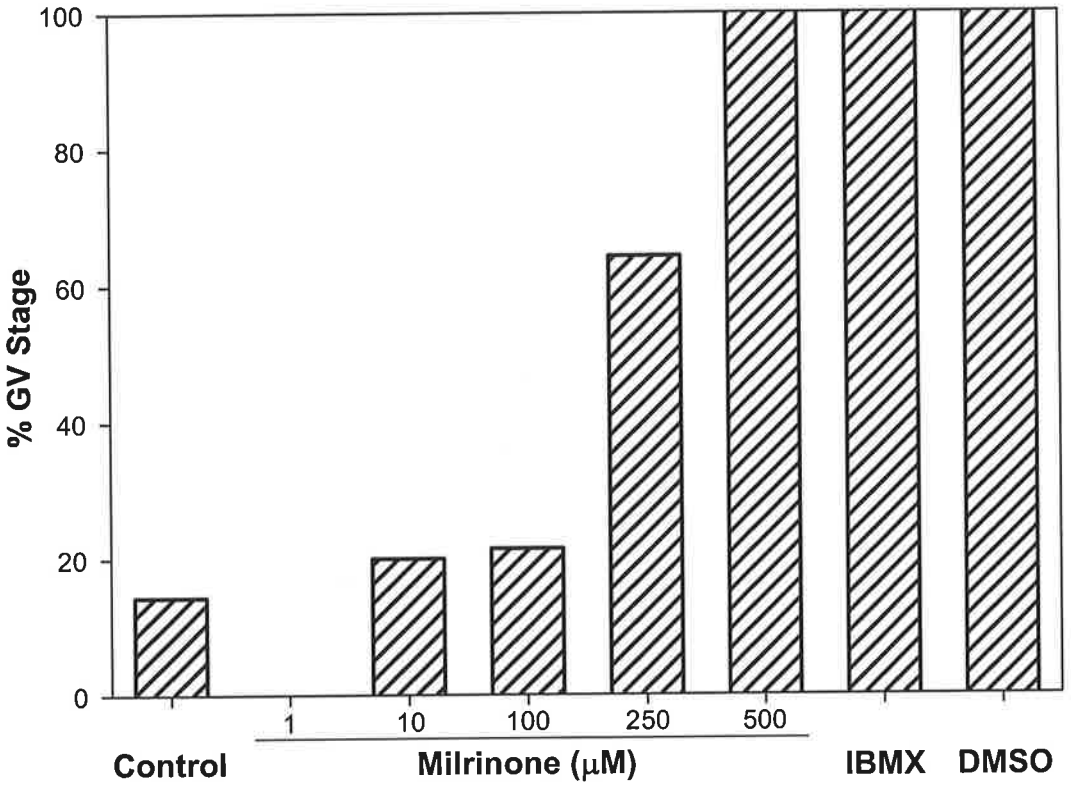


*Figure A3.*
The effects of increasing doses to 500μM of the PDE type 3 inhibitor milrinone with 5mM IBMX as a positive control and DMSO as a negative control after 16hrs of incubation. Shows percentage of oocytes remaining in the GV stage of meiosis. A mean number of 50 oocytes were used in each treatment. Each column represents a mean of 2 replicates following culture.

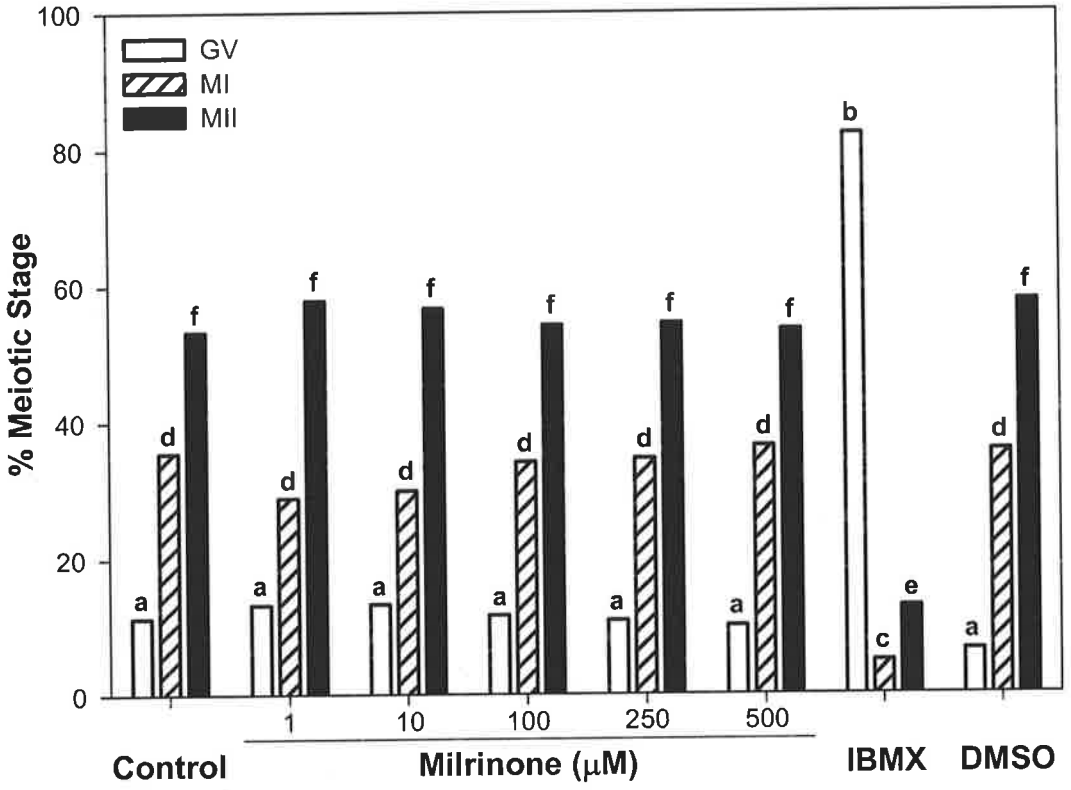


***Figure A4.***
The effect of increasing concentrations of milrinone on the spontaneous maturation of isolated bovine oocytes following 16h of incubation. Oocytes were assessed and grouped into GV, MI, and MII stages. Values with different superscript letters indicate significant differences between frequencies of oocytes in the same meiotic stage from different treatment groups (Chi squared analysis, INSTAT package, $P < 0.0001$). A mean number of 40 oocytes were used in each treatment. Each column represents a mean of 3 replicates. [a,b]Significant differences between frequencies of GV stage oocytes; [c,d]Significant differences between frequencies of MI stage oocytes; [e,f]Significant differences between frequencies of MII stage oocytes.

