

7-5-2018

## Characterizing Cytoplasmic Maturation and Improving Competence of Bovine In Vitro Matured Oocytes

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**CHARACTERIZING CYTOPLASMIC MATURATION AND  
IMPROVING COMPETENCE OF BOVINE *IN VITRO*  
MATURED OOCYTES**

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The School of Animal Sciences

by

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August 2018

## **ACKNOWLEDGMENTS**

I greatly appreciate having been given the opportunity to pursue my doctoral degree with the School of Animal and dairy sciences at Louisiana State University. I thank my advisor, Dr. Kenneth Bondioli, whose faith in my abilities, willingness to take me into his program and whose continued support and guidance have been instrumental in my success. Members of my advisory committee, Dr. Cathleen Williams and Dr. Carlos Pinto, your varied expertise, advice, and friendship have been sincerely appreciated.

Thank you to the reproductive biology team; Emilio Gutierrez, Paige Hardin, Fabian Diaz and many more these past years, you have been not only my lab partners without whose assistance my research would not be possible, but also my friends throughout. I am also grateful to Steven Blair and the RBC team as well as the many helpful staff within the AFSL for all of your help on my many requests. Also to the undergraduate students, Giuliana Bays, Samantha Lanjewar and others, thank you for volunteering your time and so often being willing to work to assist with the animals and oocyte recovery. Dr Sokolova and Dr Jiang, thank you for your expertise and for your patience in teaching me your skills.

To my friends, family and fellow graduate students, you have been a wonderful support system, providing encouragement and, more importantly, you have been there to listen and entertain me, keeping me sane and happy through the more stressful days. A special thanks to my parents, who never expected to have to support me throughout so many years of school, but who have done so both financially and psychologically without fail or complaint. Your unending love and support have been instrumental in making this success possible.

# TABLE OF CONTENTS

Acknowledgments .....	ii
Abstract.....	v
I. Introduction .....	1
II. Review of the Literature .....	4
2.1. Overview.....	4
2.2. In Vivo Oocyte Development and Folliculogenesis .....	5
2.2. Cytoplasmic Maturation .....	8
2.3. Oocyte Retrieval Methods.....	16
2.4. In Vitro Oocyte Maturation.....	18
2.5. Advancing Technologies in Oocyte Maturation.....	21
III. The Effect of Delaying Maturation on Bovine Oocyte Cytoplasmic Maturation.....	25
3.1. Introduction.....	25
3.2. Methods .....	27
3.3. Results.....	33
3.4. Discussion.....	37
IV. The Effect of Follicular Wave Phase at Time of OPU on Bovine Oocyte Cytoplasmic Maturation and Developmental Competence .....	41
4.1. Introduction.....	41
4.2. Methods .....	43
4.3. Results.....	48
4.4. Discussion.....	51
V. The Effects of Recovery Methods on Gene Expression Profiles in Bovine Oocytes .....	57
5.1. Introduction.....	57
5.2. Methods .....	59
5.3. Results.....	65
5.4. Discussion.....	72
VI. The Effect of Bovine Oocyte Recovery Method On Oocyte Ultrastructure .....	79
6.1. Introduction.....	79
6.2. Methods .....	81
6.3. Results.....	84
6.4. Discussion.....	91
VII. Summary .....	97

References.....	101
APPENDIX. Supplemental Materials .....	120
Vita.....	139

## ABSTRACT

As the necessity to improve *in vitro* embryo production efficiency is ever increasing, so too is the prerequisite obligation to improve oocyte competence. The ability of an oocyte to successfully produce a viable embryo requires that it both completes meiotic maturation and synchronously achieves cytoplasmic competence; an encompassing term that refers to the oocytes correct accumulation and storage of mRNAs, proteins and factors as well as the development and reorganization of organelles and the cytoskeleton. The current research was designed to analyze those methods that go into oocyte collection and pre-maturation handling to determine if there are ways to ensure the cohort of oocytes being placed in *in vitro* maturation (IVM) are those with the highest potential to develop. It was found that collection during different phases of the follicular wave had negligible effects on oocyte competence. However oocytes collected from growing follicles had increased ability to respond to calcium release signals compared to those recovered from atretic follicles, which was found to be important for embryo production rates. Oocytes retrieved from post-mortem ovaries exhibited irregular gene expression in immature oocytes, but expression patterns were more similar to ovum pick-up (OPU) oocytes following IVM. However, oocytes that were recovered following super-stimulation, while initially similar in gene expression to other recovery methods, displayed a dramatic increase in the level of gene transcripts present in mature oocytes. Looking only at OPU oocytes, incubation in meiotic delay was not only irreversible in some oocytes but was also only mildly successful at improving cytoplasmic maturation dynamics. Further, mitochondrial abnormalities in both distribution (projects 1, 2 and 4) and shape (project 4) were frequent. This suggests that recovering oocytes prematurely arrests mitochondrial development, which may be a

major factor in low embryo production rates. Current research found that oocyte pre-incubation is unnecessary, and that oocytes recovered from post-mortem ovaries are likely of the greatest developmental competence. When recovering oocytes by OPU, timing is unimportant but super-stimulation causes irregular gene expression, the long-term effect of which is yet to be established.

## CHAPTER I INTRODUCTION

*In vitro* oocyte maturation is the process of removing an oocyte from an antral follicle, inducing precocious meiotic maturation and supporting this development through oocyte culture. Because oocytes are removed from the support and developmental signals provided by the follicular environment, maturation not only proceeds earlier than natural, but often involves irregularities that result in *in vitro* matured oocytes having compromised developmental competence (Hyttel et al., 1997; Thomas et al., 2004b). Because the organelles, proteins and factors present in the ooplasm are required for embryo development for the first four days, the limiting factor in *in vitro* embryo production is the intrinsic competence of the oocyte itself (Lonergan et al., 2000). Many of these factors are only gained through interactions with the follicular milieu in the final stages of oocyte development, or later during final maturation.

As oocytes undergo meiotic maturation, they simultaneously undergo molecular changes, and organelle and cytoskeletal reorganization collectively termed cytoplasmic maturation. The successful completion of cytoplasmic maturation, although thus far difficult to measure, has been identified as one of the leading factors in determining if an oocyte can successfully produce offspring. As a result, the best practice towards improving *in vitro* embryo production efficiency is to improve our ability to both recover and mature highly competent oocytes.

However, there is inconclusive evidence on what best practices for producing the best oocytes might be. Although it has been recognized that oocytes from larger follicles have completed a higher degree of development and so are of greater competence, there is conflicting evidence on the potential effects of exogenous hormones and follicular atresia on oocyte

competence. An improved understanding of which oocyte retrieval processes lead to recovery of a cohort of oocytes with the greatest cytoplasmic competence, and thus the highest probability of producing offspring, could be significant for improving the efficiency of reproductive technologies in bovines. Both researchers and the cattle industry make use of oocyte collection and *in vitro* embryo production, and improving the intrinsic competence of the oocytes used would only stand to increase the efficacy and ultimate usefulness of these procedures.

In an effort to address the requirement for oocytes of optimal competence, these studies were designed to determine the effect of initial oocyte handling and recovery methods on oocyte developmental competence and on markers of cytoplasmic maturation. In the first study, oocytes were exposed to the cyclic adenosine monophosphate (cAMP) modulators IBMX and forskolin for 2 hours prior to *in vitro* maturation. It was found that while the pre-maturation protocol was successful at maintaining oocytes in meiotic arrest and at maintaining gap junction communication, this did not have an effect on improving either mitochondrial distribution characteristics of F-actin polymerization in matured oocytes. Results indicated that the meiotic arrest caused by cAMP modulation was not beneficial in improving cytoplasmic maturation, and that the meiotic arrest was partially irreversibly decreasing maturation rates rather than improving maturation characteristics.

The second study was designed to determine the effect of the phase of the follicular wave at the time of oocyte retrieval on oocyte developmental competence and embryo production parameters. It was found that oocyte collection during follicular wave growth, peak, or atretic phases did not affect oocyte recovery rates. However, as determined by measuring inositol 1,4,5-trisphosphate receptor 1 expression and mitochondrial location oocytes collected during follicle

atresia were of decreased competence than oocytes recovered during follicle growth or peak phases.

Finally, in studies three and four differences in oocyte competence, as determined by embryo production rates, global gene expression patterns and oocyte ultrastructure, were assessed as functions of the method of oocyte collection. This study was designed to determine if oocyte recovery method plays a role in the developmental competence of oocytes used for *in vitro* embryo production. It was determined that *in vitro* matured oocytes collected following super-stimulation with exogenous follicle stimulating hormone had increased transcript abundance, especially in genes regulating energy production and mitochondrial structure compared to oocytes collected following unstimulated ovum pick-up (OPU) or those recovered post-mortem. Oocytes recovered following stimulation had some mitochondrial irregularities, which were also seen in unstimulated OPU oocytes, but had high embryo production rates compared to OPU oocytes indicating that the high transcript abundance was beneficial rather than detrimental to embryo development.

## CHAPTER II

### REVIEW OF THE LITERATURE

#### 2.1 Overview

Oocytes are produced from primordial germ cells during fetal development in cattle. After initial propagation, the entire cohort of oocytes is arrested within the ovary of the fetus throughout the remainder of gestation and pre-pubescent life. Following puberty, oocytes are activated in small cohorts each estrous cycle, whereupon they complete development. Within each estrous cycle only one follicle, containing a single oocyte, will achieve dominance, supporting the oocyte in completing maturation for eventual ovulation and fertilization. The remaining oocytes from the activated cohort will undergo atresia.

The journey of the oocyte from stasis through to ovulation is a complex event that requires communication between the oocyte, the surrounding somatic cells and even factors found within the follicular fluid to properly coordinate the necessary steps of both meiotic and cytoplasmic maturation. For an oocyte to be fertile and capable of producing an embryo, it is essential that the proper signals be received for each of these steps to occur and for the oocyte to finally garner competence by the completion of maturation, at the time of ovulation.

Oocytes used in reproductive technologies, are harvested using a variety of manners, most of which involve removing the oocyte from the follicular environment prior to the attainment of full competence. This is followed by *in vitro* culture under conditions that attempt to simulate physiological conditions but which have been found to fall short of fully supporting the cytoplasmic changes necessary in attaining full developmental potential. Because so much of embryo developmental rates are based on the oocyte's competence, research has been focused on

methods of recovering those oocytes most likely to have attained a degree of competence that would allow them to complete maturation and prevail in current *in vitro* maturation conditions.

## **2.2 In Vivo Oocyte Development and Folliculogenesis**

Oocyte development, or oogenesis, begins in the fetus. In cattle this first occurs at approximately day 82 of gestation (Bilodeau-Goeseels, 2012), but the journey of oocyte development and maturation is not completed until after fertilization occurs in the adult animal. Oocytes are the gametes found in all female animals originating from primordial germ cells derived from the epithelium of the yolk sac (Byskov, 1986). Once established in the primitive ovary of the fetus, the oogonia undergo many rounds of mitotic division, multiplying to produce the animal's entire lifetime supply of gametes. Following multiplication, they differentiate and enter meiosis, after which they are termed primary oocytes. Primary oocytes undergo meiotic arrest at the dictyate stage of prophase one, which is maintained throughout the remainder of fetal development, and the animal's life until just prior to ovulation, when meiotic resumption occurs. Oocytes arrested in the dictyate stage are referred to as being in the germinal vesicle stage, as the nucleus is still compact and contained within a nuclear membrane.

Even before final maturation begins, oocytes gradually and sequentially acquire the capacity to mature, become fertilized and to support early embryonic development, throughout the oocyte growth phase (Eppig et al., 1994; Mermillod et al., 1999; Brevini Gandolfi and Gandolfi, 2001; Krisher, 2004; Hussein et al., 2006; Sirard et al., 2006a). Oocyte growth is largely controlled by interactions between the oocyte, granulosa cells, and factors found within the follicular fluid (Reader et al., 2017). Oocyte growth begins in primordial follicles and ends prior to ovulation, with the initiation of maturation. During this time they undergo many changes

necessary for supporting oocyte activation and early embryonic development including; organelle replication and accumulation of proteins and RNAs (Fair et al., 1997; Swain and Pool, 2008). For this reason, oocytes recovered from larger follicles are more fully developed and more competent to produce embryos (Blondin and Sirard, 1995; Hagemann, 1999). Alternately, oocytes from prepubertal animals have not received adequate support from follicular fluid, or granulosa cells to stimulate growth, and as a result even those oocytes harvested for *in vitro* maturation have poor developmental competence when recovered from prepubertal animals (Armstrong, 2001; Reader et al., 2017).

The granulosa cells directly surrounding the oocyte take on special functions, involving many close interactions with the oocytes, and are referred to as cumulus cells (CCs). Cumulus cells have significant influence on oocyte development through the supply of nutrients and small molecules involved in regulating oocyte meiotic maturation and metabolism (Chian and Sirard, 1995; Dunning et al., 2015; Brown et al., 2017). Cumulus cells are also responsible for delivering cytoplasmic signaling molecules that are necessary for oocyte development, for inducing sperm penetration and capacitation (Brown et al., 2017) (Dode and Graves, 2002) and for regulating the diffusion of metabolites and nutrients from the surrounding milieu into the oocyte (Dunning et al., 2012). Communication is bi-directional between CCs and the oocyte through gap junctions, a type of trans-membrane channel. This communication also plays an active role in controlling CC function, growth, differentiation and metabolism through release of oocyte secreted factors (Gilula et al., 1978; Gilchrist et al., 2004; Krisher, 2004; Thomas et al., 2004b; Hussein et al., 2006; Gilchrist and Thompson, 2007). Either removal of the cumulus cells or the disruption of their communication with the oocyte prior to completion of maturation has a

significant effect on decreasing the developmental competence of the oocytes (Zhang et al., 1995; Fatehi et al., 2002; Gilchrist and Thompson, 2007; Brown et al., 2017).

Because of the importance of cumulus cells in regulating oocyte development, one of the best indicators of oocyte competence is the presence of cumulus cells completely surrounding the oocyte. Oocytes recovered with few to no associated cumulus cells have a significantly decreased chance at normal maturation and fertilization (Shioya et al., 1988). Oocytes with few cumulus cells are most frequently recovered from large follicles, which is believed to be indicative of follicular atresia (Fukui and Sakuma, 1980). However, even in cumulus-oocyte complexes (COCs) with a normal cumulus cell investment, the connection between the oocyte and cumulus cells changes as maturation proceeds, in fact is necessary for maturation completion (Larsen et al., 1986). As meiotic maturation advances, the gap junctions permitting communication between the oocyte and cumulus cells are broken down, severing the connection completely by 13 to 24 hours in IVM, before the oocyte reaches metaphase II (Hyttel, 1987). This gap junction breakdown is critical for successful maturation, as cumulus cells produce and secrete meiosis arresting factors and when the oocyte fails to receive these signals meiotic maturation resumes. Throughout oocyte growth, factors found within follicular fluid and cumulus cells maintain the oocyte in a state of meiotic arrest. Immediately prior to ovulation, the luteinizing hormone surge causes an increase in secretion of epidermal growth factor-like peptides from mural granulosa cells (Leibfried-Rutledge et al., 1987; Bilodeau-Goeseels, 2012). These factors stimulate the oocyte to resume meiosis and undergo meiotic maturation in preparation for fertilization (Gilchrist and Thompson, 2007; Bilodeau-Goeseels, 2011).

While oocytes are maintained in meiotic arrest, in prophase I, throughout the entirety of growth and development, oocyte maturation is primarily characterized by their release from meiotic arrest. At this point, oocytes resume meiosis, progressing to metaphase II where a second arrest occurs until fertilization and activation takes place to stimulate the completion of the second stage of meiosis. The primary indicator of oocytes having successfully completed maturation is the progression from the germinal vesicle stage to the second post-ovulatory arrest at metaphase II, following extrusion of the first polar body. Based on the ease of polar body identification and the absolute necessity of its extrusion prior to fertilization, this is the most frequently used method of identifying mature oocytes. Successful nuclear maturation requires formation of the meiotic spindle and centrosomal proteins to guide chromosomal segregation (Reader et al., 2017).

Following ovulation and completion of maturation, meiosis is arrested a second time in metaphase II until the oocyte is stimulated to undergo meiotic division. This second activation is achieved through penetration of the oolemma with a spermatozoan and initiates activation of the oocyte to prevent polyspermy, and initiate meiotic completion followed by embryo development (Bilodeau-Goeseels, 2012).

