# Bovine in vitro embryo production (IVP): assessment of two new approaches to improve the efficiency of embryo production

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# Abstract

Assisted reproductive technologies within the cattle industry are used to rapidly increase genetic gains within a herd. Technologies range from artificial insemination (AI), where only the male genetics are exploited, to embryo production technologies that exploit the genetics of both the sire and dam. The aim of my research was to evaluate two approaches to improve efficiency of *in vitro* embryo production; the first focus was the oocyte and the second the sperm.

Removal of oocytes from the follicle results in spontaneous resumption of nuclear maturation (meiosis) but not cytoplasmic maturation. A lack of synchronisation between the cytoplasm and nucleus has been related to a decreased developmental potential of oocytes used for *in vitro* embryo production (IVP). Meiotic inhibition through cAMP modulation results in improved embryo development and pregnancy rates. Acting through cAMP modulation, meiosis can be inhibited in the mouse with a significant improvement in post-fertilisation development. However, bovine oocytes resume meiosis after only 6 hours, which led to small improvements in developmental parameters. We hypothesised that increasing the time of meiotic inhibition of bovine oocytes to 18 hours would lead to greater improvements to bovine IVP. Various meiotic inhibitors were investigated alone and in combination following 18 hours of incubation. The combination of IBMX and oestradiol resulted in the greatest improvements in the proportion of bovine oocytes that were delayed in reaching the M2 stage of meiosis. The use of a specific PDE8 inhibitor with IBMX did not extend the period of meiotic inhibition. Synthetic cAMP (dbcAMP) alone did not prevent meiotic progression. Addition of CNP did not significantly shift the proportion of oocytes remaining at the GV stage. Further research as to why bovine oocytes fail to maintain meiotic arrest through cAMP manipulation is required.

Cryopreserved semen is used widely for AI programs, as well as IVP. Cryopreservation and thawing is associated with more dead and damaged sperm, and increased levels of Reactive Oxygen Species (ROS), especially superoxide. Semen quality is normally assessed by motility and morphology; improvements to semen fertility predictors would benefit the industry. We aimed to determine better fertility markers in cryopreserved bull sperm by assessing levels of zinc, ROS, and superoxide, IVP results, non-return to service (NRS) rates and computer assisted sperm analysis. In addition, ROS, superoxide and zinc levels were compared in sperm of *bos taurus* and *bos indicus*. Superoxide in the midpiece of sperm was positively correlated to cleavage ($P = 0.025$) and blastocyst ($P = 0.005$) development rates. The CASA measure of beat cross frequency (BCF) was positively correlated to cleavage rates ($P = 0.027$).

Total ROS in the midpiece negatively correlated to motility ($P = 0.02$). Zinc levels in the midpiece were significantly higher in *bos taurus* compared to *bos indicus* spermatozoa ($P < 0.05$), which possibly reflects diet and/or environment. These results may lead to better semen markers for AI and IVP success.

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# Declaration of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name 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 has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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# Abbreviations

| | |
|---|---|
| µL | Microlitres |
| µM | Micro Molar |
| AI | Artificial Insemination |
| ART | Assisted Reproductive Technologies |
| ATP | Adenosine Triphosphate |
| BCF | Beat Cross Frequency |
| BCS | Body Condition Score |
| bFF | bovine follicular fluid |
| BR | Butylrolatone-1 |
| BSA | Bovine Serum Albumin |
| cAMP | Cyclic Adenosine 3', 5' Monophosphate |
| CASA | Computer Assisted Sperm Analysis |
| cGMP | Cyclie Guanosine 3', 5' Monophosphate |
| CHX | Cycloheximide |
| CI | Confidence Interval |
| CL | Corpus Luteum |
| CNP | C-type Natriuretic Peptide |
| COC | Cumulus Oocyte Complex |
| dbcAMP | Dibutyryl cyclic Adenosine Monophosphate |
| dbcGMP | Dibutyryl cyclic Guanosine Monophosphate |
| DHE | Dihydroethidium |
| DMAP | Dimethylaminopurine |
| DMSO | Dimethyl Sulfoxide |
| DNA | Dinucleotide amino acid |
| EGF | Epidermal Growth Factor |
| ET | Embryo Transfer |
| FAD | Flavin Adenine Dinucleotide |
| FCS | Fetal Calf Serum |
| FGF | Fibroblast Growth Factor |
| FSH | Follicle Stimulating Hormone |
| g | Gravitational force |
| GJC | Gap Junctional Communication |
| GPx | Glutathione peroxidase/redutase |
| GV | Germinal Vesicle |
| GVBD | Germinal Vesicle Break Down |
| $H_2O_2$ | Peroxide |
| hCG | Human Chorionic Gonadotropin |
| IBMX | 3-Isobutyl-1-methylxanthine |
| IETS | International Embryo Technology Society |
| IVC | In Vitro Culture |
| IVF | In Vitro Fertilisation |
| IVM | In Vitro Maturation |

| | |
|---|---|
| IVP | In Vitro embryo Production |
| kDa | Kilodaltons |
| L | Litres |
| LH | Luteinising Hormone |
| M1 | Metaphase 1 |
| M2 | Metaphase 2 |
| mL | Millilitres |
| mM | Milli Molar |
| MOET | Multiple Ovulation and Embryo Transfer |
| MPF | Maturation Promoting Factor |
| MR | Milrinone |
| mSPOM | Modified SPOM |
| NADH | Nicotinamide adenine dinucleotide |
| nM | Nano Molar |
| nm | Nanometers |
| NPPC | Natriuretic Peptide Precursor Type-C |
| NPR | Natriuretic Peptide Receptor |
| NPR2 | Natriuretic Peptide Receptor type 2 |
| NRS | Non Return to Service |
| ODGF | Oocyte-derived Growth Factor |
| OMI | Oocyte Maturation Inhibitor |
| OPU | Ovum Pick Up |
| PBITU | Phenylene-bis (1,2-ethanediyl)-bis-isothiourea |
| PDE | Phosphodiesterase |
| ROS | Reactive Oxygen Species |
| rpm | revolutions per minute |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| SCNT | Somatic Cell Nuclear Transfer |
| SCR | Sire Conception Rates |
| SEM | Standard Error of the Mean |
| SOD | Superoxide Dismutase |
| SPOM | Simulated Physiological Oocyte Maturation |
| TBARS | Thiobarbituric acid reactive substances |

# Chapter 1

# The Literature Review

## 1.1 Assisted Reproductive Technologies used in the Bovine Industry

Assisted reproductive technologies (ART) are utilised by breeders of elite cattle to improve the overall genetic profile of their herds more rapidly than if they were solely using natural breeding methods. Reproductive technologies used within the beef and dairy industries include artificial insemination (AI), multiple ovulation and embryo transfer (MOET), somatic cell nuclear transfer (SCNT, cloning) and *in vitro* production (IVP) of embryos (Choudhary et al. 2016; Wu & Zan 2012). Artificial insemination allows elite male genetics to be exploited. Fresh or frozen semen is inseminated into cows or heifers that have either come into heat naturally or have had their oestrous cycles synchronised (fixed-time AI) (Borges-Silva et al. 2016). This technology has been widely adopted within dairies as a method of reproduction without the need to feed and maintain bulls. AI also allows for the introduction of diverse genetics into the herd. Cloning does not result in genetic improvement. Instead it is used to replicate an animal that is already considered to be of very high genetic merit (Choudhary et al. 2016). Utilising IVP and MOET enable both the sire and dam's genetics to be exploited and promotes rapid genetic gain within a herd (Choudhary et al. 2016).

For IVP, immature oocytes from donors are aspirated by a technique called "ovum pick up" (OPU) (Callesen, Greve & Christensen 1987; Pieterse, Vos, Kruip, Wurth, et al. 1991). These oocytes are visually sorted on site based on morphology, and the oocytes deemed to be good quality are transported to an IVP laboratory where they undergo *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* culture (IVC). Approximately one week after oocyte aspiration, maturation and fertilisation, blastocysts can either be cryopreserved or transferred in the synchronised recipient cows (Hasler 2014). For MOET, the oestrus of donor cows is hormonally synchronised, and a series of follicle stimulating hormone (FSH) injections are administered to stimulate superovulation. Around the time of ovulation, the donor cows undergo AI, and embryos are flushed a week later. These embryos can either be cryopreserved and stored or transferred into synchronised recipient cows (Callesen, Liboriussen & Greve 1996).

Bovine embryo production and transfer is increasing in popularity worldwide. According to data compiled for the International Embryo Technology Society (IETS) in 2000, approximately 40,000 bovine IVP embryos were transferred into recipients worldwide. In contrast, in 2015, more than 900,000 bovine embryos were transferred into synchronised recipients and more than 400,000 of these embryos were produced *in vitro* (Perry 2016; Thibier 2001). Development and conception rates continue to be lower when embryos are cultured *in vitro* (IVP) compared to *in vivo* (MOET) (Rizos et al. 2002). Therefore further

investigation is required to improve IVP results, so that the technology will be more attractive to cattle stud breeders.

### 1.1.1 Embryo production technologies

Both IVP and MOET have advantages over the other. For MOET, embryos can be cryopreserved and exported as long as appropriate protocols have been followed (Ponsart & Pozzi 2013). IVP embryos tend not to be exported, due to the cryopreservation methods utilised, that do not meet export standards. An advantage to IVP is that the valuable donor animals are not subjected to hormonal stimulation, therefore, there is less chance of causing fertility issues due to the use of ovine or porcine derived FSH. The use of porcine or ovine derived FSH has been correlated with causing an immune response. Therefore the dosage of FSH needs to be increased regularly to continue stimulating the ovaries. Often a long term consequence is that the ovaries stop responding to the FSH stimulation (Drion et al. 1998; Kanitz, Schneider & Becker 1996). Synchronisation of the donors is also time consuming and requires donors to be put through the cattle yards regularly. Additionally, the quantity of semen used to fertilise oocytes *in vitro* is greatly reduced; one straw can often be utilised to fertilise the oocytes of several donor cows. Until recently the ability to cryopreserve embryos was a major advantage to using MOET, however, many commercial bovine IVP laboratories are now successfully cryopreserving embryos (Reyes & Jaramillo 2016). While both MOET and IVP will allow the number of calves produced per donor per year to be greatly increased, IVP has the advantage. OPU can be performed within as little as four day intervals, although many laboratories prefer to keep the intervals to approximately 2 weeks (Merton et al. 2003; Pieterse, Vos, Kruip, Willemse, et al. 1991). Additionally, oocytes can be collected from donors that are up to 4-months pregnant. Conversely, a minimum of 6 weeks is required between completing one MOET program and beginning another (Pontes et al. 2009). Therefore each donor cow has the ability to produce more embryos through IVP compared to MOET each year.

### 1.1.2 Factors affecting the success of embryo technologies

The environment during early embryogenesis can have lasting effects on the offspring. These conditions not only affect gene expression and morphology of the embryo, but also birthweight and the length of gestation. For example, when high concentrations of fetal calf serum are included in culture medium, “Large Offspring Syndrome” can occur; where *in vitro* derived offspring could be 20 percent larger

compared to *in vivo* derived offspring (Thompson et al. 1995; Walker, Hartwich & Seamark 1996). In addition, overall successful implementation of any of the assisted reproductive technologies can be affected by a range of conditions including: diet, climate, age, genetics and skill of the technicians.

Several factors have been shown to affect fertility of cattle including: nutrition, body condition score (BCS, a score of 1-5), climate, photoperiod, age and breed. These various factors have been shown to affect oocyte quality, semen quality, embryo quality and pregnancy outcomes. It has been established that for maximum reproductive performance, cattle should be on a rising plain of nutrition while undergoing OPU, AI, MOET or ET (Adamiak et al. 2005; Parr et al. 2015). Heat stress has negative effects on fertility of cattle. Several studies have reported decreased oocyte maturation and embryo production rates in summer compared to winter (Gendelman et al. 2010; Gendelman & Roth 2012; Pavani et al. 2015; Rocha et al. 1998; Torres-Junior et al. 2008). The percentage of Holstein cows that became pregnant following their first insemination after calving was higher in winter (63.8±0.4%) compared with summer (40.2±1.5%) (Pavani et al. 2015). Heat stress also resulted in an increased proportion of denuded and/or degenerated oocytes collected during OPU. There was a delayed negative effect on *in vitro* blastocyst development rates, suggesting that long term exposure to heat stress was required to illicit an effect (Torres-Junior et al. 2008). *Bos indicus* breeds are well known for their resilience in hot and humid environments compared to the *bos taurus* breeds that thrive more in temperate environments (Rocha et al. 1998). Conversely, *bos indicus* cows have been reported to produce fewer embryos and with a lower implantation rate during the winter months (Bastidas & Randel 1987). However, another study found no difference in the quality of oocytes or blastocyst development rates from Brahmans collected in summer or winter (Rocha et al. 1998). When comparing the results of the two studies by Bastidas and Randel (1987) and Rocha et al. (1998) is seems that the negative effect of summer on *bos taurus* cattle is much more extreme than the negative effect of winter on *bos indicus* cattle. Blastocyst development rates fell from approximately 30% in winter to near 0% during summer for the *bos taurus* cows. However, development rates of transferable blastocysts per Brahman donor dropped approximately 30% from a maximum of 4.2 to 2.9 in winter.

### 1.1.3 Identifying gamete quality

Currently, oocytes and sperm used in commercial settings are assessed visually to determine whether they are good quality or not (Bo & Mapletoft 2013; Cetica, Dalvit & Beconi 1999; Fitzpatrick et al. 2002). Many studies have shown this method to be flawed, and therefore determining a more rigorous method to assess gamete quality would be ideal. Some studies have assessed textures on the surface of the

oocyte while others have focused on the oocyte's metabolism, for example mitochondrial copy number and also ATP production (El Shourbagy et al. 2006; Fragouli et al. 2015; Olexikova et al. 2017; Thompson, Brown & Sutton-McDowall 2016). Studies focused on identifying sperm fertility markers have focused on several factors including mitochondrial activity, reactive oxygen species (ROS) production, and zinc content (Agarwal et al. 2014; Chatterjee & Gagnon 2001; Kerns et al. 2018).

## 1.2 Biology of bovine oocyte maturation, fertilisation and embryo development

### 1.2.1 Oocyte maturation

Female mammals develop all of their oocytes during the fetal stage. The oocyte develops the germinal vesicle at meiotic prophase, where their development remains arrested until they are either exposed to the LH surge prior to ovulation or undergo atresia. Following exposure to the LH surge, meiosis resumes and the oocyte progresses to metaphase 2 (M2), reducing the number of chromosomes from diploid to haploid. The oocyte remains at M2 until fertilisation (Lonergan & Fair 2016).

It is clear that oocytes matured *in vivo* compared to *in vitro* have higher developmental competence (Pontes et al. 2009). The decrease in competency for *in vitro* matured oocytes has been attributed to a lack of coordination between nuclear and cytoplasmic maturation. *In vivo*, the surge in LH resumes meiosis and triggers ovulation. It is believed that this transitory arrest allows time for the cytoplasmic maturation to occur. When immature oocytes are aspirated from the follicles and matured *in vitro*, they spontaneously restart meiosis and develop to M2. The result is an imbalance between the maturity of the nucleus and the cytoplasm (Kawamura et al. 2011; Rizos et al. 2002). It has been demonstrated that the size of the follicles being aspirated also affects the capability of that oocyte to develop into a viable embryo. Bovine oocytes aspirated from follicles >6 mm in diameter and fertilized and cultured in vitro produced significantly more blastocysts compared with oocytes that were aspirated from follicles 2-6 mm in diameter ($P<0.01$, 65.9% vs. 34.3% respectively) (Lonergan et al. 1994).

### 1.2.2 Fertilisation

Fertilization takes place at the ampulla-isthmus junction of the oviduct *in vivo*. The sperm adhere to the epithelial cells lining the caudal isthmus (sperm reservoir). The role of the causal isthmus is to increase the lifespan of the sperm, control capacitation and to limit the number of sperm at the site of fertilisation to reduce the likelihood of polyspermy (Miller 2015). Final capacitation of the sperm occurs when it meets the cumulus cells and the acrosome reaction occurs (Chian, Okuda & Niwa 1995).

Sperm are believed to trigger $Ca^{2+}$ oscillations within the oocyte which play an important role in activating the oocyte to resume meiosis and to promote cell divisions (mitosis) to commence. It also plays a role in preventing polyspermy (He et al. 1997; Homa, Carroll & Swann 1993). The significance of the intracellular $Ca^{2+}$ oscillations are not completely understood. However it is accepted that the $Ca^{2+}$ ions play an important role in release of the cortical granules and promoting cell division. There are two hypotheses for how the sperm promotes $Ca^{2+}$ oscillations within mammalian oocytes. The first is that there is an interaction between the sperm and the oocyte's plasma membrane. The second suggests that a sperm-bound factor is released into the oocyte (identified as phospholipase C zeta, PLCz (Homa, Carroll & Swann 1993)), and $Ca^{2+}$ is released following the interaction of this factor with unknown cytosolic targets. The second hypothesis tends to have more support within the literature (White, Pate & Sessions 2010).

### 1.2.3 Embryo development

Successful development of preimplantation embryos is dependent on the environment. From the time the matured cumulus-oocyte complex (COC) ovulates into the infundibulum of the oviduct, it is exposed to a complex environment containing the appropriate levels of gases (Fischer & Bavister 1993), energy substrates, amino acids, hormones and minerals etc. A variety of external and internal factors have been found to affect development from the zygote to the blastocyst stage. The oxygen content (Thompson, Partridge, Houghton, Cox, et al. 1996; Thompson, Partridge, Houghton, Kennedy, et al. 1996), acidity, access to energy substrates (sucrose, lactate and glucose) (Thompson, Partridge, Houghton, Cox, et al. 1996), amino acids, ions and growth factors, are some of the factors that have been found to influence embryonic development *in vitro* and *in vivo*.

As the embryo develops *in vivo*, it moves down the oviduct until it develops into a morula. It enters the uterus around the time it undergoes its first cellular differentiation and develops into a blastocyst. During

this transition, the embryo encounters an environment that matches its developmental requirements. Energy requirements of the early preimplantation embryo, prior to compaction, are relatively low compared to the later stage preimplantation embryos, which would be found in the uterus (Thompson, Partridge, Houghton, Cox, et al. 1996). This increase in energy production relates to both cellular differentiation and the activity of the Na-K ATPase pump which is responsible for forming the blastocoel cavity (Houghton et al. 2003). Following expansion, bovine embryos undergo a period of elongation prior to attaching to the uterine wall.

Although IVP has improved in many ways over the last few decades, embryos that have been developed *in vitro* continue to be inferior in quality compared to *in vivo* developed embryos (Pontes et al. 2009). Blastocysts that have been developed *in vitro* tend to have lower total cell numbers and also a smaller ratio of inner cell mass to trophectoderm cells compared to *in vivo* derived blastocysts. In addition, *in vivo* derived embryos continue to have a higher success rate of implanting and developing into viable offspring (Ealy, Wooldridge & McCoski 2019). It has been well documented that energy requirements of the early embryo from the zygote to the compact morula are very low (Thompson, Partridge, Houghton, Cox, et al. 1996; Trimarchi et al. 2000). The source of energy also changes to a reliance on the glycolytic pathway post compaction, as opposed to the oxidative phosphorylation pathway pre-compaction (Thompson, Partridge, Houghton, Cox, et al. 1996). The oviduct and uterus, where the *in vivo* embryo develops, is a highly complex environment compared to the semi defined culture media used for most mammalian embryonic culture *in vitro* (Ferraz et al. 2017). *In vitro* systems attempt to mimic the uterine environment as best as possible; incubators tend to be set to the species' core body temperature. Gaseous environments are often mimicked with the use of special gas mixes, and acidity of media is controlled with the use of buffers.

## 1.3 Two approaches to improve bovine embryo development *in vitro*

### 1.3.1 Meiotic inhibition

Developmental competence of oocytes following OPU, IVM and IVF remains low compared to oocytes that have been hormonally stimulated to ovulate. Some believe a lack of homogeneity among oocytes that have been aspirated from follicles of varying developmental stages could lead to decreased competence of oocytes used in IVM. Therefore, oocytes may not have completed the process of gaining all the essential components for their development; compromising their ability to mature, fertilise and

develop (Gilchrist & Thompson 2007). Evidence suggests that if meiosis is arrested during IVM for a limited period of time using cAMP modulators, cytoplasmic maturation can be supported, resulting in improved overall competency of oocytes matured *in vitro* (Nogueira et al. 2003).

Various meiotic inhibitors have been investigated as a way of improving cytoplasmic maturation *in vitro*, and improving the overall ability of the oocyte to fertilise and develop *in vitro* (Barretto et al. 2007; Santiquet et al. 2017; Zeng et al. 2014). Some suggest that meiotic inhibition may also improve cryotolerance of bovine oocytes (Monteiro et al. 2017). To maintain the oocyte arrested in meiosis, it is generally accepted that the intracellular concentration of cAMP needs to be maintained at a high level. Several methods of arresting meiosis have been investigated, including the use of C-Type Natriuretic Peptide, Phosphodiesterase inhibitors, Adenylate Cyclase activators and phosphorylation inhibitors. Several meiotic inhibitors have been shown to only be effective at arresting meiosis while the oocyte is still at the germinal vesicle stage. Once Germinal vesicle breakdown (GVBD) has occurred, none of the inhibitors had any effect (Sun et al. 1999)

#### 1.3.1.1 Regulation of cAMP for meiotic inhibition

Methods to prevent spontaneous resumption of meiosis involve modulation of intracellular concentrations of the messenger molecule, cyclic adenosine 3', 5' monophosphate (cAMP). Cyclic AMP is produced by the actions of the adenylate cyclase within the oocyte and granulosa cells. The cAMP produced by granulosa cells is transported into the oocyte via GJC. Ideally cAMP concentrations need to be maintained at a high level and gap junction communication (GJC) between the oocyte and cumulus cells needs to remain open; this is critical for developmental competence to be improved. This can be achieved by either increasing the concentration or production of cAMP or preventing its degradation. Phosphodiesterase (PDE) enzymes within the oocyte decrease intracellular cAMP levels by degrading cAMP (fig. 1). *In vivo*, PDE enzyme activity is inactivated by cyclic guanosine 3', 5' monophosphate (cGMP) that is produced by mural granulosa cells and transported into the oocyte through GJC (Norris et al. 2009). *In vivo*, the LH surge causes closure of the GJC, therefore preventing the inhibition of the PDE enzyme by cGMP and the transportation of externally produced cAMP into the oocyte (Norris et al. 2008). A decreased concentration of cAMP results in resumption of meiosis (Vivarelli et al. 1983). *In vitro*, degradation of cAMP can be prevented by inhibiting the phosphodiesterase enzymes either through specific or non-specific PDE inhibitors (such as 3-isobutyl-1-methylxanthine (IBMX)) or through stimulating the production of cGMP. Stimulation of the Natriuretic Peptide Receptor type-2 (NPR2) with C-type Natriuretic Peptide (CNP) results in the production of cGMP from the cumulus cells (Azari-Dolatabad et al. 2016; Soares et

al. 2017; Thomas et al. 2004). Options for increasing production of cAMP include addition of activators of adenylate cyclase activity, such as forskolin or addition of synthetic analogues of cAMP (dibutyryl cAMP (dbcAMP)) (Bagg et al. 2006; Paschoal et al. 2016; Zhang, JH et al. 2015).

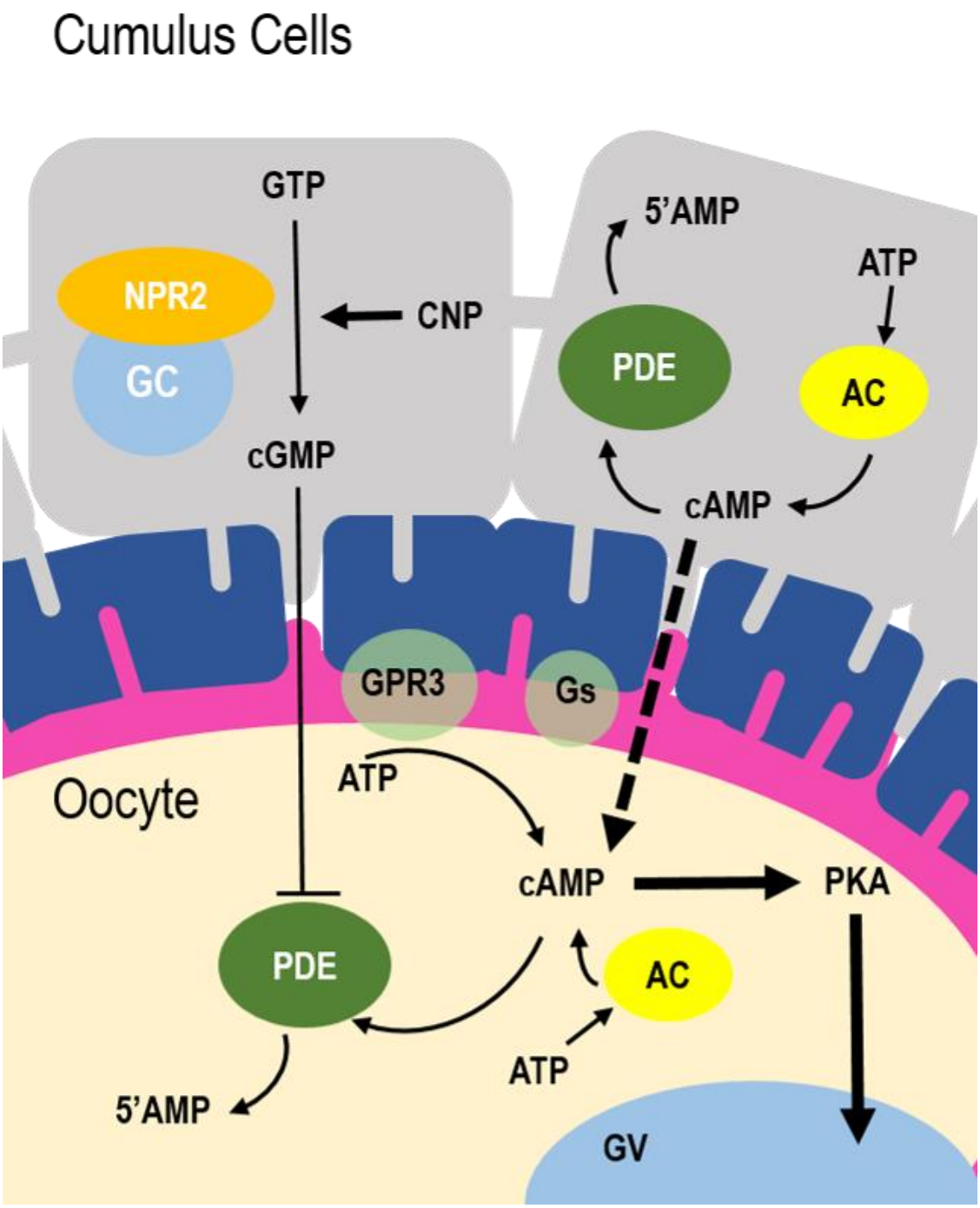


**Figure 1:** A schematic of cAMP modulation within the oocyte (adapted from Russell et al. 2016). Adenylate cyclase (AC) produces cAMP within the cumulus cells and oocytes. In addition, cAMP is synthesised from ATP within the oocyte via G-protein-coupled receptor type 3 (GPR3) activity. Phosphodiesterase (PDE) enzymes reduce cAMP to 5'AMP in the oocyte and cumulus cells. Transportation of cAMP from the cumulus cells to the oocyte occurs via gap junctions. C-type Natriuretic Peptide (CNP) binds to its receptor NPR2 on the guanylate cyclase (GC) to activate production of cGMP within the cumulus cells. Through gap junctions, cGMP is transported to the oocyte, where is binds to PDE enzymes, inhibiting the degradation of cAMP.