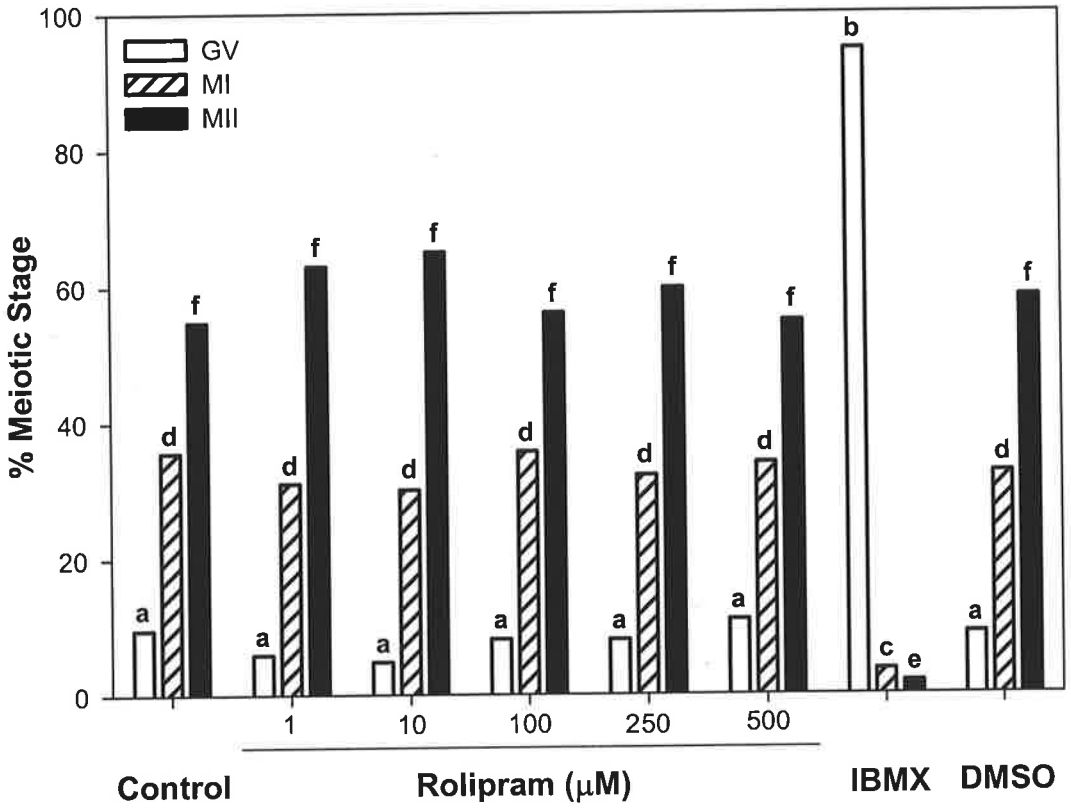


*Figure A5.*
Increasing concentrations of rolipram and its effects on the spontaneous maturation of isolated bovine oocytes following 16h of incubation. Oocytes were assessed and grouped into GV, MI, and MII stages. Each column represents a mean of 3 replicates. Values with different superscript letters indicate significant differences between frequencies of oocytes from different treatment groups in the same meiotic stage (Chi squared analysis, INSTAT package, $P < 0.0001$). A mean number of 40 oocytes were used in each treatment. Each column represents a mean of 3 replicates. [a,b]Significant differences between frequencies of GV stage oocytes; [c,d]Significant differences between frequencies of MI stage oocytes; [e,f]Significant differences between frequencies of MII stage oocytes.

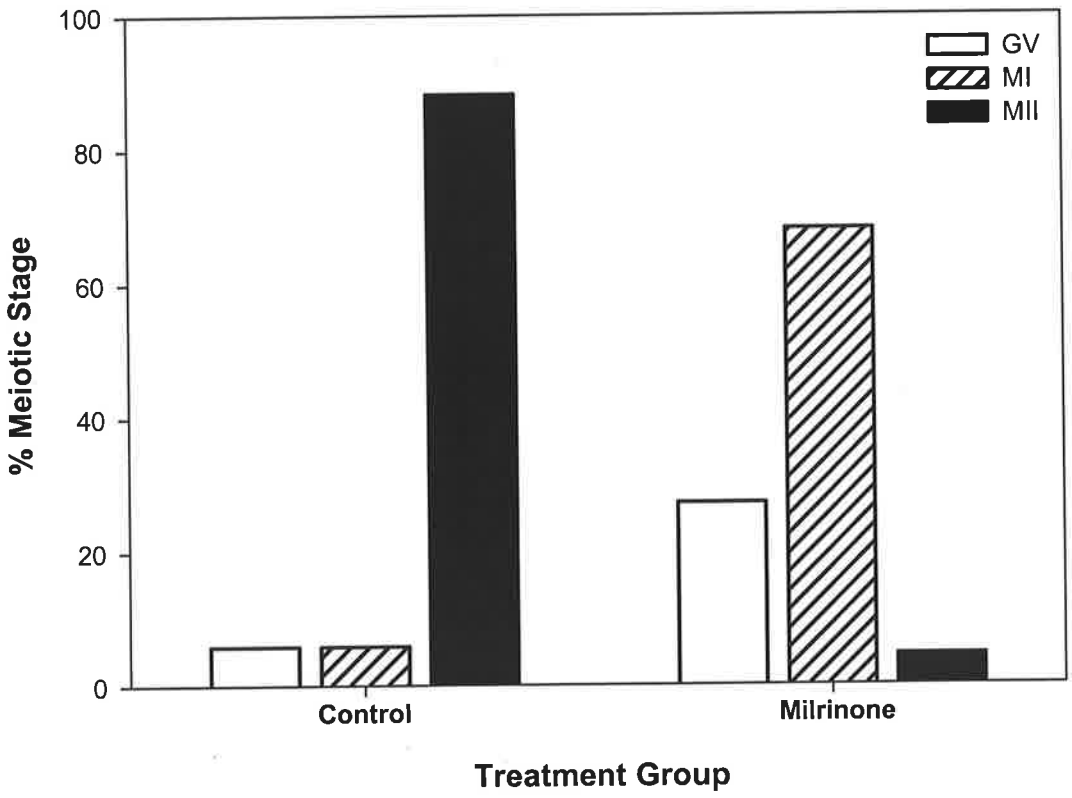


***Figure A6.***
The effects of MR on the spontaneous maturation of isolated bovine oocytes following 16h of incubation. Culture oocytes were obtained from the follicular aspirate of only four ovaries and following culture, were assessed and grouped into GV, MI, and MII stages. Each column represents a mean of only one replicate.

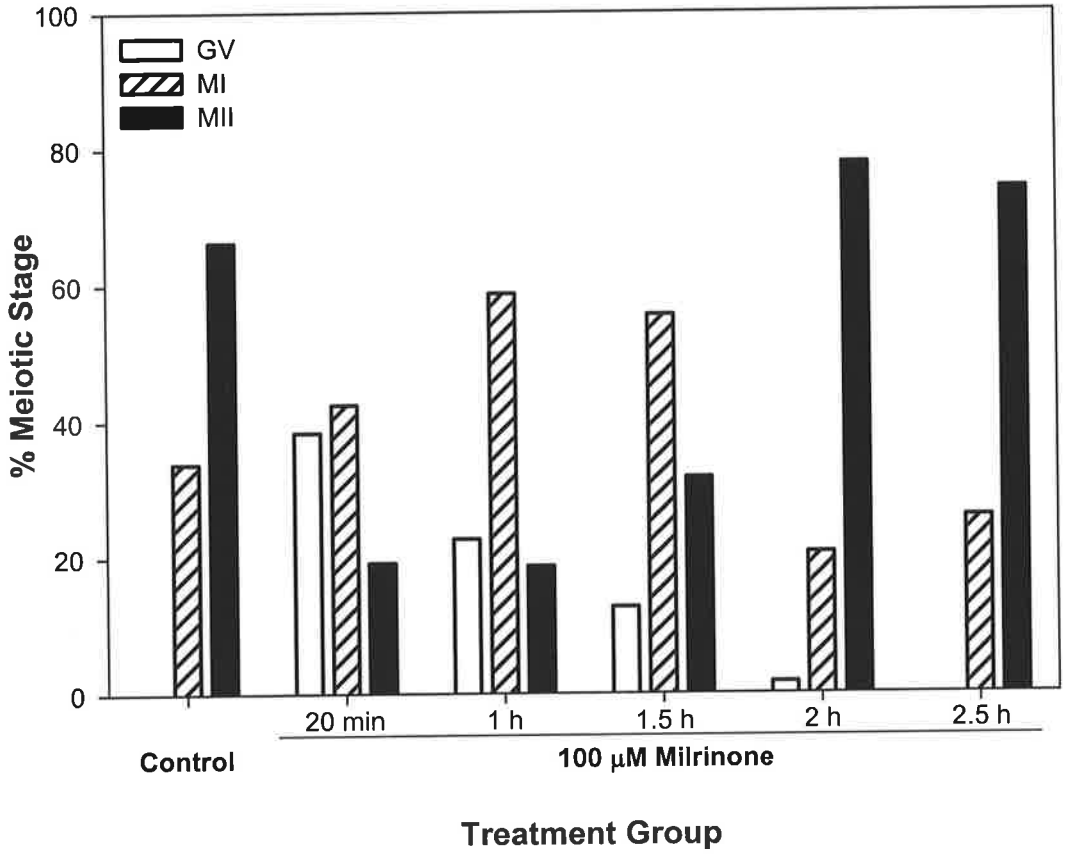


***Figure A7.***
The effect of increasing the length of time COC were left in IBMX-supplemented follicular fluid before transfer to culture medium containing milrinone on the spontaneous maturation of isolated bovine oocytes following 16h of incubation. Oocytes were assessed and grouped into GV, MI, and MII stages. A mean number of 40 oocytes were used in each treatment. Each column represents a mean of 3 replicates.