## **2.2 Cytoplasmic Maturation**

Oocyte maturation occurs *in vivo* in response to a surge of gonadotropins, and involves cytoplasmic changes in addition to the nuclear changes involved in meiotic completion (Hyttel et al., 1989a, b). Cytoplasmic maturation refers to the many non-chromosomal changes that occur within the oocyte, prior to ovulation that are necessary for the oocyte to acquire competence to develop to a viable embryo, primarily necessary molecular changes, and the reorganization of the

cytoskeleton and organelles (Ferreira et al., 2009). While cytoplasmic maturation naturally occurs concurrent to nuclear maturation, these processes are independent and often become disjointed in *in vitro* maturation protocols (Krisher, 2004). Markers of cytoplasmic maturation include; storage of mRNA, proteins, lipids and transcription factor as well as the reorganization of organelles (Krisher, 2004; Swain and Pool, 2008; Ferreira et al., 2009; Mao et al., 2014; Reader et al., 2017). As follicles grow and oocytes develop, the oocytes are highly active in synthesizing proteins and organelles but as the oocyte approaches ovulation, the oocyte reaches a state of quiescence (Thomas et al., 2004b). In order for an oocyte to be fully competent to support embryonic development, all of the necessary factors need to be in place prior to the completion of maturation and the achievement of oocyte quiescence (Thomas et al., 2004b). However, although many of the changes involved in cytoplasmic maturation have been identified, there are suspected others as yet undefined, and those that are recognized have proven difficult to define (Swain and Pool, 2008).

### **2.2.1 Calcium**

Successful fertilization requires that oocytes have not only sequestered large amounts of calcium, but also that there be pathways in place to control calcium release (Dumollard et al., 2007). Calcium is stored within the endoplasmic reticulum (ER) of oocytes, so proper release of calcium is highly reliant on changes within the ER. Oocyte maturation requires that ER not only relocate to the subolemma region, but it also requires reorganization of the ER to facilitate calcium signaling (Coticchio et al., 2015). Smooth ER must be in close proximity to mitochondria, as calcium levels within the oocyte are regulated by cross-talk between mitochondria and ooplasmic membranes (Nottola et al., 2016).

### **2.2.2 Cortical Granules**

Cortical granules are produced from the Golgi complex during oocyte growth, and develop as secretory organelles whose primary role is in preventing polyspermic fertilization (Cran et al., 1980; Abbott and Ducibella, 2001). In immature oocytes, the cortical granules are found randomly dispersed primarily within the most central regions of the cytoplasm (Hosoe and Shioya, 1997; Coticchio et al., 2016). As oocytes mature, these relocate first to clusters near the oocyte periphery (Kafi et al., 2005) and later to become concentrated directly below the oolemma, in one or two continuous rows (Cran et al., 1980; Szollosi et al., 1988; Hosoe and Shioya, 1997; Petr et al., 2001; Coticchio et al., 2015, 2016; Nottola et al., 2016). This migration is mitigated by micro-filaments within the cytoskeleton (Connors et al., 1998; Liu et al., 2010). As maturation proceeds, cortical granules also increase in quantity, size and density (Kafi et al., 2005). When oocytes are fertilized, the consequent increase in calcium stimulates exocytosis of the cortical granules, which then release their contents, stimulating oolemma hardening and preventing polyspermic fertilization (Cran et al., 1988; Kline and Kline, 1992; Coticchio et al., 2015). Abundance of cortical granules in mature oocytes is thus important in ensuring successful monospermic fertilization and oocyte activation.

### **2.2.3 Mitochondria**

Mitochondria, are the organelle that perhaps undergo the most notable change as maturation proceeds. Oocyte developmental competence is highly reliant not only on the number of mitochondria present, but also on the mitochondrial membrane potential and their distribution throughout the ooplasm (El Shourbagy et al., 2006; Tarazona et al., 2006; Van Blerkom, 2009; Reader et al., 2015). Those oocytes with increased numbers of mitochondria, and mitochondria

with increased membrane potential are considered to be more competent. Uniform mitochondrial distribution within the ooplasm further increases embryo production potential (El Shourbagy et al., 2006; Tarazona et al., 2006; Van Blerkom, 2009). While mitochondria should be found evenly distributed throughout the ooplasm, they do tend to be concentrated somewhat more heavily in areas where high levels of energy (ATP) or calcium signaling are necessary (Campello et al., 2006; Reader et al., 2017). As part of energy production requirements, and facilitation of transporting fatty acids into mitochondria for beta-oxidation, mitochondria are often found co-located with lipid droplets in highly competent oocytes (Warzych et al., 2017).

In the immature oocyte, mitochondria are spherical in shape with few cristae, representing an inactive form (Cran et al., 1980; Fair et al., 1997; Reader et al., 2017). Many mitochondria exhibit unique shapes, wherein they may appear to be hooded or vacuolated (Cran et al., 1980; Fair et al., 1997; Hyttel, 2011; Reader et al., 2015, 2017). Mitochondria are primarily found in peripheral regions in immature oocytes and migrate to become evenly distributed within the ooplasm, and commonly associated with smooth endoplasmic reticulum in fully matured oocytes (Cran et al., 1980; Cran, 1985; Hyttel et al., 1986; Reader et al., 2015).

Hooded mitochondria are generally believed to be in a state of inactivity. These are found commonly in oocytes of sheep and cattle during later stages of oocyte growth and in increasing frequency as maturation proceeds (Cran et al., 1980; Fair et al., 1997; Reader et al., 2015). However, other studies have shown that overall mitochondrial activity and ATP production increase as oocyte maturation proceeds (Stojkovic et al., 2001; Tarazona et al., 2006; Abdoon et al., 2011; Matchatkova et al., 2012; Gutnisky et al., 2013). Mitochondrial ATP production decreases following oocyte growth completion in germinal vesicle stage oocytes, and increases

again by the time oocytes reach metaphase two (Nagano et al., 2006; Abdoon et al., 2011). Those mitochondria aggregated with smooth endoplasmic reticulum (SER) are more active, than those that are isolated and it is understood that mitochondria-SER aggregates are not only key to regulating ATP production but also free calcium levels (Nottola et al., 2016).

While the change in mitochondrial location and activity with oocyte maturation are essential for competence, there is little to no change in mitochondrial numbers as maturation proceeds (Barritt et al., 2002; May-Panloup et al., 2005; Clarke, 2012; Cotterill et al., 2013). Mitochondrial replication occurs during oocyte growth and should be completed before antral follicle development, and final oocyte maturation (Piko and Taylor, 1987; Thundathil et al., 2005). However, mitochondrial copy number is an important indicator of oocyte developmental competence, with oocytes containing more copies of mitochondrial DNA, and thus more mitochondria, having higher developmental competence (Barritt et al., 2002; May-Panloup et al., 2005; Clarke, 2012; Cotterill et al., 2013).

Overall; the redistribution of mitochondria, and their activation to ensure ATP production and energy accumulation are necessary for oocyte activation and for successful embryo development (Thompson et al., 2000; Lubarda, 2005; Gasparrini et al., 2006).

#### **2.2.4 Gene Expression, Protein and Factor Accumulation**

Although it is well recognized that gene expression changes as oocytes progress through maturation, there is not currently a clearly defined set of genes whose expression is required for oocyte competence (Brevini et al., 2007; Labrecque and Sirard, 2014). However, it is widely accepted that the acquisition of developmental competence requires that oocytes produce and

store the mRNAs and proteins necessary for early embryonic development, prior to the completion of maturation.

As oocytes grow and mature, they produce a large quantity of a set of factors referred to as 'oocyte secreted factors' (OSFs). These OSFs, including growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-6 (BMP-6), are critical to oocyte competence as they regulate a broad spectrum of cumulus cell functions including; growth, survival, steroidogenesis and metabolism (Gilchrist et al., 2004; Bilodeau-Goeseels, 2012). Through controlling functions of the cumulus cells, OSFs indirectly control the viability and competence of the oocyte as well.

### **2.2.5 Other Cytoplasmic Factors**

Oocyte maturation also requires changes in many other factors in order for embryonic development to occur. Included amongst these are production of adequate ATP and redox homeostasis (Dumollard et al., 2007).

Energy production is also critical to oocyte developmental competence. Oocytes of greater developmental competence, have increased glucose metabolism rates, through both glycolysis and the pentose phosphate pathway (Sutton-McDowall et al., 2006). This is particularly true of mature oocytes, and it is common to find higher levels of ATP and lower concentrations of glutathione in metaphase II oocytes than in germinal vesicle (GV) stage oocytes (Abdoon et al., 2011). Meanwhile, glycolytic deficiencies have been shown to have a negative effect on oocyte developmental potential (Brown et al., 2017). Oocytes are extremely active, producing energy while undergoing growth. As they complete growth, energy production decreases. One method that this is visualized is as a decrease in glucose-6-phosphate dehydrogenase activity (El Shourbagy et al., 2006).

Mammalian oocytes contain high quantities of stored lipids, used for  $\beta$ -oxidation and energy production (Dunning et al., 2010; Reader et al., 2017). To ease fatty acid transport for  $\beta$ -oxidation, mitochondria and lipid droplets are often found co-located in highly competent oocytes (Reader et al., 2017). As such, lipid droplets are commonly found in a relatively peripheral orientation in immature oocytes, and become more evenly distributed throughout the ooplasm as maturation proceeds (Jeong et al., 2009). Lipids appear dark on a light microscope, and an oocyte containing a large volume of homogeneously distributed lipid should thus have an evenly dark cytoplasm (Jeong et al., 2009; Reader et al., 2017). Because lipidation is an indicator of oocyte competence, gross cytoplasm appearance is often used as a simple method of estimating oocyte developmental competence (Jeong et al., 2009).

Oocytes also contain a large number of vesicles, occupying 15-35% of the cytoplasm (Cran, 1985; O'Brien et al., 1996; Reader et al., 2015). As maturation proceeds, vesicles increase in size and are found in increasingly large groups along with those mitochondria not at the immediate oocyte periphery (Kafi et al., 2005). However, overall vesicle volume tends to decrease as oocytes mature, possibly due to the oocyte metabolizing the vesicle content throughout maturation (Auclair et al., 2013). Due to the large volume that they occupy, vesicles are found approximately evenly distributed throughout the entire ooplasm with the exception of the most peripheral regions in immature oocytes (Reader et al., 2015). As maturation proceeds, vesicles migrate to a more peripheral distribution (Pederson et al., 2016). On transmission electron microscopy, these vesicles appear translucent, but may contain membranous material, in addition to large quantities of metabolites used in oocyte maturation and embryonic development (Reader et al., 2017). As such high quantities of vesicles in immature oocytes are necessary for

competence, while lower vesicle volume following maturation indicates a metabolically active oocyte of high developmental competence.

Vacuoles, on the other hand, are rare or entirely absent in competent mature oocytes, and when identified are found to be empty but associated with lysosomes (Coticchio et al., 2016; Nottola et al., 2016). These are likely derived from swelling of Golgi and SER membranes and by cytoskeletal defects (Sathananthan et al., 1987; Nottola et al., 2008). In human oocytes, high numbers of vacuoles have been correlated with early degeneration and oocyte aging and is associated with failure of fertilization in vitro (Nottola et al., 2007, 2008, 2009; Coticchio et al., 2010; Bianchi et al., 2014; Nottola et al., 2016).

In general, the ooplasm of highly competent oocytes should be relatively homogenous, and organelles not mentioned above should be evenly distributed throughout (Shahedi et al., 2013; Nottola et al., 2016). The oolemma is continuous, with numerous long microvilli projecting into the perivitelline space in highly competent oocytes (Shahedi et al., 2013; Nottola et al., 2016). As maturation proceeds, the perivitelline space becomes larger, and the microvilli both increase in number and change shape to become longer and thinner (Kafi et al., 2005). Cytoskeletal microtubules are distributed in a largely uniform manner throughout the cytoplasm of the oocyte at GV, and microfilaments are sparsely scattered (Sun and H, 2006). Following maturation, both microfilaments and microtubules are primarily found in the cortical regions of the cytoplasm (Sun and H, 2006; Verlhac, 2011). The redistribution of these cytoskeletal polymers is critical for oocyte competence as they are both involved in maintaining oocyte structure and, microtubules are involved in mediating the relocalization of other organelles while microfilaments play a key role in chromosome migration and polar body extrusion (Sun and H,

2006). Finally, oocytes that have a greater diameter are believed to be more competent, as this indicates that they are further through the growth phase and are more likely to have undergone the above listed changes that occur during this phase (Fair et al., 1995).

Table 2.1. Summary of organelle features in highly competent oocytes. Adapted from (Ferreira et al., 2009; Mao et al., 2014; Reader et al., 2017).

Organelle	Germinal Vessicle	Metaphase II	Low Competence MII Oocyte
<b>Cortical Granule Distribution</b>	Distributed sparsely throughout cytoplasm	Peripheral	Clustered or even throughout ooplasm
<b>Endoplasmic Reticulum</b>	Uniformly distributed through cytoplasm	Forms clusters	—
<b>Golgi Network</b>	Present as fragments	Largely transformed into vesicles	—
<b>Lipid Volume</b>	High	High	Low
<b>Lipid Distribution</b>	Peripheral	Even	Peripheral
<b>Mitochondrial Distribution</b>	Peripheral	Even	Peripheral
<b>Mitochondrial Morphology</b>	Spherical or hooded, light matrix	Light matrix, hooded, few vacuoles or granules	Dense matrix, vacuoles and granules
<b>Ribosomes</b>	Many, associated with endoplasmic reticulum	Few- dissociate	—
<b>Vacuole Volume</b>	Low	Low	High
<b>Vesicle Volume</b>	Low	High	Low

## 2.3 Oocyte Retrieval Methods

In the cattle industry there are multiple methods of retrieving oocytes from follicles, each with its own set of benefits and drawbacks. The two primary methods of oocyte collection can be differentiated based on whether the donor cow is living or not. Oocytes can be recovered by either scraping or aspirating follicles from ovaries that are collected within the first four hours post-mortem (Blondin and Sirard, 1995). Since cattle are slaughtered for meat regularly, and ~20 to 60 oocytes can be recovered from a single ovary, these make a reliable method of retrieving

large numbers of oocytes with minimal cost compared to other oocyte collection methods (Hamano and Kuwayama, 1993). However, the greatest drawback is that collection can only be performed on either termination or ovariectomy of the cows, and so is a single opportunity per animal. While post-mortem ovary harvesting can provide a reliable supply of oocytes for research, where genetics are relatively unimportant, or as a last-ditch effort to recover gametes from an individual, this is not an ongoing opportunity to produce increased offspring from females of genetic merit. As a result, abattoir-derived oocytes are commonly used in research but are only rarely applicable to commercial cattle production systems.

Of use for more applications where individual genetic input is important, oocytes can also be collected from live animals, using a technique called ovum pick-up (OPU). This has the increased cost of maintaining the cows, but the advantage that oocytes can be harvested as frequently as twice weekly throughout much of the cows adult lifespan, with an average of five oocytes recovered per ovary (Broadbent et al., 1997). Because of this, this method of oocyte collection is commonly used to increase the number of offspring generated from genetically valuable females. In many species, live animal oocyte recovery requires that, at the least invasive, a laparoscopic surgery be performed to access the ovaries and aspirate follicles. However, the large size of cattle, permitted the design of a transvaginal oocyte collection technique, termed transvaginal ultrasound-guided oocyte aspiration (TUGA) , in the late 1980s (Pieterse et al., 1988). Using this method, ovaries are grasped transrectally, and held in place against the vaginal wall. An ultrasound probe, fitted with an aspiration needle, is inserted vaginally and used to visualize and aspirate antral follicles. Oocytes are aspirated along with the follicular fluid (Pieterse et al., 1988; Looney et al., 1994; Hasler et al., 1995).

## 2.4 In Vitro Oocyte Maturation

*In vitro* maturation is the process of removing germinal vesicle stage oocytes from the follicular environment prior to the luteinizing hormone surge initiating maturation. Oocytes are instead incubated in a laboratory to facilitate the completion of maturation. This is becoming increasingly common, both in research and in reproductive assistance for human fertility and that of many animal species, as it is the first step in production of embryos *in vitro*. There are two primary ways in which *in vivo* and *in vitro* oocyte maturation differ;

1. Cumulus-oocyte-complexes (COCs) used in IVM are often collected from mid-sized antral follicles, at an earlier stage of development than they would naturally be ovulated.
2. Having been removed from the follicular environment, COCs used in IVM are removed from natural meiosis inhibiting factors and spontaneously resume meiotic maturation, independent of a gonadotropin surge and despite potentially not having completed cytoplasmic maturation (Gilchrist and Thompson, 2007; Bilodeau-Goeseels, 2012).

This issue becomes particularly apparent in oocytes collected from small to mid-sized follicles (2-6mm), which would naturally remain in meiotic arrest for another four days, while continuing to undergo cytoplasmic changes necessary to support development, before the follicle would become pre-ovulatory and a gonadotropin surge would occur to stimulate *in vivo* maturation (Bilodeau-Goeseels, 2012). Oocytes recovered from mid-sized to larger follicles have had more time to receive necessary developmental cues and are more likely to be of higher competence than those recovered from small follicles (Pavlok et al., 1992).