*1.3.1.1.1 Phosphodiesterase (PDE) enzyme inhibition*

The majority of studies investigating meiotic inhibition have focussed on mouse COCs. It has been demonstrated that the major PDE within the mouse oocyte is PDE3, and specific PDE3 inhibitors alone are effective at preventing meiotic resumption (Nogueira et al. 2003; Tsafriri et al. 1996). Non-specific PDE inhibitors, such as IBMX, or specific PDE3 inhibitors are only moderately effective at preventing meiotic resumption of the bovine oocyte (Aktas et al. 1995; Barretto et al. 2007; Sirard & First 1988b; Thomas, Armstrong & Gilchrist 2002). Investigation into the types of cAMP specific PDEs present within the bovine oocyte found there were two main types; PDE3 (approximately 80%) and PDE8 (approximately 20%). All other cAMP specific PDEs make up less significant proportions (Sasseville et al. 2009). Initial studies investigating PDE8 and its role in maintaining bovine oocytes in meiotic arrest utilised a non-specific PDE8 inhibitor, Dipyridamole. The addition of 50 or 250 μM Dipyridamole, in conjunction with 100 μM forskolin, to oocyte maturation medium significantly increased cAMP levels within the bovine oocyte after 4 hours and reduced the proportion of COCs progressing through to M2 after 16 hours of treatment. When Dipyridamole and IBMX were used alone or in conjunction, there was no significant difference in the proportion of COCs that remained at the GV stage after 9 hours of treatment, and by 18 hours, very few COCs remained at the GV stage. Furthermore cleavage and blastocyst development rates were significantly impaired when dipyridamole was used during IVM as part of an IVP protocol (Sasseville et al. 2009). This result implies that this particular PDE8 inhibitor is toxic and further investigation of the effects of alternative PDE8 inhibitors during oocyte maturation is necessary.

*1.3.1.1.2 Guanylate cyclase activation*

An alternative method of inhibiting meiotic resumption within oocytes is through the use of CNP to stimulate guanylate cyclase to increase the production of cGMP. In the mouse, an increased expression of Natriuretic peptide precursor type-C (NPPC) in the granulosa cells of pre-ovulatory follicles has been demonstrated. The expression of NPPC rapidly declined following stimulation with LH or human chorionic gonadotropin (hCG) to promote ovulation. Culture of mouse COCs with CNP suppressed meiotic resumption. In humans, CNP levels decreased in the follicular fluid after treatment with hCG to stimulate ovulation (Kawamura et al. 2011). The use of 10 nM CNP alone is able to prevent GVBD in approximately 80% of mouse COCs after 24 hours of treatment (Wei et al. 2017). Alternatively higher doses of 100 nM CNP are required to prevent meiotic resumption in cat (Zhong et al. 2016) and pig (Blaha, Nemcova & Prochazka 2015) oocytes for 24 hours. In contrast, 200 nM CNP alone has been shown to maintain bovine COCs at the GV stage of meiosis for 6 hours, with a significant proportion of COCs undergoing GVBD after 8 hours of culture (Franciosi et al. 2014). Encouragingly, a 6 hour prematuration period with

200 nM CNP followed by 28 hours IVM resulted in a more than 50% increase in bovine blastocyst development rates and more than 30% increase in total blastocyst cell numbers compared to the control (Zhang, T et al. 2017). Follicular steroids (oestradiol, progesterone and androstenedione) with CNP increased the percentage of bovine COCs maintained at the GV stage for 9 hours, compared to if CNP was used alone, with an improved effect when *bos indicus* COCs were used compared to *bos taurus* (Soares et al. 2017). Expression of the receptor for CNP, NPR2, has been shown to be increased when 10 µg/mL oestradiol is included in the media (Xi et al. 2018). The combination of 100 nM CNP and 500 µM IBMX increased the proportion of bovine COCs arrested at the GV stage after 6 hours of treatment compared to when 100 nM CNP was used alone (approximately 90% compared to 75% respectively) (Soto-Heras, Paramio & Thompson 2019). Further investigation into the use of combinations of CNP with PDE inhibitors and steroids is required to increase the timeframe that bovine COCs can be maintained at the GV stage.

*1.3.1.1.3 Adenylate cyclase activation*

Adenylate cyclase activators regulate the concentration of cAMP within cells. They work by activating the enzyme responsible for producing cAMP. Forskolin, a known adenylate cyclase activator, inhibits meiotic resumption by activating adenylate cyclase to maintain high cAMP levels within the oocyte. Six hours of pre-maturation with 50µM Forskolin is effective at maintaining approximately 90% of sheep oocytes at the germinal vesicle stage. By 10 hours only 50% of the sheep oocytes remained arrested in meiosis, and by 22 hours, very few oocytes, treated with or without forskolin remained at GV (Azari-Dolatabad et al. 2016). However, another study utilising bovine COCs, showed that similar concentrations of forskolin (25, 50 or 100 µM) were ineffective at arresting meiotic resumption (Paschoal et al. 2016). Furthermore, treatment of sheep COCs with 50 µM forskolin for 6 hours prior to IVM resulted in significantly higher blastocyst development rates *in vitro* (37.7% vs. 23.5% respectively) (Azari-Dolatabad et al. 2016). The bovine study reported no significant difference in blastocyst production or total cell numbers when different concentrations of forskolin were used in the 6 hour pre-maturation period (Paschoal et al. 2016).

*1.3.1.1.4 Synthetic cAMP analogues (dibutyryl cyclic adenosine monophosphate (dbcAMP))*

Another method of inhibiting GVBD is by increasing the cAMP concentration within the oocyte by including a synthetic cAMP analogue. Recently, 1mM dbcAMP has been reported to successfully maintain porcine oocytes in meiotic arrest for 22 hours and this led to significant improvements in cleavage and blastocyst development rates *in vitro* (approximately 200% increases compared to the control) (Gil et al. 2017). The

majority of studies using dbcAMP, to date, have focused on porcine COCs (Bagg et al. 2006; Gil et al. 2017; Park & Yu 2013). While others have reported that dbcAMP is effective at maintaining meiotic arrest in porcine oocytes, not all have resulted in improvements to embryonic development *in vitro* (Bagg et al. 2006; Park & Yu 2013). Furthermore, an earlier study investigating the impact of exposing porcine oocytes to 1 mM dbcAMP during the first 20 hrs of culture found no significant difference in GVBD rate at 28 hr or maturation rate at 44 hrs. However, they did report an increased blastocyst development rate when oocytes were exposed to 1 mM dbcAMP (21.5 ± 2.5%) compared with the control (9.2 ± 1.6%). The study reported transferring embryos into 4 gilts, of which 3 became pregnant. Unusually, only embryos that were produced following exposure of the oocyte to dbcAMP were transferred. Reportedly, 19 live piglets were farrowed, but the total number of embryos that had been transferred was not disclosed (Funahashi, Cantley & Day 1997). Another study utilising porcine COCs, found that effectiveness of dbcAMP treatment was dependent on the size of follicles that the COCs were derived from. Medium sized antral follicles (>4–6 mm diameter) produced COCs that responded better to the 1 mM dbcAMP treatment compared to small follicles (2-4 mm diameter). Improved blastocyst development rates following dbcAMP treatment were only obtained from COCs derived from medium sized follicles. The greatest improvement to blastocyst development rates was achieved when 1 mM dbcAMP was combined with 500 ng/ml amphiregulin (AREG), 100 ng/mL human recombinant bone morphogenetic protein 15 (BMP 15) and 100 ng/mL human recombinant growth differentiation factor 9 (GDF9) (Sugimura et al. 2015). To date, there have been limited studies published regarding the treatment of bovine COCs with dbcAMP. A recent study investigated the effect of a 2 hour pre-maturation period in the presence of 1 mM dbcAMP and 500 µM IBMX on the developmental competence of bovine oocytes (Sugimura et al. 2018). Blastocyst development rates were significantly improved when COCs were pre-matured with 1 mM dbcAMP and 500 µM IBMX compared with the control group (62.4±4.9% vs 40.4±2.2% respectively). Combining 1mM dbcAMP and 500 µM IBMX was more effective at maintaining GJC compared with 1 mM dbcAMP alone (Sugimura et al. 2018). Further investigation into whether dbcAMP can be used to prevent meiotic resumption of bovine COCs on its own or combined with other inhibitors is required.

*1.3.1.1.5 Effects of combining multiple cAMP modulators in vitro for meiotic inhibition*

Previous studies have shown that delaying the resumption of meiosis, through the use of cAMP and cGMP modulators such as PDE inhibitors, adenylate cyclase activators, and CNP can lead to improvements in oocyte developmental competence; as evidenced by improved cleavage and embryo development rates, increased blastocyst cell numbers and improved implantation rates (Nogueira et al. 2003; Soares et al. 2017; Zeng et al. 2014; Zhang, T et al. 2017). Oocytes from mouse (Nogueira et al. 2003), pig (Gil et al.

2017) and human (Shu et al. 2008) can be prevented from resuming meiosis for extended periods of up to 24 hours, and this delay in meiotic resumption is associated with improved developmental rates. Mouse oocytes can be maintained in meiotic arrest through the use of a single inhibitor specific to PDE3, the major intraoocyte PDE in mouse oocytes (Tsafriri et al. 1996). Preventing meiotic resumption for 24 hours in mouse COCs with a specific PDE3 inhibitor (Org 9935) led to a more than 50% improvement in fertilisation rates, blastocyst development rates and live birth rates compared to the conventional *in vitro* control (Nogueira et al. 2003). Delaying meiotic resumption of bovine oocytes for 2 hours with 100 μM forskolin (adenylate cyclase activator) and 500 μM IBMX (inhibits all cAMP specific PDEs except PDE8) followed by 28 hours of IVM containing 20 μM cilostamide, also resulted in improvements in embryo production rates and blastocyst cell numbers (Albuz et al. 2010). However, these were not of the same magnitude as observed following 24 hour meiotic inhibition in other species (Nogueira et al. 2003). There was approximately a 20% improvement in fertilisation rates following the extended maturation period to 30 hours, followed by a more than 200% increase in blastocyst development rates from oocytes that had cleaved (Albuz et al. 2010). Live birth rates were not investigated, however, blastocyst cell numbers were increased by approximately 30% when oocytes underwent a delayed maturation *in vitro* (Albuz et al. 2010). To date, combinations of PDE inhibitors and or adenylate cyclase activators have only been effective at maintaining bovine oocytes at GV for short periods (Albuz et al. 2010), with few studies demonstrating maintenance of bovine oocytes at the GV stage *in vitro* for more than 6 hours (Dode & Adona 2001; Santiquet et al. 2017). Increasing the time that bovine oocytes can be maintained at GV through cAMP modulation would likely result in greater improvements to oocyte developmental competence *in vitro*.

Studies that were able to maintain bovine oocytes at the GV stage for up to 24 hours involved culturing COCs in hemi-sections of follicles (in contact with thecal cells) (Richard & Sirard 1996a), culturing in follicular fluid (Ayoub & Hunter 1993), or in the presence of 2 mM 6-dimethyl aminopurine (6-DMAP, an inhibitor of Maturation Promoting Factor (MPF)) (Dode & Adona 2001). Although COCs progressed to M2 when removed from culture with 2 mM 6-DMAP, subsequent blastocyst development was impaired by almost 50% (Dode & Adona 2001). Inhibition of germinal vesicle breakdown (GVBD) was more effective when the follicular fluid had been collected from small (2-4 mm) or medium (5-9 mm) follicles compared to large follicles (10-20 mm) during oestrus. Inhibition of GVBD was also increased if the follicular fluid was collected during oestrus (>50% of COCs at GV) compared to mid dioestrus (approximately 40%) or early proestrus (approximately 35%) when they originated from small or medium sized follicles. In contrast, the ability of follicular fluid from large follicles (10-20 mm) to inhibit meiosis was lower when collected at oestrus (approximately 35% of oocytes at GV after 24 hours) compared to late metoestrus (>50%) or early dioestrus (>50%) (Ayoub & Hunter 1993). Another more recent study investigated the

effect of inhibiting resumption of meiosis using follicular fluid on oocyte developmental potential. Nuclear maturation to metaphase II (M2) at 22 and 24 hours was lower when 75% or 100% follicular fluid was used, compared to 0, 25% or 50%. However, subsequent cleavage rates were impaired by 10% and blastocyst development rates were impaired by more than 50% when a concentration of 75% or more follicular fluid was used during oocyte maturation. The cell numbers within the inner cell mass were also increased when COCs had been cultured for 24 hours with 50% follicular fluid (approximately 35 cells) compared to 25% (approximately 29 cells) or no follicular fluid (approximately 30 cells) prior to IVF and IVC (Cruz et al. 2014). A more recent study managed to maintain bovine oocytes at GV *in vitro* for 21 hours with the combination of 100 µM cilostamide (PDE3 inhibitor), 100 nM CNP, 100 µM sildenafil (PDE5 inhibitor), 100 ng/mL bone morphogenetic protein 15 (BMP15, an oocyte secreted factor), 100 µM hypoxanthine (a purine), 100 nM oestradiol and 1 x $10^{-5}$ IU/mL FSH. However, no improvements to *in vitro* fertilisation rates, blastocyst development rates or total cell numbers of blastocysts were reported (Santiquet et al. 2017). This result contrasted with the improvements made to bovine embryo development rates and blastocyst cell numbers following a 2 hour pre-treatment with IBMX and forskolin (Albuz et al. 2010). However, for a commercial operation involving on-farm oocyte collection an optimal protocol would enable COCs to remain in the pre-maturation media from the time they are processed until they arrive in the laboratory, which in Australia, for example, could vary from 2 to 18 hours. Therefore further investigation is required to develop a protocol that enables meiosis of bovine oocytes to be inhibited for 18 hours or longer while also improving oocyte developmental competence.

*1.3.1.1.6 Maturation Promoting Factor (MPF) inhibitor*

Maturation promoting factor (MPF) is made up of cyclin dependent kinase 1 (CDK1) and cyclin B1 (Doree & Hunt 2002; Gautier et al. 1988). 6-dimethylaminopurine (6-DMAP) works as an inhibitor of MPF. It has been demonstrated to arrest more than 90% of bovine oocytes at the GV stage for 24 hours when used at 2 mM (Dode & Adona 2001). It was demonstrated that the meiotic inhibition was reversible, where more than 90% of COCs reached the M2 stage following 24 hours of IVM, regardless of whether they had been pre-incubated with 2 mM of 6-DMAP for 0, 12, 18 or 24 hours. Unfortunately, blastocyst development rates were significantly impaired when COCs had been pre-incubated with 2 mM 6-DMAP for 12, 18 or 24 hours compared to the control. Time of exposure to 6-DMAP did not affect blastocyst development rates (Dode & Adona 2001). Another earlier study used 5 mM DMAP to arrest meiosis. The results differed a little; Saeki et al. (1997) maintained 72% of COCs at the GV stage of meiosis for 24 hours, while Dode and Adona (2001) reported that the majority of COCs had undergone GVBD by 12 hours.

Inhibitors of CDK1 have been used effectively to prevent meiotic resumption in buffalo COCs (Kumar et al. 2018). Roscovitine, an inhibitor of cyclin dependent kinase 1 (CDK1) was used in conjunction with cilostamide (PDE 3 inhibitor) to successfully inhibit meiotic resumption of buffalo COCs for 24 hours. Cleavage and blastocyst development rates *in vitro* were not affected when the buffalo COCs went through IVP following meiotic inhibition (Kumar et al. 2018). It was promising that the use of both roscovitine and cilostamide for a 24 hour period did not cause toxicity to the embryonic development. Further investigation of a combination of an MPF inhibitor and cAMP modulators might also lead to extended periods of meiotic inhibition and improved bovine oocyte developmental competence *in vitro*.

Dall'Acqua et al. (2017) attempted to replicate a commercial setting when trialling the use of various meiotic inhibitors. They attempted to block meiosis during the time that COCs would routinely spend during transport to the lab (6 hrs of duration). Four treatments were tested: 100 µM Butyrolatone-I (BR (inhibitor of cyclin dependent kinase), modified SPOM (500 µM IBMX and 100 µM Forskolin, mSPOM), 100 mM Milrinone (MR) and bovine follicular fluid (bFF). In addition they had 3 control groups: IVM in a CO2 incubator in the lab in IVM media containing either fetal calf serum, or BSA, and IVM in a portable incubator in IVM media containing BSA. The results were a little unusual. The GV rates after 6 hours in transport conditions did not differ significantly between the 4 treatments containing meiotic inhibitors. However, the groups blocked by BR or mSPOM for 6 hours had a higher percentage of oocytes maintained at GV compared to two of the control groups containing no inhibitors; the control matured in the $CO_2$ incubator with FCS, and the control matured in the portable incubator with BSA. In addition after 24 hrs (control groups 24 hrs IVM, treatments 6 hrs pre-IVM with an inhibitor plus 18 hrs in IVM), there was no significant difference in the M2 rates between any of the groups. Furthermore, the cleavage rates following *in vitro* fertilisation were significantly higher in the control group matured in the $CO_2$ incubator in media containing FCS compared with both other controls, and all other pre-maturation treatments. Blastocyst development rates were significantly higher in the control group matured in the incubator with FCS compared to the group pre-matured with MR, and the control group matured in the portable incubator with BSA for the initial 6 hrs. Blastocyst development rates did not different significantly between any of the other groups. Total cell counts and apoptosis rates of day 7 blastocysts was also assessed. Whilst there were no significant differences for either assessment between any groups, overall cell counts were very low across the board compared to those reported for day 7 bovine blastocysts in other studies (Albuz et al. 2010; Thomas et al. 2004). This suggests a low quality of the systems utilised. In addition it was noted that the oocytes were incubated in the portable incubator, under transport conditions for 6 hours in a gaseous environment containing 5%$O_2$ 5% $CO_2$ and then moved to 5% $CO_2$ in air for the remainder of IVM in the laboratory incubator. Most other studies of pre-IVM systems using meiotic inhibitors are

conducted with 5% $CO_2$ in air, therefore this different environment may have had an impact on the result (Albuz et al. 2010; Barretto et al. 2007).

*1.3.1.1.7 Hypoxanthine*

A purine found naturally within follicles, Hypoxanthine, has been shown to successfully inhibit meiosis in oocytes derived from many species including mouse (Eppig, Wardbailey & Coleman 1985), rat (Tornell et al. 1990), pig (Miyano et al. 1995), and monkeys (Warikoo & Bavister 1989). However, it was found to only transiently inhibit meiosis in bovine (Sirard & First 1988a) and goat oocytes (Ma et al. 2003). Hypoxanthine (4 mM) maintained the majority of goat oocytes at the GV stage for 6 hours (56%), but by 8 hours, only 33% of goat COCs remained at the GV stage (Ma et al. 2003). Renewing the hypoxanthine within the culture media after 4 hours did not have any effect on reducing the GVBD rate at later timepoints compared to when hypoxanthine was not renewed indicating that the decrease in meiotic inhibition with time was not due to declining concentrations of hypoxanthine within the media (Ma et al. 2003). When the renewal media included 300 μM dbcAMP, the percentage of oocytes remaining at the GV stage after 18 and 24 hours of culture was higher than groups in which hypoxanthine only was renewed, or groups that didn't receive a renewal (Ma et al. 2003). Hypoxanthine levels in follicular fluid decrease as antral follicle size increases in the goat (Ma et al. 2003). An early investigation of using hypoxanthine as a meiotic inhibitor found it reduced the percentage of bovine COCs that underwent GVBD following 6 hours of incubation. Using 2 mM hypoxanthine alone reduced the number of bovine COCs undergoing GVBD from 80% to 60% after 6 hours of treatment. The addition of 0.05 or 0.2 mM adenosine further reduced the number of bovine COCs undergoing GVBD to approximately 30% (Sirard & First 1988a).

### 1.3.1.2 Conclusions and areas where further investigation is needed for meiotic inhibition of bovine COCs

Further investigation is required for improved to bovine oocyte competency by extended meiotic inhibition for use in IVP. For pre-maturation benefits to be adapted in a commercial setting, meiotic inhibition of bovine oocytes needs to be extended to at least 18 hours. This would make it more practical to users and increase the effect of improved embryo quality should be compounded if patterns match other species, such as the mouse (Nogueira et al. 2003). Implantation data relating to bovine blastocysts produced *in vitro* following a pre-maturation treatment with meiotic inhibitors is needed to assess if pre-maturation is beneficial bovine embryonic developmental competence. Inhibition of all major cAMP modulating PDEs within the bovine oocyte has be suggested as a method of increasing meiotic inhibition rates. The major

PDEs within the bovine oocyte has previously been reported at PDE3 (80%) and PDE8 (20%) (Sasseville et al. 2009). Inhibiting PDE3 but not PDE8 is only transiently effective at preventing meiotic resumption of the bovine oocyte. Using a non-specific PDE8 inhibitor (Dipyridamole) was reported to have some effect at inhibiting meiosis of bovine oocytes, however it had a negative effect on embryo development (Sasseville et al. 2009). Investigation of an alternative PDE8 inhibitor in combination with the broad spectrum PDE inhibitor (IBMX) could lead to improvements to the rates of inhibition of meiotic resumption and improve developmental competence. While dbcAMP is effective at inhibiting meiotic resumption of porcine oocytes, few studies have investigated its use with bovine oocytes. Investigation of dbcAMP alone and in conjunction with other meiotic inhibitors needs to be attempted with bovine oocytes. Furthermore, combining several meiotic inhibitors may result in extended periods of meiotic inhibition, even if each component is not effective on its own. Utilising MPF inhibitors is effective at inhibiting meioisis of bovine COCs, however the treatments tend to be detrimental to developmental competency *in vitro (*Dode & Adona 2001*)*. Future studies should investigate combining MPF inhibitors with cAMP modulators to extend the period of meiotic inhibition and improve bovine oocyte competency *in vitro*.

## 1.3.2 Sperm

### 1.3.2.1 Sperm use in the bovine industries

Cryopreserved bull semen is normally stored in straws under liquid nitrogen for use in AI and IVF programs. Alternatively, semen can be maintained at ambient temperatures or chilled, although this is less common, and tends to be favoured in small geographical regions such as New Zealand. Long distances between semen collection locations and insemination locations makes chilled semen unattractive, as it needs to be utilised within 2 days for reliable results (reviewed by Vishwanath & Shannon 2000). Cooling or freezing of semen increases shelf life by reducing the metabolic rate of sperm (Fu et al. 2019). Not only does the percentage of motile sperm decrease over days of chilled storage, the ability to fertilise oocytes *in vitro* also decreases as storage time increases (Krzyzosiak et al. 2001). Cryopreservation enables storage of semen over extended periods of time, even years, however the process causes significant levels of sperm death. Semen extenders (diluents) for cryopreserved semen are generally made to contain cryoprotectants, antioxidants, energy sources (proteins and sugars) and antibiotics (reviewed by Vishwanath & Shannon 2000). Diluents for fresh chilled semen tend to be simpler and contain a buffered solution, egg yolk or milk, and antibiotics. Egg yolk is an excellent energy source in sperm diluents. Using egg yolk combined with a phosphate buffered solution, enables motility to be maintained for up to 150 hours when stored at 10˚C. Maintaining the pH of the diluent to 6.75 was reported

as the most optimal (Phillips & Lardy 1940). Several semen extenders exist on the market, ranging from simple salt solutions to more complex buffered solutions with the addition of egg yolk or milk (reviewed by Vishwanath & Shannon 2000).

The concentration of sperm in storage has been demonstrated to affect sperm survival. Storage of fresh sperm at higher densities (200 million sperm per mL compared to 12.5 million sperm per mL) resulted in longer survival times after being warmed to 37˚C ($P<0.01$) (further diluted at the time of insemination). The presence of dead sperm and seminal plasma also negatively affected survival of sperm following warming to 37˚C (Shannon 1965). Furthermore, calving rates are affected by the concentration of sperm at the time of AI. Fresh semen stored at 3 or 4 x $10^6$ sperm per dose for 2 days resulted in significantly lower calving rates than fresh semen that was stored in 5 x $10^6$ sperm per dose for 1 day (Murphy, EM et al. 2018). Semen that has been stored chilled contains a higher proportion of live sperm compared to cryopreserved semen (Shannon & Vishwanath 1995). Furthermore, the concentration of sperm (per mL) that semen is stored at was shown to affect the levels of oxidative stress. Sperm stored at ambient temperature at 1 x $10^6$ sperm per insemination dose had less oxidative stress than sperm stored at the usual 5 x $10^6$ sperm per insemination dose, and oxidative stress increased with each day that the sperm were stored in a nitrogen saturated extender (Caprogen) (Murphy, C et al. 2013). Furthermore, glucose concentration in the diluent was lower after 5 days when sperm were stored at 5 x $10^6$ compared compared to 1 x $10^6$ sperm per insemination dose. However, individual sperm consumed 4 times more glucose over the 5 days when they were stored at 1 x $10^6$ sperm per mL compared to 5 x $10^6$ sperm per insemination dose (Murphy, C et al. 2013).