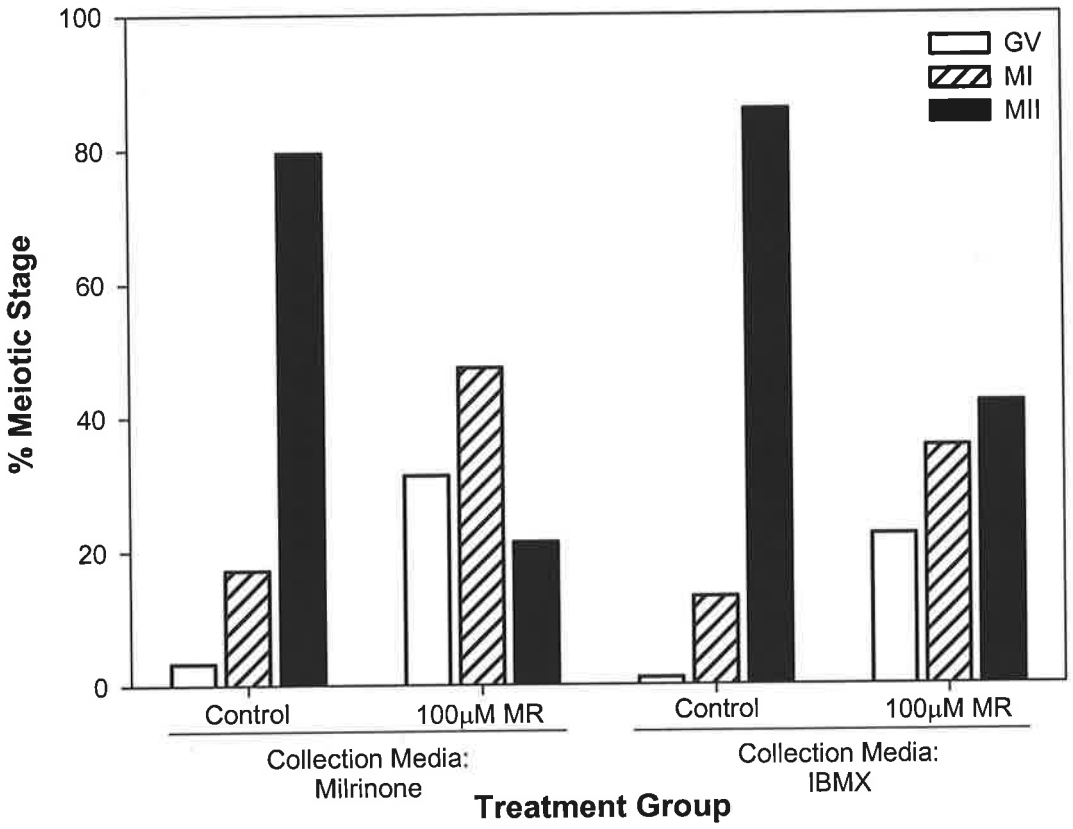


***Figure A8.***
Comparison of the effects of using MR- or IBMX-supplementation in the collection medium on the ability of milrinone to inhibit the spontaneous maturation of isolated bovine oocytes following 16h of incubation. Oocytes were assessed and grouped into GV, MI, and MII stages. Each column represents a mean of 2 replicates. A mean number of 40 oocytes were used in each treatment.

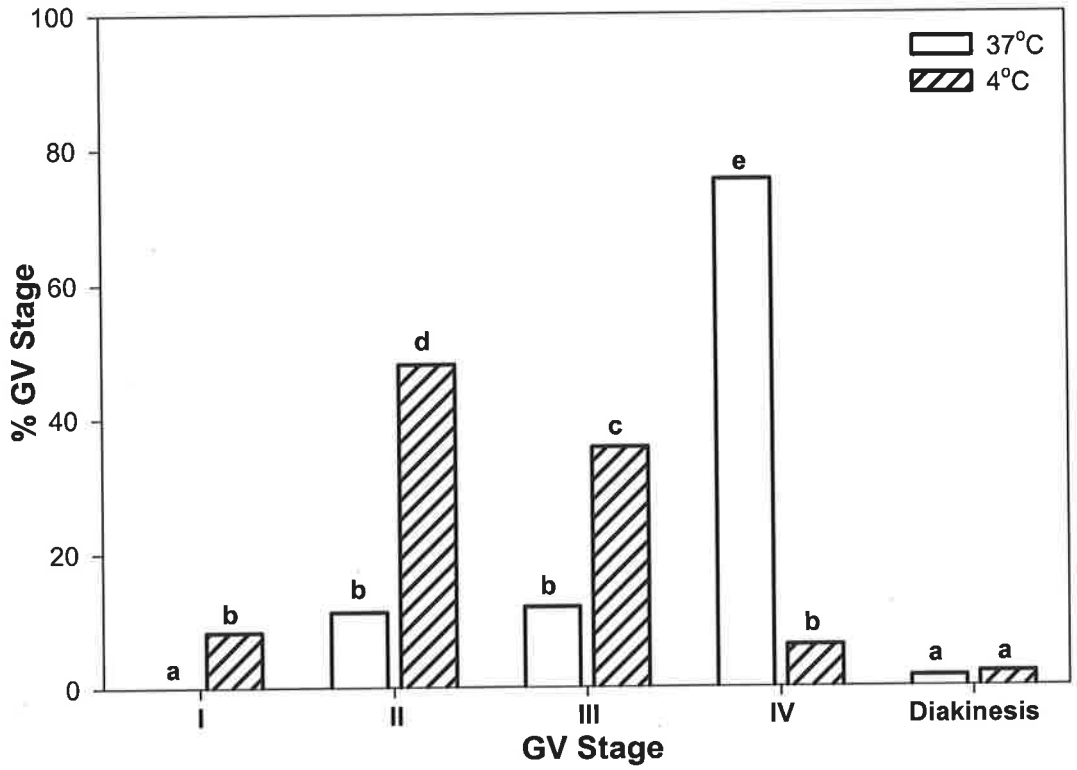


***Figure. A9.***
Effect of temperature of ovaries during their collection at the abattoir on the germinal vesicle configurations of the oocytes at time = 0; collection time approximately 3-7 hours. Oocytes were immediately fixed following aspiration from the follicles and then assessed for GV configuration. A mean number of 90 oocytes were used in each treatment group from three replicate experiments. Means with no common superscripts are significantly different (Chi squared analysis, $P<0.05$).