Current IVM culture media, while complex, is not an optimal media to simulate follicular fluid, and is instead based off of somatic cell culture media (Gilchrist and Thompson, 2007). The

composition of IVM media, and thus the environment that these oocytes are exposed to, is significantly different from the fluid environment oocytes would naturally be maturing in within follicles (Sutton et al., 2003; Sutton-McDowall et al., 2005; Gilchrist and Thompson, 2007; Salhab et al., 2013). These environmental differences caused by IVM media shortcomings, are one factor driving the often reported decreased developmental competence seen in *in vitro* matured oocytes compared to their *in vivo* matured counterparts (Greve et al., 1987; Rizos et al., 2002; Sutton et al., 2003; Thompson, 2006; Phongnimitr et al., 2013).

An optimal media has not yet been devised, and as a result there are many variations on the market in which to mature oocytes. However, all contain the same base constituents; a balanced salt solution to maintain appropriate osmolality between 265 to 300 mOsmol (Wright and Bondioli, 1981; Rosenkrans et al., 1993), amino acids, a pH buffer system to maintain consistent pH of 7.2 to 7.4, and an energy source; most commonly pyruvate, all in a purified water base. Bovine COCs are placed in the desired balanced oocyte maturation medium, and using current protocols are cultured for 18-24 hours before maturation can be expected (Ward et al., 2002; Lequarre et al., 2005; Agung et al., 2006). Oocytes are cultured in a humidified incubator at internal body temperature (38.5°C) and a low pCO<sub>2</sub> environment (~5%), and low pO<sub>2</sub> (5-7%) to simulate the anticipated conditions within the ovary (Thompson et al., 1990). Under normal, *in vitro*, conditions meiotic maturation is completed with the oocyte reaching metaphase II by 18 to 24 hours of culture, and it can be expected that 80% of cultured oocytes will successfully complete maturation (Suss et al., 1988; Sirard et al., 1989). However, overall *in vitro* matured oocytes are noted to be less competent than those produced *in vivo*, with decreased embryo production rates resulting (Rizos et al., 2002; Krisher, 2004; Walls et al., 2012, 2015).

Ultrastructural comparisons between *in vivo* and *in vitro* matured oocytes show general similarity (Coticchio et al., 2016), but it is not uncommon to find abnormalities in oocyte maturation, due to IVM, especially in indicators of cytoplasmic maturation and oocyte competence. IVM oocytes frequently exhibit aberrations in mitochondria-smooth endoplasmic reticulum (SER) aggregates, with mitochondria being found isolated or in association with the oocyte membrane rather than in close proximity to SER (Coticchio et al., 2016). *In vitro* matured bovine oocytes tend to have decreased lipid levels compared to *in vivo* matured oocytes (Auclair et al., 2013; Warzych et al., 2017). Although the number of mitochondria do not generally differ between *in vivo* and *in vitro* matured oocytes (Coticchio et al., 2016), IVM oocytes often show aberrations in the mitochondrial distribution, with increased probabilities of mitochondrial clustering throughout the ooplasm (Abdoon et al., 2011). It is probable that one reason for these abnormalities found in *in vitro* matured oocytes, is due to irregularities in cumulus cell function, resulting in oocyte exposure to dysregulated metabolites and signaling molecules during maturation (Brown et al., 2017).

Although IVM has not yet been optimized for cytoplasmic maturation, parameters for meiotic maturation are well known. Within 16-20 hours of IVM most oocytes will reach metaphase II (Lequarre et al., 2005; Agung et al., 2006). When the IVM culture period exceeds 20-24 hours oocyte competence is decreased, resulting in impaired development and decreased embryo production rates (Ward et al., 2002; Lequarre et al., 2005; Agung et al., 2006). However, so long as the maturation period is within this critical range, oocyte competence is not hindered and oocytes collected during a single OPU session can be fertilized together, despite the potential for oocytes having matured at different times (Merton et al., 2012). Still, with current practices,

although IVM increases the number of oocytes that can be recovered and fertilized, only a small portion of those oocytes that mature to metaphase two have the potential to produce fully competent embryos (Schroeder and Eppig, 1984).

## **2.5 Advancing Technologies in Oocyte Maturation**

Studies attempting *in vitro* fertilization (IVF) in cattle began in the 1970s (Sreenan, 1970; Hunter et al., 1972), with the first successful bovine IVF being reported in 1977 (Iritani and Niwa, 1977). Progress continued rather rapidly in the initial years, and the first calf was born following embryo transfer of an *in vitro* matured oocyte in 1982 (BG et al., 1982), followed by continued successes throughout the 1980s (Critser et al., 1986; Hanada et al., 1986; Lu et al., 1987).

Based on meiotic progression, IVM rates are currently high, approaching 90% (Leal et al., 2012). However, with current protocols, although *in vitro* embryo production (IVP) does increase embryo production rates compared to *in vivo* development, IVP is still quite inefficient. While 30-40% of matured bovine oocytes successfully develop to the blastocyst stage *in vitro* (Lonergan et al., 2000; Bilodeau-Goeseels, 2012), only 20-30% of retrieved oocytes develop into highly competent, transferable embryos (Van Wagtendonk-de Leeuw, 2006). This is suggestive of a deficiency in the developmental competence of *in vitro* matured oocytes, be it an innate oocyte competence issue or one caused by suboptimal culture conditions (Trounson et al., 2001; Jurema and Nogueira, 2006).

Regardless, *in vitro* oocyte maturation has the potential to be a revolutionary reproductive technology. This allows the female reproductive capacity to be maximized through recovering the entire supply of oocytes available within an ovary at any given time point (either living or

post-mortem) for genetic preservation, or production of multiple offspring (Gilchrist and Thompson, 2007). In recent years, the focus has been directed towards improving the efficiency of *in vitro* embryo production by attempting to delay meiotic maturation of oocytes, while allowing cytoplasmic maturation to proceed. In theory, this would result in oocytes more similar to those matured *in vivo*, and hence more developmentally competent (Wu et al., 2006). The primary method of delaying meiotic maturation has been through artificially maintaining high cAMP levels within the oocyte, blocking the pathway resulting in M phase promoting factor (MPF) activation, and hence reversibly preventing oocyte maturation. Meiotic resumption is largely controlled by the activity of M phase promoting factor (MPF), which is composed of a regulatory subunit; cyclin B, and a catalytic subunit; CDK1 (Bilodeau-Goeseels, 2012). Methods to delay maturation, look at preventing activation of MPF, through maintaining high levels of its primary inhibitor, cyclic adenosine monophosphate (cAMP) (Homa, 1988; Bilodeau-Goeseels, 2012). Common methods of doing so are either the inhibition of specific cyclin-dependent kinases to prevent activation of oocyte MPF (Gilchrist and Thompson, 2007), or the use of cAMP promoting/elevating agents to similarly inhibit meiotic resumption (Sirard and First, 1988; Bilodeau-Goeseels, 2003a, b, 2012). Artificially elevating cAMP will temporarily block meiotic resumption in ungulates, and has a longer-lasting effect in mice and humans (Sirard and First, 1988; Thomas et al., 2002, 2004a). Cyclic AMP works by activating protein kinase A (PKA) when cAMP levels are high. Protein kinase A inactivates CDC25b preventing its action and activates WEE1B which in turn phosphorylates CDK1 and inactivates the MPF complex. When cAMP levels drop, PKA inactivates WEE1B, and activates CDC25b which then acts to dephosphorylate CDK1, activating MPF and allowing meiosis to proceed (Bilodeau-Goeseels,

2012). Thus, artificially maintaining high levels of cAMP will block the meiotic activation pathway by preventing MPF phosphorylation. Although use of cAMP, or other chemicals aimed at altering the above pathway to inhibit MPF, have shown success at delaying maturation, there has not been an associated increase in embryo production (Kulbeka et al., 2000; Lonergan et al., 2000; Ponderato et al., 2001; Hashimoto et al., 2002; Ponderato et al., 2002).

A second method of delaying meiotic maturation in bovine oocytes is to prevent germinal vesicle breakdown, through inhibition of the protein synthesis necessary for MPF activation (Sirard et al., 1989; Lonergan et al., 1997; Avery et al., 1998). Cyclohexamide is one such protein synthesis inhibitor, and is commonly and successfully used in inhibiting meiosis in oocytes (Kastrop et al., 1991; Saeki et al., 1997). As with cAMP, inhibition with cyclohexamide is reversible and oocytes will resume meiosis when they are moved to a maturation promoting medium free of inhibitors (Lonergan et al., 1997). Although cycloheximide has been used in the past to successfully delay maturation, the resultant embryo production rates are decreased compared to those from oocytes subjected to conventional IVM, suggesting that this treatment is otherwise decreasing oocyte competence (Kastrop et al., 1991; Saeki et al., 1997).

There are three different methods of cAMP modulation in use; adenylate cyclase activators (Thomas et al., 2002), cAMP analogues (Homa, 1988), and phosphodiesterase inhibitors (Bilodeau-Goeseels, 2003a). However, research to date has been inconclusive as to the benefits of cAMP modulators, whether alone or in combination. Meiosis is largely only transiently inhibited in bovines, yet many studies continue to find insignificant differences in blastocyst development rates between conventionally matured oocytes and those pre-incubated for meiotic delay (Bagg et al., 2006, 2007; Luciano et al., 2011; Gharibi et al., 2013).

In regards to oocyte collection, there is also dispute as to the best method for retrieving oocytes of the greatest innate competence. There has been evidence from recent research that oocytes recovered from ovaries four hours post-mortem are of optimal competence due to early atretic factors (Blondin et al., 1997). Based off of this understanding, super-stimulation protocols currently in use, and with the greatest degree of success, rely on a 24-48 hour coasting period following the last follicle stimulating hormone (FSH) injection, before oocytes are recovered. This coasting period causes atresia to begin without progressing far enough to be detrimental to oocyte competence (Sirard et al., 1999).

One of the greatest challenges in improving IVM is to first improve our understanding of oocyte cytoplasmic maturation in order to best determine which factors are most influential on oocyte competence (Gilchrist and Thompson, 2007). From there, being able to collect and mature oocytes with the greatest degree of competence would be making great strides towards improving *in vitro* embryo production efficiency, and eventually applicability. The current study was geared towards using a variety of cytoplasmic markers to identify those oocytes of the greatest developmental competence, applied towards selecting between different oocyte collection and maturation treatments in determining those best able to provide highly competent oocytes.

## **CHAPTER III**

### **THE EFFECT OF DELAYING MATURATION ON BOVINE OOCYTE CYTOPLASMIC MATURATION**

#### **3.1 Introduction**

One of the biggest failings of *in vitro* maturation is that oocytes are released from follicular fluid, and the natural meiotic inhibiting factors found within, prior to completion of development. This induces oocytes to undergo spontaneous meiotic maturation without first accumulating all of the cytoplasmic factors necessary to sustain early embryonic development (Pincus and Enzmann, 1935; Edwards, 1965). Exacerbating this issue is the fact that while 24-hour maturation is designed to promote nuclear maturation (Merton et al., 2012), conditions are still sub-optimal for cytoplasmic maturation completion (Hagemann, 1999). Because an oocyte cannot be held in the mature state without risking degeneration, there has been an emphasis on research into methods for temporarily inhibiting spontaneous meiotic maturation, allowing cytoplasmic maturation to proceed and “catch up” first. It is believed that doing so will allow oocytes more time in communication with cumulus cells to accumulate and store molecules necessary for embryonic development, before meiotic maturation resumes and the connections are broken (Bilodeau-Goeseels, 2012). However current knowledge of how of how meiotic arrest is maintained *in vivo* is limited and so methods *in vitro* are based on delaying meiotic resumption instead (Bilodeau-Goeseels, 2011).

There are three different methods of cAMP modulation in use; adenylate cyclase activators (eg; forskolin) (Thomas et al., 2002), cAMP analogues (eg; dibutyryl cAMP) (Homa, 1988), and phosphodiesterase inhibitors (eg; 3-isobutyl-1-methylxanthine) (Bilodeau-Goeseels, 2003a). However, research to date has been inconclusive as to the benefits of cAMP modulators.

While meiosis is only transiently inhibited and gap junctional communication is maintained, theoretically supporting cytoplasmic maturation, studies continue to find insignificant differences in blastocyst development rates between conventionally matured oocytes and those pre-incubated for meiotic delay (Bagg et al., 2006, 2007; Luciano et al., 2011; Gharibi et al., 2013).

One current practice, referred to as “simulated physiological oocyte maturation” (SPOM), maintains meiotic arrest through combining two methods of cAMP maintenance (Albuz et al., 2010). Incubation of oocytes for two hours in a pre-maturation medium containing a combination of both IBMX and forskolin causes nearly a 67-fold spike in cAMP within the oocyte (from 3 fmol to 200 fmol), effectively preventing meiotic resumption by mimicking the increase in cAMP levels that occur during the pre-ovulatory gonadotropin surge (Albuz et al., 2010). The combined effect both increases cAMP production (forskolin) and prevents cAMP degradation (IBMX), to maintain maximal cAMP levels. Once removed from this pre-maturation medium, oocytes in the SPOM protocol are cultured in an extended maturation of 30 hours, where the IVM medium is supplemented with another cAMP modulator (cilostamide, allowing only gradual cAMP degradation) with the aim of meiotic resumption being restricted to a decreased rate. The SPOM system has increased bovine embryo production rates compared to IVM (26% and 8% respectively) as well as implantation rates (53% cf 28%).

One of the major problems associated with the use of meiosis promoting factor (MPF) inhibitors, is that they have been shown to cause changes in the oocyte ultra-structure. By interrupting cumulus cell-oocyte communication, and causing organelle disruptions the effects of MPF inhibitors may be long-lasting and debilitating for oocyte competence towards embryo development (Fair et al., 2002; Lonergan et al., 2003). To date, however, those factors that work

by directly maintaining cAMP levels, cAMP modulators, appear to be the best candidate for delaying meiotic maturation, potentially causing fewer aberrations than do other methods of MPF inhibition (Bilodeau-Goeseels, 2003a, b).

This project compared oocytes that were matured using a conventional 24-hour maturation, and those using a modified SPOM method, with a 2-hour pre-incubation and a 24-hour maturation period to determine the effects of cycloheximide and IBMX on select oocyte cytoplasmic maturation indicators. The hypotheses were that; 1) holding oocytes in cAMP modulators would reversibly maintain both meiotic arrest and gap junction communication and that cytoplasmic maturation would proceed during this period. 2) Oocytes undergoing extending IVM, including both pre-maturation and IVM, would have more complete cytoplasmic maturation than those oocytes undergoing conventional IVM. The aim was to determine if the 2-hour pre-incubation period was, in fact, adequate at maintaining gap junction-mediated communication between cumulus cells and oocytes for a long enough period to improve cytoplasmic maturation, and thus oocyte competence.

## **3.2 Methods**

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

### **3.2.1 Animals**

All procedures in this experiment were approved by the Louisiana State University Agricultural Center's Institutional Animal Care and Use Committee and were carried out at the LSU Reproductive Biology Center, St. Gabriel, LA, USA. Thirty-five mixed breed beef cows, housed on pasture with ad libitum access to mixed native grasses supplemented with hay and full

access water were used for intermittent oocyte collection throughout autumn 2014 and spring 2015.

### 3.2.2 Experimental Design

The effect of *in vitro* maturation system on completion of oocyte cytoplasmic maturation was assessed through comparison of immature oocytes to oocytes at different stages of *in vitro* maturation. Oocytes were recovered via transrectal ultrasound-guided oocyte aspiration on 12 days, and were randomly assigned by day to one of four treatment groups (Table 3.1).