Sperm can be divided into 3 segments: head, midpiece and tail. The midpiece is where mitochondria are located. Mitochondria impact several sperm functions including motility, hyperactivation, capacitation, acrosome reaction, and fertilisation. Furthermore ROS production is a by-product of mitochondrial metabolism (reviewed by Moraes & Meyers 2018). The variety of ROS produced by sperm include nitric oxide, peroxide and superoxide (Aitken, De Iuliis & McLachlan 2009). It has also been suggested that intracellular zinc ions within sperm mitochondria may also play a role in regulation of sperm motility (Sorensen, Stoltenberg, et al. 1999). Mitochondrial status is important for sperm functionality. Production of cellular energy is related to spermatozoa motility and therefore fertility (Mazur et al. 2000). Therefore the midpiece could be an ideal area to focus on when looking for fertility markers of sperm.

### 1.3.2.2 Effects of Reactive Oxygen Species (ROS) to semen

Concentrations of ROS accumulate over time in store semen. Furthermore, the process of thawing damages the plasma membrane of the spermatozoa and results in an increased production of ROS, especially in the form of superoxide (Chatterjee & Gagnon 2001). Consequently, a dose of frozen semen for AI needs to contain approximately 4 times the sperm dose of fresh chilled semen (20 x $10^6$ compared to 5 x $10^6$ sperm per mL) (Shannon & Vishwanath 1995). Therefore fewer insemination doses are obtained when semen is frozen. Furthermore it is not clear how long sperm can be stored in a frozen state before fertility begins to decline. A decrease in motility, viability and a reduced pregnancy rate following AI have been reported from thawed bull semen compared to fresh semen (Murphy, EM et al. 2018). One study comparing 32 year-old cryopreserved semen with 2 year-old frozen semen reported reduced fertilisation rates *in vitro* and a higher proportion of necrotic sperm from the 32 year old samples (Akyol, Ertem & Varisli 2019). The presence of dead sperm has been demonstrated to have a toxic effect on the live sperm (Shannon & Curson 1972). When sperm die, the enzyme L-amino acid oxidase is activated. This leads to the production of peroxide ($H_2O_2$), which is responsible for the toxicity of dead sperm. Egg yolk within the extender increases the toxicity caused by dead sperm due to the presence of aromatic amino acids, especially phenylalanine, which L-amino oxidase has an affinity for. However, reducing the proportion of yolk in the extender from 20% to 5% reduces the effect (Shannon & Curson 1972). Furthermore, the addition of catalase counters the negative effects of dead sperm by enzymatically reducing $H_2O_2$ to $H_2O$ (Shannon & Curson 1972). Saturating the extender with nitrogen prior to use increases the viability of fresh-chilled semen to 8 days, due to the reduced oxygen content in the extender (Shannon 1965). The inclusion of caproic acid within semen extenders reduces the metabolic rate of sperm resulting in reduced production of ROS (Vijayaraghavan, Bhattacharyya & Hoskins 1989). One of the more complex extenders commercially available is Caprogen, which includes both catalase and caproic acid (Shannon, Curson & Rhodes 1984).

### 1.3.2.3 Zinc in sperm

Recently, zinc ion concentration has been identified as a marker for sperm capacitation with a signature that has been replicated between three species (bovine, porcine and human) (fig.2). Signature 1 is characterised with zinc staining in the head, midpiece and tail of the sperm (uncapacitated), Signature 2 is characterised by zinc staining in the head and midpiece (capacitating), Signature 3 is characterised by zinc staining in the midpiece only (capacitated) and Signature 4 is characterised by no zinc staining (after

capacitation with a remodelled plasma membrane). Signature 1 was most common in freshly ejaculated sperm, while sperm with the zinc signature 2 were hyperactivated and have the capability to bind to the zona pellucida of the oocyte. Adding ZnCl to the capacitation medium maintained boar sperm at Signature 1, while the addition of a zinc chelator was able to alter the signature to 3 or 4. Boars with previously determined high fertility had twice the proportion of zinc signature 3 sperm after capacitation *in vitro* compared with sperm from boars of low fertility (Kerns et al. 2018). Similarly, another study found the concentration of zinc within human sperm to be negatively correlated with progressive motility rates (Henkel, RR et al. 2003). Progressive motility is often used as a measure of predictive fertilisation ability for sperm (Li et al. 2016). The addition of either 0.01 or 0.1 mg/mL nano zinc oxide to sheep semen extender improved post-thaw progressive motility and plasma membrane integrity compared with the control. Alternatively, motility was significantly decreased when the semen extender contained a higher concentration (1 mg/mL) of nano zinc oxide (Heidari et al. 2019). The nano-form of zinc oxide was used because its properties enable easier absorption while producing fewer free radicals and therefore this form was expected to have a lower toxicity effect on the sperm (Heidari et al. 2019). Therefore, zinc levels within sperm may be an indicator of fertilisation potential. Potentially this information could be used to develop a diagnostic test of sperm for use at the time of semen collection or after cryopreservation. Alternatively it may lead to further investigation of nutritional management prior to semen collection.

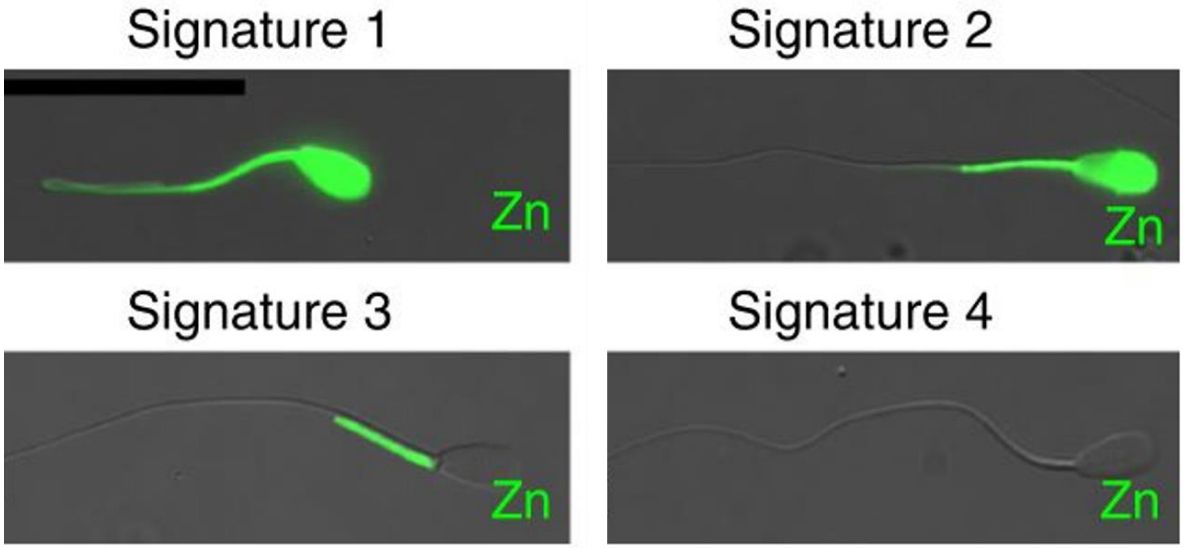


**Figure 2**: Examples of the zinc signature relating to the sperm capacitation are adapted from Kerns et al. (2018). Signature 1 is characterised with zinc staining in the head, midpiece and tail (uncapacitated). Signature 2 is characterised with zinc staining in the head and midpiece (capacitating). Signature 3 is characterised with zinc staining in the midpiece (capacitated). Signature 4 is characterised by no zinc staining (after capacitation).

### 1.3.2.4 Effects of climate and genotype on semen quality

Season and breed has been shown to affect semen quality. The commercial cattle breeds generally divide into two sub-species; *bos taurus* and *bos indicus* (Greenwood, Gardner & Ferguson 2018). These sub-species are adapted to live in different environments. The *bos taurus* cattle tend to be more suited to the cooler drier environments whereas *bos indicus* cattle are adapted to thrive in warmer tropical environments with increased skin to body ratios, long ears and a smaller body frame (Turner 1980). The number of major sperm defects tends to be higher in summer than in winter. This effect tends to be more pronounced in sperm collected from *bos taurus* (Simmental) bulls compared to *bos indicus* (Nellore) bulls (Nichi et al. 2006). Oxidative damage has also been reported as higher in sperm collected in summer compared to winter. The same study also reported that lipid peroxidation (ROS production) was higher in semen from Simmental *(bos taurus)* bulls compared with Nellore (*bos indicus*) bulls independent of season. Thiobarbituric acid reactive substances (TBARS) were higher in semen collected during summer compared to winter, while Glutathione peroxidase/reductase (GPx) activity was higher in Simmental semen compared to Nellore semen. A negative correlation was also reported between superoxide dismutase (SOD) (responsible for extinguishing superoxides) in semen and sperm defects ($r$=-0.51, $P$=0.041) in Simmental sperm during summer, but not during the other seasons (Nichi et al. 2006). It has been suggested that heat tolerance of *bos indicus* breeds may extend to increased resistance to heat-induced lipid peroxidation within their semen; resulting in a lower increase in the production of ROS and superoxides during the summer; therefore fewer sperm defects (Nichi et al. 2006). A study in tropical regions of Brazil demonstrated that the ejaculate from *bos indicus* bulls is approximately 20 percent more concentrated compared to *bos taurus* ejaculates (approximately 1.5 x $10^9$ sperm per mL compared to 1.2 x $10^9$ sperm per mL, respectively), however, the volume was not different between the sub-species. Furthermore they demonstrated that the number of total sperm defects (acrosome damage, coiled or bent tails, irregularly shaped heads, presence of vacuoles etc.) tended to be higher in sperm from *bos indicus* semen ($P < 0.1$). The percentage of motile sperm was not affected by the genotype of the bull (Brito, LFC et al. 2002). An earlier study based in semi tropical Florida also reported that semen from Brahman (*bos indicus*) bulls was inferior for all tested traits (% motility, % normal and % abnormal) when compared to temperate breeds such as Angus and Hereford (*bos taurus*). Senepol bulls (a tropically adapted *bos taurus* breed) also outperformed Brahman bulls (Chenoweth et al. 1996). In contrast, another study in a tropical area of Nigeria found no breed effect on ejaculate volume or sperm quality. Their study investigated the indigenous *bos indicus* breeds of Bunaji and Sokoto Gundali as well as the *bos taurus* breed of Freisian and Freisian cross Bunaji. They found that the environment had a significant impact on ejaculate volume and semen quality parameters. During the wet season, they reported 15 percent less dead sperm and less than half the number of defected sperm across all bulls studied. Furthermore, the

volume of the ejaculate increased by more than 50 percent, the concentration of sperm almost doubled, and the total sperm numbers in ejaculates almost tripled in the wet season (Rekwot et al. 1987). It is most likely that the dry season had a negative impact on semen quality and quantity due to reduced grazing quality, coupled with hot ambient temperature in the study by Rekwot et al. (1987). Spermatogenesis in bulls occurs over 61 days (reviewed by Staub & Johnson 2018). Therefore environment at the time of and prior to semen collection can have a big impact on the quality and quantity of the ejaculate obtained.

#### 1.3.2.5 Conclusions and future direction of studies investigating sperm fertility markers

Identification of accurate bull sperm fertility markers will improve the efficiency of assisted reproductive technologies for cattle. Many studies have demonstrated that high ROS levels negatively affect motility of sperm (Baumber et al. 2000). Few studies have investigated the effect ROS has on the ability of bull sperm to fertilise COCs *in vivo* and *in vitro*. This is especially true for superoxide, which has been demonstrated to be one of the main forms of ROS in thawed bovine sperm (Chatterjee & Gagnon 2001). Therefore the relationship between ROS and the ability of sperm to fertilise oocytes needs further investigation. Zinc content and localisation in sperm has been demonstrated to differ at various stages of capacitation, and also to affects sperm motility (Kerns et al. 2018; Sorensen, Stoltenberg, et al. 1999). Assessment of whether zinc levels affect bovine embryonic development *in vitro* and *in vivo* is required. Bull semen fertility has been reported to differ depending if semen originated from *bos taurus* or *bos indicus* bulls (Nichi et al. 2006). Whether this is due to different levels of ROS and or zinc is unclear. These levels could be related to environmental adaptions. The identification of accurate bull sperm fertility markers could lead to future studies focused on optimising bull management for collection of high quality semen.

## 1.4 Hypothesis and Aims

Study 1: Meiotic inhibition of bovine COCs *in vitro*:

Hypothesis 1*:* Pre-maturation of bovine COCs with a specific PDE8 inhibitor combined with a broad spectrum PDE inhibitor (IBMX) will delay meiotic progression in 80% of bovine oocytes for at least 18 hours.

Hypothesis 2: Pre-maturation of bovine COCs with dbcAMP will delay the meiotic progression in 80% of bovine oocytes for at least 18 hours.

Hypothesis 3: Pre-maturations of bovine COCs with a combination of IBMX and oestradiol will delay meiotic progression in 80% of bovine COCs for at least 18 hours.

Hypothesis 4: Pre-maturation of bovine COCs with the addition of CNP to IBMX and oestradiol will further delay meiotic progression in 80% of bovine COCs for at least 18 hours.

Aim 1: To arrest meiosis of the bovine oocyte at the germinal vesicle stage for 18 hours

Study 2: Investigation of potential fertility markers of bovine sperm:

Hypothesis 1: Fertilisation potential of bull sperm (*in vivo* and *in vitro*) is impacted negatively when ROS and zinc levels are increased within the midpiece of sperm.

Hypothesis 2: ROS and zinc levels will be significantly different between sperm or *bos taurus* and *bos indicus* bulls.

Aim 2: To identify more accurate fertility markers of bull sperm than is currently used within the bull semen collection industry.

# Chapter 2
# Meiotic inhibition of bovine COCs *in vitro*

## 2.1 Introduction

### 2.1.1 Oocyte maturation and developmental competence

All oocytes develop during female fetal development, and are arrested in the fetal ovary at prophase 1 of meiosis, also referred to as the germinal vesicle (GV) stage. *In vivo*, nuclear maturation resumes following the LH surge that triggers ovulation of the dominant follicle, and the oocyte is ovulated at the metaphase II stage of meiosis (Kawamura et al. 2011; Rizos et al. 2002). For *in vitro* embryo production, immature bovine oocytes are collected from antral follicles of various sizes, and generally at random stages during the oestrous cycle. Nuclear maturation of the oocyte spontaneously resumes when the cumulus-oocyte complex (COC) is aspirated from the ovarian follicle (Pincus & Enzmann 1935). However, the cytoplasmic organelles, including the mitochondria and endoplasmic reticulum, may not have 'matured' sufficiently to allow for the optimal developmental potential of the oocyte following fertilisation *in vitro*; often referred to as "asynchronised maturation". Appropriate maturation of the oocyte's cytoplasm has been demonstrated as a major factor that results in higher developmental potential of oocytes that have been matured *in vivo,* compared to those matured *in vitro (*Gilchrist & Thompson 2007*)*. In recent years, a concept of arresting meiotic progress of aspirated oocytes, while supporting the development of cytoplasmic organelles, has been investigated as a means to improve developmental competence of *in vitro* matured oocytes (Barretto et al. 2007; Santiquet et al. 2017; Zeng et al. 2014).

### 2.1.2 Objectives

Further investigations are required into the specific mechanisms required for inhibition of meiotic progression in bovine oocytes for extended periods of time. The current study aimed to extend the time that bovine oocytes could be arrested *in vitro* at the GV stage of meiosis to 18 hours. Eighteen hours was selected as an optimal time period that could be readily incorporated into commercial bovine *in vitro* embryo production (IVP) programs. The initial experiments aimed to determine the effects of inhibiting PDE8 activity, using a new specific PDE8 inhibitor (PF-04957325 from MedChem Express), on meiotic progression of bovine COCs. Subsequent experiments aimed to examine the effects of dbcAMP, oestradiol and CNP, in combination with the PDE inhibitor, IBMX, on meiotic progression of bovine COCs.

## 2.2 Methods

### 2.2.1 Ovary collection

Bovine ovaries from cross bred (*bos taurus* and *bos indicus*) cattle were collected from a local abattoir (JBS Swift, Rockhampton, Australia). They were transported to the laboratory (Australian Reproductive Technologies, Rockhampton, Australia) in 500 mL of 0.9% saline at 35˚C within 3 hours of collection. Antral follicles with a diameter between 2 and 8 mm were manually aspirated with a 19G needle and 10 mL syringe. Follicular fluid was searched using a dissecting microscope and COCs with an even coloured ooplasm and at least three complete layers of cumulus cells were collected and divided between treatment groups. Each group was then washed twice in 2 mL of VitroMat (Art Lab Solutions, Adelaide, Australia) and COCs were incubated in groups of 10 COCs per 50 µL drop of VitroMat (ART Lab Solutions). VitroMat medium used for washing and incubation contained 4 mg/ml BSA and the meiotic inhibitors appropriate for each treatment group, with no added serum or gonadotropins. COCs were incubated under mineral oil (M5310, Sigma-Aldrich, Merck, Germany) in 35 mm Falcon dishes (35-1008, Corning, New York, USA), at 38.8˚C in 5% $CO_2$ in air for various times depending on the experimental design.

### 2.2.2 Orcein staining for determination of stage of meiosis

At 2, 4 or 6 hours COCs were denuded for staining by gently pipetting up and down with a 25 µL pipette in media drops of approximately 50 µL. For later observation times, COCs were placed in a 1.5 ml microtube containing 1 mL of 2 mg/mL hyaluronidase with 6 mg/mL BSA in VitroWash (ART Lab Solutions). A pipette set to 500 µL was used to gently pipette up and down until the cloud of cumulus cells became invisible. The microtube was then vortexed for 3 minutes. Oocytes were removed from the microtube and gently pipetted up and down in 50 µL drops of VitroWash (ART Lab Solutions) to remove any remaining cumulus cells. Oocytes were arranged in a line on a microscope slide (n=10 per slide) in 10 µL of media. The slide was placed on the heated (38˚C) microscope stage for 30 seconds for some of the media to evaporate. A coverslip with Vaseline added to the top and bottom edge was placed on the slide, and gently tapped with a needle to flatten the oocytes. Ten microliters of 1% orcein (O7380-5g, lot# BCBR9195, Sigma-Merck) in 45% glacial acetic acid (ARK-2183, lot# GLAA132-34, Sigma-Merck) was pipetted under the coverslip from one open end with a 10 µL pipette. Oocytes were visualised on an inverted microscope (Olympus IX70) at 400x magnification with Hoffman Modulation Contrast. Oocytes were classified as being in the Germinal Vesicle (GV) stage if the nuclear envelope was still present. Germinal Vesicle Breakdown (GVBD) was classified when the nuclear envelope had disappeared and the chromatin had condensed. Metaphase 1 (M1) was classified when the condensed chromatin had met at

the metaphase plate, and Metaphase 2 (M2) was classified when two distinct groups of chromatin were visible, with one group in an extruded polar body (fig. 3).

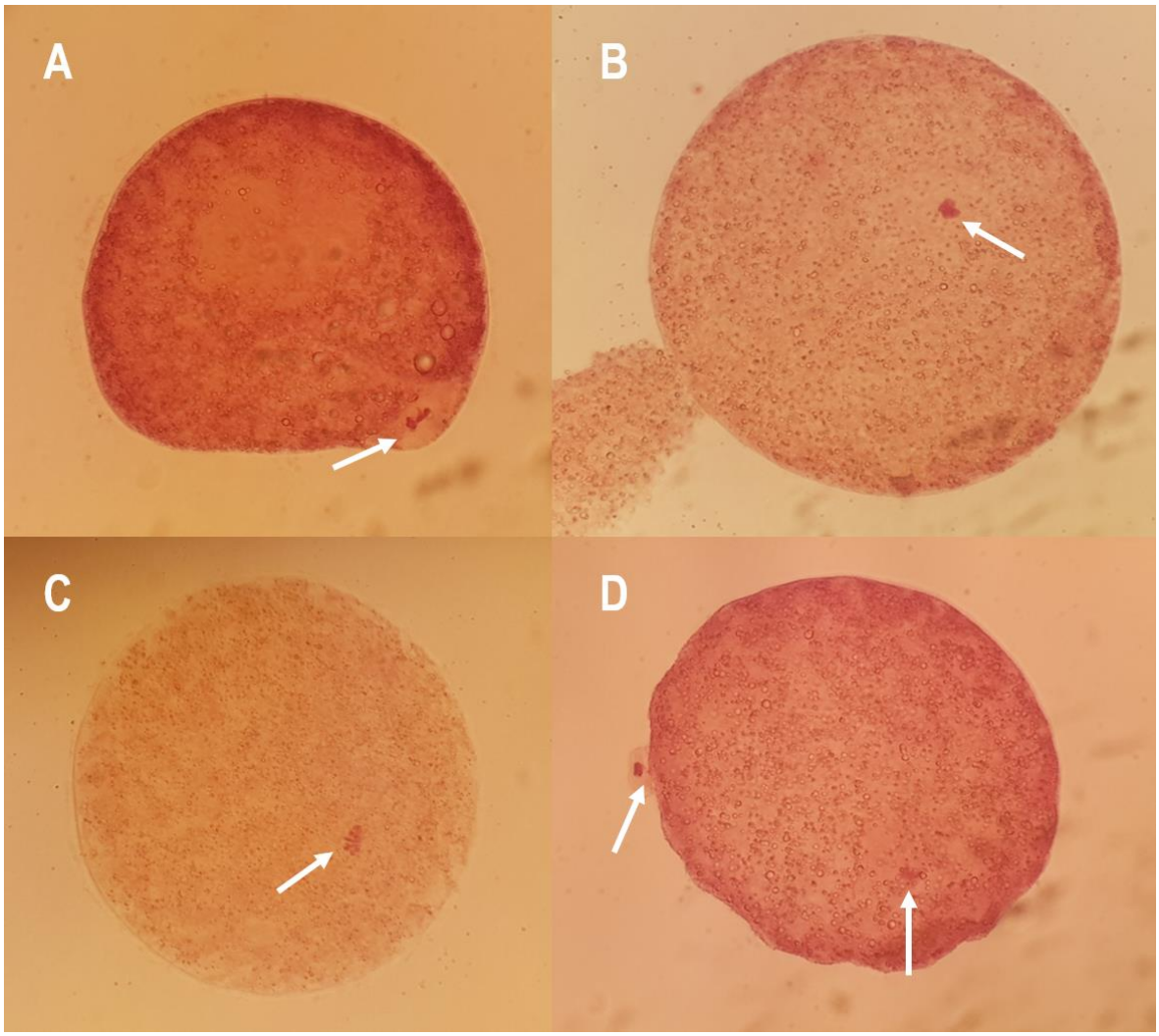


**Figure 3:** Examples of orcein staining of bovine oocytes for meiotic progression determination. Arrows point to the chromatin. The GV stage (A) is characterised by chromatin enclosed by the nuclear envelope. The GVBD (B) stage is characterised by the disappearance of the nuclear envelope and condensation of the chromatin. The M1 stage (C) is characterised by the condensed chromatin lining up on the metaphase plate. The M2 stage (D) is characterised by two distinct groups of chromatin with one extruded in the first polar body.

## 2.2.3 Inhibitor Preparations

### 2.2.3.1 PF-04957325 (PDE8 inhibitor)

PF-04957325 (MedChem Express, catalogue # HY-15426, batch # 23381, molecular weight 400.38 g/mol) was dissolved in dimethyl sulfoxide (DMSO) prior to use. Throughout all experiments DMSO concentrations within the final media were maintained to be no more than 0.1%. Stock solutions of 10 mM PF-04957325 were prepared by diluting 5 mg of PF-04957325 in 1.25 mL of DMSO. Aliquots were stored in microtubes, wrapped in aluminium foil and maintained at -20 degrees Celsius for no more than 4 weeks.

### 2.2.3.2 3-isobutyl-1-methylxanthine (IBMX)

Stock solutions of 500 mM IBMX (Sigma, catalogue # 15879, lot # 0001416650) were prepared for all experiments. Fifty milligrams of IBMX (molecular weight 222.24 g/mol) was dissolved in 450 µL of DMSO. Aliquots were stored in microtubes, wrapped in aluminium foil and maintained at -20 degrees Celsius for no more than 4 weeks.

### 2.2.3.3 Dibutylryl Cyclic AMP (dbcAMP)

Stock solutions of 100 mM dbcAMP (Sigma D0627-250MG, Molecular Weight 491.37 g/mol) were prepared by dissolving 50 mg of dbcAMP in 1018 µL of VitroWash (ART Lab Solutions). Aliquots were stored in microtubes at -20˚C for no more than 4 weeks.

### 2.2.3.4 C-Type Natriuretic Peptide (CNP)

A 100 µM stock solution of CNP (Sigma N8768-.5MG, Molecular Weight = 2197.60 g/mol) was prepared by dissolving 0.5 mg of CNP in 2280 µL VitroMat base media (ART Lab Solutions) without any gonadotropins included. Aliquots were stored in microtubes at -20 degrees Celsius for no more than 4 weeks.

#### 2.2.3.5 β-Oestradiol

β-Oestradiol (Sigma, E2257, Molecular Weight = 272.38 g/mol) was dissolved in ethanol to produce a 10 µM stock solution via a double dilution. Firstly 1 mg of β-oestradiol was dissolved in 3.67 mL of ethanol to make a 1 mM solution then 100 µL of the 1 mM solution was added to 9.9 mL ethanol to make a 10 µM stock. The 10 µM stock solution was stored at -20 degrees Celsius for no more than 3 months.

### 2.2.4 Experimental Designs

#### 2.2.4.1 Experiment 1

##### *2.2.4.1.1 PF-04957325 (PDE8 inhibitor) dose response (0, 1 µM, 5 µM and 10 µM)*

Cumulus oocyte complexes were divided into 4 groups: 1) control (no inhibitors), 2) 1 µM PF-04957325 with 500 µM IBMX, 3) 5 µM PF-04957325 with 500 µM IBMX, 4) 10 µM PF-04957325 with 500 µM IBMX. COCs were incubated in their treatment group, as described above, for 2, 4, 6, 18 or 24 hours. Oocytes (15 to 20 COCs per treatment) were removed at each time point and meiotic stage was determined by orcein staining.