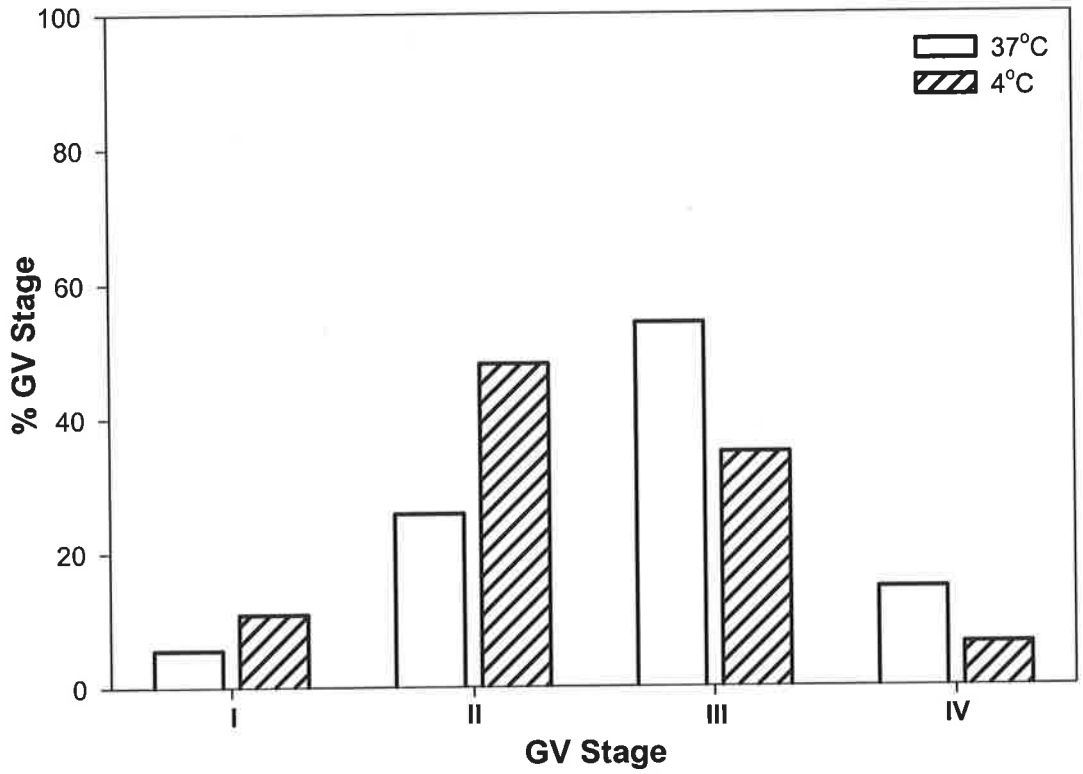


***Figure A10.***
Effect of temperature of ovaries during their collection at the abattoir on the germinal vesicle configurations of the oocytes at time = 0; collection time approximately 1.5-2 hours. Oocytes were immediately fixed following aspiration from the follicles and then assessed for GV configuration. A mean number of 90 oocytes were used in each treatment group from three replicate experiments.

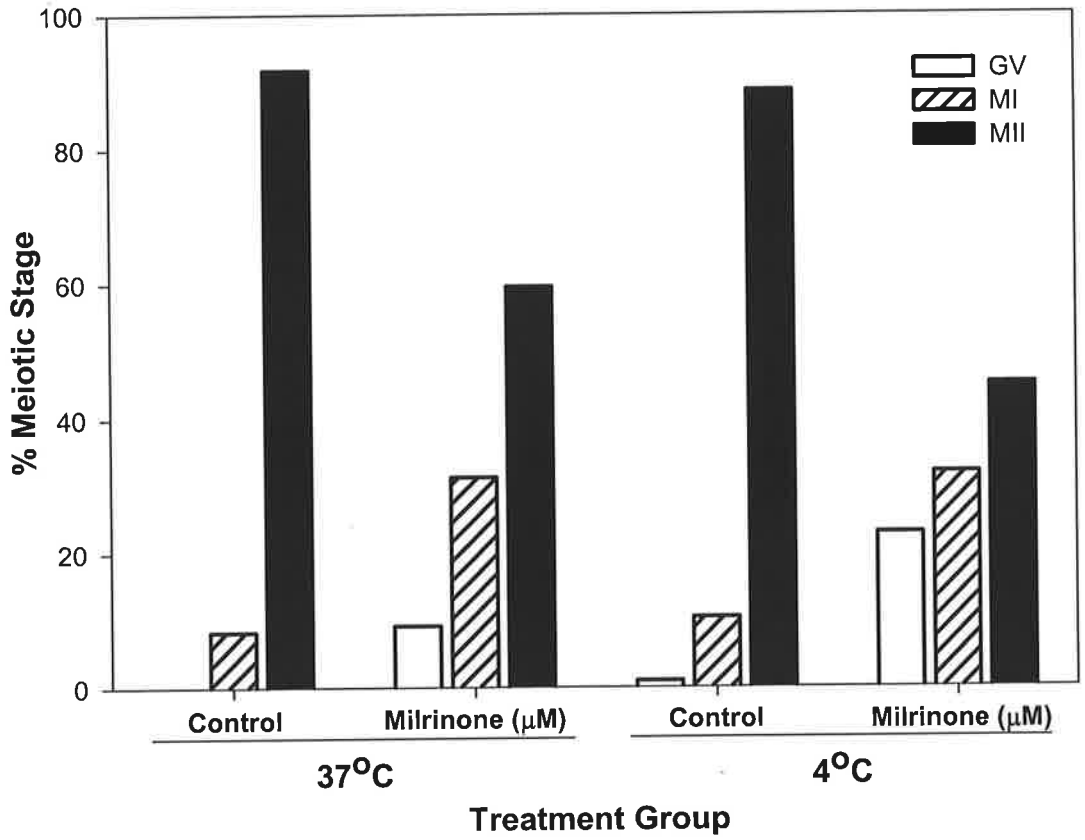


***Figure A11.***
Effect of the type 3 PDE inhibitor (milrinone; 100μM) on the spontaneous meiotic maturation of cumulus-oocyte complexes aspirated from ovaries collected at 4 or 37°C. COCs were cultured in control or milrinone-supplemented media and were then assessed for meiotic progression and classified as GV, MI, or MII stage. A mean number of 80 oocytes were used in each treatment group from three replicate experiments.

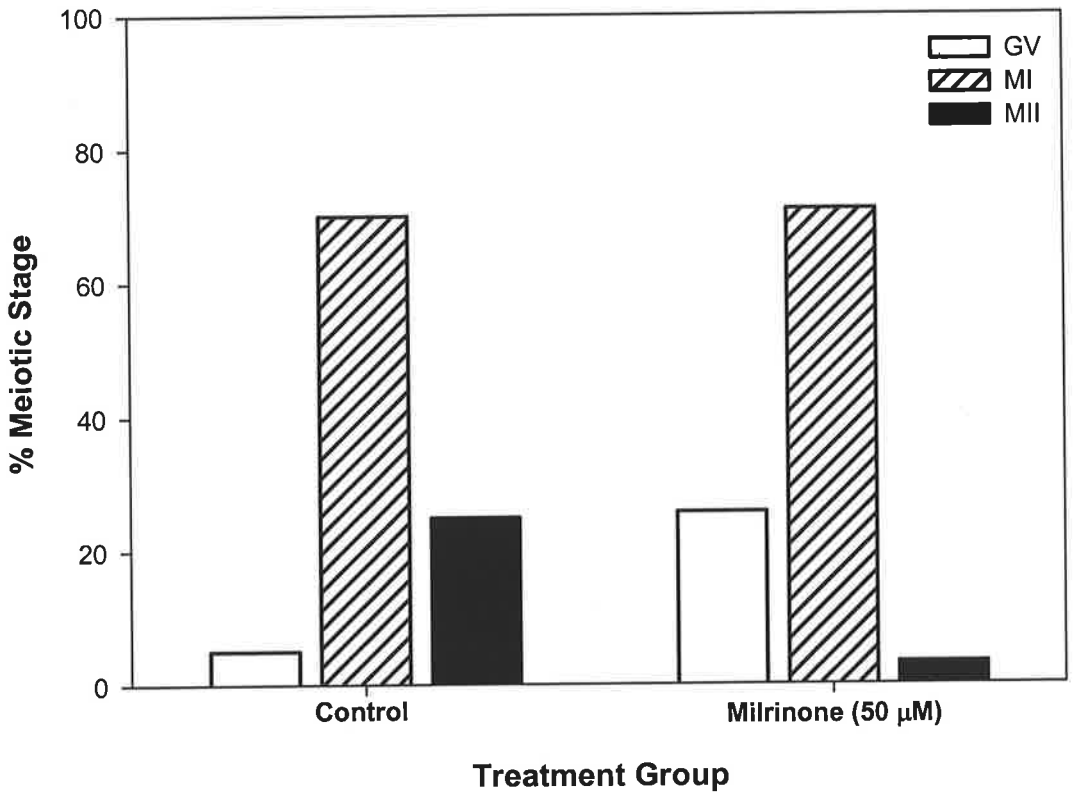


***Figure A12.***
The efficacy of milrinone in inhibiting spontaneous oocyte meiotic progression in vitro after 16 h of culture, following use of un-supplemented follicular aspirate as collection media. Oocytes were assessed and grouped into GV, MI, and MII stages. Each column represents a mean of 3 replicates. A mean number of 50 oocytes were used in each treatment.