Table 3.1 Treatment groups for analysis of the effect of *in vitro* maturation protocols on completion of oocyte cytoplasmic maturation

	Control	Pre-Maturation
<b>Immature (GV)</b>	<b>1;</b> Immediate Fixation (n=285)	<b>2;</b> Pre-maturation (n=280)
<b>Mature (MII)</b>	<b>3;</b> Conventional IVM (n=300)	<b>4;</b> Extended IVM (n=290)

### 3.2.3 Oocyte Retrieval

Oocytes were recovered, as cumulus-oocyte complexes (COCs), from live cattle using ovum pick-up (OPU, Figure 3.1). Methods were adapted from those used by Bailey (Bailey, 2014). For oocyte recovery, cows were restrained in a manual squeeze chute and were administered 30 mg xylazine (Xylamed™, VetOne®, Boise, ID, USA) intramuscularly, as a mild general sedative. They were then given a 5mL, 2% lidocaine (VetOne®, Boise, ID, USA) epidural as a local anesthetic. Ovaries were palpated rectally and held against the vaginal wall so follicles could be visualized on ultrasound. The ultrasound probe, an 8.5MHz convex transducer, within a hard plastic needle guide (Boland Vet Sales, Keller, TX, USA), and connected to a SonoSite® MicroMaxx® ultrasound, was inserted through the vaginal canal and held against the vaginal wall against the ovary. Once follicles were visualized all follicles greater than 3 mm in

diameter were aspirated, using an 18-gauge, three inch long disposable needle (AirTite Products N183) connected to the ultrasound through the needle guide system. Aspiration was performed using negative pressure, achieved by connecting a vacuum pump (Cook Veterinary Products, Australia). The needle was connected to an embryo collection filter (Agtech Inc., Manhattan, KS, USA) which was first primed with collection medium (Appendix). Aspiration system was rinsed with collection medium after each ovary to prevent oocytes from being held in the aspiration lines. The needle was replaced, and ultrasound probe cleaned after each cow. Once recovered, oocytes were placed in HEPES-TALP (Appendix) at 38.5°C until further processing.

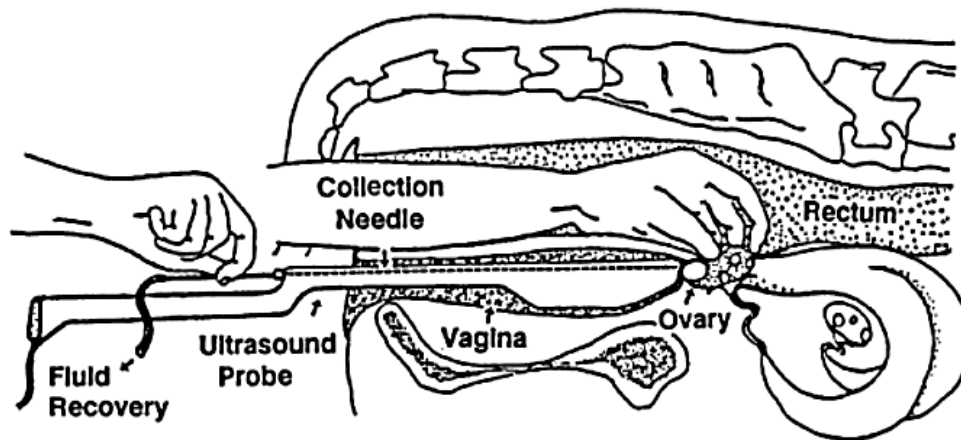


Figure 3.1. Drawing by E. Meintjes depicting the transvaginal ultrasound-guided follicular aspiration procedure (Godke et al., 2014).

### 3.2.4 Pre-Maturation

Oocytes in treatment groups 2 and 4 were incubated for two hours in a pre-maturation medium, at 38°C in a non-CO<sub>2</sub> incubator prior to further processing. This medium was based on HEPES-TALP, supplemented with 0.1µM Forskolin and 0.5µM IBMX (3-Isobutyl-1-methylxanthine) (Appendix). Oocytes were then removed from the cAMP modulating medium,

and washed three times in Hepes-TALP before being moved to either IVM medium (Treatment 4) or proceeding with the staining protocol (Treatment 2).

### **3.2.5 Oocyte Culture**

Oocytes in treatments 3 and 4 were put through a standard *in vitro* maturation (IVM) protocol. Those oocytes in treatment 3 were placed immediately into IVM following retrieval, while those in treatment 4 had a 2-hour pre-maturation incubation described above prior to IVM.

Oocytes were washed 3 times in IVM medium (Appendix) before being split into cohorts of 8-12 and placed into 35 $\mu$ L drops of IVM medium under oil. IVM dishes were then placed in a humidified incubator at 38.5°C under 5% CO<sub>2</sub> for 22 hours.

### **3.2.6 Staining and Fixation**

Oocytes in each treatment group (one through four) were split into two different analysis methods, such that half of the oocytes for each treatment were analyzed by each method.

In analysis method A, oocytes were stained with Calcein AM to identify gap junction closure between the oocyte and the surrounding cumulus cells. Cumulus-oocyte complexes were incubated in 0.1 $\mu$ g/mL Calcein AM (Life Technologies, Eugene OR) for 15 minutes. Following this oocytes were vortexed for two minutes in 1mg/mL hyaluronidase in Hepes-TALP to remove cumulus cells. Denuded oocytes were then incubated for 30 minutes at 38°C in 1 $\mu$ g/mL Hoechst 33342 (Life Technologies) for nuclear identification. The stained oocytes were mounted on slides under 10 $\mu$ L of vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA). Slides were viewed on a wide field fluorescent microscope at 400X magnification and images were saved for each oocyte (Figure 3.2).

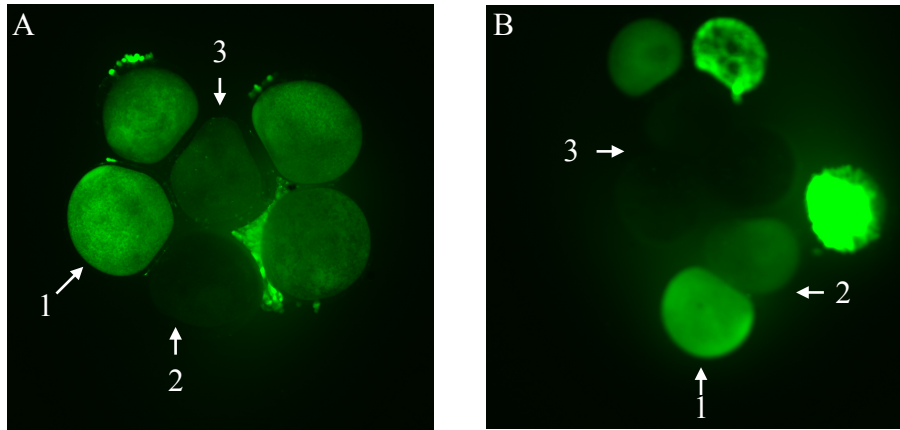


Figure 3.2. Calcein AM staining of oocytes. A. Six oocytes stained after a two-hour pre-incubation period in IBMX and forskolin. B. Eight oocytes stained after extended *in vitro* maturation, including a two-hour preincubation and 24-hour maturation. In both A and B, arrows indicate gap junction functional status as below; 1- open, 2- partially open, 3- closed.

Oocytes assigned to analysis method B were denuded using the above mentioned protocols prior to staining. They were then incubated for 40 minutes in 400nM MitoTracker deep red (Life Technologies, M22426), diluted in Hepes-TALP at 38°C in a non-CO<sub>2</sub> environment (Figure 3.3). MitoTracker stained oocytes were rinsed and fixed in 4% paraformaldehyde for one hour at room temperature. They were then placed in an 0.5% Triton X-100 blocking solution for 30 minutes at room temperature before being incubated for 30 minutes at 38.5°C with 1µg/mL Alexa Flour 488 conjugated Phalloidin (Life Technologies) stain, for identification of F actin (Figure 3.4). Finally, oocytes were washed for 10 minutes and stained with 1µg/mL Hoechst 33342 for 30 minutes before rinsing and mounting on slides under 10µL of Vectashield anti-fade mounting medium. Oocyte staining was analyzed on a wide field fluorescent microscope (Nikon H600L equipped with a Zyla SCMOS camera) at 600X magnification and images were saved for each oocyte.

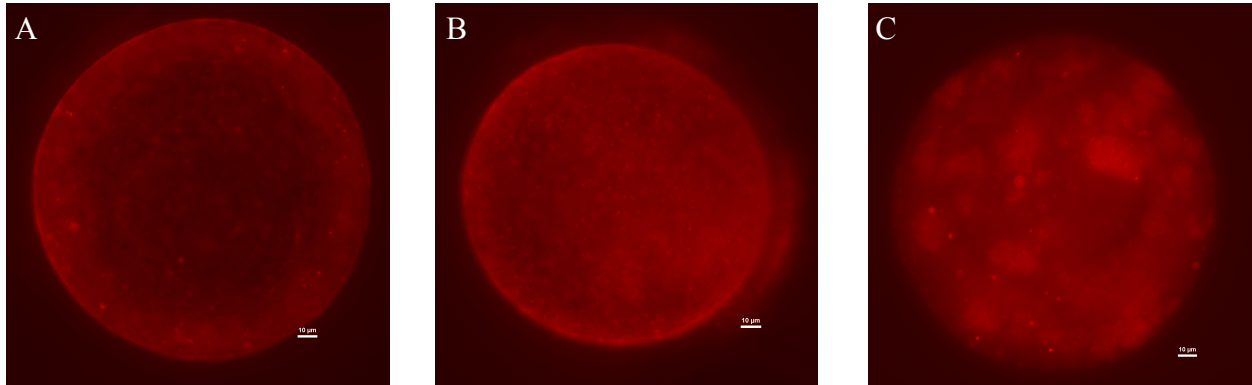


Figure 3.3. Mitotracker Deep red staining of oocyte mitochondria. A. An oocyte displaying peripheral mitochondria. B. An oocyte with mitochondria found in diffuse clusters throughout ooplasm. C. An oocyte with mitochondria evenly diffuse throughout ooplasm.

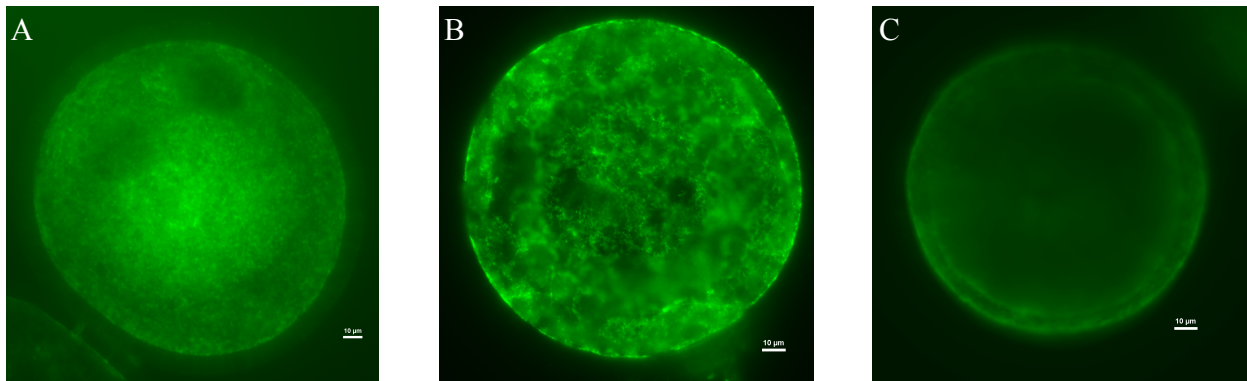


Figure 3.4. Phalloidin staining of oocyte F-actin, representing tubulin aggregation. A. An oocyte with diffuse, unpolymerized F-actin. B. An oocyte with disaggregated F-actin forming short polymers. C. An oocyte displaying fully networked, densely aggregated F-actin.

Images from both analysis methods were later analyzed by an observer blinded to treatment, and numerically coded for stain parameters (Table 3.2) that corresponded to previously documented indications of oocyte maturity (Thomas et al., 2004b, b; Adona et al., 2008; Abdoon et al., 2011; Matchatkova et al., 2012).

Table 3.2 Criteria for classification of oocyte maturation status for each parameter analyzed.

	Assigned Score	Meiotic Status	Gap Junctions	Mitochondria	F-Actin
<b>Immature</b>	1	Germinal vesicle	Open	Peripheral	Disaggregated
<b>Maturing</b>	2	Metaphase I	Partially open	Diffuse	Partially aggregated
<b>Mature</b>	3	Metaphase II	Closed	Diffuse clusters	Polymerized network

### 3.2.7 Statistics

All statistical tests were performed in RStudio (Team, 2016). Results for analysis method A were compared using chi square analysis to determine if there were any differences in the proportion of oocytes at each level of gap junction communication between treatments and meiotic maturation. Results for analysis method B were assessed to determine significant interactions between treatment, meiotic maturation, mitochondrial location and F-actin aggregation, using chi square analysis of the proportion of oocytes representing each characteristic. Specific differences were determined using the Kruskal-Wallis Nemenyi post-hoc test. Significance was set at  $P < 0.05$ .

### 3.3 Results

The majority of oocytes that were fixed immediately following retrieval were found in the germinal vesicle (GV) stage of meiotic arrest, and this was not changed over the course of the 2 hour pre-incubation period ( $P = 0.62$ ). Oocytes in both groups that underwent *in vitro* maturation (IVM) progressed through meiosis, with oocytes that underwent conventional meiosis having a slightly higher proportion of oocytes completing meiosis than those that were held in pre-incubation prior to IVM (63% and 54% of oocytes reaching metaphase II, respectively). Results outlined in Table 3.3 show that treatment had an overall significant effect on meiotic stage

( $P < 0.001$ ). Both methods of maturation showed significantly different maturation profiles than did immature fixed oocytes or oocytes exposed to a pre-maturation period only ( $P < 2 \times 10^{-16}$  for all comparisons). It is important to note that there was a significant difference in maturation profiles between conventional and extended IVM groups as well ( $P = 0.0042$ ) with conventional IVM having a slightly improved maturation rate.

Table 3.3. Proportion table depicting the meiotic status of oocytes in each treatment group and the overall average meiotic phase within each treatment.

Meiotic Status	Immediate Fixation (n)	Pre-Maturation (n)	Conventional IVM (n)	Extended IVM (n)	Total (n)
<b>GV</b>	0.77 (219)	0.79 (221)	0.11 (33)	0.12 (35)	0.44 (508)
<b>MI</b>	0.11 (31)	0.08 (22)	0.15 (45)	0.2 (58)	0.14 (162)
<b>MII</b>	0.05 (14)	0.02 (6)	0.63 (189)	0.54 (157)	0.32 (370)
<b>Unknown/ Degenerate</b>	0.05 (14)	0.11 (31)	0.11 (33)	0.14 (41)	0.1 (115)
<b>Most Frequently Occurring Meiotic Stage</b>	GV <sup>A</sup>	GV <sup>A</sup>	MI <sup>B</sup>	MI <sup>C</sup>	

Superscripts<sup>(A,B,C)</sup> indicate significant differences in the overall distribution of oocyte meiotic status between treatments at  $p < 0.005$ .

Gap junctions were found to be open in the majority of immature fixed oocytes. This function was maintained, though not entirely throughout the pre-maturation incubation ( $P = 0.0036$ ) with a 13% increase in the number of oocytes with closed gap junctions occurring following the pre-incubation (Table 3.4). Following extended IVM, gap junctions continued to close showing a lower proportion with open gap junctions than immediately following pre-maturation (34% and 40% open, respectively), but without a significant change to the overall average profile ( $P = 0.79$  and  $P = 0.0021$  for comparison to pre-incubation and immature, respectively). When compared to all other treatment groups, oocytes cultured in a conventional

IVM treatment were the most likely to have completely closed gap junctions (43%), which was significantly different from all other treatments ( $P=2.6 \times 10^{-9}$ ,  $P=0.0038$ , and  $P=0.0066$  for immediate fixation, pre-maturation and extended IVM, respectively). As well as treatment having a significant overall effect ( $P<0.001$ ) on gap junction functional status, meiotic phase ( $P=1.43 \times 10^{-13}$ ) and the interaction between treatment and meiotic phase ( $P=7.68 \times 10^{-6}$ ) were also significantly correlated to gap junction closure, with mature, MII, oocytes being the most likely to have closed gap junctions.

Table 3.4. Proportion table depicting the functional status of gap junctions of oocytes in each treatment group and the overall average gap junction function level within each treatment.

<b>Gap Junction Communication</b>	<b>Immediate Fixation</b>	<b>Pre-Maturation</b>	<b>Conventional IVM</b>	<b>Extended IVM</b>	<b>Total</b>
<b>Open</b>	0.59 (77)	0.4 (50)	0.2 (26)	0.34 (44)	0.38 (196)
<b>Partially Open</b>	0.22 (28)	0.32 (40)	0.37 (48)	0.4 (57)	0.32 (165)
<b>Closed</b>	0.19 (25)	0.32 (40)	0.43 (56)	0.26 (34)	0.3 (154)
<b>Most Frequently Occurring GJ Status</b>	Open <sup>A</sup>	Open <sup>B</sup>	Closed <sup>C</sup>	Partial <sup>B</sup>	

Superscripts(<sup>A,B,C</sup>) indicate significant differences in the overall distribution of oocyte gap junction functional status between treatments at  $p<0.01$ .