##### *2.2.4.1.2 PF-04957325 (PDE8 inhibitor) and IBMX*

Based on the results from experiment 1, the effect of IBMX and PF-04957325 were tested alone and in combination. A single time point of 18 hours was assessed because that was the time point that we aimed to arrest meiosis until. COCs were divided into 4 groups: 1) control (no PDE inhibitors), 2) 500 µM IBMX, 3) 10 µM PF-04957325 4) 10 µM PF-04957325 with 500 µM IBMX. COCs were incubated in their treatment group for 18 hours, and meiotic stage was then determined by orcein staining.

##### *2.2.4.1.3 PF-04957325 (PDE8 inhibitor) dose response (0, 50 µM and 100 µM)*

Based on the results from the previous experiment, the effects of higher concentrations of PF-04957325 were assessed. COCs were divided into 4 groups: 1) control (no PDE inhibitors), 2) 500 µM IBMX, 3) 50 µM PF-04957325 with 500 µM IBMX, 4) 100 µM PF-04957325 with 500 µM IBMX. COCs were incubated in their treatment group for 18 hours, and meiotic stage was then determined by orcein staining.

#### 2.2.4.2 Experiment 2

##### *2.2.4.2.1 Dibutyryl cyclic AMP (dbcAMP) dose response (0, 1 mM and 5 mM)*

Cumulus oocyte complexes were incubated in VitroMat (ART Lab Solutions), with or without the addition of dbcAMP (0 mM, 1 mM or 5 mM) for 18 hours and meiotic stage was then determined by orcein staining.

#### 2.2.4.3 Experiment 3

##### *2.2.4.3.1 5 mM Dibutyryl cyclic AMP (dbcAMP) with or without oestradiol (100 nM) and IBMX (500 μM)*

Cumulus oocyte complexes were divided into 4 groups all containing 5 mM dbcAMP: 1) control (no added β-oestradiol or IBMX), 2) 100 nM β-oestradiol, 3) 500 μM IBMX, 4) 100 nM β-oestradiol and 500 μM IBMX. Meiotic stage was determined by orcein staining after 18 hours incubation.

#### 2.2.4.4 Experiment 4

##### *2.2.4.4.1 5 mM Dibutyryl cyclic AMP (dbcAMP), 100 nM oestradiol and 500 μM IBMX with or without 200 nM C-type Natriuretic Peptide (CNP)*

Cumulus oocyte complexes were divided into 3 groups: 1) control (no added inhibitors), 2) 5 mM dbcAMP, 100 nM Oestradiol and 500 μM IBMX, and 3) 5 mM dbcAMP, 100 nM Oestradiol, 500 μM IBMX and 200 nM CNP. The dose of CNP was selected based on results from other published studies investigating the use of CNP for meiotic inhibition of bovine COCs (Zhang, T et al. 2017). Meiotic stage was determined after 12 and 18 hours of incubation by orcein staining.

### 2.2.5 Statistical Analysis

The statistical analysis was completed by Jana Maria Bednarz, a Statistician from the Adelaide Health Technology Assessment (AHTA) at the University of Adelaide. All statistical analysis was performed using Stata (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC) at

the $\alpha = 0.05$ level of statistical significance. To calculate the percentage of oocytes at each meiotic stage, for each treatment, the number of oocytes counted at that stage was divided by the total number of oocytes within the treatment, and multiplied by 100. For experiment 1, Chi Squared test was used to assess if the distribution of COCs at the meiotic stages varied between treatments. For experiments 2, 3 and 4, Fisher's Exact Test of Association was used to assess if the distributions of COCs at each meiotic stage differed between treatments. 95% confidence intervals for the population percentage were calculated using the Exact (Clopper-Pearson) method.

## 2.3 Results

### 2.3.1 Experiment 1*:*

#### 2.3.1.1 Dose response of 1, 5 and 10 µM PDE8 inhibitor (PF-04957325) with 500 µM IBMX at 2, 4, 6, 18 and 24 hours.

There was no difference between treatments in the proportion of oocytes at the Germinal Vesicle (GV) stage after 2 or 4 hours of culture with 1, 5 or 10 µM PDE8 inhibitor and 500 µM IBMX or no meiotic inhibitors (control). More than 98% of oocytes were at the GV stage at 2 hours, and more than 84% of oocytes were at the GV stage at 4 hours (Table 1). After 6 hours of culture more COCs were at the GV stage when cultured with 1, 5 or 10 µM PDE8 inhibitor and 500 µM IBMX ($p<0.0001$) compared to COCs cultured without inhibitors. The proportion of COCs at GV did not differ between groups exposed to 1, 5 or 10 µM of PDE8 inhibitor and 500 µM IBMX (Table 1). After 18 hours of culture, very few COCs remained at the GV stage of meiosis within all treatment groups (1.8 - 2.9%) and the proportion of GV stage oocytes did not differ between treatments. A higher proportion of COCs were at the M1 stage following 18 hours of culture with 1, 5 or 10 µM PDE8 inhibitor and 500 µM IBMX ($p\leq0.001$) compared to COCs in the control group. A higher proportion of COCs in the control group had reached the M2 stage, compared to oocytes matured in the presence of the meiotic inhibitors (Table 1). After 24 hours of culture, there was no significant difference in the distribution of COCs at the various meiotic stages whether COCs had been cultured with or without the meiotic inhibitors (Table 1).

#### 2.3.1.2 2x2 factorial with 10 µM PDE8 inhibitor and 500 µM IBMX at 18 hours

There were no COCs remaining at the GV stage of meiosis following 18 hours of culture with 10 µM PDE8 inhibitor, 500 µM IBMX or the combination of 10 µM PDE8 inhibitor and 500 µM IBMX. After 18 hours of culture, the distribution of meiotic stages did not differ between COCs cultured with 10 µM PDE8 inhibitor alone and the control group. More COCs remained at the M1 stage of meiosis when cultured with 500 µM IBMX alone or with 500 µM IBMX and 10 µM PDE8 inhibitor, compared to culture with the PDE8 inhibitor alone or the control group ($p<0.001$). Meiotic distribution did not differ between groups cultured with IBMX with or without the 10 µM PDE8 inhibitor (Table 2).

**Table 1:** The effect of the 1 µM, 5 µM and 10 µM PF-04957325 (PD8) with 500 µM IBMX on meiotic progression after 2, 4, 6, 18 and 24 hours of treatment of bovine COCs.

| Time (hours) | Treatment | Total oocytes n | GV | | GVBD | | M1 | | M2 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | n (%) | 95% CI | n (%) | 95% CI | n (%) | 95% CI | n (%) | 95% CI |
| 2 | Control | 61 | 61 (100) | (94.1, 100) | 0 | - | 0 | - | 0 | - |
| | 1 µM PD8 + 500 µM IBMX | 65 | 64 (98.46) | (91.7, 100) | 1 (1.54) | (2.11-10.35) | 0 | - | 0 | - |
| | 5 µM PD8 + 500 µM IBMX | 66 | 66 (100) | (94.6, 100) | 0 | - | 0 | - | 0 | - |
| | 10 µM PD8 + 500 µM IBMX | 67 | 67 (100) | (94.6, 100) | 0 | - | 0 | - | 0 | - |
| 4 | Control | 65 | 57 (87.69) | (77.18, 94.53) | 8 (12.31) | (6.22, 22.90) | 0 | - | 0 | - |
| | 1 µM PD8 + 500 µM IBMX | 55 | 46 (83.64) | (71.20, 92.23) | 9 (16.36) | (8.66, 28.77) | 0 | - | 0 | - |
| | 5 µM PD8 + 500 µM IBMX | 55 | 47 (85.45) | (73.33, 93.50) | 8 (14.55) | (7.37, 26.69) | 0 | - | 0 | - |
| | 10 µM PD8 + 500 µM IBMX | 59 | 53 (89.83) | (79.17, 96.18) | 6 (10.17) | (4.59, 21.04) | 0 | - | 0 | - |
| 6 | Control | 145 | 80 (55.17)[a] | (46.70, 63.43) | 65 (44.83)[a] | (36.90, 53.03) | 0 | - | 0 | - |
| | 1 µM PD8 + 500 µM IBMX | 127 | 103 (81.10)[b] | (73.20, 87.50) | 24 (18.90)[b] | (12.97, 26.70) | 0 | - | 0 | - |
| | 5 µM PD8 + 500 µM IBMX | 135 | 112 (82.96)[b] | (75.54, 88.89) | 23 (17.04)[b] | (11.56, 24.39) | 0 | - | 0 | - |
| | 10 µM PD8 + 500 µM IBMX | 129 | 113 (87.60)[b] | (80.64, 92.74) | 16 (12.40)[b] | (7.72, 19.34) | 0 | - | 0 | - |
| 18 | Control | 102 | 3 (2.94) | (0.94, 8.79) | 8 (7.84) | (3.95, 14.98) | 18 (17.65)[a] | (11.37, 26.36) | 73 (71.57)[a] | (62.00, 79.52) |
| | 1 µM PD8 + 500 µM IBMX | 106 | 3 (2.83) | (0.91, 8.48) | 7 (6.60) | (3.16, 13.28) | 42 (39.62)[b] | (30.72, 49.27) | 54 (50.94)[b] | (41.44, 60.38) |
| | 5 µM PD8 + 500 µM IBMX | 105 | 2 (1.90) | (0.47, 7.37) | 7 (6.67) | (3.19, 13.40) | 35 (33.33)[b] | (24.94, 42.94) | 61 (58.10)[b] | (48.40, 67.20) |
| | 10 µM PD8 + 500 µM IBMX | 112 | 2 (1.79) | (0.44, 6.92) | 11 (9.82) | (5.50, 16.93) | 45 (40.18)[b] | (31.46, 49.56) | 54 (48.21)[b] | (39.06, 57.49) |
| 24 | Control | 90 | 5 (5.56) | (1.83, 12.49) | 4 (4.44) | (1.66, 11.33) | 19 (21.11) | (13.89, 30.84) | 62 (68.89) | (58.54, 77.64) |
| | 1 µM PD8 + 500 µM IBMX | 111 | 2 (1.80) | (0.22, 6.36) | 1 (0.90) | (0.12, 6.20) | 15 (13.51) | (8.28, 21.28) | 93 (83.78) | (75.65, 89.57) |
| | 5 µM PD8 + 500 µM IBMX | 116 | 4 (3.45) | (0.95, 8.59) | 0 | - | 16 (13.79) | (8.60, 21.40) | 96 (82.76) | (74.71, 88.63) |
| | 10 µM PD8 + 500 µM IBMX | 127 | 3 (2.36) | (0.49, 6.75) | 1 (0.79) | (0.11, 5.44) | 24 (18.90) | (12.97, 26.71) | 99 (77.95) | (69.86, 84.36) |

Different superscripts indicate a significant difference exists (P<0.05) within a column for that time point.
Includes 95% confidence intervals (CI).

**Table 2:** The effect of 500 µM IBMX and 10 µM PF-04957325 (PD8) alone and combined on meiotic progression after 18 hours of treatment of bovine COCs.

| Treatment | Total oocytes | GV | | GVBD | | M1 | | M2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | n (%) | 95% CI | n (%) | 95% CI | n (%) | 95% CI | n (%) | 95% CI |
| Control | 112 | 1 (0.89) | (0.12, 6.13) | 0 | - | 37 (33.04)[a] | (24.92, 42.31) | 74 (66.07)[a] | (56.77, 74.28) |
| 500 µM IBMX | 134 | 0 | - | 2 (1.49) | (0.37, 5.82) | 75 (55.97)[b] | (47.42, 64.18) | 57 (47.90)[b] | (39.03, 56.91) |
| 10 µM PD8 | 127 | 0 | - | 0 | - | 33 (25.98)[a] | (19.06, 34.35) | 94 (74.02)[a] | (65.65, 80.94) |
| 500 µM IBMX + 10 µM PD8 | 119 | 0 | - | 0 | - | 62 (52.10)[b] | (43.09, 60.97) | 57 (42.54)[b] | (34.40, 51.10) |

Different superscripts indicate a significant difference exists (P<0.05) within a column.
Includes 95% confidence intervals (CI).

2.3.1.3 Dose response of 50 µM and 100 µM PDE8 inhibitor (PF-04957325) with 500 µM IBMX after 18 hours of culture

Since 5 µM or 10 µM PDE8 inhibitor did not affect meiotic resumption, the effects of higher concentrations were assessed. No oocytes remained at the GV stage of meiosis after 18 hours of culture with 50 µM or 100 µM PDE8 inhibitor with 500 µM IBMX, 500 µM IBMX alone or no meiotic inhibitors. A greater proportion of COCs were at the M1 stage following culture with IBMX alone or combined with either dose of PDE8 inhibitor, compared to control oocytes cultured without meiotic inhibitors ($p<0.0001$). Meiotic progression did not differ between groups cultured with IBMX alone or combined with 50 µM or 100 µM of PDE8 inhibitor (Table 3).

**Table 3:** The effect of 50 µM or 100 µM PDE8 inhibitor and 500 µM IBMX on meiotic progression of bovine COCs after 18 hours of treatments.

| Treatment | Total oocytes | M1 | | M2 | |
|---|---|---|---|---|---|
| | | n (%) | 95% CI | n **(%)** | 95% CI |
| Control | 142 | 47 **(33.10)**[a] | (25.82, 41.28) | 95 **(66.90)**[a] | (58.72, 74.18) |
| 500 µM IBMX | 165 | 106 **(64.24)**[b] | (56.61, 71.22) | 59 **(35.76)**[b] | (28.78, 43.39) |
| 50 µM PDE8 + 500 µM IBMX | 131 | 80 **(61.07)**[b] | (52.42, 69.07) | 51 **(38.93)**[b] | (30.93, 47.58) |
| 100 µM PDE8 + 500 µM IBMX | 153 | 98 **(64.05)**[b] | (56.11, 71.29) | 55 **(35.95)**[b] | (28.71, 43.89) |

Note: there were no oocytes recorded at the GV or GVBD stage for any of the treatment groups.
Different superscripts indicate a significant difference exists ($P<0.05$) within a column.
Includes 95% confidence intervals (CI).

## 2.3.2 Experiment 2

2.3.2.1 Dose response with 0, 1 mM and 5 mM dbcAMP at 18 hours

After 18 hours of culture with 0 (control), 1 mM or 5 mM dbcAMP, less than 2% of COCs remained at the GV stage of meiosis. Maturation in the presence of dbcAMP did not affect the meiotic distribution at 18 hours (Table 4).

**Table 4:** Effect of 1 mM or 5 mM dbcAMP on meiotic progression of bovine COCs after 18 hours of treatment.

| Treatment | Total oocytes n | GV | | GVBD | | M1 | | M2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | n **(%)** | 95% CI | n **(%)** | 95% CI | n **(%)** | 95% CI | n **(%)** | 95% CI |
| Control | 108 | 2 **(1.85)** | (0.23, 6.53) | 0 | - | 18 **(16.67)** | (10.19, 25.06) | 88 **(81.48)** | (72.86, 88.31) |
| 1 mM dbcAMP | 103 | 1 **(0.97)** | (0.02, 5.29) | 0 | - | 17 **(16.50)** | (9.92, 25.11) | 85 **(82.52)** | (73.79, 89.30) |
| 5 mM dbcAMP | 99 | 0 | - | 1 **(1.01)** | (0.03, 5.50) | 16 **(16.16)** | (9.53, 24.91) | 82 **(82.83)** | (73.94, 89.67) |

Includes 95% confidence intervals (CI).

## 2.3.3 Experiment 3

### 2.3.3.1 2x2 factorial with 100 nM β-Oestradiol and 500 µM IBMX (5 mM dbcAMP in all treatments) at 18 hours

After 18 hours of culture with and without 100 nM β-oestradiol and 500 µM IBMX alone and combined, very few COCs remained at the GV stage. A greater proportion of COCs were at the M1 meiotic stage when cultured with β-oestradiol or IBMX alone, compared to the control group ($p<0.01$). When COCs were cultured with β-oestradiol and IBMX combined, a higher proportion remained at M1 after 18 hours, compared to COCs from the control group, or those cultured with β-oestradiol or IBMX (Table 5).

**Table 5:** Effect of 5 mM dbcAMP with the addition of 100 nM β-oestradiol and or 500 µM IBMX on meiotic progression of bovine COCs after 18 hours of treatment.

| Treatment | Total oocytes | GV | | GVBD | | M1 | | M2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | n **(%)** | 95% CI | n **(%)** | 95% CI | n **(%)** | 95% CI | n **(%)** | 95% CI |
| Control[#] | 145 | 0 | - | 0 | - | 34 **(23.45)**[a] | (16.82, 31.20) | 111 **(76.55)**[a] | (68.80, 83.18) |
| 100 nM Oestradiol[#] | 129 | 0 | - | 2 **(1.55)** | (0.19, 5.49) | 47 **(36.43)**[b] | (28.14, 45.36) | 80 **(62.02)**[b] | (53.05, 70.41) |
| 500 µM IBMX[#] | 142 | 2 **(1.41)** | (0.17, 6.00) | 0 | - | 61 **(42.96)**[b] | (34.69, 51.53) | 79 **(55.63)**[b] | (47.07, 63.96) |
| 100 nM Oestradiol + 500 µM IBMX[#] | 122 | 0 | - | 0 | - | 111 **(90.98)**[c] | (84.44, 95.41) | 11 **(9.02)**[c] | (4.59, 15.56) |

# Note: All groups contain 5mM dbcAMP

Different superscripts indicate a significant difference exists ($P<0.05$) within a column

Includes 95% confidence intervals (CI)

## 2.3.4 Experiment 4

### 2.3.4.1 5 mM dbcAMP, 100 nM Oestradiol, 500 µM IBMX with and without 200 nM CNP at 12 and 18 hours

After 12 hours of culture with 5 mM dbcAMP, 100 nM oestradiol and 500 µM IBMX with or without 200 nM CNP, less than 13% of all COCs remained at the GV stage of meiosis across all treatment groups. The proportion of COCs at the GV stage was not different between any of the treatment groups. However, COCs that had been cultured in the presence of dbcAMP, oestradiol and IBMX with or without CNP were more likely to be at the GVBD stage of meiosis compared to the control group after 12 hours ($p<0.01$). The proportion of COCs at GVBD did not differ between COCs cultured with or without CNP (Table 6).

After 18 hours of culture with 5 mM dbcAMP, 100 nM oestradiol and 500 µM IBMX with or without 200 nM CNP, no COCs remained at the GV stage of meiosis. For statistical analysis, GVBD and M1 results were combined due to low numbers in the GVBD group. A greater proportion of oocytes were at the M1 stage following culture with dbcAMP, oestradiol and IBMX, with or without CNP, compared to the control ($p<0.0001$). Oocytes cultured under control conditions, with no meiotic inhibitors, were more likely to be at M2 after 18 hours. However, culture with or without 200 nM CNP did not affect the distribution of meiotic stages at 18 hours (Table 6).

**Table 6:** Effect of 5 mM dbcAMP, 100 nM β-oestradiol, 500 µM IBMX with and without 200 nM CNP on meiotic progression of bovine COCs after 12 and 18 hours of culture.

| Time (hours) | Treatment | Total oocytes n | GV | | GVBD | | M1 | | M2 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | n (%) | 95% CI | n (%) | 95% CI | n (%) | 95% CI | n (%) | 95% CI |
| 12 | Control | 106 | 14 **(13.21)** | (7.41, 21.17) | 9 **(8.49)**[a] | (3.96, 15.51) | 76 **(71.70)** | (62.12, 80.02) | 7 **(6.60)** | (2.70, 13.13) |
| | No CNP[#] | 127 | 17 **(13.39)** | (8.00, 20.56) | 32 **(25.20)**[b] | (17.92, 33.67) | 78 **(61.42)** | (52.37, 69.92) | 0 | - |
| | 200 nM CNP[#] | 122 | 14 **(11.48)** | (6.42, 18.50) | 28 **(22.95)**[b] | (15.82, 31.43) | 80 **(65.57)** | (56.43, 73.94) | 0 | - |
| 18 | Control | 130 | 0 | - | 1 **(0.77)** | (0.02, 4.21) | 37 **(28.46)**[a] | (20.90, 37.04) | 92 **(70.77)**[a] | (62.15, 78.41) |
| | No CNP[#] | 116 | 0 | - | 0 | - | 88 **(75.86)**[b] | (67.04, 83.32) | 28 **(24.14)**[b] | (16.68, 32.96) |
| | 200 nM CNP[#] | 119 | 0 | - | 6 **(5.04)** | (1.87, 10.65) | 85 **(71.43)**[b] | (62.43, 79.33) | 28 **(23.53)**[b] | (16.24, 32.18) |

# Treated with 5 mM dbcAMP, 100 nM estradiol, 500 µM IBMX.

Different superscripts indicate a significant difference exists ($P<0.05$) within a column for that time point.

Includes 95% confidence intervals (CI).

## 2.4 Discussion

The present study aimed to inhibit meiotic resumption of bovine oocytes *in vitro* for at least 18 hours. A combination of IBMX and β-estradiol with dbcAMP led to the greatest delay in reaching the M2 stage of meiosis after 18 hours of culture. However, we were not able to prevent GVBD for 18 hours. Although that result was promising, adding an additional mediator of meiosis in the form of CNP did not lead to significant delays in meiotic progression. Assessment at 12 hours revealed that the majority of bovine COCs had progressed to M1 by that time, regardless of treatment. Inhibition of PDE8 activity, using a specific PDE8 inhibitor, had no effect on meiotic inhibition when used on its own or with IBMX. Therefore further investigation is required to achieve meiotic inhibition of bovine COCs for extended periods of up to 18 hours.

Sasseville et al. (2009) reported that 20% of PDE activity within bovine oocytes was related to PDE8, with 80% contributed by PDE3. In the current study, inhibition of PDE8 using a specific PDE8 inhibitor (PF-04957325) was therefore expected to increase the time that bovine oocytes could be maintained at the GV stage of meiosis. Dipyridamole, a non-specific PDE8 inhibitor (also inhibits PDE7, 10 and 11), has previously been shown to decrease the percentage of bovine oocytes that progressed to the M2 stage when combined with an adenosine cyclase activator, forskolin, for 16 hours, however, dipyridamole was ineffective at delaying meiotic resumption on its own. Exposure of the oocyte to dipyridamole also had a negative effect on both cleavage and blastocyst developmental rates (Sasseville et al. 2009). For these studies we tested a newly developed PDE8 inhibitor that was designed by Pfizer (Groton Laboratories, Groton, CT, USA) to target the human PDE8 protein (Vang et al. 2010). We chose the investigated doses based on other inhibitor studies and also taking into consideration the $IC_{50}$ values (0.7 nM for PDE8A and 0.3 nM for PDE8B according to MedChem Express datasheets) (Sasseville et al. 2009). Another unpublished study from our research group also demonstrated that this specific PDE8 inhibitor with IBMX was ineffective at increasing the time that sheep oocytes could be maintained at the GV stage *in vitro*. A recent study investigated the ability of PDE4 (Rolipram), 7 and 8 (PF-04957325) inhibitors to modulate cAMP concentrations within mouse preovulatory follicles. They found cAMP levels were only increased significantly when all three inhibitors were used simultaneously for 4 hours. Individually, none of the inhibitors had a significant effect on cAMP levels within the follicle. When the PDE7 and 8 inhibitors were used alone, no GVBD had occurred after 24 hours within the follicle enclosed COCs, all other combinations using the PDE4 inhibitor elicited GVBD; meiosis resumption. This suggests that both the PDE7 and PDE8 play important roles in regulating meiosis in mouse follicles, and it demonstrates that this particular PDE8 inhibitor is effective in another species (Vigone et al. 2018). It is possible that we

need to further increase the concentrations of PDE8 inhibitor for bovine studies before we will see an effect. To my knowledge, the experiments by Sasseville et al. (2009) have not been repeated, therefore it is possible that the proportions of the types of PDEs within the bovine oocytes were misrepresented. Oocytes from other species such as the mouse, human and pig do appear to be easier to arrest at the GV stage of meiosis using single or multiple PDE inhibitors, dbcAMP or CNP for up to 24 hours (Gil et al. 2017; Kawamura et al. 2011; Vigone et al. 2018). Further investigation is required to extend the time that bovine oocytes can be arrested in meiosis. Perhaps further investigation is necessary into combinations of various doses of PDE inhibitors, guanylate cyclase activators and adenylate cyclase activators.

Rather than focus on inhibiting PDEs our further studies investigated the use of dbcAMP to inhibit meiotic resumption of bovine oocytes. Recently, dbcAMP has been shown to act as a very effective inhibitor of meiotic resumption of pig oocytes over an extended period of 22 hours (Gil et al. 2017). However, in the current study, the addition of dbcAMP during bovine COC culture had no effect on delaying the resumption of meiosis when comparable doses were investigated after 18 hours. A previous study has shown that the effectiveness of dbcAMP treatment during pig IVM to improve embryonic development is dependent on the size of the follicles that the COCs were derived from. COCs from small follicles (2-4 mm diameter) did not have improved development regardless of treatment with dbcAMP or not, while COCs from medium sized follicles (>4-6 mm diameter) had improved blastocyst development rates when treated with dbcAMP for 22 hours followed by a further 22 hours IVM without any meiotic inhibitors. However, meiotic progression was investigated after the cumulative 44 hour IVM period which included a final 22 hours without inhibitors (Sugimura et al. 2015). The current study pooled all COCs derived from follicles ranging from approximately 2-8 mm in diameter. Further studies could investigate the effect of dbcAMP on meiotic progression of bovine COCs derived from defined follicle sizes of small, medium and large follicles. An early study investigated a 6 hour pre-treatment of bovine COCs with 0.01 mM, 0.1 mM and 1 mM dbcAMP with or without cycloheximide prior to 24 hours of conventional IVM without any meiotic inhibitors. They found no effect of dbcAMP on cleavage rates and a small, but significant, improvement to embryo developmental rates to the 64 cell stage when 0.1 mM dbcAMP had been used with cycloheximide (Guixue et al. 2001). Further investigation of the effects of dbcAMP at lower concentrations than those used in the current study may therefore be warranted. Alternatively, dibutyryl cyclic guanosine monophosphate (dbcGMP) may be more effective at inhibiting PDEs within the oocyte, to maintain high intra-oocyte concentrations of cAMP. However, the dbcGMP analog seems to lack potency on its own in terms of preventing the resumption of meiosis from other species (starfish and rat) (Karaseva & Khotimchenko 1991; Tornell, Brannstrom & Hillensjo 1984). It appears that mechanisms to prevent meiotic resumption in bovine oocytes differs to other species, therefore it does not make sense to rule

dbcGMP out as a potential meiotic inhibitor due to results obtained from rat and starfish studies. Alternatively, a combination of dbcGMP and dbcAMP could be more effective.