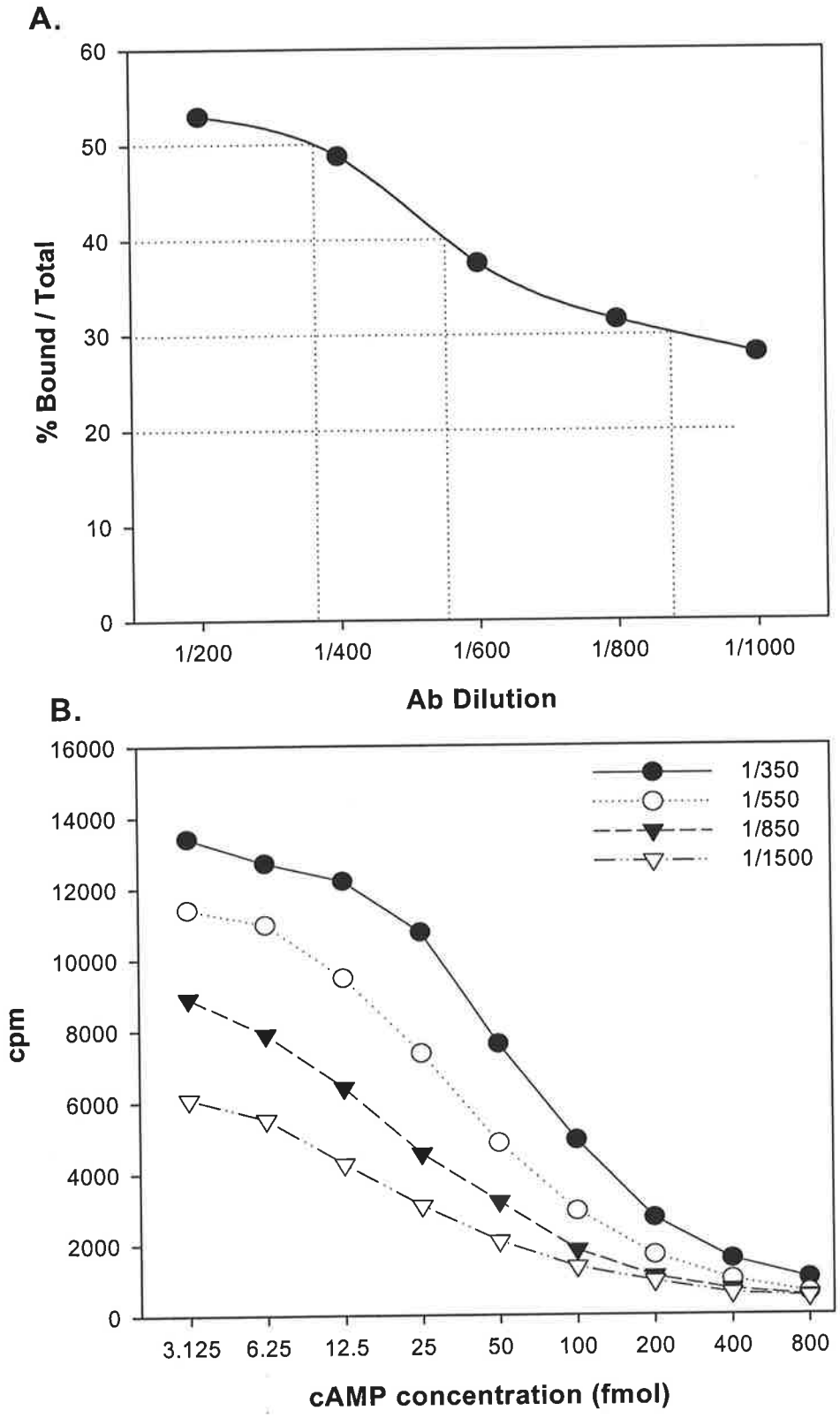


*Figure A13.*
Increasing the sensitivity of the cAMP radioimmunoassay. A. Determination of the percentage of bound Ab at dilutions of 1/200, 1/400, 1/600, 1/800, and 1/1000. Dilutions of Ab that gave 50, 40, 30, and ~20% bound Ab were then used to create standard curves, shown in B. Standard curves produced using 1/350, 1/550, 1/850, and 1/1500 dilutions.

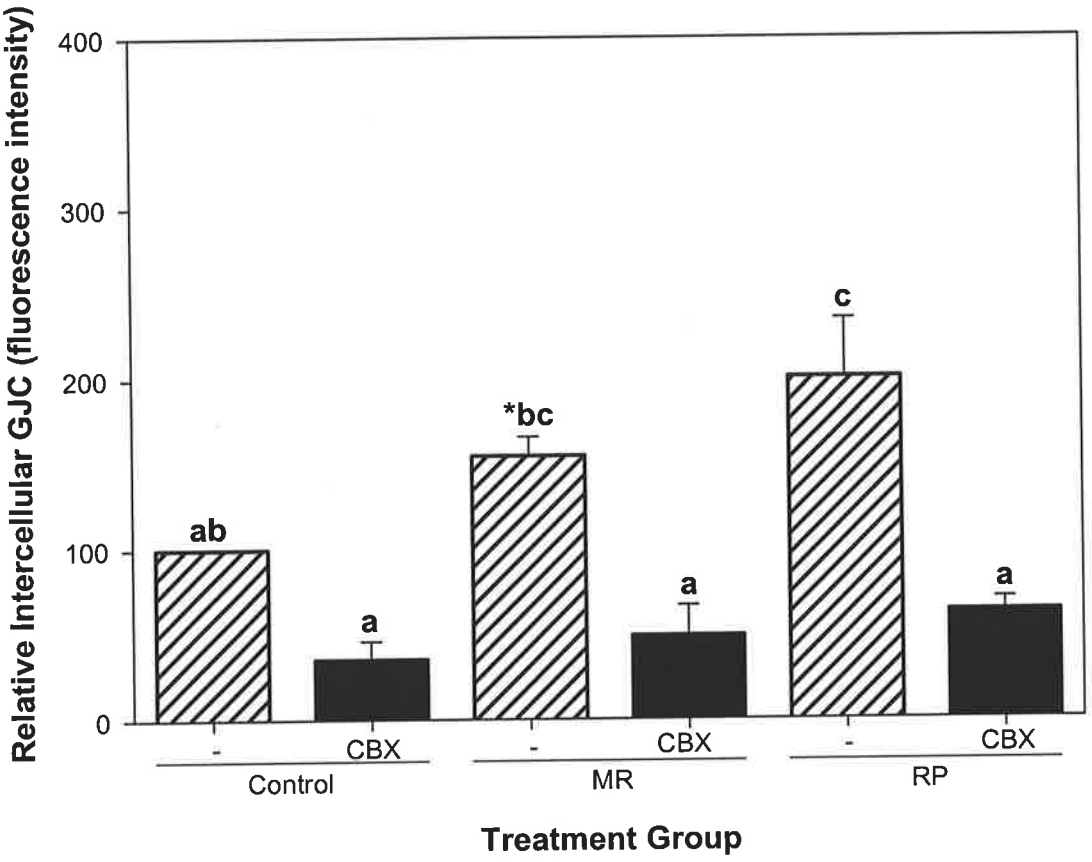


*Figure A14.*
Effect of the gap junction blocker Carbenoxolone on oocyte-cumulus cell GJC after 6 h of culture. COC were cultured with either the type 3 or the type 4 PDE inhibitor, with or without 30 μM carbenoxolone for 6 h and were then assessed for levels of GJC between the two cell types. A mean number of 10 oocytes were used in each treatment group in each of three replicate experiments. Means with no common superscripts are significantly different (Chi squared analysis, $P<0.05$).