All three distinct patterns in mitochondrial distribution were found within each treatment group, and while treatment did have an overall effect on mitochondrial location within the ooplasm ( $P<0.001$ ), pairwise post-hoc comparisons found no significant differences between the proportion tables of any two specific treatments ( $P>0.1$ ). The most frequently observed orientation for all treatments groups was for mitochondria to be found in clusters throughout the entire ooplasm, however oocytes subjected to pre-maturation alone showed the highest proportion of oocytes with clustered, diffuse mitochondrial patterning (48%, compared to

33-43%, Table 3.5). As with gap junction closure, the interaction between treatment and meiotic completion had a significant impact ( $P=0.0072$ ) on mitochondrial translocation, although nuclear status alone was an insignificant factor ( $P=0.45$ ). Treatment with conventional IVM did result in oocytes with the lowest incidence of both peripheral mitochondria and limited mitochondrial numbers, indicating that this treatment had the greatest effect on supporting mitochondrial maturation.

Table 3.5. Proportion table depicting the mitochondrial location patterns found in oocytes in each treatment group and the overall average mitochondrial location within each treatment.

<b>Mitochondrial Location</b>	<b>Immediate Fixation</b>	<b>Pre-Maturation</b>	<b>Conventional IVM</b>	<b>Extended IVM</b>	<b>Total</b>
<b>Peripheral</b>	0.07 (11)	0.12 (19)	0.05 (9)	0.17 (27)	0.11 (70)
<b>Diffuse</b>	0.23 (36)	0.15 (23)	0.42 (71)	0.23 (37)	0.26 (166)
<b>Diffuse Clusters</b>	0.37 (57)	0.48 (74)	0.43 (73)	0.33 (53)	0.41 (262)
<b>Unknown</b>	0.33 (51)	0.25 (39)	0.1 (17)	0.27 (43)	0.22 (141)
<b>Most Frequently Occurring Mitochondrial Location</b>	Diffuse Clusters <sup>A</sup>	Diffuse Clusters <sup>A</sup>	Diffuse Clusters <sup>A</sup>	Diffuse Clusters <sup>A</sup>	

Superscripts(<sup>A</sup>) indicate significant differences in the overall distribution of oocyte mitochondrial location between treatments at  $p<0.05$ . There was no significant difference found between treatment groups.

Tubulin patterns, as represented by F-actin staining, primarily changed in aggregation rather than distribution patterns. Regardless of treatment, few oocytes had tubulin in its aggregated form of long filamentous networks, but this was most frequently seen following extended IVM (23% cf 9-13%, Table 3.6). Treatment did have a significant effect on the changes in tubulin ( $P<0.01$ ), but the only treatment that showed a significant difference in patterns was extended IVM. Oocytes in extended IVM had similar tubulin characteristics to those that were fixed immediately in the GV stage ( $P=0.23$ ) but different characteristics from either oocytes that

had been pre-incubated only (P=0.0047), or cultured in conventional IVM without pre-incubation (P=0.010).

Table 3.6. Proportion table depicting the tubulin aggregation patterns in oocytes in each treatment group and the overall average tubulin patterns within each treatment.

<b>Tubulin Aggregation</b>	<b>Immediate Fixation</b>	<b>Pre-Maturation</b>	<b>Conventional IVM</b>	<b>Extended IVM</b>	<b>Total</b>
<b>Disaggregated</b>	0.35 (54)	0.49 (76)	0.51 (87)	0.31 (48)	0.43 (275)
<b>Partially Aggregated</b>	0.44 (68)	0.35 (54)	0.34 (58)	0.43 (69)	0.38 (243)
<b>Filamentous Network</b>	0.09 (14)	0.11 (17)	0.13 (22)	0.23 (37)	0.14 (90)
<b>Unknown</b>	0.12 (19)	0.05 (8)	0.02 (3)	0.03 (5)	0.05 (32)
<b>Most Frequently Occurring Tubulin Aggregation</b>	Partially Aggregated <sup>AB</sup>	Disaggregated <sup>B</sup>	Disaggregated <sup>B</sup>	Partially Aggregated <sup>A</sup>	

Superscripts<sup>(A,B)</sup> indicate significant differences in the overall distribution of oocyte tubulin aggregation patterns between treatments at p<0.005.

### 3.4 Discussion

Since so much of oocyte competence is determined by successful, and concurrent, meiotic and cytoplasmic maturation, and since *in vitro* maturation results in maturation occurring in an environment quite different from that naturally occurring within the follicle, it is not unexpected that the *in vitro* maturation system is considered to be one of the most critical factors in determining the competence of IVM oocytes (Kafi et al., 2005). Although the effect of low concentrations of cAMP is transient in bovines and has been shown to improve overall meiotic maturation rates and resultant blastocyst rates by as much as 30% over traditional IVM alone (Thomas et al., 2004b), including a high concentration either before or during IVM could decrease oocyte developmental competence (Luciano et al., 1999).

A previous study in our lab found that this pre-maturation protocol delayed meiotic maturation with 85% of oocytes having already resumed meiosis at the start of conventional IVM, and only 25% when cAMP modulators were present (Farmer, 2014). However, this meiotic inhibition was found to be largely irreversible, with 80.6% of oocytes reaching MII by completion of conventional IVM, and only 43.8% of oocytes in extended IVM completing meiotic maturation (Farmer, 2014), with similar results seen in other studies (Gharibi et al., 2013). Results in this study were in agreement with these previous studies, in that meiotic maturation rates were decreased after exposure to IBMX and Forskolin. These results corroborate the belief that cAMP modulation may be less reversible in the bovine than was originally believed. However, meiotic maturation rates were universally lower than anticipated in this study, indicating that there may have been unrecognized confounding effects decreasing the oocyte competence.

The goal of exposing oocytes to cAMP modulators is that by blocking the MPF pathway, gap junctions between oocytes and cumulus cells will be maintained (Thomas et al., 2004b). This will allow continued communication, while holding oocytes in meiotic stasis, which should encourage oocyte cytoplasmic changes necessary for early embryonic development to occur (Thomas et al., 2004b). However, results of the current study indicated that incubation in IBMX and forskolin was only partially successful at maintaining gap junctional communication and that this was largely irreversible. There was a significant decrease in the proportion of oocytes with open gap junctions between post-collection and following the pre-maturation incubation, but no notable difference in gap junction function between incubation and following IVM after the pre-incubation protocol. This was significantly different from the results seen for oocytes that were

matured *in vitro* without a pre-incubation, which experienced a more extensive cessation of gap junctional communication. These results suggest that cAMP modulators, specifically IBMX and forskolin, may be less effective than intended for improving oocyte competence. These results are in corroboration with those published by Razza et al, wherein oocytes cultured in extended IVM were found to exhibit ultrastructural characteristics more similar to immature oocytes than to conventionally *in vitro* matured oocytes (Razza et al., 2015). Oocytes exposed to IBMX and forskolin had decreased smooth endoplasmic reticulum, mitochondrial and lipid volume than IVM oocytes, but increased vesicle volume (Razza et al., 2015).

The major problem with gap junctions closing is that molecules and factors from cumulus cells that are necessary for oocyte cytoplasmic maturation are blocked from entry into the oocyte (Thomas et al., 2004b). The two cytoplasmic factors analyzed in this study, mitochondrial relocation and F-actin polymerization, both showed insignificant improvements in measurable maturation following the pre-incubation protocol.

One unexpected result of this study is that a high degree of vacuolization was viewed within the oocytes, recognizable as well defined roughly circular areas within the cytoplasm that were unstained with either Mitotracker or Phalloidin (Figure 3.4). While vacuoles were present in large numbers in oocytes of all treatment groups, they appeared to be far more common in matured oocytes, both those with and without the pre-incubation prior to maturation. High levels of vacuoles have previously been associated with oocytes of decreased developmental competence (Coticchio et al., 2016; Nottola et al., 2016). The fact that these are developing over the course of maturation, and not present in high numbers in immature oocytes, may be further indicative of sub-optimal maturation conditions resulting in declining oocyte competence.

Further, it is possible that the development of this high degree of vacuolization may be interfering with proper migration of organelles, disrupting measurement of these cytoplasmic maturation parameters.

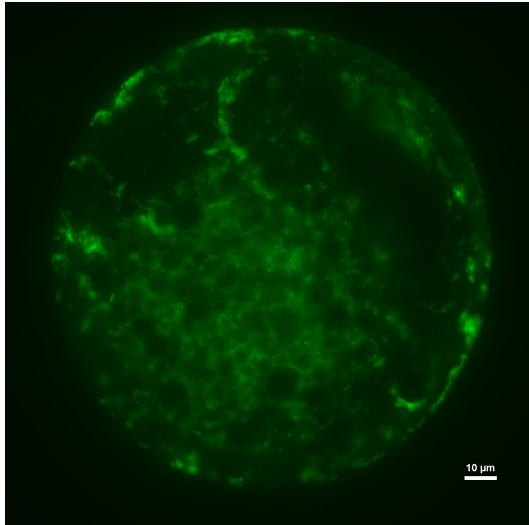


Figure 3.5. Image of an oocyte stained with phalloidin, showing a high degree of vacuolization visible as dark unstained circles within ooplasm.

Overall, results indicate that a two-hour incubation in forskolin and IBMX is likely to be insufficient for increasing *in vitro* embryo yield. Although gap junctions are maintained and meiotic stasis is achieved in some oocytes, both results are only achieved in a small proportion of oocytes, and both are seen to be largely irreversible, preventing proper resumption of meiosis and development following removal of cAMP modulators. Further, the inability of modulators to maintain full gap junction communication results in irregular results at permitting cytoplasmic maturation as indicated by the fact that neither mitochondrial migration nor tubulin aggregation was significantly improved by the pre-maturation protocol. Finally, the formation of vacuoles during both IVM protocols is further proof that current *in vitro* culture conditions are suboptimal and must be improved upon before we will see increased embryo production rates.

## **CHAPTER IV**

### **THE EFFECT OF FOLLICULAR WAVE PHASE AT TIME OF OPU ON BOVINE OOCYTE CYTOPLASMIC MATURATION AND DEVELOPMENTAL COMPETENCE**

#### **4.1 Introduction**

Success of any *in vitro* embryo production program requires first that oocytes collected be not only numerous, but of optimal developmental competence (Seneda et al., 2001). The follicular environment may play a key role in regulating oocyte growth and development. As such, oocytes recovered from larger follicles, which have had more time to grow and receive developmental signals from follicular fluid and granulosa cells, are considered to be more competent, producing 5-10% more embryos, than those recovered from small growing follicles (Fair et al., 1995; Hagemann et al., 1999; Hagemann, 1999). As the follicle continues to grow and becomes dominant, it produces estradiol and inhibin, both of which exert negative feedback activity on follicle stimulating hormone (FSH), causing apoptosis and regression of subordinate follicles (Ireland et al., 1984; Kaneko et al., 1997; Ginther et al., 2000; Bleach et al., 2001). The follicular wave is commonly divided into three distinct phases; at the start of the wave all follicles are growing together (follicle growth), but by mid-wave (peak) a single dominant follicle has emerged which deviates and grows to pre-ovulatory sizes while inhibiting further development of subordinate follicles (Sunderland et al., 1994). The third phase, referred to as the atretic phase, is dominated by regression of all subordinate follicles. These effects combine to have the result that both the total number, and average developmental competence of oocytes recovered can vary greatly based on follicle wave phase at the time of collection (Seneda et al., 2001).

Despite the benefits of waiting to collect oocytes from large pre-ovulatory follicles, previous studies have found that oocytes collected during the follicle growth phase had a higher degree of competence than those collected during the dominance phase (Hagemann et al., 1999; Seneda et al., 2001; Machatkova et al., 2004). In fact, the presence of a dominant follicle has been found to not only decrease the competence of oocytes recovered from subordinate follicles but also from the oocytes within the dominant follicles themselves (Hagemann, 1999). Oocytes collected during the regression phase were not necessarily of depressed competence, and even a high degree of follicle atresia had no negative impact on oocyte competence (Hagemann et al., 1999). However, oocytes collected during the growth phase of follicular waves, when there is no atresia present, have been shown to be of an overall greater competence, as well as improved recovery rates (Seneda et al., 2001; Machatkova et al., 2004).

Despite this understanding, most commercial oocyte collection programs rely on performing follicular aspirations on random days of the cows' estrous cycles, rather than employing a system to time oocyte recovery (Morotti et al., 2014; Cavalieri et al., 2017). In practices that do try to perform timed oocyte pick-up (OPU), there is disagreement on whether or not follicular atresia has negative effects on oocyte competence. As such, there is a lack of consensus on when OPU is best performed for maximal oocyte competence and quantity. This project was designed with the objective of determining the role of follicular wave phase and ovarian dynamics on oocyte collection rates, and oocyte cytoplasmic competence. The hypotheses were that; 1) Oocyte recovery rates would be lowest at wave peak but that oocyte competence would be lowest during follicle atresia. 2) Oocytes recovered during wave peak

would be of greater developmental competence than those collected from either growing or atretic follicles.

## **4.2 Methods**

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

### **4.2.1 Animals**

All procedures in this experiment were approved by the Louisiana State University Agricultural Center's Institutional Animal Care and Use Committee and were carried out at the LSU Reproductive Biology Center, St. Gabriel, LA, USA. Thirty-six mixed breed beef cows, housed on pasture with ad libitum access to mixed native grasses supplemented with hay and full access water were used for oocyte collections from June 2016 through March 2017.

### **4.2.2 Experimental Design**

This project was designed as a repeated measures, treatment cross-over design. Oocytes were collected from live cattle, at 3 different points within the follicular wave to represent the cohort of oocytes available during follicle growth (day-4 of a new wave), peak (day-8), and atresia (day-12). Each cow was used on three sequential cycles, so that collections would occur during each wave phase for every cow. The entire project was repeated twice with different groups of cows.

Stimulation of a new follicular wave was achieved through performing a dominant follicle removal (DFR) on all cows. During the DFR, follicles greater than 6 mm in diameter were aspirated using the same transvaginal ultrasound-guided follicle aspiration (TUGA) techniques as for oocyte collection. Cows were then randomly assigned to one of three groups, to

determine oocyte collection day, though all cows were housed on the same pasture. Using a cross-over design, this DFR followed by oocyte retrieval was repeated three times so that each cow was collected during each follicular phase.

#### **4.2.3 Oocyte Retrieval**

Cumulus-oocyte complexes (COCs), from live cattle using transrectal ultrasound-guided oocyte aspiration, using methods described above in section 3. Cows were restrained in a manual squeeze chute, administered 30 mg xylazine and given a 5mL, 2% lidocaine epidural. Ovaries were palpated rectally and held against the vaginal wall so follicles could be visualized on ultrasound and aspirated using an ultrasound probe that had been inserted vaginally. Once follicles were visualized all follicles greater than three mm in diameter were aspirated. Follicular fluid was aspirated directly into a 50mL Falcon™ tube that had been primed with collection medium. Tubes were labelled with each cow's identification number and a new collection tube was used for each cow. The aspiration system was rinsed with collection medium after each ovary to prevent oocytes from being held in the aspiration lines.

During the oocyte retrieval process, parameters for each ovary were recorded including; the number of follicles aspirated and the average size of follicle present, whether or not a dominant follicle was present and its size if noted, and whether or not a corpus luteum was present. Once recovered, oocytes were placed in Hepes-TALP at 38.5°C until further processing. The total number of oocytes recovered per cow was recorded as well as their visible grade, based on cumulus cell enclosure and cytoplasm appearance. All oocytes for each treatment from a single day were pooled at the end of the recovery process for all allotted cows, and were then randomly divided into level two treatment groups.

#### **4.2.4 Oocyte Maturation**

One-third of the recovered oocytes were fixed immediately following retrieval to represent germinal vesicle stage oocytes for the staining analyses. The remaining two-thirds of the recovered oocytes were put through a standard *in vitro* maturation (IVM) protocol following retrieval. Oocytes were washed 3 times in TCM-199 based IVM medium (Appendix) before being split into cohorts of 8-12 and placed into 35 $\mu$ L drops of IVM medium under oil. IVM dishes were then placed in a humidified incubator at 38.5°C under 5% CO<sub>2</sub> for 22 hours.

Following maturation, half of the matured oocytes were fixed and stained using the same protocols as for the immature oocytes. The remaining oocytes were subjected to parthenogenetic activation and were cultured to determine embryo production rates.

#### **4.2.5 Parthenogenetic Activation and Embryo Culture**

Following completion of maturation, cumulus-oocyte complexes were rinsed in Hepes-TALP before being denuded by vortexing for two minutes in 1mg/mL hyaluronidase. Denuded oocytes were recovered from hyaluronidase, rinsed three more times in Hepes-TALP and stained in 5 $\mu$ M Fluo 3-AM (Molecular Probes, F1242), to identify free cytoplasmic calcium. Oocytes were incubated in 20 $\mu$ L droplets of Fluo 3-AM under oil for 45 minutes at 38.5°C before being washed in Hepes-TALP again. Pre-activation images were taken on an inverted microscope (Nikon Eclipse TE200 equipped with a Hitachi camera) at 400X magnification.