Individually both IBMX and oestradiol had an effect on delaying COCs reaching the M2 stage, and an additive effect was seen when IBMX and oestradiol were combined. IBMX prevents the breakdown of cAMP by inhibiting all cAMP specific PDEs except PDE8 (Sasseville et al. 2009). Oestradiol has been shown to increase the expression of *Npr2*, the receptor for CNP, in bovine oocytes and cumulus cells (Xi et al. 2018). This additive effect was promising for us, it suggested that we might be able to further delay COCs from reaching M2 by combining multiple cAMP modulators. Given the results with IBMX and β-oestradiol, we expected an additional effect from adding CNP to the media. However, while the proportion of COCs that had reached M2 at 18 hours was reduced when cultured in the presence of IBMX and β-oestradiol, this proportion did not differ with or without the addition of CNP. Another study from within our research group assessed the use of CNP and IBMX on meiotic progression of bovine oocytes after 6 hours. The group treated with 500 μM IBMX alone or with 100 nM CNP were more likely to still be at the GV stage after 6 hours compared to the control or if 100 nM CNP had been used alone. No significant difference in the GV rate after 6 hours was reported between the groups treated with 100 nM CNP or 500 μM IBMX alone (Soto-Heras, Paramio & Thompson 2019). These results and our own results suggest that CNP alone is not a potent meiotic inhibitor for bovine COCs

Studies from the 1990's demonstrated that bovine oocytes could be arrested at the GV stage for 24 hours if they were cultured in follicular hemi-sections containing thecal cells (Richard & Sirard 1996a). It was also demonstrated that bovine COCs could be maintained at the GV stage for 12 hours if they were cultured in media that had been pre-conditioned by follicular hemi-sections containing thecal cells, without direct contact to thecal cells (Richard & Sirard 1996b). Later, a study published in the early 2000's partially identified a thecal secreted factor that may be responsible for regulating meiotic resumption of COCs. They concluded that the factor could not be a peptide or fatty acid, by treating media that had been pre-conditioned with thecal cells with proteases and chloroform. They also identified that the responsible factor is a small molecule, at less than 5 kDa in size, and stable following 5 minute 100˚C heat treatment (Van Tol & Bevers 2001). A purine may fit the criteria. Purines found naturally within follicular fluid, hypoxanthine and adenosine, have been shown to successfully arrest meiosis in several species including the oocytes from mice, rats, pigs and monkeys (Eppig, Wardbailey & Coleman 1985; Miyano et al. 1995; Tornell et al. 1990; Warikoo & Bavister 1989). However, hypoxanthine has only been able to hold bovine and goat COCs at the GV stage for approximately 6 hours (Ma et al. 2003; Sirard & First 1988a), and adenosine has been shown to be ineffective at preventing meiotic resumption in bovine COCs (Sirard 1990).

Furthermore Van Tol and Bevers (2001) suggested that hypoxanthine was not the thecal cell secreted factor inhibiting meiotic resumption of bovine oocytes, as charcoal treatment of the <5kDa fraction of thecal cell conditioned media did not have any effect on the meiotic inhibitory response suggesting the molecule is not hydrophobic (Dang & Lowik 2005; Van Tol & Bevers 2001). Charcoal treatment has previously been demonstrated to inhibit hypoxanthine (unpublished, but referred in Van Tol & Bevers 2001). Therefore the thecal cell secreted factor modulating meiotic resumption/inhibition in bovine oocytes remains elusive, and requires further investigation. It would be interesting to find out if combining an inhibitor of MPF (such as DMAP) with cAMP modulators would help to increase the time bovine oocytes can be arrested at the GV stage and also improve developmental potential. We are unsure if using a combination of inhibitors instead of a single inhibitor, such as IBMX, to maintain the oocytes at GV for 6 hours would be beneficial in terms of development potential. We also are unsure if using the inhibitors for a whole 24 hour maturation period would have any effect on development rates. Alternatively it is possible that embryo quality might be improved (for example higher cell numbers) even if development rates are not improved.

In conclusion, we were unable to extend the time that bovine oocytes could be arrested at the GV stage of meiosis to 18 hours. We significantly decreased the proportion of COCs that had progressed to the M2 stage of meiosis after 18 hours of treatment with the combination of IBMX and oestradiol. Future investigations of combinations of PDE inhibitors, adenylate cyclase activators, dbcAMP, guanylates cyclase activators and or dbcGMP with and without inhibitors of MPF may lead to successes in extending meiotic inhibition of bovine oocytes.

# Chapter 3
# Investigation of potential fertility markers of bovine sperm

## 3.1 Introduction

### 3.1.1 Semen use in the cattle industry

Bull genetics are spread widely following semen collection and preservation. Semen frozen in straws can be maintained for long periods of time in liquid nitrogen tanks or for shorter periods stored fresh-chilled or fresh-ambient (reviewed by Vishwanath & Shannon 2000). Most agree that fresh chilled or ambient stored semen should be used within up to 4 days of collection, as the quality and motility of the samples decreases linearly after this time (Murphy, EM et al. 2018; Murphy, EM et al. 2017). The ability of chilled sperm to fertilise oocytes *in vitro* also decreases as storage time increases (Krzyzosiak et al. 2001). Semen can be used in artificial insemination or embryo technologies such as *in vitro* embryo production (IVP) (Choudhary et al. 2016; Wu & Zan 2012) and the benefits include introducing elite genetics to a herd without the cost of purchasing and maintaining the bull. This also allows genetics to be transferred nationally and internationally with ease (Harris & Newman 1994).

Conventional methods of assessing sperm fertility focus on motility and morphological assessments (Fitzpatrick et al. 2002).These methods are not always a reliable indicator of sperm fertility potential. Therefore further investigation is required to develop a more accurate assessor of sperm fertility. Sperm fertility is affected by several factors including climate, nutrition and collection and storage methods (Amann & Waberski 2014). The majority of semen used in the bovine industry is cryopreserved. The process of cryopreserving and thawing semen has been shown to result in increased production of Reactive Oxygen Species (ROS), especially in the form of superoxide (Chatterjee & Gagnon 2001). Motility and mitochondrial membrane potential has been shown to be impacted by ROS levels in semen (Gibb, Lambourne & Aitken 2014; Johannisson et al. 2018). Recently a zinc signature relating to sperm capacitation has been reported. Zinc ion concentrations decrease as sperm undergo capacitation (Kerns et al. 2018). Another study reported a negative correlation between zinc ion concentrations of human sperm and progressive motility (Henkel, RR et al. 2003). Further assessment of whether ROS or Zinc in bull sperm could be used as a fertility marker are needed.

### 3.1.2 Objectives

The first objective of this study was to assess if levels of zinc, superoxide and ROS in cryopreserved bull semen correlates with the fertilisation potential *in vitro* and *in vivo*. Due to the environmental adaptions, thresholds of ROS, superoxides and zinc may differ between the bovine sub-species. Fertilisation

potential may also be affected at differing thresholds of ROS, superoxide and zinc in cryopreserved bull sperm of each sub-species. Therefore the second objective of this study was to determine whether the potential markers of fertility differ between *bos indicus* and *bos taurus* cryopreserved bull sperm.

## 3.2 Methods

### 3.2.1 Sperm used in this study

Cryopreserved semen of 13 bulls was donated for this study. The cryopreserved semen from 6 *bos indicus* bulls (5 Brahman and 1 Droughtmaster) was donated by Beef Breeding Services (Greg Fawcett, Rockhampton, Queensland, Australia). We were not informed of the fertilisation potential of these bulls through artificial insemination or *in vitro* fertilisation. In addition, semen from 6 *bos taurus* bulls (breeds unknown) were donated by Kiri Beilby and Peter Thurn from Genetics Australia, Cooperative Bacchus Marsh (Maddingley, Victoria). The *bos taurus* semen arrived with a "non-return to service (NRS) rate" based on results from AI under commercial conditions. The NRS rate informs us of the likely conception rate following insemination compared to an average bull. For example, a NRS rate of +2 can be interpreted as being likely to result in a 2% higher conception rate than an average bull following AI. The semen of the 6 *bos indicus* bulls and a known quality control bull (*bos taurus*) were assessed for *in vitro* embryo production results. All donated sperm underwent Computer Assisted Sperm Analysis (CASA) using Androvision software and a Zeiss Lab.1A inverted microscope at the Equine Health and Performance Centre, School of Animal and Veterinary Sciences (Roseworthy Campus, University of Adelaide, Australia) to obtain detailed sperm concentration and motility information.

### 3.2.2 *In vitro* production of embryos

#### 3.2.2.1 Ovary collection and in vitro maturation of bovine oocytes

Ovaries were collected from a local abattoir from *bos taurus* cattle (M.C. Herd, Corio, Victoria, Australia). They were transported to the laboratory in 0.9% saline solution at 35˚C within 3 hours of collection. At the laboratory, follicles (2-8 mm diameter) were manually aspirated using an 18 G needle attached to a 10 mL syringe. Follicular fluid was searched using a dissecting microscope, and COCs with an even coloured ooplasm and at least 3 complete layers of cumulus cells were selected for use. All COCs were washed twice with 2 mL of VitroWash (ART Lab Solutions, Adelaide, Australia) and once with 2 mL of VitroMat (ART Lab Solutions) media. Groups of no more than 30 COCs per 300 µL of VitroMat (ART Lab Solutions) under paraffin oil in Nunc four-well dishes (product code 144444, Thermofisher Scientific) were matured at 38.8˚C in 5% $CO_2$ in air for 22-24 hours.

#### 3.2.2.2 In vitro fertilisation of bovine oocytes

Matured oocytes (presumed MII) were washed in VitroFert (ART Lab Solutions) supplemented with 10 IU/ml heparin, 25 µM penicillamine, 12.5 µM hypotaurine, and 1.25 µM epinephrine before being transferred into four-well dishes (product code 144444, Thermofisher Scientific, USA) containing 300 µL of IVF media under paraffin oil. Semen from 6 unknown *bos indicus* bulls and a known *bos taurus* bull (control) were thawed at 35˚C for 60 seconds, and layered over a 40%:80% gradient of Bovipure (Nidacon, International AB, Sweden) in a 15 mL sterile polystyrene conical tube (product code 6272, Globe Scientific Inc., New Jersey, USA). Semen was then centrifuged for 15 minutes at 300 x g. The supernatant was removed, and the pellet of sperm was resuspended in 1 mL of warmed VitroWash (ART Lab Solutions). The diluted sperm was centrifuged for 5 minutes at 300 x g. The supernatant was removed and 2.5 µL of the pellet was diluted in either 100 µL of IVF media or water. This was used to assess the percent of motile sperm and to obtain a sperm count. The remaining pellet was then diluted with IVF media, and approximately 1 million live sperm per mL were added to wells containing 300 µL of IVF media containing no more than 30 matured COCs. The fertilisation dishes were maintained at 38.8˚C in 5% $CO_2$ in air for 16-22 hours.

#### 3.2.2.3 In vitro culture of bovine embryos

Sixteen to 22 hours after IVF, the presumptive zygotes were denuded by vigorously pipetting zygotes up and down in 50 µL microdrops of VitroFert (ART Lab Solutions) media with a micropipette set at 25 µL. Groups of no more than 15 presumptive zygotes were moved into 50 µL drops of VitroCleave (ART Lab Solutions) under paraffin oil in 35 mm Falcon dishes (product code 351008, Corning, New York, USA). The dishes were incubated at 38.8˚C in 5% $O_2$ 6% $CO_2$ with a balance of $N_2$ for approximately 96 hours. After 96 hours, no more than 15 cleaved embryos were moved to 50 µL drops of VitroBlast (ART Lab Solutions) under paraffin oil in 35 mm Falcon dishes (product code 351008, Corning). The embryos were further incubated at 38.8˚C in 5% $O_2$ 6% $CO_2$ in a balance of $N_2$ under paraffin oil for approximately 48 hours.

### 3.2.3 Preparation of semen for fluorescent staining

Semen straws were thawed at 35˚C for 60 seconds and loaded on a 40% (1 mL) and 80% (1 ml) gradient of BoviPure in BoviDilute (Nidacon, Sweden) in a 15 mL sterile, polystyrene conical tube (Globe Scientific Inc., USA) that had been allowed to reach ambient temperature. The samples were centrifuged at 300 x

g for 15 minutes to separate motile sperm from non-motile sperm. The supernatant was then removed, and 1 mL of VitroWash, (ART Lab Solutions, warmed at 38.8˚C) was added to each sample, gently mixed and centrifuged for 5 minutes at 300 x g. The supernatant was removed and the sperm pellet was divided between media containing all the specified stains and the negative control which only contained Hoescht 33342. Hoescht 33342 and MitoTracker staining were used as positive controls for head and midpiece staining respectively. Hoescht 33342 stains DNA which is located in the head of sperm and MitoTracker Deep Red stains mitochondria which are located in the midpiece of the sperm

3.2.3.1 Triple Stain for Sperm: Zinc (Fluo-Zin-3AM)

To investigate zinc levels, the pellet containing motile sperm was divided between 1) 500 µL of VitroFert (ART Lab Solutions) without any added sperm capacitators (heparin), but containing 100 nM MitoTracker Deep Red (Invitrogen, CAT# M22426, diluted in DMSO, stock solution 1 mM), 5 µM Fluo-Zin-3AM (Invitrogen, CAT# F24195, diluted in DMSO, stock solution 500 µM), and 90 µM bisbenzimide H33342 trihydrochloride (Hoescht 33342, Sigma-Aldrich, CAT# B2261, diluted in Milli-Q water, stock solution 18 mM); or 2) 500 µL of fertilisation media containing only 90 µM Hoescht 33342 as a negative control. Sperm were incubated with the stains for 30 minutes at 38.8˚C. The solution containing the stained sperm was then gently mixed, and 3 µL was placed onto a slide and covered with a coverslip without any mounting solution. Slides were imaged using a confocal microscope (Olympus FV10i) at 120x magnification with 3 channels Hoescht 33342 (Ex: 352 nm, Em: 455 nm), FluoZin3AM (Ex: 494 nm, Em: 516 nm) and MitoTracker Deep Red (Ex: 642 nm, Em: 661 nm) at consistent settings. Hoescht 33342 was imaged with sensitivity and laser settings of 40%. MitoTracker Deep Red was imaged with sensitivity settings of 50% and a laser setting of 20%. FluoZin3AM was imaged with sensitivity settings of 60% and laser at 40%. Phase contrast images were captured with a sensitivity setting of 20% (fig.4). All images were captured with the largest (1024x1024 pixels) and highest quality (x16) settings available with the aperture at x2.5 and cross talk communications on.

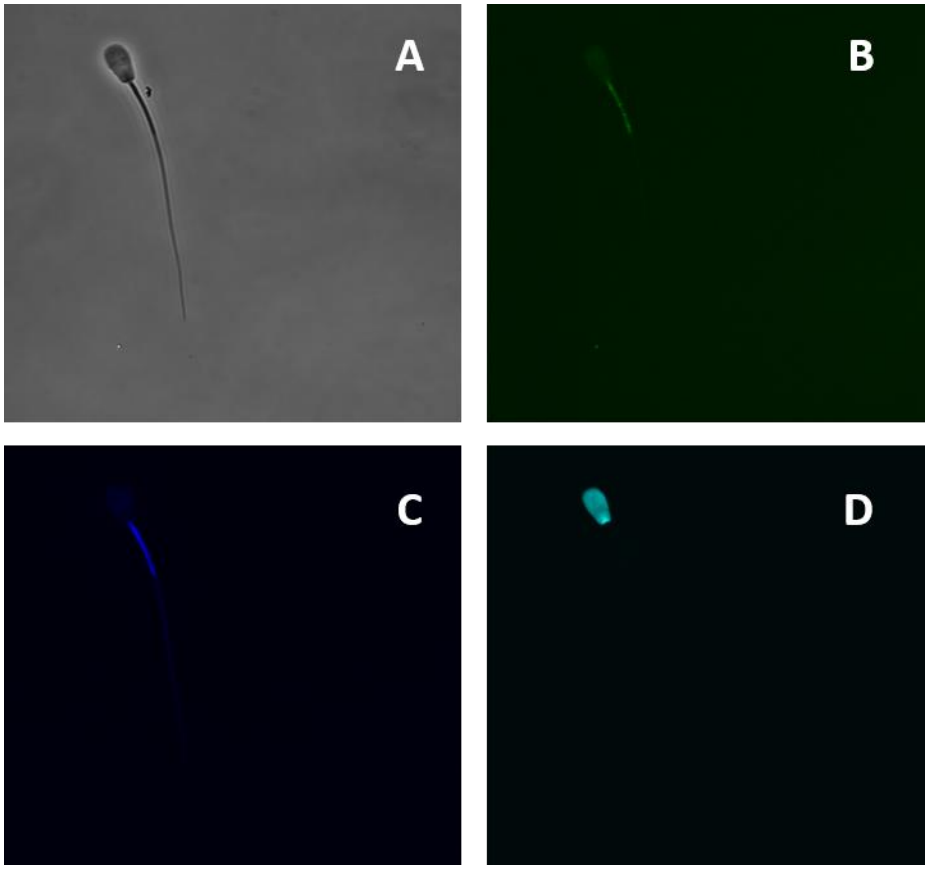


**Figure 4:** Images obtained following confocal microscopy for triple staining of sperm with Fluo-Zin-3AM, MitoTracker and Hoescht 33342. Phase contrast (A), Fluo-Zin-3AM (B), MitoTracker (C) and Hoescht 33342 (D).

3.2.3.2 Double Stain for Sperm: ROS detection (CellRox)

To investigate total ROS levels, the pellet was divided between 1) 500 µL of VitroFert (ART Lab Solutions) without any added sperm capacitation promoters, but containing 5 µM CellROX (Invitrogen, CAT# C10422, diluted in DMSO, stock solution 2.5 mM), and 90 µM bisbenzimide H33342 trihydrochloride (Hoescht 33342, Sigma-Aldrich, CAT# B2261, diluted in Milli-Q water, stock solution 18 mM) or 2) 500 µL of VitroFert containing 90 µM Hoescht 33342 (negative control) and incubated for 30 minutes at 38.8˚C. The stained sperm solution was then gently mixed, and 3 µL was added to a slide and covered with a coverslip without any mounting solution. Slides were imaged using a confocal microscope (Olympus FV10i) at 120x magnification with 2 channels Hoescht 33342 (Ex: 352 nm, Em: 455 nm), and CellROX Deep Red (Ex: 635 nm, Em: 660-760 nm) at consistent settings. Hoescht 33342 was imaged with sensitivity and laser settings of 40%. CellROX was imaged with a sensitivity of 49.6% and the laser at 40%. Phase contrast images were imaged with a sensitivity of 20% (fig.5). All images were captured with the largest (1024 x 1024 pixels) and highest quality (x16) settings available with the aperture at x2.5 and cross talk communications on.

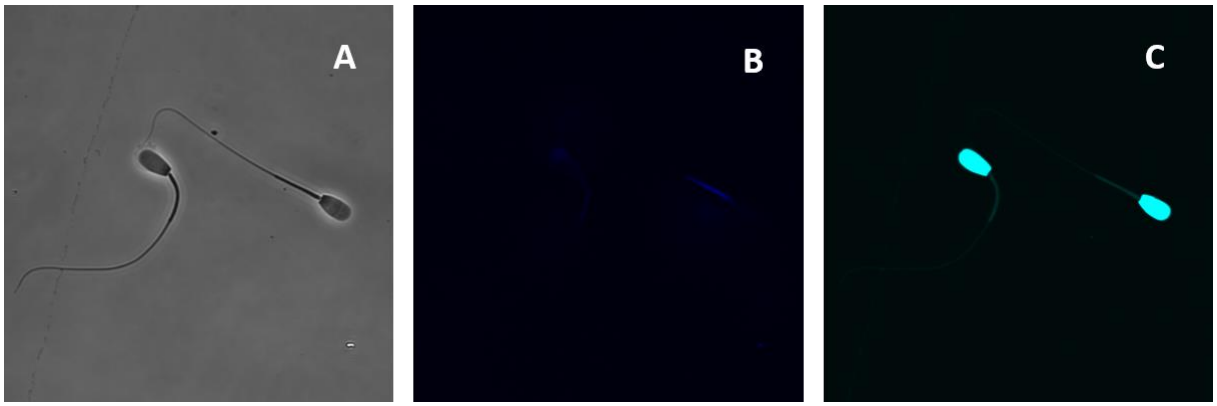


**Figure 5:** Examples of images obtained following confocal microscopy of CellROX and Hoescht 33342. Phase contrast (A), CellROX (B) and Hoescht 33342 (C).

3.2.3.3 Double stain for detection of superoxide (dihydroethidium (DHE))

To investigate superoxide levels, the pellet was divided between 1) 500 µL of VitroFert (ART Lab Solutions) without any added sperm capacitation promoters (heparin), but containing 2 µM dihydroethidium (DHE)(Invitrogen, CAT# D11347, diluted in DMSO, stock solution 2 mM), and 90 µM bisbenzimide H33342 trihydrochloride (Hoescht 33342, Sigma-Aldrich, CAT# B2261, diluted in Milli-Q water, stock solution 18 mM); or 2) 500 µL of VitroFert containing 90 µM Hoescht 33342 (negative control), and incubated for 30 minutes at 38.8˚C. The stained sperm solution was then gently mixed, and 3 µL was added to a slide and covered with a coverslip without any mounting solution. Slides were imaged using a confocal microscope (Olympus FV10i) at 120x magnification with 2 channels Hoescht 33342 (Ex: 352 nm, Em: 455 nm (light blue / aqua)), and DHE (Ex: 549 nm, Em: 574 nm) at consistent settings. Hoescht 33342 was imaged with sensitivity and laser settings of 40%. DHE was imaged using the Calcium Orange channel with a sensitivity of 49.6% and the laser setting at 40%. Phase contrast images were imaged with a sensitivity of 20% (fig.6). All images were captured with the largest (1024 x 1024 pixels) and highest quality (x16) settings available with the aperture at x2.5 and cross talk communications on.

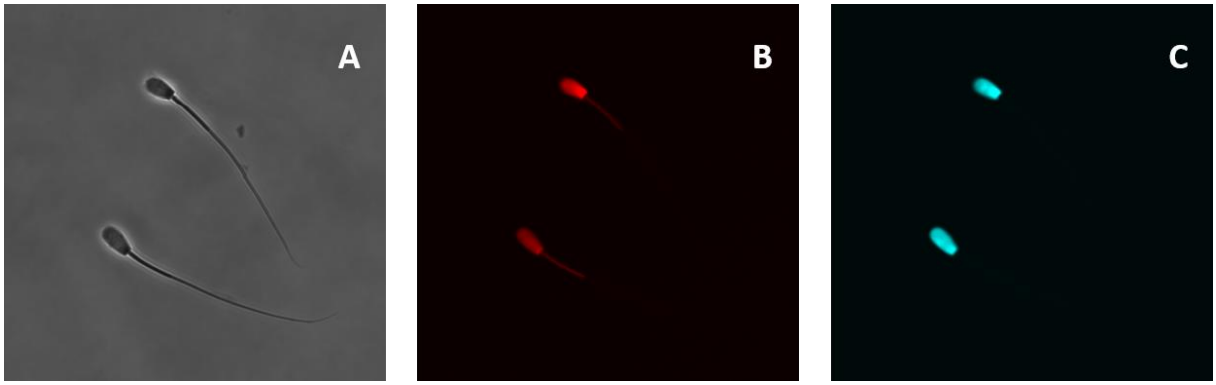


**Figure 6:** Examples of images obtained following confocal microscopy of sperm double stained for Dihydroethidium (DHE) and Hoescht 33342. Phase contrast (A), DHE (B), and Hoescht 33342 (C).

### 3.2.4 Image analysis of sperm

Fluorescence images were prepared and relatively quantified using ImageJ software. Approximately 520 sperm per stain were imaged for the analysis. Phase contrast images were used to trace around the various segments of the sperm (head, midpiece and tail). The background was then cleared. An in-house macro was used to convert the image to 8 bit and measure mean fluorescence intensity of all the images (fig.7). To account for auto-fluorescence, fluorescence was measured from each quadrant of the background of 5 original images per stain, per bull and per day of stain to enable total fluorescence to be quantified relative to background fluorescence. The background fluorescence was averaged for each bull and stain per day, and subtracted from fluorescence of sperm images.

Fluorescence of the head and midpiece were investigated for Fluo-Zin-3AM, while head, midpiece and tail were investigated for CellROX and DHE.

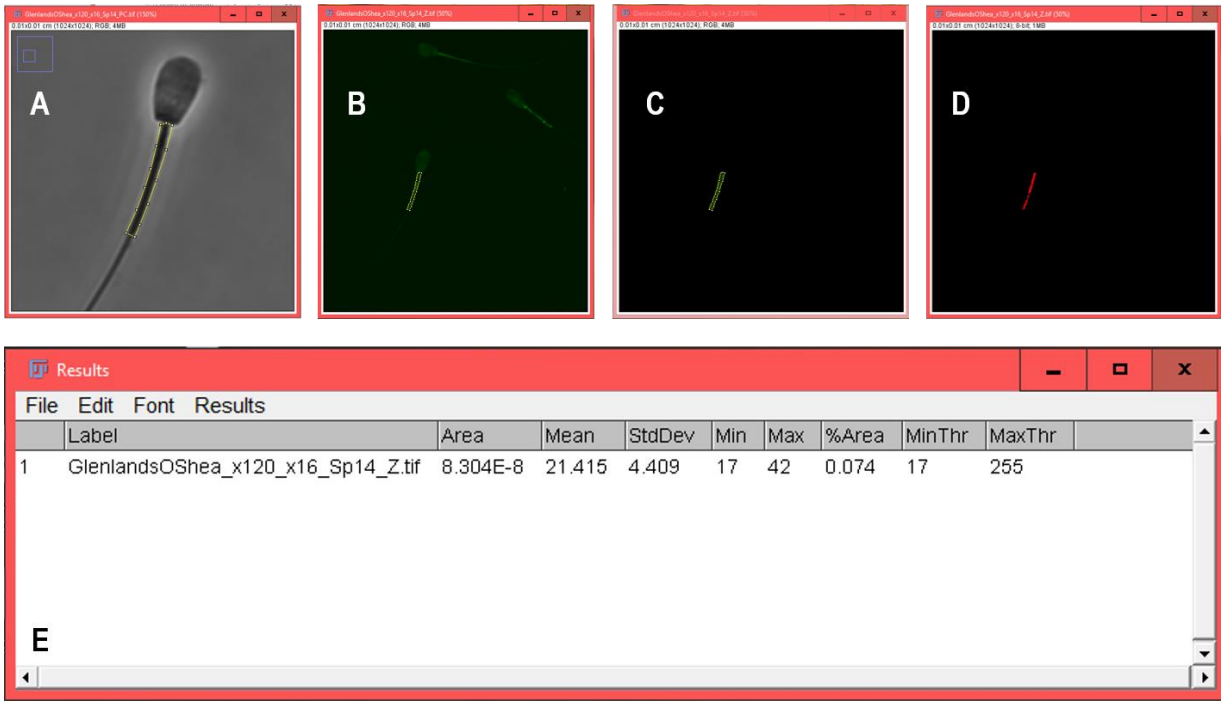


**Figure 7:** Images were prepared and the mean fluorescence intensity was quantified using ImageJ software and a macro developed in-house. The region of interest was traced in the phase contrast image (A) and saved. Then the region of interest was selected on the fluorescent image (B). The background was deleted, so that only the region of interest remained (C). The image was then converted to an 8 bit image and the threshold was selected (D). The mean fluorescent intensity was then measured on the selected area, and a report from the macro was generated (E).