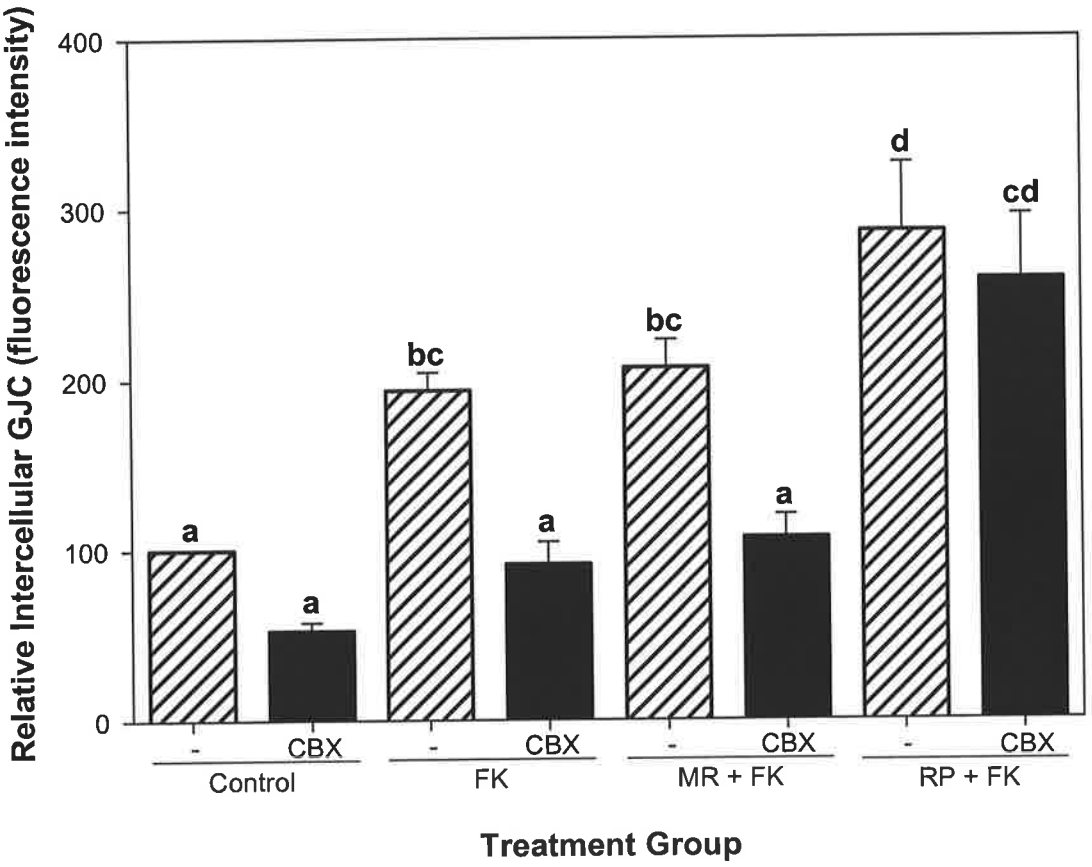


*Figure A15.*
Effect of the gap junction blocker Carbenoxolone on oocyte-cumulus cell GJC after 6 h of culture. COC were cultured in the presence of forskolin with either the type 3 or the type 4 PDE inhibitor, with or without 30 μM carbenoxolone for 6 h and were then assessed for levels of GJC between the two cell types. A mean number of 10 oocytes were used in each treatment group in each of three replicate experiments. Means with no common superscripts are significantly different (Chi squared analysis, $P<0.05$).

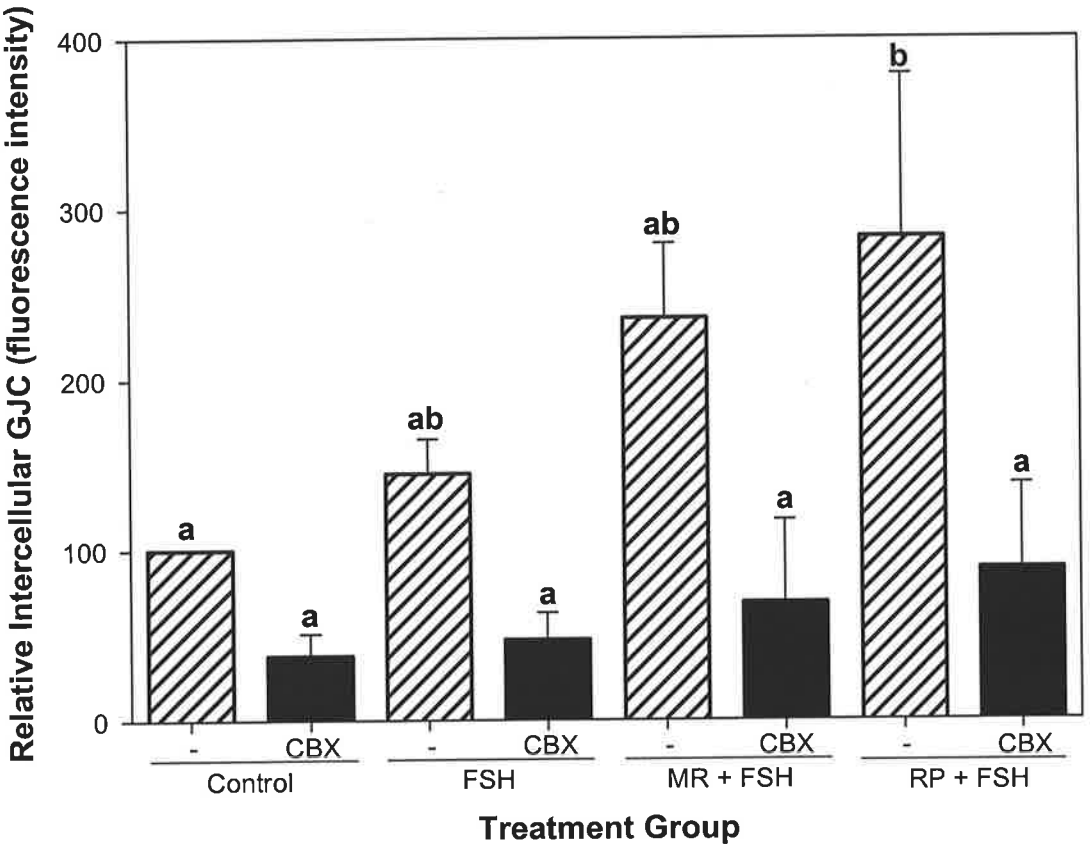


***Figure A16.***
Effect of the gap junction blocker Carbenoxolone on oocyte-cumulus cell GJC after 6 h of culture. COC were cultured in the presence of FSH with either the type 3 or the type 4 PDE inhibitor, with or without 30 μM carbenoxolone for 6 h and were then assessed for levels of GJC between the two cell types. A mean number of 10 oocytes were used in each treatment group in each of three replicate experiments. Means with no common superscripts are significantly different (Chi squared analysis, $P<0.05$).