Oocytes were then chemically activated in a 20 $\mu$ L droplet of 20 $\mu$ M ionomycin for five minutes. They were washed again in Hepes-TALP and post-activation images were taken, and staining intensity recorded. Oocytes were cultured for four hours in 30 $\mu$ L drops of 200mM DMAP under oil and at 38.5°C, to complete activation. Activated parthenotes, were rinsed in

synthetic oviductal fluid (SOF) medium (Appendix) before being transferred to fresh 35 $\mu$ L drops of SOF under oil. Presumptive embryo were cultured at 38.5°C, in a 5% CO<sub>2</sub> humidified incubator for 7 days. Embryo cleavage was recorded on day-2 of culture, development to the 8-cell stage was recorded on day-4.

Fluo 3-AM images were coded on a 0 to 4 scale based on staining intensity, with 0 indicating no fluorescence visible, and 4 representing high intensity fluorescence throughout the entire cytoplasm. Fluorescence was compared between oocytes pre- and post-ionomycin activation, and average fluorescence levels at both time points were compared across the different treatment groups (follicular phases at time of oocyte recovery). Oocytes were separated into different culture drops based on their post-activation staining intensity, so effect of cytoplasmic calcium levels on embryo development rates could be tracked.

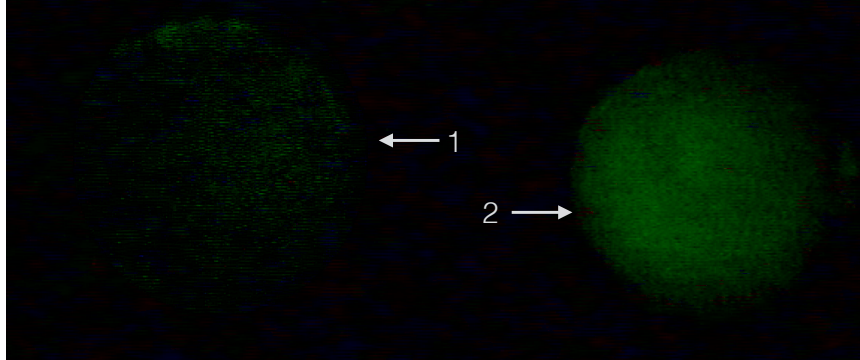


Figure 4.1. Staining reference for Fluo3-AM levels. Image 1 on the left represents an oocyte graded “1” or “slight” and image 2 on the left represents an oocyte graded “3” or “high” fluorescence.

#### **4.2.6 Staining and Fixation**

Both immature and mature oocytes, with the exception of those that were parthenogenetically activated for embryo production, underwent the same fixation and staining protocol (Appendix for detailed protocol). Cumulus-oocyte complexes were denuded either

immediately following collection (germinal vesicle stage) or following 22-hours of IVM (MII stage), by vortexing in 200mL of 1mg/mL hyaluronidase in a 1.5mL microcentrifuge tube for 2 minutes. Denuded oocytes were stained with 400nM Mitotracker deep red (Molecular Probes, M22426) for 30 minutes at 38.5°C before fixation in 2% paraformaldehyde. Inositol 1,4,5-trisphosphate receptor 1 (IP<sub>3</sub>R1) identification was performed using a permeabilization protocol followed by a one hour incubation at 38.5°C in a primary antibody against IP<sub>3</sub>R1 (1:500, Abcam, 5804). Oocytes were then washed followed by incubation in a secondary goat antibody against rabbit IgG and conjugated with Alexa Fluor 488 (1:500, Abcam, 150077). Finally oocytes were mounted on slides under 10μL of Vectashield containing DAPI. Oocytes were viewed on a wide-field Nikon microscope at 600X, and images were deconvolved 10 times before being analyzed by a blinded observer for staining characteristics.

Using similar guidelines to those outlined in section 3, oocytes were coded for meiotic maturation based on DAPI staining of the chromatin (germinal vesicle, metaphase I or metaphase II) and for mitochondrial maturation as determined by Mitotracker identification of mitochondrial location within the ooplasm (peripheral, clustered, diffuse). Inositol 1,4,5-trisphosphate receptor 1 expression was measured as total cell fluorescence in Image J, corrected by subtracting individual image background fluorescence intensity.

#### **4.2.7 Statistics**

All statistical tests were performed in RStudio (Team, 2016). Results involving embryo development rates were compared using chi square tests to determine if there were any interactions between follicular phase, cytoplasmic calcium, and embryo development. Results of oocyte staining were assessed to determine significant interactions between follicular wave

phase, meiotic maturation, mitochondrial location and IP<sub>3</sub>R1 expression, using linear models with a step-function analysis of variance (ANOVA) to determine the best model. Chi square analysis was performed comparing the proportion of oocytes at each mitochondrial maturation level against follicular wave phase. Pairwise interactions were analyzed using the Kruskal-Wallis Nemenyi post-hoc test. Included into the step-wise ANOVA model, were factors recorded during OPU to determine if follicular phase had an effect on apparent oocyte grade or oocyte collection rates.

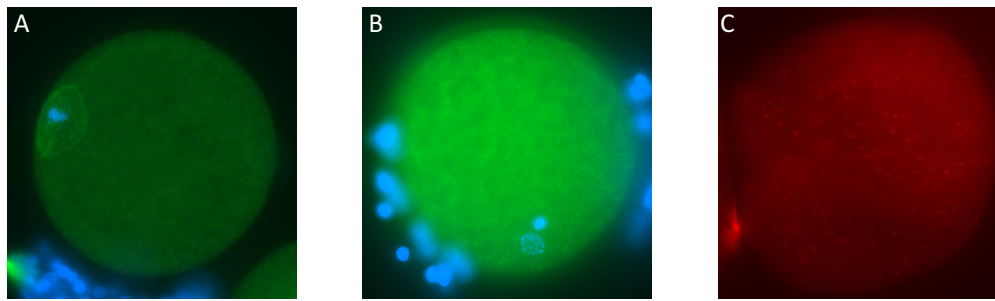


Figure 4.2. A. In immature oocyte stained to identify chromatin (blue) and IP<sub>3</sub>R1 (green). B. A metaphase II oocyte after 24 hours of IVM stained to identify chromatin (blue) and IP<sub>3</sub>R1 (green). C. An oocyte stained with Mitotracker deep red to identify chromatin, found in the diffuse orientation here.

### 4.3 Results

A total of 1,054 oocytes were collected over the course of this trial (364, 374, and 316 on day-4, day-8 and day-12 respectively). Of these 812 were used for analysis (Table 4.1), with the remainder being either lost during handling or discarded for visible degeneration.

Table 4.1. Number of oocytes collected and allocated to each analysis method

Collection Day	Germinal Vesicle	Metaphase II	Embryo Production
<b>Follicle Growth</b>	n=63	n=76	n=107
<b>Follicle Peak</b>	n=86	n=101	n=117
<b>Follicle Atresia</b>	n=96	n=74	n=92

With the exception of a positive correlation between number of follicles aspirated and oocyte recovery (recovery rate=61%,  $P=2 \times 10^{-16}$ ,  $r^2=0.75$ ), none of the recorded variables had an effect on the number of oocytes that were recovered (Table 4.2). Follicular phase did not affect either the number of follicles present or the number of oocytes recovered, and there was no tendency for oocytes from larger follicles to be higher grade at the time of recovery ( $P=0.23$ ,  $r^2=0.11$ ). Further, follicular wave phase had no bearing on oocyte grade  $P=0.786$ ).

Table 4.2. Oocyte collection parameters by follicle phase at time of OPU.

Collection Parameters	Follicle Growth	Follicle Peak	Follicle Atresia
<b>Number of Follicles Present (mean per cow)</b>	17.4	18.1	15.4
<b>Number of Oocytes Collected (mean per cow)</b>	10.1	10.4	8.8
<b>Proportion of A/B Grade Oocytes (mean per cow)</b>	0.53	0.45	0.48
<b>Mean Oocyte Recovery Rate Per Cow</b>	0.65	0.62	0.58

\*There were no significant interactions between follicular phase and any of the parameters measured ( $P>0.05$ ).

Completion of meiotic maturation was strongly correlated to whether oocytes had been subjected to IVM or not ( $P=0.132 \times 10^{-11}$ ,  $r^2=0.68$ ), but was not significantly affected by follicle phase ( $P=0.9$ ). Mitochondrial migration appeared to occur independently of either IVM status or meiotic maturation (Table 4.3), but IP<sub>3</sub>R1 expression significantly increased over the course of IVM ( $P=0.013$ ), and in correlation with meiotic completion ( $P=0.007$ , Table 4.4). Overall, the most important factor affecting IP<sub>3</sub>R1 expression levels was the follicular phase at the time of OPU ( $P=0.0056$ ). The highest IP<sub>3</sub>R1 expression was found in oocytes collected during follicular growth (day-4 post-DFR) and the lowest expression levels were found in oocytes recovered from atretic follicles (day-12 post-DFR).

Table 4.3. The effect of follicular phase and IVM on mitochondrial distribution patterns in bovine oocytes.

<b>Mitochondrial Distribution Proportions</b>	<b>Follicle Growth</b>		<b>Follicle Peak</b>		<b>Follicle Atresia</b>	
	<b>GV (n)</b>	<b>MII (n)</b>	<b>GV (n)</b>	<b>MII (n)</b>	<b>GV (n)</b>	<b>MII (n)</b>
<b>Peripheral</b>	0.164 (6)	0.169 (9)	0.295 (14)	0.17 (9)	0.215 (13)	0.059 (3)
<b>Diffuse</b>	0.5 (20)	0.677 (37)	0.464 (23)	0.508 (28)	0.545 (34)	0.677 (33)
<b>Clusters</b>	0.064 (2)	0.016 (1)	0.142 (7)	0.152 (8)	0.073 (5)	0.019 (1)
<b>Few Mitochondria</b>	0.272 (11)	0.138 (7)	0.099 (5)	0.17 (9)	0.167 (10)	0.245 (12)

\*Neither maturational status nor follicular phase at the time of OPU had a significant effect on mitochondrial location ( $P>0.05$ ).

Table 4.4. The effect of follicular phase and IVM on IP<sub>3</sub>R1 expression levels in bovine oocytes.

<b>IP<sub>3</sub>R1 Accumulation</b>	<b>Follicle Growth<sup>A</sup></b>		<b>Follicle Peak<sup>AB</sup></b>		<b>Follicle Atresia<sup>B</sup></b>	
	<b>GV (n)</b>	<b>MII (n)</b>	<b>GV (n)</b>	<b>MII (n)</b>	<b>GV (n)</b>	<b>MII (n)</b>
<b>Low</b>	0.59 (23)	0.407 (22)	0.551 (27)	0.436 (24)	0.468 (29)	0.633 (31)
<b>Slight</b>	0.282 (11)	0.315 (17)	0.347 (17)	0.436 (24)	0.468 (29)	0.245 (12)
<b>Moderate</b>	0.051 (2)	0.204 (11)	0.082 (4)	0.128 (7)	0.064 (4)	0.122 (6)
<b>High</b>	0.077 (3)	0.074 (4)	0.02 (1)	0	0	0
<b>Total (n)</b>	39	54	49	55	62	49

\*The interaction between maturation status and follicular phase had an overall significant effect on IP<sub>3</sub>R1 expression ( $P=0.013$ ). Oocyte maturation alone was not a significant factor in IP<sub>3</sub>R1 expression ( $P=0.13$ ). <sup>AB</sup>When looking only at the effect of follicular phase, there was a significant difference in IP<sub>3</sub>R1 expression between oocytes collected during follicle growth and atresia ( $P=0.013$ ), but not between oocytes from follicle peak and either growth or atresia ( $P=0.179$  and  $P=0.974$  respectively).

Embryo development rates for this project were uniformly low, with cleavage rates of 19.4%, 17.6% and 12.2% for oocytes collected during follicle growth, peak and atresia respectively ( $P=0.0.14$ , Table 4.5). Development beyond the 8-cell stage was also low across all collection groups at 1.9%, 7.2%, and 2.8% or follicle growth, peak and atresia respectively

( $P=0.44$ ), but was slightly better from oocytes collected during wave peak. However, Fluo 3-AM fluorescence levels had a significant effect on cleavage rates ( $P<0.01$ ) and this was maintained for embryo development rates ( $P<0.001$ ). Both cleavage and embryo development rates were higher in oocytes with low to no Fluo 3-AM staining at the time of parthenogenetic activation, with cleavage rates of 40%, 11%, 20%, and 2% and embryo development rates of 13%, 3%, 2% and 0% for nil fluorescence, slight, moderate and high fluorescence levels respectively.

Table 4.5. Embryo development rates as a function of follicular phase at the time of OPU, and oocyte cytoplasmic Fluo 3-AM fluorescence levels at the time of parthenogenetic activation.

	No Fluorescence		Slight		Moderate		High	
	2-Cell (n)	8-Cell (n)	2-Cell (n)	8-Cell (n)	2-Cell (n)	8-Cell (n)	2-Cell (n)	8-Cell (n)
<b>Follicle Growth</b>	0.2 (3)	0.067 (1)	0.12 (2)	0	0.38 (8)	0	0.06 (1)	0
<b>Follicle Peak</b>	0.6 (26)	0.26 (11)	0.2 (2)	0.1 (1)	0	0	0	0
<b>Follicle Atresia</b>	0.4 (12)	0.07 (2)	0	0	0.21 (6)	0.07 (2)	0	0

#### 4.4 Discussion

In addition to factors integral to the oocyte itself, previous studies have found that oocyte competence can be affected by follicle size and estrous cycle stage (Gilchrist et al., 1995). Estrous cycle synchronization in cows prior to oocyte retrieval through the use of exogenous gonadotropins to control follicle wave emergence, has been reported to have an overall effect of improving oocyte quantity and developmental competence, as well as embryo production rates when compared to unsynchronized cows (Cavalieri et al., 2017). The study presented here aimed to look at oocytes collected from follicles of similarly staged cohorts, at three specified points in the follicular wave to analyze the direct effect of the follicular environment prior to OPU, on oocyte competence. On initial analysis, we didn't find that a significant effect of follicular phase

on any of the parameters recorded; oocyte number, recovery rate, or initial grade Presence or absence of either a corpus luteum or a dominant follicle also failed to significantly affect on oocyte collection rates or competence. These results suggest that, all other factors being equal, timing OPU for specific estrous stages may be less beneficial for optimizing oocyte collection rates and more important for ensuring that follicles are of a homogenous developmental stage. One early study found that, contrary to more recent studies, neither follicle size nor ovarian activity at the time of oocyte recovery had any effect on maturation rate (Fukui and Sakuma, 1980). While current studies were in agreement with this, with regard to meiotic maturation, results of the current study indicated that the effect of follicle size on cytoplasmic maturation and oocyte competence was rather more complicated. This lends evidence to the concept that while meiotic maturation occurs early in the maturation process, prior to the completion of oocyte cytoplasmic maturation and asynchronously with the attainment of full developmental competence.

Methods were based on the previously reported findings that completion of cytoplasmic maturation is absolutely required before oocytes acquire the competence to support early embryonic development (Watson, 2007). Of the many different factors involved in cytoplasmic maturation, it has not yet been determined if any one is the best indicator of competence, being acquired last or most reliably (Sirard et al., 2006b). Three cytoplasmic determinants were used as potential indicators of developmental competence. The changes in mitochondrial distribution that occur during oocyte maturation are well described (Matchatkova et al., 2012) and the function of mitochondria in energy production, make their redistribution to ensure equal mitochondrial allocation to each embryonic cell critical in ensuring developmental competence. It was found

that follicular phase did not significantly affect mitochondrial distribution patterns, suggesting that oocytes from all follicular stages are equally competent, in this regard. However, although insignificant, there was an apparent increase in the number of oocytes with mitochondria distributed in clusters when they were collected during follicle peak. Of interest, this study also failed to find a significant difference in mitochondrial allocation between GV and MII stage oocytes, with the majority of oocytes across all collection days, and both maturational stages having the mitochondria already spread diffusely throughout the ooplasm, an orientation that is more commonly associated with partially mature oocytes (Matchatkova et al., 2012). These results suggest that, while oocyte mitochondrial maturation has already begun by the time oocytes are recovered, especially when collected from large follicles, IVM conditions are inadequate to stimulate the completion of mitochondrial redistribution. This result has been corroborated by past studies, although with the finding that GV oocytes with diffusely distributed mitochondria were most frequently recovered from larger follicles (Matchatkova et al., 2012).