### 3.2.5 Statistical Analysis

Results were analysed using Graph Pad Prism 8.0. Correlations of cleavage and blastocyst development rates with CASA results and fluorescent stain results were assessed for the 7 semen samples (1 laboratory control (*bos taurus)* and 6 *bos indicus* bulls) that were used for *in vitro* embryo production. Semen from a further six *bos taurus* bulls had NRS rate scores correlated against the CASA results and the fluorescent stain results. For each stain; sperm segments were analysed against the bovine subspecies they originated from (*bos taurus* or bos *indicus*). Data for fluorescent intensities of stains were assessed for normal distribution. If the data were normally distributed, an unpaired parametric test was used to compare intensity between *bos indicus* and *bos taurus* sperm. If the data were not normally distributed, the Mann-Whitney test was used. Paired t-tests were used to assess differences against the negative control for each stain to account for autofluorescence. Again, parametric testing was used when the data were normally distributed, and non-parametric testing was used when the data were not normally distributed. Results were considered statistically significant if the P-value was less than 0.05.

## 3.3 .Results

### 3.3.1 *In vitro* embryo production results

*In vitro* embryo production results are displayed in Table 7. Bull 1 is a *bos taurus* bull with known high fertility regularly used for quality control for IVP in our laboratory. The other 6 semen samples were from *bos indicus* bulls that had unknown fertility (*in vitro* or *in vivo*) prior to these experiments. These results were used in subsequent analysis to assess if correlations exist between *in vitro* cleavage and blastocyst development rates with both CASA results and content of zinc, ROS and superoxide in the cryopreserved spermatozoa.

**Table 7:** *In vitro* embryo production using semen from seven individual bulls for IVF. Bull 1 is a *bos taurus* bull routinely used as a control in the laboratory. Bulls 2-7 were *bos indicus* bulls with unknown fertility.

| Bull | Number of replicates | Number of COCs | Number Cleaved (% of COCs) n (%) | Number of Blastocysts (% of cleaved) n (%) | Number of Blastocysts (% of COCs) n (%) |
|---|---|---|---|---|---|
| **1** | 4 | 97 | 76 **(78)** | 40 **(53)** | 40 **(41)** |
| **2** | 3 | 82 | 40 **(49)** | 18 **(45)** | 18 **(22)** |
| **3** | 3 | 80 | 45 **(56)** | 14 **(31)** | 14 **(18)** |
| **4** | 3 | 84 | 34 **(40)** | 5 **(15)** | 5 **(6)** |
| **5** | 3 | 72 | 66 **(92)** | 28 **(42)** | 28 **(39)** |
| **6** | 3 | 78 | 41 **(53)** | 14 **(34)** | 14 **(18)** |
| **7** | 3 | 80 | 76 **(95)** | 44 **(58)** | 44 **(55)** |

### 3.3.2 Correlation of CASA and cleavage rates

Table 8 displays the *in vitro* cleavage rates following IVF using semen from 7 bulls, and CASA parameters for the semen samples. CASA measures of total motility and progressive motility were the same for each bull, therefore only total motility was interrogated during statistical analysis. The CASA program included circle motility with progressive and total motility percentages. Total motility (fig.6), and slow progressive motility tended to be positively correlated with embryo cleavage rates (r=0.691, P=0.085, n=7; and r=0.713, P=0.072, n=7 respectively), while beat cross frequency (Mean BCF (Hz)) was positively

correlated with embryo cleavage rates (r=0.81, P=0.027, n=7, fig 8). Cleavage rates *in vitro* were not related to the concentration of sperm in the straw or fast progressive motility of the sperm sample. Other CASA outputs including curvilinear velocity and straight line velocity did not correlate with cleavage rates (data not shown).

**Table 8:** Correlation between embryo cleavage rates following IVP and Computer Assisted Sperm Analysis results utilising cryopreserved semen of 7 bulls. Results were considered significant with a P < 0.05.

| Bull | Cleavage Rate (%) | Number of sperm analysed | Concentration ($10^6$/mL) | Total Motility (%) | Progressive Motility (%) | Fast Prog. Motility (%) | Slow Prog. Motility (%) | Mean BCF (Hz) |
|---|---|---|---|---|---|---|---|---|
| **1** | 78 | 682 | 36.89 | 46.33 | 46.33 | 6.6 | 39.3 | 2.28 |
| **2** | 49 | 1632 | 88.27 | 19.42 | 19.42 | 0.98 | 18.44 | 1.11 |
| **3** | 56 | 2574 | 139.21 | 45.57 | 45.57 | 2.84 | 42.74 | 2.98 |
| **4** | 40 | 997 | 53.92 | 27.88 | 27.88 | 1.91 | 25.98 | 1.58 |
| **5** | 92 | 1143 | 61.82 | 66.49 | 66.49 | 5.34 | 60.89 | 2.97 |
| **6** | 40 | 1289 | 69.72 | 61.06 | 61.06 | 9.78 | 50.97 | 1.85 |
| **7** | 95 | 1492 | 80.69 | 58.45 | 58.45 | 7.04 | 51.41 | 3.75 |
| **r** | | | -0.202 | 0.691 | 0.691 | 0.441 | 0.713 | **0.810** |
| ***P* Value** | | | 0.663 | 0.085 | 0.085 | 0.322 | 0.072 | **0.027** |

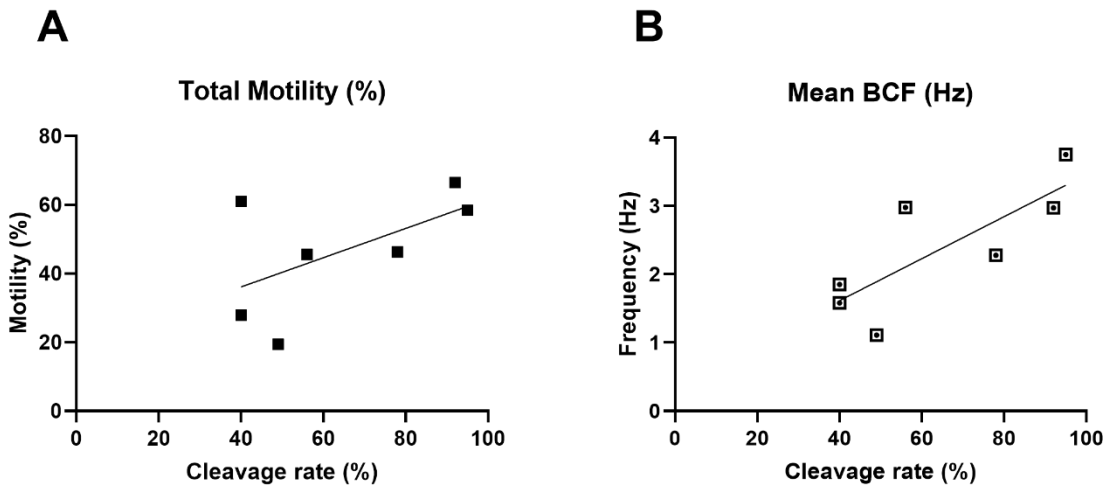


**Figure 8:** Relationship of cleavage rate in vitro with total sperm motility (r=0.691, P=0.085, A) and mean beat cross frequency (BCF, r=0.81, P=0.027, B) for 7 individual bulls determined by CASA analysis.

### 3.3.3 Correlation of CASA with blastocyst development rates

Table 3 displays the blastocyst development rates from oocytes that cleaved *in vitro* and the CASA analysis results for semen samples from each of the bulls. None of the measures of semen quality were related to blastocyst development rates (Table 9, fig 9).

**Table 9:** Correlation between blastocyst development rates following IVP and Computer Assisted Sperm Analysis results utilising cryopreserved semen of 7 bulls. Results were considered significant with $P < 0.05$.

| Bull | Blastocyst Rate (%) | Number of sperm analysed | Concentration ($10^6$/mL) | Total Motility (%) | Progressive Motility (%) | Fast Prog. Motility (%) | Slow Prog. Motility (%) | Mean BCF (Hz) |
|---|---|---|---|---|---|---|---|---|
| **1** | 53 | 682 | 36.89 | 46.33 | 46.33 | 6.6 | 39.3 | 2.28 |
| **2** | 45 | 1632 | 88.27 | 19.42 | 19.42 | 0.98 | 18.44 | 1.11 |
| **3** | 31 | 2574 | 139.21 | 45.57 | 45.57 | 2.84 | 42.74 | 2.98 |
| **4** | 15 | 997 | 53.92 | 27.88 | 27.88 | 1.91 | 25.98 | 1.58 |
| **5** | 42 | 1143 | 61.82 | 66.49 | 66.49 | 5.34 | 60.89 | 2.97 |
| **6** | 34 | 1289 | 69.72 | 61.06 | 61.06 | 9.78 | 50.97 | 1.85 |
| **7** | 58 | 1492 | 80.69 | 58.45 | 58.45 | 7.04 | 51.41 | 3.75 |
| **r** | | | -0.133 | 0.335 | 0.335 | 0.386 | 0.307 | 0.459 |
| ***P* Value** | | | 0.777 | 0.463 | 0.463 | 0.393 | 0.503 | 0.301 |

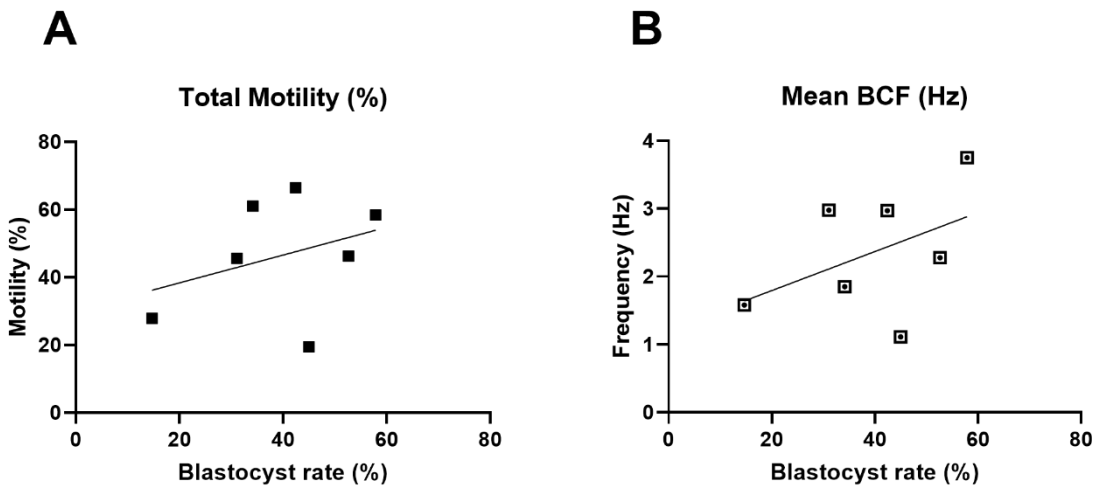


**Figure 9:** Relationship of blastocyst development rates from cleaved oocytes following IVP with total motility (r=0.335, P=0.463, NS, A) and mean Beat Cross Frequency (BCF) (Hz) (r=0.459, P=0.301, NS, B) of the semen sample used for IVF.

### 3.3.4 Correlation of sperm analysis measures and Non Return to Service (NRS) rates from Artificial Insemination with bos taurus semen

Semen from six *bos taurus* bulls used in the study had NRS rates supplied by a commercial bovine reproduction company, Genetics Australia (Table 4). No correlation existed between the NRS rate for these 6 bulls and CASA scores for sperm concentration, total motility, progressive motility, fast progressive motility, slow progressive motility and mean BCF (Table 10).

**Table 10:** Correlation of Non Return to Service (NRS) rates and Computer Assisted Sperm Analysis results of 6 bos taurus bulls. Results were considered significant with $P < 0.05$.

| Bull | NRS Rate (%) | Number of sperm analysed | Concentration ($10^6$/mL) | Total Motility (%) | Progressive Motility (%) | Fast Prog. Motility (%) | Slow Prog. Motility (%) | Mean BCF (Hz) |
|---|---|---|---|---|---|---|---|---|
| **1** | 3.55 | 611 | 33.05 | 30.44 | 30.44 | 6.87 | 23.57 | 1.47 |
| **2** | 2.6 | 561 | 30.34 | 34.58 | 34.58 | 11.76 | 22.46 | 1.21 |
| **3** | 2.53 | 842 | 45.54 | 10.21 | 10.21 | 1.54 | 8.43 | 1.03 |
| **4** | -3.27 | 664 | 35.91 | 40.96 | 40.96 | 5.57 | 35.09 | 2.49 |
| **5** | -3.17 | 782 | 42.29 | 4.99 | 4.99 | 0.9 | 4.09 | 0.29 |
| **6** | -2.76 | 988 | 53.44 | 44.13 | 44.13 | 13.16 | 30.06 | 1.26 |
| **r** | | | -0.469 | -0.136 | -0.136 | 0.059 | -0.191 | -0.076 |
| ***P* Value** | | | 0.349 | 0.797 | 0.797 | 0.912 | 0.717 | 0.886 |

### 3.3.5 Correlation of IVP results and Zinc, total Reactive Oxygen Species and Superoxide in sperm used for IVF

#### 3.3.5.1 Zinc

Correlations between cleavage rates *in vitro* and zinc content in sperm are displayed in Table 11. Embryo cleavage rates during IVP were not related to the zinc content of the sperm as assessed by mean fluorescence intensity of FluoZin-3AM in the head or midpiece. Intensity of staining with Hoescht (control for the head) and MitoTracker (control for the midpiece) was not correlated with cleavage rates. Similarly, there was no correlation between blastocyst development rates and the intensity of Fluo-Zin-3AM, Hoescht or MitoTracker (Table 12).

**Table 11:** Embryo cleavage rates and mean fluorescence intensities of Fluo-Zin-3AM, Hoescht 33342 and Mitotracker utilising cryopreserved semen from 7 bulls. Results were considered significant with $P < 0.05$.

| Bull | Cleavage Rate % | FluoZin Head (Mean + SEM) | FluoZin Midpiece (Mean +SEM) | Hoescht (Mean + SEM) | Mitotracker (Mean +SEM) |
|---|---|---|---|---|---|
| 1 | 78 | 13.36 ± 1.93 | 24.92 ± 2.22 | 98.63 ± 2.57 | 47.94 ± 2.06 |
| 2 | 49 | 9.08 ± 1.72 | 11.38 ± 1.03 | 79.36 ± 2.33 | 34.67 ± 2.15 |
| 3 | 56 | 10.90 ± 2.18 | 11.28 ± 1.01 | 99.35 ± 2.29 | 24.76 ± 2.66 |
| 4 | 40 | 5.62 ± 0.92 | 7.23 ± 0.52 | 88.41 ± 3.00 | 44.73 ± 2.17 |
| 5 | 92 | 14.37 ± 2.34 | 11.28 ± 1.13 | 95.97 ± 2.75 | 28.50 ± 2.52 |
| 6 | 40 | 8.04 ± 0.79 | 13.32 ± 0.99 | 94.79 ± 2.93 | 47.65 ± 1.55 |
| 7 | 95 | 5.31 ± 1.23 | 5.56 ± 0.74 | 64.59 ± 3.83 | 10.63 ± 2.03 |
| r | | 0.271 | -0.002 | -0.319 | -0.608 |
| P Value | | 0.557 | 0.997 | 0.486 | 0.148 |

**Table 12:** Blastocyst development rates from cleaved oocytes and mean fluorescence intensities of Fluo-Zin-3AM, Hoescht 33342 and MitoTracker utilising cryopreserved semen from 7 bulls. Results were considered significant with $P < 0.05$.

| Bull | Blastocyst Rate % | FluoZin Head (Mean + SEM) | FluoZin Midpiece (Mean +SEM) | Hoescht (Mean + SEM) | Mitotracker (Mean +SEM) |
|---|---|---|---|---|---|
| 1 | 53 | 13.36 ± 1.93 | 24.92 ± 2.22 | 98.63 ± 2.57 | 47.94 ± 2.06 |
| 2 | 45 | 9.08 ± 1.72 | 11.38 ± 1.03 | 79.36 ± 2.33 | 34.67 ± 2.15 |
| 3 | 31 | 10.90 ± 2.18 | 11.28 ± 1.01 | 99.35 ± 2.29 | 24.76 ± 2.66 |
| 4 | 15 | 5.62 ± 0.92 | 7.23 ± 0.52 | 88.41 ± 3.00 | 44.73 ± 2.17 |
| 5 | 42 | 14.37 ± 2.34 | 11.28 ± 1.13 | 95.97 ± 2.75 | 28.50 ± 2.52 |
| 6 | 34 | 8.04 ± 0.79 | 13.32 ± 0.99 | 94.79 ± 2.93 | 47.65 ± 1.55 |
| 7 | 58 | 5.31 ± 1.23 | 5.56 ± 0.74 | 64.59 ± 3.83 | 10.63 ± 2.03 |
| r | | 0.232 | 0.289 | -0.431 | -0.437 |
| P Value | | 0.617 | 0.530 | 0.334 | 0.327 |

3.3.5.2 Reactive Oxygen Species (ROS)

No correlation existed between cleavage rates and the intensity of CellROX fluorescence in the head, midpiece or tail of the sperm (Table 13) or the intensity of Hoescht 33342 (control) fluorescence. Likewise there was no correlation between blastocyst development rates and CellROX or Hoescht 33342 fluorescence intensities (Table 14).

**Table 13:** Embryo cleavage rates and mean fluorescence intensities of CellROX and Hoescht 33342 utilising cryopreserved semen from 7 bulls. Results were considered significant with $P < 0.05$.

| Bull | Cleavage Rate % | CellROX Head (Mean + SEM) | CellROX Midpiece (Mean + SEM) | CellROX Tail (Mean + SEM) | Hoescht (Mean + SEM) |
|---|---|---|---|---|---|
| 1 | 78 | 3.25 ± 0.42 | 8.21 ± 1.30 | 0.72 ± 0.21 | 128.03 ± 1.60 |
| 2 | 49 | 10.06 ± 1.82 | 17.89 ± 2.02 | 3.40 ± 0.68 | 116.73 ± 2.68 |
| 3 | 56 | 9.81 ± 1.23 | 19.85 ± 1.90 | 3.10 ± 0.34 | 130.91 ± 2.09 |
| 4 | 40 | 3.73 ± 1.08 | 10.38 ± 2.22 | 2.55 ± 0.83 | 116.41 ± 3.44 |
| 5 | 92 | 5.34 ± 1.75 | 9.88 ± 2.88 | 6.23 ± 3.02 | 121.59 ± 3.57 |
| 6 | 40 | 8.14 ± 2.26 | 6.77 ± 1.75 | 1.30 ± 0.19 | 130.90 ± 1.42 |
| 7 | 95 | 4.21 ± 0.81 | 9.82 ± 1.71 | 1.79 ± 0.41 | 140.55 ± 1.03 |
| r | | -0.488 | -0.355 | 0.215 | 0.539 |
| P Value | | 0.267 | 0.435 | 0.644 | 0.212 |

**Table 14:** Blastocyst development rates and mean fluorescence intensities of CellROX and Hoescht 33342 utilising cryopreserved semen from 7 bulls. Results were considered significant with $P < 0.05$.

| Bull | Blastocyst Rate (%) | CellROX Head (Mean + SEM) | CellROX Midpiece (Mean + SEM) | CellROX Tail (Mean + SEM) | Hoescht (Mean + SEM) |
|---|---|---|---|---|---|
| **1** | 53 | 3.25 ± 0.42 | 8.21 ± 1.30 | 0.72 ± 0.21 | 128.03 ± 1.60 |
| **2** | 45 | 10.06 ± 1.82 | 17.89 ± 2.02 | 3.40 ± 0.68 | 116.73 ± 2.68 |
| **3** | 31 | 9.81 ± 1.23 | 19.85 ± 1.90 | 3.10 ± 0.34 | 130.91 ± 2.09 |
| **4** | 15 | 3.73 ± 1.08 | 10.38 ± 2.22 | 2.55 ± 0.83 | 116.41 ± 3.44 |
| **5** | 42 | 5.34 ± 1.75 | 9.88 ± 2.88 | 6.23 ± 3.02 | 121.59 ± 3.57 |
| **6** | 34 | 8.14 ± 2.26 | 6.77 ± 1.75 | 1.30 ± 0.19 | 130.90 ± 1.42 |
| **7** | 58 | 4.21 ± 0.81 | 9.82 ± 1.71 | 1.79 ± 0.41 | 140.55 ± 1.03 |
| **r** | | -0.165 | -0.150 | -0.144 | 0.532 |
| **P Value** | | 0.7233 | 0.748 | 0.7585 | 0.2188 |

3.3.5.3 Superoxide

Mean fluorescence of DHE (superoxide) in the midpiece was positively correlated with both cleavage ($r=0.817$, $P=0.025$, Table 15) and blastocyst development rates ($r=0.908$, $P=0.005$, Table 16) (fig.10). DHE levels in the sperm head or tail were not correlated with cleavage or blastocyst development rates. Fluorescence intensity of Hoescht 33342, the control, did not correlate with cleavage or blastocyst development rates.

**Table 15:** Cleavage rates and mean fluorescence intensities of Dihydroethidium (DHE) and Hoescht 33342 from 7 bulls. Results were considered significant with $P < 0.05$.

| Bull | Cleavage Rate % | DHE Head (Mean + SEM) | DHE Midpiece (Mean + SEM) | DHE Tail (Mean + SEM) | Hoescht (Mean + SEM) |
|---|---|---|---|---|---|
| **1** | 78 | 26.85 ± 1.08 | 9.65 ± 0.69 | 0.34 ± 0.04 | 64.61 ± 2.73 |
| **2** | 49 | 18.31 ± 0.91 | 7.55 ± 0.71 | 0.34 ± 0.05 | 72.42 ± 2.58 |
| **3** | 56 | 15.57 ± 0.95 | 5.61 ± 0.59 | 0.44 ± 0.02 | 87.26 ± 2.57 |
| **4** | 40 | 29.72 ± 1.23 | 3.59 ± 0.48 | 0.32 ± 0.02 | 114.27 ± 2.16 |
| **5** | 92 | 23.99 ± 0.84 | 8.40 ± 0.61 | 0.44 ± 0.05 | 87.07 ± 2.58 |
| **6** | 40 | 3.92 ± 0.34 | 3.50 ± 0.31 | -0.02 ± 0.06 | 80.70 ± 2.71 |
| **7** | 95 | 30.03 ± 1.58 | 10.28 ± 0.75 | 0.48 ± 0.08 | 81.56 ± 2.92 |
| **r** | | 0.454 | **0.817** | 0.514 | -0.320 |
| **P Value** | | 0.307 | **0.025** | 0.238 | 0.484 |

**Table 16:** Blastocyst development rates (of cleaved COCs) and mean fluorescence intensities of Dihydroethidium (DHE) and Hoescht 33342. Results were considered significant with $P < 0.05$.

| Bull | Blastocyst Rate (%) | DHE Head (Mean + SEM) | DHE Midpiece (Mean + SEM) | DHE Tail (Mean + SEM) | Hoescht (Mean + SEM) |
|---|---|---|---|---|---|
| **1** | 53 | 26.85 ± 1.08 | 9.65 ± 0.69 | 0.34 ± 0.04 | 64.61 ± 2.73 |
| **2** | 45 | 18.31 ± 0.91 | 7.55 ± 0.71 | 0.34 ± 0.05 | 72.42 ± 2.58 |
| **3** | 31 | 15.57 ± 0.95 | 5.61 ± 0.59 | 0.44 ± 0.02 | 87.26 ± 2.57 |
| **4** | 15 | 29.72 ± 1.23 | 3.59 ± 0.48 | 0.32 ± 0.02 | 114.27 ± 2.16 |
| **5** | 42 | 23.99 ± 0.84 | 8.40 ± 0.61 | 0.44 ± 0.05 | 87.07 ± 2.58 |
| **6** | 34 | 3.92 ± 0.34 | 3.50 ± 0.31 | -0.02 ± 0.06 | 80.70 ± 2.71 |
| **7** | 58 | 30.03 ± 1.58 | 10.28 ± 0.75 | 0.48 ± 0.08 | 81.56 ± 2.92 |
| **r** | | 0.216 | **0.908** | 0.313 | -0.807 |
| **P Value** | | 0.642 | **0.005** | 0.495 | 0.028 |

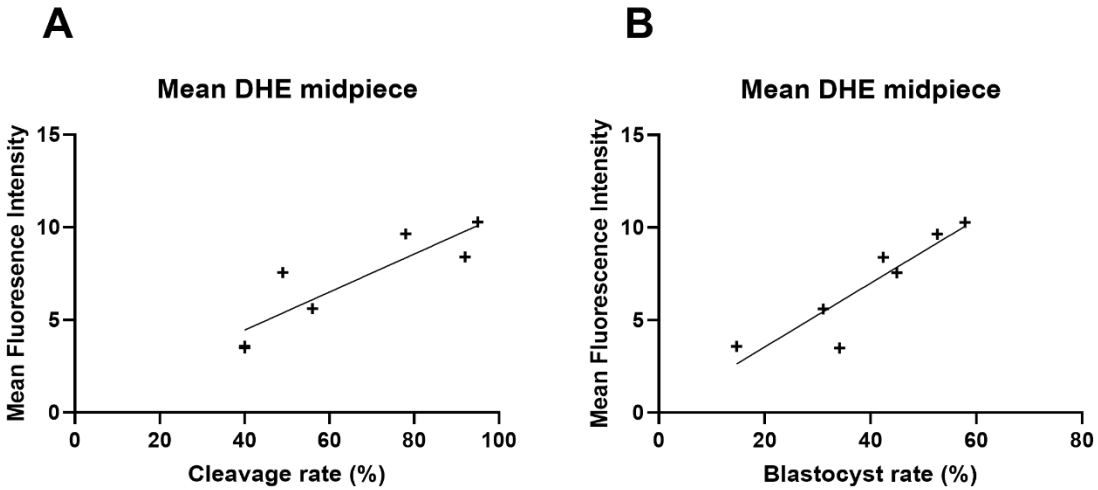


**Figure 10:** Correlation between embryo cleavage rate ($r = 0.817$, $P = 0.025$, A) and blastocyst development rate (from cleaved) ($r = 0.908$, $P = 0.005$, B) following IVP and the mean fluorescence intensity of DHE in the midpiece of the sperm used for IVF.