**cAMP Radioimmunoassay**

Cyclic AMP content of COC, complex-derived oocytes (CDO), DO and supernatants of granulosa cell cultures were measured using a radioimmunoassay method described and validated previously [119]. CDO (COCs cultured with their cumulus mass intact and then denuded prior to assay) were used to evaluate the effect of the surrounding cumulus cells on intra-oocyte cAMP concentrations. After 5hrs of culture, 6-10 COC, 10-16 CDO, and 16-24 DO were washed in H-TCM + 2mM IBMX, transferred to 0.5ml of ethanol (100%) and stored at –20°C. Before cAMP measurements, samples were vortex agitated for 30 seconds and then centrifuged at 3000g for 15min at 4°C. Briefly, supernatants were removed, evaporated, resuspended in assay buffer (50mM sodium acetate, pH 5.5) and assayed following acetylation [the addition of triethylamine (AJAX Chemicals, Sydney, Australia) and acetic anhydride (BDH Laboratory Supplies, Poole, England) 2:1 v/v] and appropriate dilution. For assay of cAMP produced by MGCs after culture, media was removed from wells and stored at -20°C until diluted, acetylated, and assayed. $^{125}$I-labelled cAMP (specific activity of 2175 Ci/mM, prepared by iodinating 2'-0-monosuccinyladenosine-3':5'-cyclic monophosphate tyrosyl methyl ester (Sigma) using the chloramine T method [120]) and cAMP antibody (as prepared by Reddoch et al [119]) were added and duplicate samples covered and left overnight at 4°C. The following day, 1ml cold 100% ethanol was added and the samples were centrifuged at 3000g. The supernatant was removed and the pellet dried and counted using a gamma counter. Triplicate samples were used to produce a standard curve (0-800 fmol cAMP).

**Oocyte-Cumulus Cell Gap Junctional Communication Assay**

To assess the level of inter-cellular gap-junctional connection between the oocyte and its cumulus vestment during IVM, gap-junctional dye transfer from the cumulus cells to the oocyte was measured using the acetoxymethyl (AM) ester derivative of the fluorescent indicator calcein (calcein-AM; 3',6'-Di(O-acetyl)-2',7'-bis[N,N-bis(carboxymethyl) amino methyl]-fluorescein, tetraacetoxy methyl ester; C-3100; Molecular Probes; Eugene, OR, USA). Calcein-AM (MW 994.87) is non-fluorescent, electrically neutral and highly lipophilic because of the acetoxymethyl groups in the molecule and can rapidly permeate into the cytoplasm through the cell membrane [121-123]. Once inside the cell, non-specific endogenous esterases cleave the lipophilic acetoxymethyl groups, producing calcein (MW 622.54) – a fluorescent, negatively charged molecule that is unable to leak out of cells across the plasma membrane, but is able to pass between cells connected via gap junctions. Cumulus-oocyte gap junctional communication was measured by quantitative fluorescence microscopy as the amount of calcein in the oocyte, transferred from the cumulus cells through gap junctions via passive diffusion (see below).

Individual vials (50 μg) of calcein-AM were stored desiccated at -20°C and reconstituted at a concentration of 5 mM in DMSO fresh for each experiment (calcein-AM in solution is gradually hydrolysed over time to generate fluorescent calcein). At the concentrations of calcein-AM used, the final concentration of DMSO in any treatment well was <0.1%.

COCs were cultured in B-TCM + BSA (4 mg/ml) with or without either PDE inhibitor (100 μM) for 0, 1, 2, 3, 4, 6, 8, 16, or 20 h, after which they were transferred to a solution of 1 μM calcein-AM freshly made up in a modified phenol red- and protein-free B-TCM (CAM-

BTCM) + polyvinyl alcohol (PVA; 0.3 mg/ml; Sigma) ± PDE inhibitor for 15 minutes. Phenol red and protein were excluded from the standard oocyte culture media to avoid interference with fluorescence measurement due to non-specific cleavage of the AM group from calcein-AM. COCs were cultured with the dye for 15 min and were then transferred to calcein-AM-free media ± the various treatments and cultured for a further 25 minutes to allow for dye exchange between the cumulus cells and the oocyte. Unincorporated dye was then removed by three washes in calcein-AM-free CAM-BTCM with or without the various treatments. Prior to fluorescence analysis, COCs were completely denuded of their surrounding cumulus cells using vigorous pipetting so that only dye confined within the denuded oocyte after transport via gap junctions was measured. Carbenoxolone (CBX; 3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate; Sigma), a known gap junction blocker [124], was used to confirm intra-oocyte fluorescence was dependent on and due to conducting gap junctions between the cumulus cells and the oocyte.

Within 30 minutes of denuding, the intra-oocyte fluorescence emission of calcein in pulsed oocytes was measured using a fluorophotometric-inverted microscope (Leica, Wetzlar, Germany). DO in the experimental field of view were analysed singularly and independently from neighbouring oocytes. Fluorescence readings of DOs in each replicate experiment are represented as relative fluorescence intensity compared to the t=0 control DO reading (%).

## Appendix 3 - Tissue Culture Media

**Hepes-TCM (100ml)**

| | |
|---|---|
| 2X TCM stock | 50 ml |
| Milli Q $H_20$ | 46.5 ml |
| Stock B | 800 µl |
| Stock C | 700 µl |
| Hepes Acid | 298 mg |
| Hepes Na salt | 325 mg |
| Penicillin-Streptomycin (100 IU/ml) | 2 ml |

Adjust pH to 7.4 and osmolality to 265-285 mOsm. Filter sterilise through a 0.22 µM filter and store at 4°C.

**Bicarbonate-TCM (100ml)**

| | |
|---|---|
| 2X TCM stock | 50 ml |
| Stock B | 10 ml |
| Stock C | 0.5 ml |
| Glutamine | 1 ml |
| Milli Q $H_20$ | 38.5 ml |
| Penicillin-Streptomycin (100 IU/ml) | 2 ml |

If the 2X TCM stock is more than 2 weeks old add glutamine, otherwise omit glutamine and increase the volume of Milli Q water added to 39.75 ml. Filter sterilise through a 0.22µM filter and store at 4°C.

**IVM Media (10ml)**

| | |
|---|---|
| B-TCM | 9.89 ml |
| FSH (10 IU/ml) | 100 µl |
| Cysteamine (100 µM) | 10 µl |

Filter sterilise through a 0.22 µM filter. Equilibrate for a minimum of 2 hours at 38.5°C and 96% humidity in an atmosphere of 5% $CO_2$ in air.

**F-SOF**

Cook Bovine In Vitro Fert, Cook Veterinary Products.

**IVF media**

| | |
|---|---|
| F-SOF | 10 ml |
| Stock Penicillamine | 100 µl |
| Stock Hypotaurine | 100 µl |
| Stock Heparin | 10 µl |

Make up fresh on day required. Equilibrate for a minimum of 2 hours at 38.5°C and 96% humidity in an atmosphere of 5% $CO_2$ in air.

**Percoll**

90% Percoll:

| | |
|---|---|
| Percoll | 4.5 ml |
| Stock SH | 400 µl |
| Stock B | 50 µl |
| 100X SPAD | 50 µl |

45% Percoll:
Dilute 2 mL of 90% Percoll with 2 mL H-SOF. Warm solutions used to make up Percoll gradients to room temperature before use. Make up fresh on day required.

**H-SOF**
Cook Bovine In Vitro Wash; Cook Veterinary Products.

**E-SOF**
Cook Bovine In Vitro Cleave; Cook Veterinary Products.

**L-SOF**
Cook Bovine Blast Media, Cook Veterinary Products.

## Appendix 4 - Tissue Culture Media Stocks

**Stock B (50 ml)**

| | |
|---|---|
| $NaHCO_3$ | 1.05 g |
| Phenol Red | 5 mg |
| Milli Q $H_20$ | 50 ml |

Filter sterilise through a 0.22 μM filter and store at 4°C. Solution to be used within 2 weeks.

**Stock C (10 ml)**

| | |
|---|---|
| Na Pyruvate | 51 mg |
| Milli Q $H_20$ | 10 ml |

Filter sterilise through a 0.22 μM filter and store at 4°C. Solution to be used within 1 week.