Inositol 1,4,5-trisphosphate receptor 1 is recognized as undergoing a marked increase in expression as oocytes mature. Since IP<sub>3</sub>R1 is expressed on the surface of smooth endoplasmic reticulum (SER), by measuring the expression of IP<sub>3</sub>R1, information about the location and density of the SER network as well as the presence of IP<sub>3</sub>R1 themselves can be postulated. IP<sub>3</sub>R1 is a receptor within the SER which, when phosphorylated, initiates release of calcium stores from the SER (Wakai et al., 2012). This efflux of Ca<sup>2+</sup> from the SER results in the increased cytoplasmic Ca<sup>2+</sup> levels responsible for initiating oocyte activation at the time of fertilization (Malcutt et al., 2006; Whitaker, 2006). As a result, without a high level of IP<sub>3</sub>R1 expression, successful fertilization would not be feasible. Oocytes in this study were found to exhibit a

dramatic and reliable increase in IP<sub>3</sub>R1 expression as maturation proceeded, across all follicular phases. However, this increase was the least recognizable in oocytes collected during the atresia phase. In fact, there was a significant difference in IP<sub>3</sub>R1 expression across follicular phases, with oocytes from follicle growth having the highest expression and those from atresia exhibiting the lowest expression rates. These results indicate that follicle atresia may carry over to have negative effects on oocyte IP<sub>3</sub>R1 expression, resulting in oocytes having irregularly high levels in immature oocytes at the time of recovery, and a lack of increased expression to the normal levels following completion of IVM.

Fluo 3-AM binds calcium and is often used as a viability marker for cultured cells. The staining intensity is directly and positively correlated to cytoplasmic calcium levels. Embryo development rates were found to be significantly, negatively correlated to stain intensity, and as such lower cytoplasmic calcium levels was indicative of mature oocytes of greater competence. Because both oocyte activation and successful fertilization rely on calcium signaling pathways, it is possible, that these low cytoplasmic calcium levels are correlated to higher levels of sequestered calcium and resultant stronger signaling potential in oocytes with low Fluo 3-AM stain intensity. Alternately, it is possible that the high cytoplasmic calcium levels indicated by high-intensity Fluo 3-AM staining, is a signal of calcium leaking either from the endoplasmic reticulum stores, or through the oolemma from the extracellular media. This calcium leakage through membranes could be indicative of atresia of the oocytes, which would explain the failed embryonic development from oocytes with high cytoplasmic calcium levels.

One previous study demonstrated that, while oocyte competence varied with estrous cycle stage, this was independent of follicle size or atretic status (Hagemann, 1999), while

another study reported that early atresia has no effect on embryo production rates but that heavily atretic follicles contain oocytes with decreased developmental potential (de Wit et al., 2000). This is indicative that systemic hormones may play a role in oocyte competence (Robertson et al., 1996; de Wit et al., 2000). As such, it is feasible, that stimulation protocols, by altering the systemic hormones, may have a significant impact on oocyte competence and embryo production. Results of the current study disagree with previous results on the importance of follicular environment in ensuring successful oocyte maturation and instead lend credence to the belief that having a cohort of oocytes at the same developmental stage at the initiation of maturation may be of the utmost importance in determining success of *in vitro* embryo production. However, both IP<sub>3</sub>R1 and Fluo 3-AM staining, as well as embryo development rates indicated that oocytes collected during follicular growth had significantly improved developmental competence over those collected from atretic follicles, and somewhat improved competence compared to oocytes collected during wave peak.

The biggest downfall to performing oocyte retrieval on random days is that, although cows with dominant follicles can easily be identified, it is not possible to differentiate between growth stage and atretic follicles (Cavalieri et al., 2017). This potentially high proportion of atretic follicles aspirated in unstimulated OPU can effectively decrease the overall average competence of collected oocytes, decreasing embryo production rates (Machatkova et al., 2004). Because dominant follicles have a negative effect on competence of oocyte from subordinate follicles, but there is an apparent increase in oocyte competence as follicles grow, it has been suggested that the optimal oocyte recovery period is from large follicles just prior to deviation (Hagemann, 1999). It is possible that the poor results seen in oocytes collected during wave peak

in this study were due to a heterogenous population of oocytes from dominant follicles and early atretic follicles.

Another possibility is a result of the slightly increased proportion of A/B grade oocytes collected during wave emergence, compared to other follicular phases. Initial grades are based largely on the cumulus cell investiture surrounding the oocytes. As cumulus cells are a major factor in regulating the signals from the external environment that reach the oocyte, and for supporting oocyte function (Wrenzycki and Stinshoff, 2013), it is possible that a slight difference in the number of cumulus cells surrounding an oocyte can have strong effects on resultant embryonic development rates. As oocytes grow, the number of mitochondria increase from at few as 10 in germ cells, and 200 in oogonium to an increase to 6,000 mitochondria in primary oocytes and 100,000 or more by the completion of maturation (Ferreira et al., 2009; Mau et al., 2014). It is possible that, in correlation with the low grades apparent, the number of oocytes with few mitochondria present may be indicative of oocytes being retrieved prior to the completion of the growth phase, lacking the cytoplasmic factors necessary to attain cytoplasmic competence.

However, until these factors can be verified, and a method of reliably collecting large numbers of pre-deviation oocytes can be determined, results here would indicate that timed OPU performed frequently enough to collect oocytes only during the follicular growth phase (every 4 days in 2-wave cows) is the best method for ensuring recovery of maximal numbers of highly competent oocytes.

## **CHAPTER V**

### **THE EFFECTS OF RECOVERY METHODS ON GENE EXPRESSION PROFILES IN BOVINE OOCYTES**

#### **5.1 Introduction**

As *in vitro* embryo production (IVP) becomes increasingly more popular, the demand to improve the efficiency of oocyte recovery also increases. It has become mandatory to not only maximize the number of oocytes that can be collected but also to ensure that recovered oocytes are of optimal developmental competence to ensure high developmental competence. Following the success of exogenous hormone stimulation at improving *in vivo* embryo production rates, many different groups have aimed to improve oocyte recovery rates and competence through manipulating their recovery protocols to incorporate different intervals between OPU sessions or by using exogenous hormones (Gibbons et al., 1994; Goodhand et al., 1999; Chaubal et al., 2007). Previous studies have found that use of exogenous hormones to stimulate follicle growth improves oocyte development and competence (Sirard et al., 2006a; Cavalieri et al., 2017), while others have found that super-stimulation had no effect on oocyte retrieval rates or oocyte developmental competence (Oliveira et al., 2016). Super-stimulating females prior to collection has been shown to increase embryo production rates anywhere from not at all (Oliveira et al., 2016) to as much as 7.3% as compared to unstimulated collections (Cavalieri et al., 2017). Oocytes recovered following stimulation are from a more homogenous cohort of follicles, often further along in development than follicles aspirated in conventional OPU. Thus these super-stimulated oocytes are believed to be more developmentally competent and to result in higher embryo production rates (Cavalieri et al., 2017). However, there is also the potential that the high doses of exogenous hormones being administered may negatively affect the normal progression

of oocyte development by hastening follicle growth rates, thus being detrimental to oocyte competence and embryo production (Lee et al., 2006; Zeng et al., 2009; De los Reyes et al., 2011).

Meanwhile follicular atresia, such as that seen in ovaries collected post mortem, has been shown to be potentially beneficial to oocyte competence, but the mechanism is as of yet undetermined (Blondin and Sirard, 1995; Blondin et al., 1997). It is possible that the beneficial effect of post-mortem oocyte recovery is simply attributable to the fact that there is more room for selection of mid- to large-sized follicles and that a larger proportion of oocytes recovered have a high quality cumulus cell investment when compared to oocytes recovered through ovum pick-up (OPU) (Souza-Fabjan et al., 2014).

While many proteins found in oocytes, are stored during oocyte growth, the period of final oocyte maturation is a highly active period during which the oocyte is transcribing mRNA and producing, modifying and storing proteins, many of which are necessary for embryonic development (Dielman et al., 2002). It is these mRNAs and proteins, accumulated primarily during oocyte growth, which drive early embryonic development until the embryonic genome is activated at the 8-cell stage (Fair et al., 2007; Ferreira et al., 2009). As a result of oocytes matured *in vitro* are often removed from the follicular environment prior to the completion of this mRNA accumulation, *in vitro* and *in vivo* matured oocytes have been shown to have not only differences in the overall transcriptional profiles but primary differences in pathways effecting early embryonic development (Mamo et al., 2011). However, despite the known potential for oocyte retrieval interfering with normal developmental dynamics, there is little information available on the effects of oocyte recovery methods on gene expression. This study was

designed to answer the query on whether super-stimulation or post-mortem oocyte collection change the global gene expression of *in vitro* matured oocytes in comparison to those collected following conventional OPU. It was anticipated that comparing differences in gene expression dynamics along with embryo production rates, would help to settle the question of if oocyte source could impact developmental competence and *in vitro* survival. It was hypothesized that; 1) Super-stimulation would cause irregular gene expression patterns, but post-mortem oocyte recovery would not affect transcript profiles. 2) Post-mortem effects will be beneficial for oocyte developmental competence resulting in increased embryo production rates.

## **5.2 Methods**

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

### **5.2.1 Animals**

All procedures in this experiment were approved by the Louisiana State University Agricultural Center's Institutional Animal Care and Use Committee and were carried out at the LSU Reproductive Biology Center, St. Gabriel, LA, USA. Thirty mixed breed beef cows, housed on pasture with ad libitum access to mixed native grasses supplemented with hay and full access water were used for oocyte collections from May through November of 2017.

### **5.2.2 Experimental Design**

Oocytes were collected via three different collection methods in an attempt to determine if the oocyte source affects gene expression or the innate developmental competence of the oocytes. Because they are the three most commonly employed methods of oocyte retrieval in bovine IVP research, oocytes were collected from live cows via ovum pick-up (OPU) either at

random time points in the estrous cycle (Group 1; OPU), or following a super-stimulation protocol (Group 2; FSH) and oocytes were retrieved post-mortem from abattoir derived ovaries (Group 3; PM). Recovered oocytes from all three treatments were then randomly allocated to one of three treatment groups, where they were either snap frozen immediately at the germinal vesicle (GV) stage, matured *in vitro* to the metaphase II (MII) stage and then snap frozen, or matured and fertilized. Both groups of snap frozen oocytes were subjected to RNA-Seq to allow comparison of gene expression of both immature and mature oocytes between groups, as well as the change of gene expression over the course of maturation in each group. Fertilized oocytes were then cultured to the blastocyst stage as a measure of developmental competence.

In all there were six treatment groups compared for sequencing. Table 5.1 shows coding for each group.

Table 5.1. Notation for each of the six treatments used in this study.

Collection Method	Germinal Vesicle	Metaphase II
Oocyte Pick-up (OPU)	IO	MO
Super-stimulation (FSH)	IS	MS
Post-mortem (PM)	IP	MP

### 5.2.3 Super-stimulation

Cows assigned to superstimulation were subjected to a dominant follicle removal (DFR) where all follicles greater than 6mm in diameter were ablated, using the same methods described below for transvaginal ultrasound-guided follicle aspiration (TUGA). This ensured all cows were in the follicular growth phase at the start of the stimulation program. Five mL of prostaglandin F<sub>2α</sub> (Lutalyse<sup>®</sup>, dinoprost tromethamine, 25mg, Zoetis Inc, Kalamazoo, MI, USA ) was administered intramuscularly (im) to lyse any corpora lutea present, and an intravaginal

progesterone releasing device was inserted (Eazi-Breed™ CIDR®, Zoetis Laboratories, Kalamazoo, MI, USA). Cows were administered follicle stimulating hormone (FSH, Folltropin®-V, Bioniche Animal Health, Athens, GA, USA) im every twelve hours for a total of six doses, beginning 36 hours after DFR. A constant dose schedule was used with injections of 1.7 mL each to a total of 200mg FSH per cow (20mg/mL). Twenty-four hours after the last FSH injection CIDRs were removed, and oocyte retrieval was performed 24 hours after CIDR removal, allowing a 48-hour coasting period between the last FSH injection and oocyte recovery (Figure 5.1).

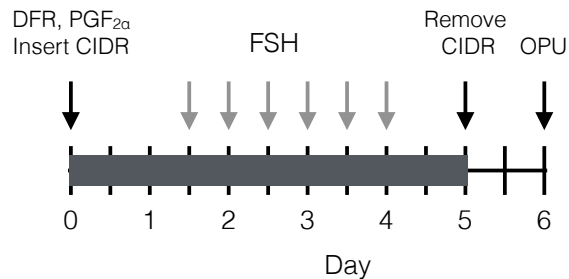


Figure 5.1. Schedule used for the super-stimulation protocol.

#### 5.2.4 Oocyte Retrieval

Oocytes collected from both FSH and OPU groups were recovered as cumulus-oocyte complexes (COCs), from live cattle using transrectal ultrasound-guided oocyte aspiration, using methods described above in section 3.3.3. Cows were restrained in a manual squeeze chute, administered 30 mg xylazine im and given a 5mL, 2% lidocaine epidural. Ovaries were palpated rectally and held against the vaginal wall so follicles could be visualized on ultrasound and aspirated using the aid of an ultrasound probe that had been inserted vaginally. Once visualized, all follicles greater than three mm in diameter were aspirated. Follicular fluid was aspirated directly

into a 50mL Falcon™ tube that had been primed with collection medium. The aspiration system was rinsed with collection medium after each ovary to prevent oocytes from being held in the aspiration lines. Once recovered, oocytes were placed in Hepes-TALP at 38.5°C, transported 15 minutes to the lab and held briefly until further processing. The total number of oocytes recovered was recorded as well as their visible grade, based on cumulus cell enclosure and cytoplasm appearance (Appendix). All oocytes from a single day were pooled at the end of the recovery process, and were then randomly separated into cohorts for each analysis method.

For post-mortem collection of oocytes, ovaries were harvested at a local abattoir following slaughter of cows. They were stored in an insulated cooler to maintain temperature for a maximum of two hours before being transported 15 minutes to the laboratory. On arrival at the laboratory, ovaries were trimmed and rinsed three times with warmed saline. All visible follicles were then aspirated by inserting an 18-gauge, 1.5 inch needle connected to a vacuum line. Lines containing follicular fluid were rinsed with Dulbecco's phosphate buffered saline to ensure oocytes were recovered from the collection lines. Once recovered oocytes were rinsed and moved directly to treatment.

Oocytes from all three retrieval methods that were assigned to analysis at the GV stage were denuded of cumulus cells by vortexing for two minutes in 200µL of BO-Wash (IVF Biosciences product #61008) and were snap-frozen as per methods outlined below in section 5.3.6 immediately following initial processing.

### **5.2.5 Oocyte Maturation and Embryo Culture**

Oocytes from both OPU and super-stimulation collection methods (that were assigned to treatments requiring maturation) were rinsed three times in IVM medium (Appendix) before

being transferred in groups of 10-12 to fresh 35µL droplets of IVM medium under oil. They were then cultured for 22 hours in a humidified incubator at 38.5°C and 5% CO<sub>2</sub>.

Post-mortem recovered oocytes were purchased from a commercial source and shipped in for further processing. Those assigned to treatments requiring maturation were rinsed three times before being transferred in groups of 50 to a 2mL Falcon™ tube containing IVM medium, that had been gassed and equilibrated under 5% CO<sub>2</sub>. They were then transported overnight in a portable incubator (MiniTube product#19180/0001) and maintained at a constant temperature of 37°C. On arrival in our laboratory, they were transferred to a humidified incubator at 38.5°C, under 5% CO<sub>2</sub> for the remainder of the 22 hour maturation period.

Oocytes from all three treatment groups that were assigned to RNA-Seq at the MII stage were then denuded and snap frozen following protocols outlined below in section 5.3.6. The remaining oocytes were used for embryo culture. Oocytes were rinsed three times in drops of BO-IVF medium (IVF Biosciences, product #61003) before being transferred in groups of up to 50 to fresh 450µL wells of BO-IVF. Meanwhile, a single 0.25mL straw of frozen semen from a bull of proven fertility was thawed by brief submersion of the sealed straw in a 37°C water bath and prepared for insemination. Thawed semen was transferred to a 15mL conical tube containing 2mL of BO-Semen prep (IVF Biosciences, product #61004) and centrifuged for 5 minutes at 400G. The sperm pellet was removed, resuspended in 2mL of fresh BO-Semen prep and again centrifuged for 5 minutes at 400G. The final sperm pellet was resuspended in ~500µL of fresh BO-Semen prep to a final concentration of  $4.0 \times 10^6$ /mL. Fifty microliters of sperm suspension was then added to each droplet containing oocytes for embryo production. Oocytes and sperm

were cultured together in BO-IVF in a humidified incubator at 38.5°C under 5% CO<sub>2</sub> for 18 hours in *in vitro* fertilization (IVF).