### 3.3.6 Correlation of Non Return to Service (NRS) rates and Zinc, total Reactive Oxygen Species and Superoxide in sperm

#### 3.3.6.1 Zinc

Table 17 displays the NRS rates and mean fluorescence intensities from Fluo-Zin-3AM, Hoescht 33342 and Mitotracker staining of sperm from six *bos taurus* bulls. No correlation was found between NRS and the fluorescence intensity of Fluo-Zin-3AM in the head or midpiece of the sperm. Therefore the zinc content of the sperm did not correlate to NRS. Likewise, Hoescht 33342 and MitoTracker staining intensity was not related to the NRS rate.

#### 3.3.6.2 Reactive Oxygen Species (ROS)

No correlations were found between Non return to service (NRS) rates and mean fluorescence intensities of CellROX in the head, midpiece, and tail (Table 18). Therefore ROS levels in the sperm did not correlate with NRS rate. Fluorescence intensity of Hoescht 33342 (control) was also not correlated with NRS rate.

**Table 17:** Non Return to Service (NRS) rates and mean fluorescence intensities of Fluo-Zin-3AM, Hoescht 33342 and MitoTracker. Results were considered to be significant with $P < 0.05$.

| Bull | NRS Rate (%) | FluoZin Head (Mean + SEM) | FluoZin Midpiece (Mean +SEM) | Hoescht (Mean + SEM) | Mitotracker (Mean +SEM) |
|---|---|---|---|---|---|
| **1** | 3.55 | 12.30 ± 1.48 | 14.42 ± 1.50 | 86.078 ± 2.57 | 27.56 ± 2.11 |
| **2** | 2.6 | 4.30 ± 0.35 | 7.76 ± 1.78 | 91.48 ± 2.86 | 20.85 ± 1.10 |
| **3** | 2.53 | 9.29 ± 0.43 | 19.99 ± 0.92 | 98.38 ± 2.78 | 31.15 ± 1.69 |
| **4** | -3.27 | 5.32 ± 0.32 | 15.71 ± 0.58 | 103.83 ± 3.55 | 29.62 ± 1.61 |
| **5** | -3.17 | 14.08 ± 2.15 | 17.67 ± 1.33 | 100.55 ± 2.73 | 33.47 ± 1.41 |
| **6** | -2.76 | 5.07 ± 1.97 | 29.63 ±1.42 | 95.47 ± 2.97 | 29.02 ± 1.84 |
| **r** | | 0.098 | -0.489 | -0.752 | -0.527 |
| ***P* Value** | | 0.853 | 0.325 | 0.085 | 0.282 |

**Table 18:** Non Return to Service (NRS) rates and mean fluorescence intensities of CellROX and Hoescht 33342. Results were considered to be significant when $P < 0.05$.

| Bull | NRS Rate (%) | CellROX Head (Mean + SEM) | CellROX Midpiece (Mean + SEM) | CellROX Tail (Mean + SEM) | Hoescht (Mean + SEM) |
|---|---|---|---|---|---|
| **1** | 3.55 | 7.32 ± 0.79 | 17.10 ± 1.75 | 2.45 ± 0.35 | 117.68 ± 2.28 |
| **2** | 2.6 | 5.02 ± 0.67 | 13.55 ± 1.58 | 2.37 ± 0.58 | 127.12 ± 1.59 |
| **3** | 2.53 | 5.18 ± 0.92 | 12.17 ± 1.93 | 2.47 ± 0.49 | 126.12 ± 2.11 |
| **4** | -3.27 | 3.07 ± 0.61 | 6.84 ± 1.47 | 1.20 ± 0.59 | 126.59 ± 1.61 |
| **5** | -3.17 | 19.80 ± 2.64 | 31.19 ± 2.88 | 3.83 ± 0.84 | 126.40 ± 1.51 |
| **6** | -2.76 | 4.07 ± 0.94 | 4.52 ± 0.93 | 2.80 ± 0.95 | 119.91 ± 1.87 |
| **r** | | -0.274 | 0.003 | -0.100 | -0.205 |
| ***P* Value** | | 0.600 | 0.996 | 0.851 | 0.696 |

3.3.6.3 Superoxide

Non Return to Service (NRS) rates were not related to mean fluorescence intensities of DHE within the head, midpiece, or tail of the sperm (Table 19). Therefore superoxide levels in the sperm were not correlated with the NRS rate. NRS rates were also not correlated to intensity of Hoescht 33342 staining.

**Table 19:** Non Return to Service (NRS) rates and mean fluorescence intensities of DHE and Hoescht 33342. Results were considered to be significant when P < 0.05.

| Bull | NRS Rate (%) | DHE Head (Mean + SEM) | DHE Midpiece (Mean + SEM) | DHE Tail (Mean + SEM) | Hoescht (Mean + SEM) |
|---|---|---|---|---|---|
| **1** | 3.55 | 27.14 ± 1.22 | 8.71 ± 0.60 | 0.51 ± 0.06 | 77.95 ± 2.93 |
| **2** | 2.6 | 11.12 ± 0.94 | 5.95 ± 0.46 | 0.11 ± 0.02 | 86.78 ± 3.14 |
| **3** | 2.53 | 12.10 ± 0.75 | 4.80 ± 0.40 | 0.35 ± 0.01 | 80.70 ± 2.21 |
| **4** | -3.27 | 21.13 ± 0.73 | 6.00 ± 0.44 | 0.20 ± 0.04 | 64.72 ± 2.12 |
| **5** | -3.17 | 22.23 ± 1.25 | 6.20 ± 0.69 | 0.24 ± 0.05 | 62.65 ± 2.35 |
| **6** | -2.76 | 28.88 ± 1.29 | 9.93 ± 0.68 | 0.39 ± 0.04 | 83.01 ± 3.13 |
| **r** | | -0.432 | -0.141 | 0.259 | 0.651 |
| **P Value** | | 0.393 | 0.790 | 0.621 | 0.162 |

### 3.3.7 CASA analysis vs midpiece staining for Zinc, Reactive Oxygen Species and Superoxide

Correlations between CASA outputs and mean fluorescence intensity of stains for zinc and reactive oxygen species within the sperm midpiece were also assessed. The midpiece was further assessed following the strong positive correlation observed between superoxide in the midpiece and cleavage and blastocyst development rates *in vitro*.

#### 3.3.7.1 Total Motility

Mean fluorescence intensity of Fluo-Zin-3AM or DHE in the sperm mid piece were not correlated with total motility scores (fig.11). However the mean fluorescence intensity of CellROX in the midpiece was negatively correlated with total motility (r=-0.6192, P=0.02).

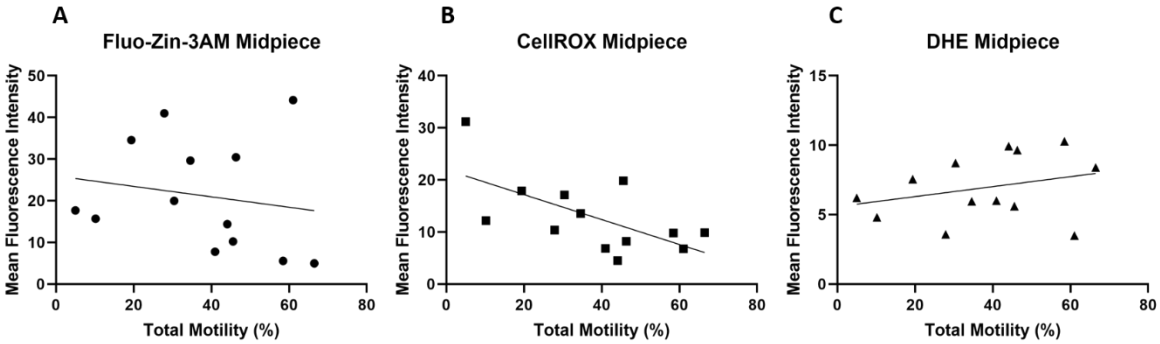


**Figure 11:** Correlations between mean fluorescence intensity of Zinc (A), CellROX (B) and DHE (C) in the midpiece with total motility. A negative correlation exists between the fluorescence intensity of CellROX in the midpiece and total motility of the sperm (r=-0.619, P=0.02). No correlation exists between the mean fluorescence intensity of FluoZin-3AM (r=-0.184, P=0.55, NS)) or DHE (r=0.288, P=0.34, NS) and total motility.

3.3.7.2 Beat Cross Frequency (BCF)

BCF was not correlated with mean fluorescence intensity of Zinc (r=-0.455, P=0.118), CellROX (r=-0.421, P=0.152) or DHE (r=0.317, P=0.29) staining of the midpiece (fig.12)

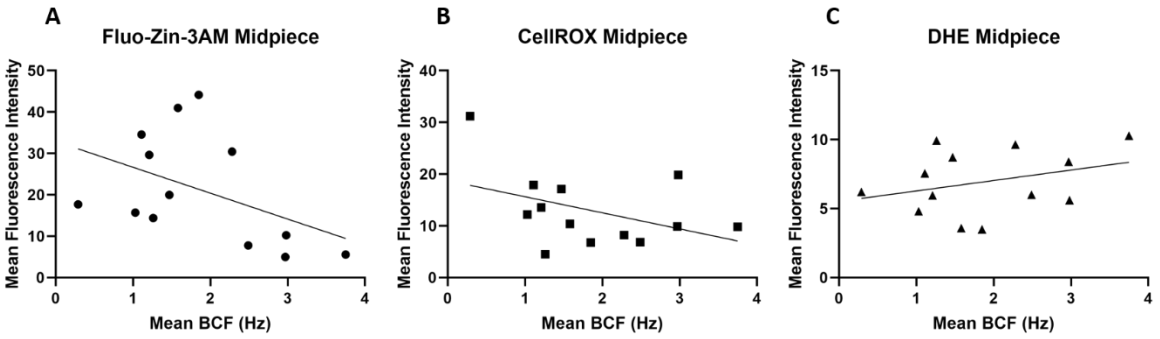


**Figure 12:** Correlations between mean fluorescence intensity of Zinc (r=-0.4548, P=0.1184, NS, A), CellROX (r=-0.4212, P=0.1518, NS, B) and DHE (r=0.3168, P=0.2917, NS, C) in the sperm midpiece and Beat Cross Frequency (BCF) of the sperm.

### 3.3.8 Comparison of Zinc, total Reactive Oxygen Species and Superoxide in sperm of *bos indicus* and *bos taurus* bulls

#### 3.3.8.1 Zinc

Fluo-Zin-3AM staining intensity of the sperm head was not different between *bos indicus* and *bos taurus* sperm (fig.13). Zinc content was lower in the midpiece of *bos indicus* sperm than *bos taurus* sperm (P=0.02) (fig. 13). Hoescht 33342 and MitoTracker fluorescence intensities did not differ between *bos indicus* and *bos taurus* sperm (fig.14).

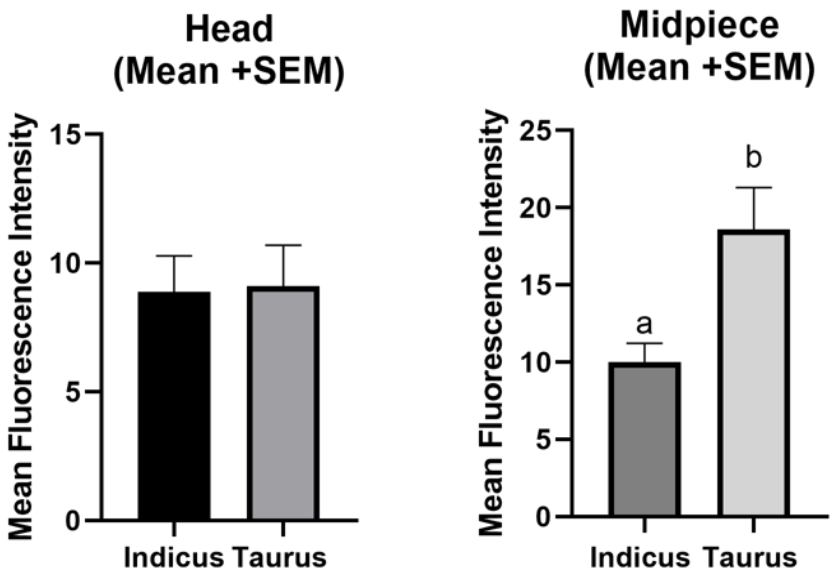


**Figure 13:** Fluo-Zin-3AM mean fluorescence intensities (+SEM), in the head and midpiece of *bos indicus* and *bos taurus* bull sperm. Different letters represent a significant difference of $P < 0.05$.

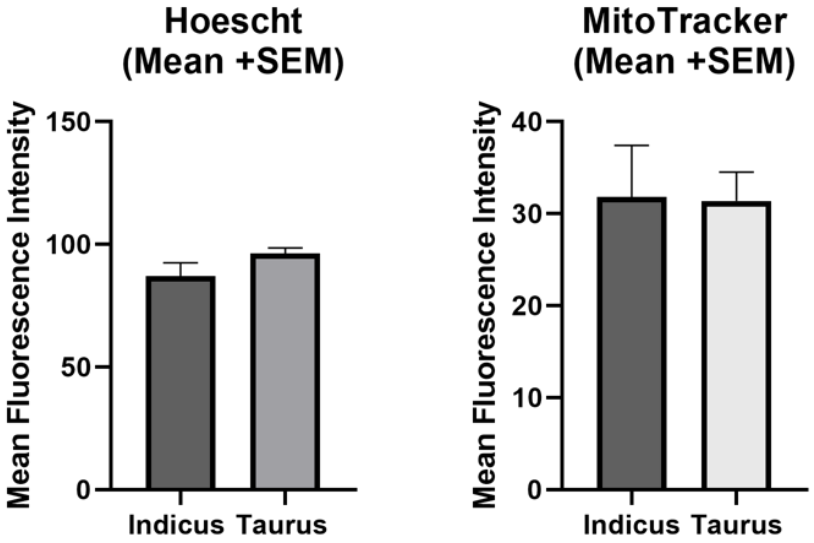


**Figure 14:** Mean fluorescence intensity (+ SEM) of Hoescht 33342 and MitoTracker in *bos indicus* and *bos taurus* bull sperm, used as a control during staining.

3.3.8.2 Reactive Oxygen Species

Reactive oxygen species levels in *bos indicus* and *bos taurus* sperm were investigated through CellROX staining (fig.15). ROS levels in the head, midpiece, and tail did not differ *bos indicus* and *bos taurus* sperm. Hoescht 33342 levels were not different between *bos indicus* and *bos taurus* sperm.

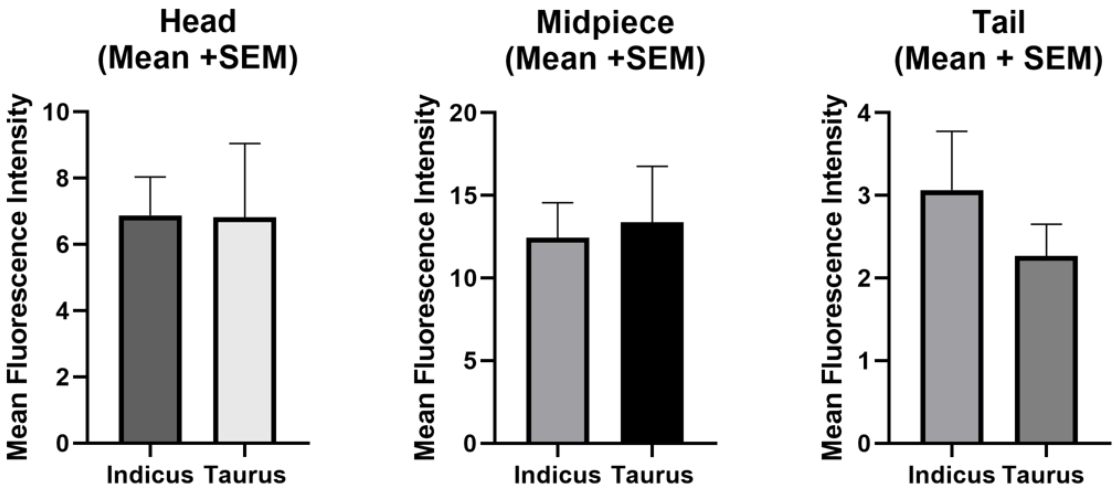


**Figure 15:** Mean fluorescence intensity (+SEM) of CellROX in the head, midpiece, tail of *bos indicus* and *bos taurus* bull sperm.

### 3.3.8.3 Superoxide

Superoxide levels were indicated by DHE fluorescence staining. Superoxide levels in the head, midpiece and tail were not different between *bos indicus* and *bos taurus* sperm (fig.16). Mean fluorescence intensity of Hoescht 33342 did not differ between *bos indicus* and *bos taurus* sperm.

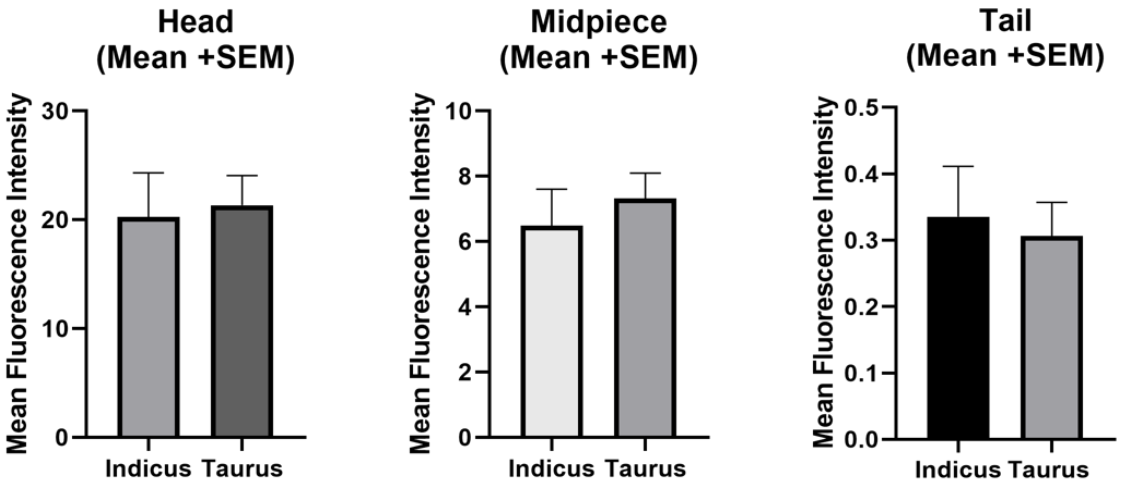


**Figure 16:** Mean fluorescence intensity (+SEM) of dihydroethidium (DHE) in the head, midpiece and tail of *bos indicus* and *bos taurus* bull sperm.

## 3.3.9 Negative Controls

A comparison of the mean fluorescence intensities between the negative controls (no fluorescent marker used) and Fluo-Zin-3AM, CellROX and DHE staining is displayed in figure 17. The negative controls were subjected to the same confocal microscopy setting as the sperm that were stained with the fluorescent markers. The purpose of the negative control was to demonstrate that the markers were effective and that we were not solely obtaining autofluorescence. Fluorescence was significantly different between the negative control and Fluo-Zin-3AM in the head and midpiece ($P<0.01$). Fluorescence was significantly different between the negative control and CellROX in the head, midpiece and tail ($P<0.01$). The negative control was also significantly different compared to DHE fluorescence in the head, midpiece and tail ($P<0.01$). This demonstrates that the fluorescent markers used were effective, and minimal autofluorescence was present.

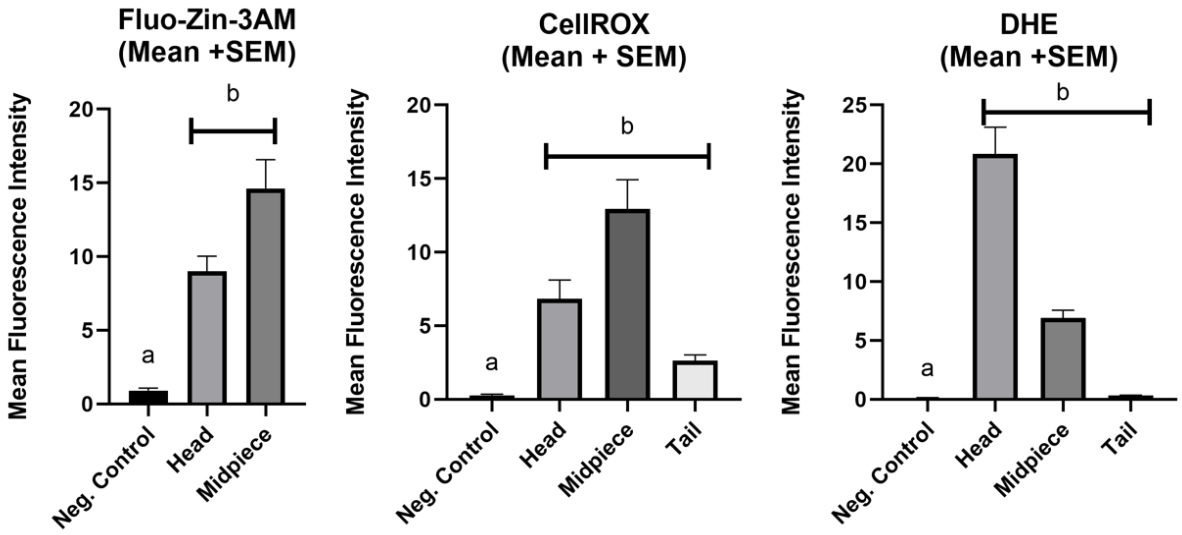


**Figure 17:** Negative controls compared to mean fluorescence intensities in the head (H), midpiece (M) and tail (T) for FluoZin 3AM, CellROX and Dihydroethidium (DHE).

### 3.3.10 Summary Tables of Results

The results of this chapter have been summarised in Table 20, 21 and 22 to clearly demonstrate where significant results were obtained. A summary of the analysed correlations between CASA outputs and mean fluorescence intensities of FluoZin 3AM, CellROX and DHE against IVP results (cleavage and blastocyst development rates) and NRS rates are displayed in Table 20. A summary of the analysed mean fluorescence intensities of FluoZin 3AM, CellROX and Superoxide in the midpiece of cryopreserved bull sperm against the total motility and BCF from CASA is displayed in Table 21. Table 22 displays a summary of the results relating to the comparison of mean fluorescence intensities of FluoZin 3AM, CellROX and DHE in the head, midpiece and tail of *bos indicus* and *bos taurus* bull sperm.

**Table 20:** A summary of investigated correlations between Computer Assisted Sperm Analysis (CASA) outputs and mean fluorescence intensities of FluoZin 3AM, CellROX and Dihydroethidium (DHE) against *in vitro* embryo production (IVP) results and Non Return to Service (NRS) rate results. Observed correlations are indicated by " ✔ ", trends are indicated by "+" and the presence of "x" indicated that no correlation was observed.

| | IVP (Cleavage rate) | IVP (Blastocyst rate) | NRS |
|---|---|---|---|
| **Concentration ($10^6$/mL)** | **x** | **x** | **x** |
| **Total Motility (%)** | **+** | **x** | **x** |
| **Progressive Motility (%)** | **+** | **x** | **x** |
| **Fast Prog. Motility (%)** | **x** | **x** | **x** |
| **Slow Prog. Motility (%)** | **+** | **x** | **x** |
| **Mean BCF (Hz)** | ✔ | **x** | **x** |
| **FluoZin 3AM Head** | **x** | **x** | **x** |
| **FluoZin 3AM Midpiece** | **x** | **x** | **x** |
| **CellROX Head** | **x** | **x** | **x** |
| **CellROX Midpiece** | **x** | **x** | **x** |
| **CellROX Tail** | **x** | **x** | **x** |
| **DHE Head** | **x** | **x** | **x** |
| **DHE Midpiece** | ✔ | ✔ | **x** |
| **DHE Tail** | **x** | **x** | **x** |

**Table 21:** A summary of investigated correlations between CASA outputs of Total Motility and Beat Cross Frequency (BCF) against mean fluorescence intensities of FluoZin 3AM, CellROX and Dihydroethidium (DHE) in the midpiece of cryopreserved bull sperm. Observed correlations are indicated by a "✔". The presence of "x" indicated that no correlation was observed.

| | FluoZin 3AM | CellROX | DHE |
|---|---|---|---|
| **Total Motility (%)** | **x** | ✔ | **x** |
| **Mean BCF (Hz)** | **x** | **x** | **x** |

**Table 22:** A summary of the comparison of mean fluorescence intensities of FluoZin 3AM, CellROX and Dihydroethidium (DHE) in the head, midpiece and tail of sperm from *bos indicus* and *bos taurus* bulls. A significant difference of fluorescence intensities between the subspecies is indicated in the table by "*". Where no significant difference of observed, it is indicated by "ns". Lack of nomenclature indicates that the investigation did not take place.

| | Head | Midpiece | Tail |
|---|---|---|---|
| **FluoZin 3AM** | **ns** | * | |
| **CellROX** | **ns** | **ns** | **ns** |
| **DHE** | **ns** | **ns** | **ns** |

## 3.4 Discussion

The present study had two aims. The first was to correlate zinc, superoxide and ROS concentrations in bull sperm with the fertilisation potential of sperm *in vitro* (IVP) and *in vivo* (NRS). The second objective was to investigate if a difference in zinc, superoxide and ROS exists between the sperm of *bos indicus* and *bos taurus* bulls that may contribute to identify markers of fertility. Results indicate that superoxide in the midpiece of bull sperm (primarily *bos indicus*) could be a marker of fertility. A positive correlation was found between the superoxide marker, DHE in the midpiece for both cleavage and blastocyst development rates *in vitro*. Zinc and total ROS in the sperm was not indicative of semen fertility and did not correlate with cleavage or blastocyst development rates *in vitro*. The current study did not identify markers of bull fertility for artificial insemination (NRS). No correlations existed between zinc, ROS or superoxide and the NRS rates. Routine methods of semen quality assessment using CASA was not effective at predicting fertility of semen *in vitro* or *in vivo*. Although trends existed for a relationship between embryo cleavage rates and sperm motility, the only parameter that was highly correlated with cleavage rates *in vitro* was beat cross frequency (BCF). None of the CASA outputs were correlated with NRS rates in the present study, however, total ROS in the midpiece was negatively correlated to total motility, suggesting high levels of ROS could be detrimental to sperm function.