**Stock S (glucose-, lactate-free SOF salts)**

| | |
|---|---|
| Milli Q Water | 100 mL |
| NaCl | 5.5 g |
| KCl | 0.3 g |
| $NaH_2PO_4$ (anhydride) | 0.036 g |
| $MgSO_4.7H_2O$ | 0.123 g |
| Kanamycin | 0.1 g |

Filter sterilise through a 0.22 μM filter and store at 4°C. Solution to be used within three months.

**Stock SH**

To 25 ml Stock S, add:

| | |
|---|---|
| Hepes free acid | 0.6 g |
| Hepes sodium salt | 0.65 g |

Store filtered at 4°C, lasts 3 months.

**SPAD (100X)**

| | |
|---|---|
| Milli Q $H_2O$ | 10 ml |
| NaCl | 0.29 g |
| $CaCl_2.2H_2O$ | 0.3 g |

Store filtered at 4°C; lasts for 3 months.

**2X TCM Stock - Standard (500ml)**

| | |
|---|---|
| Medium-199 | 9.8 g |
| Milli Q $H_20$ | 500 ml |

Filter sterilise through a 0.22 μM filter and store at 4°C.

## Appendix 5 - Reagents

**cAMP assay acetylation mix**
cAMP assay acetylation mix prepared fresh before each assay using triethylamine (Ajax Chemicals, Auburn, NSW, Australia): acetic anhydride (BDH Laboratory Supplies, Poole, Dorset, England) (2:1).

**Assay Buffer**
cAMP assay buffer prepared using 0.05 M sodium acetate (4.1 g/L sodium acetate; BDH Laboratory Supplies), pH adjusted to 5.5 with 1M Acetic acid.

**Calcein-AM**
Individual vials (50 μg) of Calcein-AM (Molecular Probes; C-3100) were stored desiccated at -20°C and reconstituted to a 5 mM solution with anhydrous dimethyl-sulphoxide (DMSO Hybrimax®; Sigma, D-2650). Calcein-AM was made up fresh for each experiment (Calcein-AM in solution is gradually hydrolysed over time to generate fluorescent Calcein).

**cAMP**
100 μl aliquots of a $10^{-8}$ mol/ml stock solution dissolved in 100% ethanol were lyophilised in a 1.6 ml Eppendorf tube ($1x10^{8}$mol per tube) for use in cAMP RIA.

**α-CAMP**
(As prepared by Reddoch et al [119]). Stock solution prepared by dissolving 100 mg of lyophilised antibody in 2.5 ml cAMP assay buffer. Stored frozen at -20°C in 0.1 ml aliquots (4 mg/100 μl). For use in cAMP RIA, one 0.1 ml aliquot diluted in 40 ml cAMP assay buffer containing 10 mg/ml BSA. 200 μl added per tube containing 100 μl standard or sample.

**Cilostamide**
1 M stock solution of Cilostamide (ICN Biomedicals Inc., Aurora, Ohio, USA) prepared by dissolving pure compound in DMSO. Stored at -20°C in desiccator in 3 and 100 μl aliquots.

**Clearing Solution**
For orcein de-staining of oocytes. Glycerol (Ajax Chemical Company): Acetic Acid (BDH Laboratory Supplies): Milli Q $H_2O$ (1:1:3)

**Dissociation Media 1**
100 ml B-TCM + 346 mg EGTA (9.1 mM; Sigma, E-4378) + 200 mg BSA (2 mg/ml; Fraction V, Sigma). Stored at 4°C. Lasts 4-6 weeks.

**Dissociation Media 2**
100 ml B-TCM + 79 mg EGTA (2.1 mM) + 17.1 g sucrose (0.5 M; Sigma) + 200 mg BSA (2 mg/ml). Stored at 4°C. Lasts 4-6 weeks.

**Forskolin**
250 mM stock solution (Sigma, F6886) prepared by dissolving pure compound in DMSO. Stored at -20°C in 1.5 and 6 μl aliquots.

**Heparin** (10 mg/ml stock)
Dissolve 20 mg Heparin (Sodium salt, Grade I-A; Sigma, H-3393) in 2 mL 0.9 % sterile saline.

**Hypotaurine** (10 mM stock)
Dissolve 10.9 mg hypotaurine (2-aminoethanesulfinic acid; Sigma, H-1384) in 10mL 0.9 % sterile saline and store filtered at 4°C. Lasts 1 week.

**$^{125}$I-cAMP**

cAMP labelled by David Casley, Department of Medicine, The University of Melbourne, Austin and Repatriation Hospital. The cAMP-tyrosine methyl-ester was iodinated using the chloramine T method [120] with resultant specific activity of 0.537 MBq/pmol. Stored in 70 % ethanol at -20°C.

**IBMX**
3-isobutyl-1-methyl-xanthine (Sigma, I-7018). 1 M stock solution prepared by dissolving pure compound in DMSO. Stored at -20°C in desiccator in 3 and 100 µl aliquots.

**Human Chorionic Gonadotrophin** (hCG)
hCG (Profasi; Serono, French's Forest, NSW, Australia) dissolved in sterile saline containing 0.1 % BSA. Stored at -20°C.

**Milrinone**
250 mM stock solution of milrinone (Sigma, M-4659) prepared by dissolving pure compound in DMSO. Stored at 4°C in desiccator in 2 µl aliquots.

**Mineral Oil**
Wash mineral oil (embryo-tested; Sigma, M-8410) with sterile Milli Q water and store at room temperature. Maintain sterility.

**Orcein Stain**
Stock solution prepared by dissolving 1 g of pure orcein powder (Sigma, O-7380) in 45 ml acetic acid with constant stirring over gentle heat for 1 h. Add ~55 ml Milli Q water to make up a final volume of 100 ml. Filter the stain twice before storing in a glass bottle at room temperature.

**Penicillamine**
20 mM stock solution prepared by dissolving DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid; Sigma, P-5125) in 0.9 % sterile saline and store filtered at 4°C. Lasts 1 week.

**Penicillin-Streptomycin**
Aliquot out Penicillin-Streptomycin solution (JRH Biosciences, Parkvelle, Victoria, Australia; 050-81901) and store at -20°C until use.

**rhFSH**
Recombinant human follicle stimulating hormone (rhFSH, Gonal-F, Serono, French's Forest, NSW, Australia; L1932701A). 10 IU/ml stock solution prepared by dissolving 75 IU in 7.5 ml sterile saline containing 0.1 % BSA. Stored at -20°C in 0.1 and 0.5 ml aliquots (1 and 5 IU respectively).

**Rolipram**
10 mM stock solution prepared by dissolving pure rolipram compound (ICN Biomedicals Inc., Aurora, Ohio, USA; Catalogue Number 159810) in DMSO. Stored at -20°C in 13, 28, and 220 µL aliquots.

## Appendix 6 - Published Version of Paper I

**'Differential Effects of Specific Phosphodiesterase Isoenzyme Inhibitors On Bovine Oocyte Meiotic Maturation'**
**Rebecca E Thomas, David T Armstrong, Robert B Gilchrist.**
**Developmental Biology; 2002; 244; 215-225.**

Thomas, R.E., Armstrong, D.T., and Gilchrist, R.B., (2002) Differential effects of specific phosphodiesterase isoenzyme inhibitors on bovine oocyte meiotic maturation. *Developmental Biology, v. 244 (2), pp. 215-225.*

## Appendix 7 - Published Version of Paper II

**'Bovine Cumulus Cell-Oocyte Gap Junctional Communication During In Vitro Maturation in Response to Manipulation of Cell-Specific Cyclic Adenosine-Monophosphate Levels'**

**Rebecca E Thomas, David T Armstrong, Robert B Gilchrist.**

**Biology of Reproduction; 2004; 70; 548-556.**

Thomas, R.E., Armstrong, D.T., and Gilchrist, R.B., (2004) Bovine cumulus cell-oocyte gap junctional communication during in vitro maturation in response to manipulation of cell-specific cyclic adenosine 3',5'-monophosphate levels. *Biology of Reproduction, v. 70 (3), pp. 548-556.*