*Bos indicus* and *bos taurus* cattle are adapted to thrive in different environments. It has been suggested that their environmental adaptation may extend to sperm function. Levels of ROS and superoxide did not differ between sperm from *bos taurus* or *bos indicus* semen, but zinc probe fluorescence within the midpiece was found to be almost double in sperm from *bos taurus* bulls compared with *bos indicus* bulls. Further investigation is required to elucidate why. Embryo production and NRS data was not available for both sub-species in the present study, therefore we could not compare embryo development rates *in vitro* or NRS rates between *bos indicus* and *bos taurus* bull semen.

Progressive motility has been shown to be a good indicator for fertilisation potential (Li et al. 2016; Morrell et al. 2016). Analysis of semen quality using CASA was developed to accurately assess many variables of semen (including sperm concentration, total motility, progressive motility, circular motility, and BCF). CASA was designed to provide an overall assessment of the quality of semen at time of collection and post-thaw. However, CASA analysis of sperm is not a reliable method for predicting fertility of a bull in isolation. This is because many factors influence pregnancy outcomes during an AI program. Some crucial factors outside of semen quality that influence pregnancy outcome include the skill of the AI technician, age, body-score index and nutrition of the recipient cattle, and season (Amann & Waberski 2014). Some

CASA systems are also able to assess sperm morphology; however, the system that was used in the current study did not have that capability. Furthermore, morphological assessment of spermatozoa has been shown to be a superior indicator of semen quality compared to assessment of motility (Fitzpatrick et al. 2002). Assessing membrane integrity of sperm using stains such as acridine orange and propidium iodide is effective at predicting the fertility of ram semen used in AI (Santolaria et al. 2015). We found no correlation between our CASA results and NRS scores. However, there was not a wide range of NRS scores for the semen used in this study. For the bull semen assessed *in vitro*, there was a positive correlation between the embryo cleavage rates and BCF as assessed by CASA. The BCF is defined as the frequency that the head crosses the forward projection line. It has been associated with hyperactivity and capacitation of spermatozoa (Chamberland et al. 2001). Total sperm motility and embryo cleavage rates also tended to be positively correlated. The semen from bulls with NRS scores were not tested *in vitro* during this study due to difficulty in accessing abattoir derived ovaries locally for IVP. Ideally these bulls should also be tested *in vitro* to enable further comparison of the semen of the *bos indicus* and *bos taurus* bulls. It would also increase sample size for correlation of the *in vitro* data with CASA outputs and measures of zinc, ROS and superoxide in sperm.

The presence of ROS and superoxide within sperm has been related to spermatozoon fertility in several species. A positive correlation between superoxide in the mitochondria of stallion sperm and motility has been reported (Gibb, Lambourne & Aitken 2014). No correlation between the motility of bull sperm and superoxide was found in the present study. However, there was a positive correlation between superoxide in the midpiece and cleavage (fig. 10) and blastocyst (fig. 11) development rates *in vitro*. This may be due to the preparation of bull semen for IVF. Centrifugation through a non-continuous gradient of BoviPure was used to separate live and dead sperm. Therefore the live sperm are more concentrated at the time of IVF compared to when initially thawed. In contrast, the CASA analysis was performed on post-thawed semen that had not undergone centrifugation. An equine study also reported that an increased production of ROS in sperm resulted in a reduced mitochondrial membrane potential (Johannisson et al. 2018). Mitochondria are located within the midpiece of sperm. The organelle impacts several sperm functions including: hyperactivation, capacitation, acrosome reaction and fertilisation. Furthermore, ROS is a by-product of mitochondrial metabolism (reviewed by Moraes & Meyers 2018). Therefore it is not surprising that the midpiece was where correlations were detected in the present study. A variety of ROS are naturally produced by spermatozoa, including superoxide, nitric oxide and $H_2O_2$. Small amounts of ROS are beneficial as they stimulate the tyrosine phosphorylation cascades that have been associated with sperm capacitation (reviewed by Aitken, De Iuliis & McLachlan 2009). An earlier study using hamster sperm demonstrated that ROS may play a role in hyperactivation of spermatozoa. This was demonstrated by inhibiting Nitric Oxide synthase with 0.5 or 5 mM of methyl-L-arginine or 1 or 10 µM Phenylene-bis

(1,2-ethanediyl)-bis-isothiourea (PBITU). This resulted in significant decreases in the percentage of motile sperm and also decreased the grade of progressive motility. Inducing hyperactivation of unstimulated hamster spermatozoa with 400 nM nitropusside was as effective as using epinephrine (Yeoman, Jones & Rizk 1998). This contrasted with our results of a negative correlation between ROS in the midpiece and total motility. Chatterjee and Gagnon (2001) reported that the production of superoxide radicals significantly increases at the time of thawing, however, nitric oxide levels are not affected. Furthermore, they demonstrated that the majority of ROS within thawed sperm were superoxides. In addition they reported $H_2O_2$ levels to be similar in both fresh and cooled semen. This is surprising as $H_2O_2$ production has been demonstrated to increase as sperm degrade over time (Murphy, C et al. 2013; Shannon & Curson 1972). A recent study reported an increase in DNA fragmentation and $H_2O_2$ production in thawed bovine semen (Gurler et al. 2016). Low levels of ROS are beneficial to the viability and functionality of sperm, however, high ROS levels have negative effects on sperm viability (Aitken 1995; Aitken et al. 1997; Baumber et al. 2000). Further investigation is needed to determine the upper threshold of ROS concentration that is beneficial to bovine sperm viability and functionality, and also how to manage or maintain these levels. Fluorescence intensity of ROS and superoxide did not differ in the sperm of *bos taurus* or *bos indicus* bulls in the present study. This suggests that ROS and specifically superoxide production within spermatozoa is not specific to the sub-species. The semen storage, as well as season, nutrition and age of the bull at the time of collection is likely to have more of an impact on the production of ROS in sperm than the sub-species of origin. These conditions were likely different for all the semen samples used in the present study.

The existence of a zinc signature throughout different stages of sperm capacitation has recently been reported. Basically, zinc levels decrease as sperm undergo capacitation until depleted (Kerns et al. 2018). The majority of sperm imaged for this study had a zinc signature 2 (approximately 70%, data not shown). The distribution of sperm throughout each zinc signature did not correlate with cleavage or blastocyst development rates *in vitro* (data not shown). It was expected that zinc content within the post-thawed bovine sperm would correlate with some of the CASA outputs, especially total motility and BCF. However, that was not the case, with no correlations detected. In addition, no correlation existed between zinc content of bovine sperm and cleavage and blastocyst development rates *in vitro* or NRS rates. Earlier studies using human spermatozoa have reported a negative correlation between zinc in the flagella and seminal plasma with motility (Henkel, R et al. 1999; Henkel, RR et al. 2003; Sorensen, Bergdahl, et al. 1999). They suggest zinc may play a role in inhibition of progressive motility, which has recently been supported by Kerns, Zigo and Sutovsky (2018). Semen collected from goats that had been supplemented with 40 mg of zinc per kg dry matter from 5 to 13 months of age resulted in improved cleavage rates *in vitro* compared with semen from goats without zinc supplementation (Hemalatha et al. 2018). However,

the zinc level within the spermatozoa at the time of insemination was not assessed. Furthermore, cleavage rates *in vitro* were extremely low in both the control group (approximately 18%) that did not receive supplementation, and the zinc supplemented group (approximately 30%). This suggests that the goats may have been deficient in dietary zinc, therefore zinc supplementation was beneficial to improve their fertility. Supplementation of ram semen extender with 0.01 or 0.1 mg/mL nano zinc oxide prior to semen cryopreservation increased the percentage of progressive motility, sperm survival and improved plasma membrane integrity post-thaw. However, supplementation of the extender with 1 mg/mL zinc oxide led to decreased motility post-thaw (Heidari et al. 2019). This suggests that some zinc is beneficial to sperm fertility, but high quantities could be detrimental. Further investigation into the optimal levels of zinc within the diet and/or semen extender is required to capitalise on this information. The intensity of fluorescence of zinc in the midpiece of sperm from *bos taurus* was approximately double that of sperm from *bos indicus* semen ($P < 0.05$). Ideally we would have had *in vitro* embryo production data from both *bos indicus* and *bos taurus* semen in the current study, as perhaps the cleavage and blastocyst development rates may differ between the groups studied. It is likely that the diets differed between the two groups (and within the groups) in the present study, and it is possible that the diet of the *bos indicus* bulls was deficient in zinc, and/or the diet of the *bos taurus* bulls may have significant zinc supplementation. It is also possible that the optimal amounts of zinc for sperm fertility differ between *bos indicus* and *bos taurus* bulls.

Reports comparing semen quality between *bos indicus* and *bos taurus* bulls have been conflicting. Semen from *bos indicus* bulls has been reported to contain a high density of sperm and increased prevalence of sperm defects (Brito, LFC et al. 2002). However, other studies have demonstrated that other parameters, such as climate and age, have more of an effect on semen quality than breed. In tropical conditions, semen from temperate breeds is affected by heat stress, while *bos indicus* bulls suffer cold stress in temperate regions (Godfrey et al. 1990). This is logical, because the different sub-species are adapted to thrive in different environments. In addition to long ears, a hump and dewlap, *bos indicus* bulls have differing scrotal physiology than *bos taurus* bulls. Differences include increased ratios of testicular artery length and volume against testicular volumes in *bos indicus* bulls compared to *bos taurus* bulls (Brito, LF et al. 2004). In locations that experience dramatic differences in rainfall between seasons, rain was positively related to semen quality (Chacon et al. 1999; Rekwot et al. 1987). This is most likely due to poor quality of grazing available during the dry season. In the study by Rekwot et al. (1987), the dry season was also coupled with very high ambient temperatures. Age of the bull at the time of semen collection has been demonstrated to affect semen quality. *Bos indicus* bulls reach puberty 3 months later than *bos taurus* bulls (Fields, Burns & Warnick 1979; Fields, Hentges & Cornelisse 1982). Bull age has been reported to positively correlate with the percentage of normal spermatozoa within an ejaculate, and

negatively correlate with the percentage of abnormal spermatozoa within an ejaculate (Chenoweth et al. 1996). Therefore it is likely that climate, Body Score Index, diet and age of the bull at the time of semen collection impact semen quality more than genotype.

Information on season, nutrition and age of the bull at the time of semen collection was not available for the semen used in the present study. We also do not know how long the semen straws had been stored cryopreserved. The *bos indicus* semen was from stud breeders in the Central Queensland region of Australia, and the *bos taurus* semen was from Victoria, Australia; two geographically different regions, but regions where these sub-species would typically be found. The semen had also been collected and processed by different bull semen collection centres. Therefore it is reasonable to assume collection and cryopreservation methods might have differed (artificial vagina or electroejaculation and different cryopreservation extenders) (Murphy, EM et al. 2017).

Due to time constraints and the loss of the local abattoir in South Australia (Thomas Foods International, Murray Bridge) due to fire in January 2018, it was difficult to obtain enough oocytes to test fertilisation and blastocyst development rates *in vitro* following IVF using semen from all the bulls. Furthermore, the number of semen samples tested *in vitro* was not large enough to allow the samples to be categorised as either 'good' or 'bad' for IVP, therefore it was only possible to analyse the data for correlations. Future studies utilising a larger number of semen samples would enable the effects of zinc, ROS and superoxide on IVF outcomes to be assessed further.

In conclusion, the present study found that superoxide levels within the midpiece of cryopreserved *bos indicus* sperm were positively correlated with *in vitro* cleavage and blastocyst development rates. Overall, ROS was negatively correlated with motility, most likely affected by $H_2O_2$ and nitric oxide. Cleavage rate *in vitro* was also positively correlated to BCF, as analysed by CASA. Zinc levels were found to be significantly higher in the midpiece of *bos taurus* sperm compared to *bos indicus* sperm. Although further investigation is required, the results are promising for the identification of potential fertility markers for cryopreserved bull sperm.

# Chapter 4
# Discussion

## 4.1 Introduction

The present study aimed to investigate two approaches to improve the efficiency of bovine *in vitro* embryo production (IVP). The use of IVP globally as a tool to rapidly increase genetic gain within a herd continues to increase in popularity (Perry 2016; Stroud 2012; Thibier 2001). Industry standard blastocyst production rates remain at about 30 percent of cumulus oocyte complexes (COCs) collected (Perry 2016). Conception rates remain less than 50 percent of embryos transferred. Therefore improvement to the efficiency of bovine IVP is required to exploit the benefits that IVP technology offers to stud cattle breeders (rapid herd genetic gains, semen economy, exploitation of sire and dam genetics etc.). This study focused on each of the gametes. The first experimental chapter focused on meiotic inhibition of bovine oocytes as a potential means to improve *in vitro* embryo production, and the second investigated potential markers of sperm fertility.

## 4.2 Meiotic inhibition of bovine oocytes

### 4.2.1 Discussion and Conclusions

Preventing meiotic resumption of COCs for a period of time prior to IVM has been associated with improved oocyte competency for IVP when cyclic adenosine monophosphate (cAMP) modulators are used (Zeng et al. 2014). Longer periods of meiotic inhibition following follicular aspiration have been associated with greater improvements to oocyte developmental competency *in vitro* (Albuz et al. 2010; Nogueira et al. 2003). However, few studies have managed to maintain bovine COCs arrested at the germinal vesicle (GV) stage of meiosis for more than 6 hours and improve developmental potential *in vitro* (Aktas et al. 1995; Albuz et al. 2010; Santiquet et al. 2017). The present study was also unable to extend the time bovine COCs can be maintained at the GV stage of meiosis. Combinations of cAMP modulators were assessed. PDE8 has previously been shown to account for 20% of all PDEs within bovine oocytes, with the majority (80%) being PDE3. Inhibiting PDE3 is only able to maintain bovine COCs at GV for short periods of time (Albuz et al. 2010; Mayes & Sirard 2002). Therefore it was hypothesised that a PDE8 inhibitor would extend the period of meiotic arrest. A non-specific PDE8 inhibitor (dipyridamole) was shown to increase cAMP production and prevent bovine COCs reaching the M2 stage after 18 hours. However it was concluded to be toxic as embryonic development was impaired after its use (Sasseville et al. 2009). Initial experiments in the present study investigated a new specific PDE8 inhibitor (PF-04957325). However, the specific PDE8 inhibitor (1, 5, 10, 50 and 100 μM) did not have any effect on

meiotic inhibition when used alone or with 500 µM IBMX. The synthetic analogue of cAMP (dibutyryl cAMP (dbcAMP)) has been reported to be effective at maintaining porcine COCs at GV for 22 hours (Gil et al. 2017). Therefore comparable doses of dbcAMP were investigated with bovine COCs. The use of 1 and 5 mM dbcAMP alone did not delay meiotic progression of bovine oocytes in the present study. Combining 500 µM 3-Isobutyl-1-methylxanthine (IBMX, a non-specific phosophodiesterase (PDE) inhibitor, except for PDE8) with 100 nM oestradiol (with 5 mM dbcAMP) was the most effective at delaying progression to the metaphase 2 (M2) stage of meiosis. Oestradiol has previously been shown to increase expression of the NPR2 receptor (receptor of C-type natriuretic peptide (CNP)), and combined with CNP has increased the percentage of COCs maintained at the GV stage of meiosis in previous studies (Soares et al. 2017; Xi et al. 2018; Zhang, MJ et al. 2011). Therefore addition of 200 nM CNP to 500 µM IBMX, 100 nM oestradiol and 5 mM dbcAMP was expected to further delay meiotic resumption in bovine COCs. However no further delay of meiotic progression was observed in the present study.

### 4.2.2 Future Directions

Further investigation is required to extend the time bovine COCs can be arrested at the GV stage of meiosis. Studies from the 1990's demonstrated that bovine oocytes could be arrested at the GV stage for 24 hours if they were cultured in follicular hemi-sections containing thecal cells (Richard & Sirard 1996a). It was also demonstrated that bovine COCs could be maintained at the GV stage for 12 hours if they were cultured in media that had been pre-conditioned by follicular hemi-sections containing thecal cells, without direct contact to thecal cells (Richard & Sirard 1996b). Later, a study published in the early 2000's partially identified a thecal secreted factor that may be responsible for regulating meiotic resumption of COCs. They concluded that the factor could not be a peptide or fatty acid, by treating media that had been pre-conditioned with thecal cells with proteases and chloroform. They also identified that the responsible factor is a small molecule, at less than 5 kDa in size, and stable following 5 minute 100˚C heat treatment (Van Tol & Bevers 2001). A purine may fit the criteria. Purines found naturally within follicular fluid, hypoxanthine and adenosine, have been shown to successfully arrest meiosis in several species including the oocytes from mice, rats, pigs and monkeys (Eppig, Wardbailey & Coleman 1985; Miyano et al. 1995; Tornell et al. 1990; Warikoo & Bavister 1989). However, hypoxanthine has only been able to hold bovine and goat COCs at the GV stage for approximately 6 hours (Ma et al. 2003; Sirard & First 1988a), and adenosine has been shown to be ineffective at preventing meiotic resumption in bovine COCs (Sirard 1990). Furthermore Van Tol and Bevers (2001) suggested that hypoxanthine was not the thecal cell secreted factor inhibiting meiotic resumption of bovine oocytes, as charcoal treatment of the <5kDa fraction of thecal cell conditioned media did not have any effect on the meiotic inhibitory response suggesting the molecule is not hydrophobic (Dang & Lowik 2005; Van Tol & Bevers 2001). Charcoal treatment has

previously been demonstrated to inhibit hypoxanthine (unpublished, but referred in Van Tol & Bevers 2001). Therefore the thecal cell secreted factor modulating meiotic resumption/inhibition in bovine oocytes remains elusive, and requires further investigation. Investigation of co-culture of thecal cells in addition to IBMX and oestradiol may aid in preventing meiotic resumption for extended periods of time and improve oocyte competency. It would also be interesting to find out if combining an inhibitor of maturation promoting factor (MPF) (such as DMAP) with cAMP modulators would help to increase the time bovine oocytes can be arrested at the GV stage and also improve developmental potential. We are unsure if using a combination of inhibitors instead of a single inhibitor, such as IBMX, to maintain the oocytes at GV for 6 hours would be beneficial in terms of development potential. We also are unsure if using the inhibitors for a whole 24 hour maturation period would have any effect on development rates. The recent study by Santiquet et al. (2017) managed to inhibit meiotic resumption of bovine COCs for 24 hours using a combination of meiotic inhibitors (PDE3 and 5 inhibitors, CNP, bone morphometric protein 15, hypoxanthine, β-oestradiol and FSH). Their treatments did not affect developmental competence of bovine COCs *in vitro* or alter cell numbers of the blastocysts. However, improvements to developmental competence and blastocyst quality have been reported in many studies when a period of meiotic inhibition was utilised (Albuz et al. 2010; Nogueira et al. 2003). Therefore, it is still possible that the meiotic inhibitor treatments trialled in the present study might improve embryo quality (for example higher cell numbers) and development rates in future studies.

## 4.3 Fertility markers of bovine sperm

### 4.3.1 Discussion and Conclusions

Bull genetics are distributed widely as cryopreserved semen and used either in artificial insemination (AI) or IVP. Cryopreservation is utilised to extend the shelf life of semen by reducing the metabolic rate of sperm (Fu et al. 2019). The process of cryopreservation and thawing of semen reduces the percentage of viable and motile sperm and increases levels of reactive oxygen species (ROS). Furthermore, conception rates are reduced following AI with frozen bull semen compared to fresh bull semen (Murphy, EM et al. 2018). Recently an identified Zinc signature relating to sperm capacitation has been reported (Kerns et al. 2018). Zinc levels in sperm have also been shown to relate to sperm motility (Henkel, RR et al. 2003). The current study investigated potential fertility markers (ROS, superoxide and Zinc) of cryopreserved bull sperm and their relationship to IVP rates, non-return to service (NRS) rates and computer assisted sperm analysis (CASA) outputs for *bos indicus* and *bos taurus* semen. Mitochondria,

located in the midpiece of sperm, produce ROS as a by-product (Moraes & Meyers 2018). Superoxide has been demonstrated to account for the majority of ROS in thawed sperm (Chatterjee & Gagnon 2001). Therefore it was not surprising that the significant results in the present study related to the midpiece of the sperm. Superoxide in the midpiece of cryopreserved bull sperm was found to be highly correlated to cleavage and blastocyst development rates *in vitro.* In contrast, total ROS and zinc levels were not correlated with cleavage or blastocyst development rates *in vitro*. In the present study, zinc, ROS and superoxide did not relate to NRS rates. The narrow range of NRS rates (3.55 to -3.27) associated with the sperm used in this study may have impacted this outcome. The routine method of using CASA to assess sperm fertility was not highly effective in this study. The output of beat cross frequency (BCF) was the only parameter that highly correlated with cleavage rates *in vitro*. Some motility parameters of the sperm tended to be related to cleavage rates *in vitro*. However, the majority of parameters were not indicative of sperm fertility for IVP. Furthermore none of the CASA outputs correlated with NRS rates. This was also likely impacted by the narrow range of NRS rates used in the study. Levels of ROS in the midpiece correlated negatively with motility in the current study. Previous reports have demonstrated that ROS plays a role in sperm capacitation and hyperactivity, and that some ROS is beneficial to sperm function (Aitken, De Iuliis & McLachlan 2009; Yeoman, Jones & Rizk 1998). Further investigation is required to identify the threshold where ROS levels change from beneficial to detrimental to sperm function and viability. The present study did not compare IVP and NRS rates between *bos indicus* and *bos taurus* bulls due to a lack of time and resources. However, zinc, ROS and superoxide levels were compared between the sub-species. We were investigating if their environmental adaptations may have extended to sperm function. Zinc levels were significantly higher in the midpiece of *bos taurus* sperm compared to *bos indicus* sperm. It is unclear why zinc levels differed between the groups, or what impact that has on sperm function. Diet may have played a role and further investigation is required. No difference existed between ROS and superoxide levels in the sperm between the sub-species. The results of the present study indicate that a more accurate predictor of sperm fertility than currently available with CASA is required.

### 4.3.2 Future Directions

*In vitro* cleavage and blastocyst development rates have been demonstrated to highly correlate to artificial insemination (AI) results when the same batch of cryopreserved semen is used in each procedure (O'Meara et al. 2005; Zhang, BR et al. 1997). However a recent study, comparing sire conception rates (SCR) (same definition as NRS) did not find a correlation between cleavage rates *in vitro* when semen

was used for IVP and conception rates following AI (Ortega et al. 2018). Their SCR rates (4.1 to -9.4) were broader than the NRS rates (3.55 to -3.27) used in the present study. Furthermore, although they reported a difference in conception rates between sperm from high and low fertility bulls used in AI, only one of the low fertility SCR bulls resulted in unacceptably low conception rates in the report: skewing the results (Ortega et al. 2018). It is possible that SCR or NRS rates are not always a highly accurate assessment for bull fertility; it is probably dependant on the scale of data available to calculate these scores. Investigation of correlations between superoxide levels in the midpiece of sperm and conception rates following AI should be conducted. The present study was based on small sample sizes of bulls in each category, and the range of NRS rates was very narrow. Therefore the highly positive correlation between superoxide within the midpiece of cryopreserved sperm and cleavage and blastocyst development rates *in vitro* requires further validation. Additional experiments could also investigate if semen of poor fertility *in vitro* can be "rescued" by treatment with superoxide prior to or during IVF.

The midpiece is where sperm mitochondria reside. Mitochondrial metabolism produces ROS as a by-product in several forms, including superoxide (reviewed by Moraes & Meyers 2018). Therefore mitochondrial activity may be indicative of sperm fertility. The negative control for Fluo-Zin-3AM had some autofluorescence that was particularly present in the midpiece. Detection of autofluorescence of flavin adenine dinucleotide (FAD) uses similar laser settings to what was used for Fluo-Zin-3AM. Along with nicotinamide adenine dinucleotide (NADH), FAD is an electron transporter in the mitochondrial electron transport chain (Bartolome & Abramov 2015). Therefore some autofluorescence during Fluo-Zin-3AM imaging may have been due to mitochondrial activity. Future studies should analyse FAD autofluorescence of the sperm to investigate if mitochondrial activity differs between the sub-species and fertility potential. This would also enable stain-free analysis of sperm. If FAD levels were found to be a reliable predictor of sperm fertility, a simple sperm analyser could be developed for the field without a need for preparing and maintaining fluorescent stains.

Bull fertility has been shown to have an impact on both cleavage rates and embryo quality (higher cell numbers) (Ortega et al. 2018). Therefore, identification of semen fertility markers can have significant economic outcomes for breeders, and accurate diagnosis of semen fertility is preferred. Currently used methods of assessing semen fertility lack accuracy. This was demonstrated by very few correlations with CASA outcomes and IVP or NRS rates in the present study. Superoxide in the midpiece was highly correlated with cleavage and blastocyst development rates *in vitro*, and therefore should be further investigated on a broader scale. Future studies on superoxide and sperm could lead to the development of a more accurate diagnostic test for sperm fertility that could extend across species. Improving the

accuracy of bull semen fertility assessment will result in improvements to the efficiency of ART including IVP for livestock breeders.

# Chapter 5
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