Following fertilization, cumulus-oocyte complexes were moved to 200µL of 1mg/mL hyaluronidase and vortexed for 2 minutes to remove all of the surrounding cumulus cells. Denuded oocytes were then rinsed three times in Hepes-Talp, and washed through three droplets of BO-IVC medium (IVF Biosciences, product #61001) before being transferred to a fresh drop of BO-IVC under oil (BO-Oil, IVF Biosciences, product #6200) and returned to the incubator. Presumptive embryos were checked on day-2, day-4 and day-7 following fertilization and embryo development was recorded.

#### **5.2.6 RNA Sequencing**

Both GV and MII oocytes from all three collection methods were subjected to the same RNA-Seq protocols. Immediately following completion of oocyte collection (GV) or IVM (MII), oocytes were denuded of all cumulus cells by vortexing for two minutes in 200µL of BO-Wash (IVF Biosciences product #61008). Denuded oocytes were rinsed three times in BO-wash and twice in PBS+1%BSA (Appendix). They were then transferred in pools of four oocytes to 1.5mL microcentrifuge tubes, in a total volume of 2µL of PBS per tube. Oocytes were snap frozen and maintained at -80°C until completion of RNA-Seq.

Thawed oocytes were dissociated in lysis buffer and cDNA libraries were prepared using SMART-Seq v4 (Takara Bio USA) following manufacturers guidelines (Appendix) (Petropoulos et al., 2016). Following oocyte lysis, cDNA was synthesized first by ligation of the 3' primer sequence, SMART-Seq CDS primer IIA, followed by reverse transcription using SMARTScribe Reverse Transcriptase. Prepared cDNA was then amplified by 16 cycles of PCR using SeqAMP

DNA Polymerase and was purified using AMPure XP beads. Quality and quantity of cDNA was measured using Qubit 3.0 and quality was validated on an Agilent TapeStation. One hundred and fifty nanograms of cDNA was fragmented and sequencing libraries were prepared using a Nextera XT DNA library preparation kit (Illumina) and multiplexed by Nextera XT Index kit (Illumina). Finally, sequencing libraries were purified by AMPure XP beads, and quantified by Qubit 3.0 and TapeStation for measurement of concentration and size distribution. Indexed libraries were diluted to 4nM and equal, pooled samples were sequenced on an Illumina NextSeq 500 Platform at Pennington's Biomedical Research Center (Baton Rouge, LA, USA).

### **5.2.7 Data Analysis**

RNA-seq reads were trimmed and aligned to the bovine reference genome (bosTau8) using STAR (2.5.3). Differentially expressed genes between different treatment groups were determined using DESeq2 package in R 5.0. In each comparison, genes were deemed differentially expressed if they showed a false discovery rate adjusted P-value  $<0.05$  and a confident Log2-fold change (FC)  $>1$ . Gene ontology (GO) and Pathways analysis were performed using IPA (Ingenuity Pathway Analysis, Qiagen).

Embryo production rates were compared across oocyte recovery methods using chi square analysis in RStudio.

## **5.3 Results**

A total of 232 OPU, 238 FSH and 375 PM oocytes were recovered. Of which, 92 were used in pools of four, for RNA-Seq (4 replications for each treatment, 3 replications for MII PM). Three hundred and six were used in IVF, and the remaining oocytes have been maintained at  $-80^{\circ}\text{C}$  for later analysis (Table 5.2).

Table 5.2. Number of samples allocated to each treatment.

Collection Method	Germinal Vesicle	Metaphase II	Embryo Production
Oocyte Pick-up	4	4	66
Super-stimulation	4	4	80
Post-mortem	4	3	160

A total of 605.8 million raw sequencing reads that passed filtering were obtained and an average of 26.3 million read pairs per sample were generated (Appendix). Reads were largely of high quality, and in the majority of samples >90% of reads were correctly aligned to exons.

During gene expression analysis, multi-dimensional scaling (MDS) plots were created to compare gene expression patterns between all samples (Figure 5.2). When assessed in two dimensions based on the biological coefficient of variation (BCV), samples were clearly broken into two groups with different gene expression patterns between immature and mature oocytes, regardless of treatment group. Within each maturation level, differences were less notable. There was a high degree of overlap in expression patterns between immature oocytes from all recovery methods. In *in vitro* matured oocytes, the oocytes collected following FSH stimulation had a different expression pattern from oocytes of either other retrieval method, with the exception of one OPU sample that was more similar to the FSH samples. On further analysis, a heatmap was created, assessing only the ten most variably expressed genes (Figure 5.3). Of these ten genes, three are transmembrane proteins, two are involved in cytoskeleton polymer changes, one is a peptidase and one is a glutathione transferase. Based on expression of these genes, oocytes were largely clustered in groups by both maturation level and collection method, with only a few samples having expression patterns dissimilar to others within their treatment. Again IVM oocytes collected following super-stimulation were on a different hierarchical branch than the

majority of the other recovered oocytes, suggesting a high degree in difference in transcript abundance.

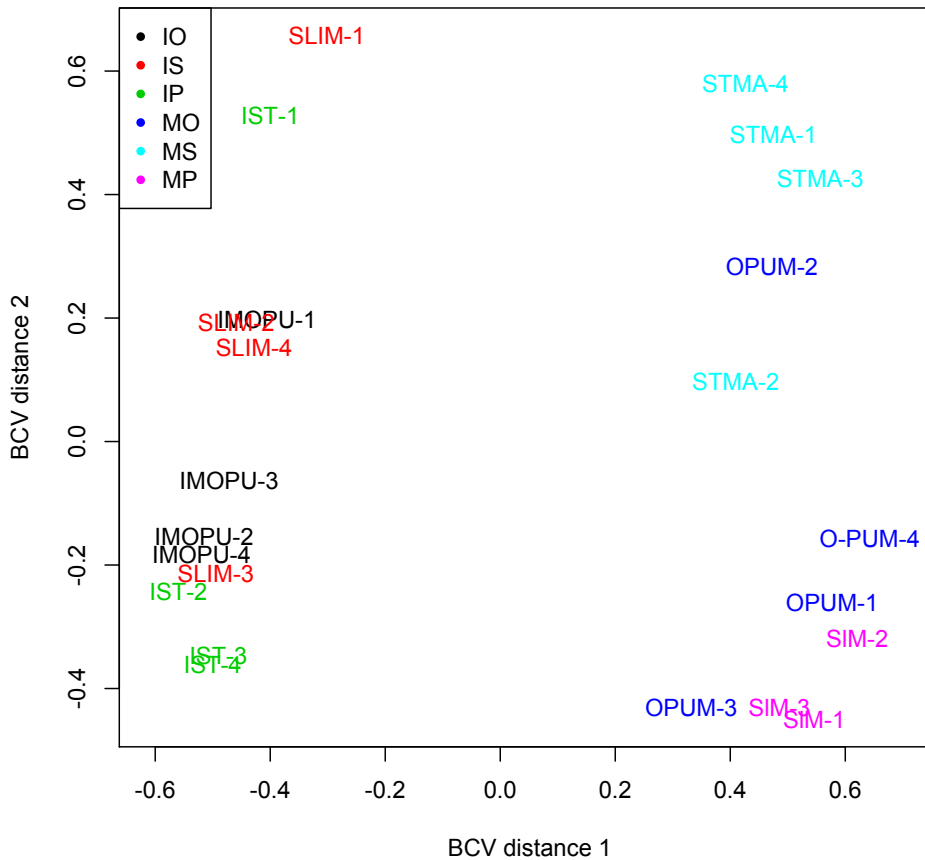


Figure 5.2. MDS plot depicting overall differences in gene expression patterns between individual samples of pooled bovine oocytes classified by oocyte recovery method and maturation level.

Oocytes within each recovery method exhibited a characteristic change in gene expression over the course of maturation. Nearly 6000 genes had altered expression between germinal vesicle (GV, immature) stage and metaphase II (MII, mature) oocytes, within each treatment, with the majority having higher transcript abundance following maturation. However, once genes were filtered based on both significance (FDR  $P < 0.05$ ) and  $> 2$ -fold difference in expression between groups, this pattern changed. Only ~2000 genes per collection method met

these qualifications, and among them there was a roughly even split between up- and down-regulation (Figure 5.4). There was a slight preference for transcript abundance to be increased in mature oocytes that was most notable in those oocytes recovered following FSH stimulation. When complete pathway analysis was performed, the pathways most affected by maturation were those involved in energy production, as was especially true of FSH oocytes.

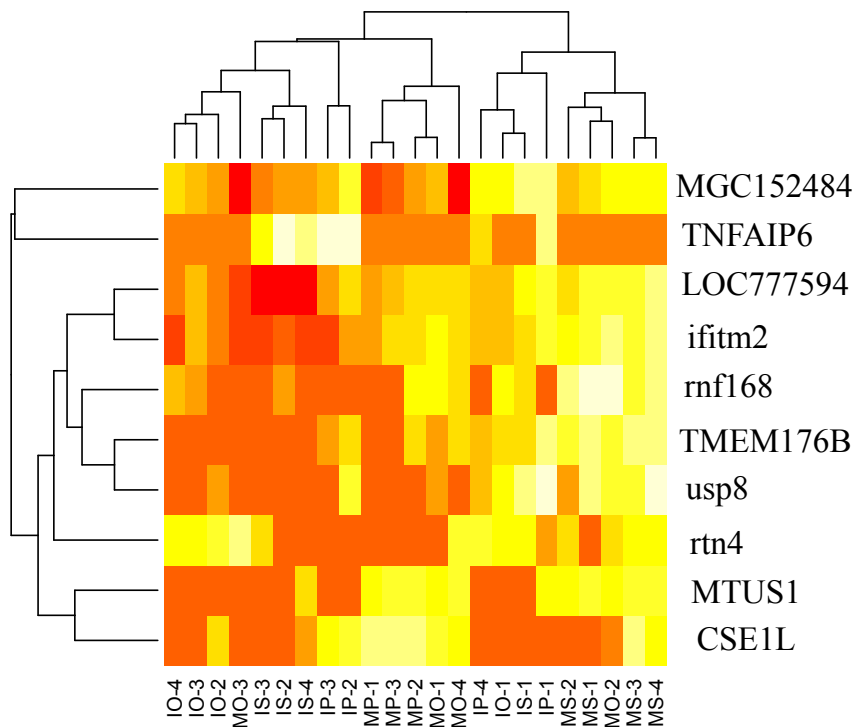


Figure 5.3. Heatmap showing relative expression levels of the ten genes that were the most variable in expression level between samples. Oocyte hierarchical clustering, shown along the top, is based on similarity on expression patterns for these ten genes. Yellow indicates low expression and Red indicates high expression.

Gene expression differences were also compared between oocytes from the different recovery methods within each maturation level. Using only those genes that had a two-fold or greater difference in expression, which were calculated as significantly differentially expressed (FDR  $P < 0.05$ ), it was found that in GV oocytes, the greatest difference was between OPU and PM

oocytes where 67 genes were up-regulated in PM oocytes compared to OPU, and 105 were down-regulated (Figure 5.5). Meanwhile, between OPU and FSH oocytes, no genes were significantly up-regulated in FSH, and only 12 were down-regulated. In the PM:FSH comparison 20 genes were up-regulated in FSH oocytes and 35 were down-regulated. Of those genes that were significantly different, the most significantly affected pathways were primarily related to the TCA cycle and ATP synthesis, GnRH receptor pathway and TGF- $\beta$  signaling. The OPU:PM comparison also showed a significant difference in the activity of multiple genes (28) regulating the zona pellucida and fertilization, all of which had decreased transcript abundance in OPU oocytes (FDR  $P < 0.05$ ). Genes regulating mitochondrial and organelle structure were found in greater abundance in OPU oocytes than FSH oocytes (FDR  $P < 0.05$ ).

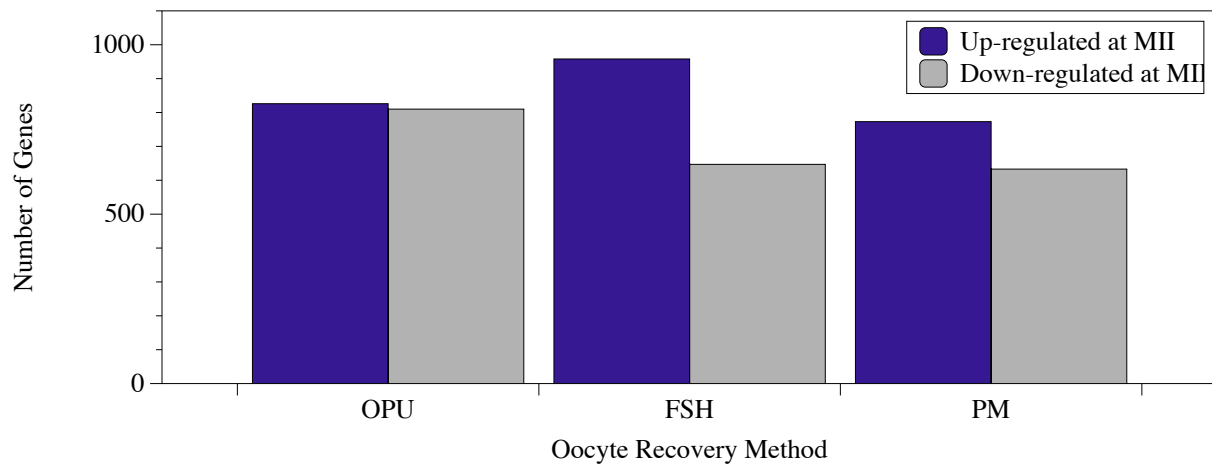


Figure 5.4. The change in gene expression within each oocyte recovery method over the course of *in vitro* maturation. Approximately 2000 genes change expression within each treatment group. In OPU oocytes there is a relatively even split between up- and down-regulated genes, while in both FSH and PM oocytes there is a greater number of genes that are down-regulated in MII oocytes.

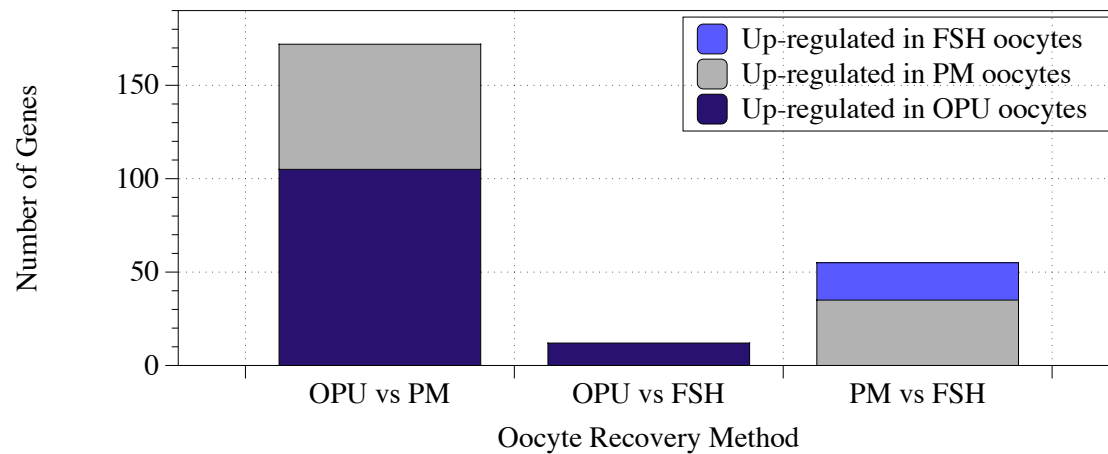


Figure 5.5. Comparison of gene expression in germinal vesicle stage bovine oocytes recovered by three different methods.

As oocytes matured, the relative gene expression patterns changed rather dramatically (Figure 5.6). In the comparison between OPU:PM oocytes the apoptosis signaling pathway was significantly up-regulated in OPU oocytes, as were genes controlling mitochondria and organelle function, and the electron transport chain. In the FSH:PM comparison there was a significant increase in the expression of genes involved in the TCA cycle, ATP synthesis, glycolysis and the pentose phosphate pathway in FSH oocytes. Similarly, in the comparison between FSH and OPU oocytes, FSH oocytes had increased transcript abundance for genes controlling the TCA cycle, ATP synthesis, cellular metabolism and the cell cycle but decreased abundance of transcripts involved in control of organelle structure and function (FDR  $P < 0.05$ ). In all, only 46 genes had significantly different expression between OPU and PM oocytes (13 up-regulated and 33 down-regulated in PM oocytes), while 296 genes had significantly different expression between OPU and FSH oocytes (269 up-regulated and 27 down-regulated in FSH), and 307 were differentially expressed between PM and FSH (305 up-regulated and 2 down-regulated in FSH).

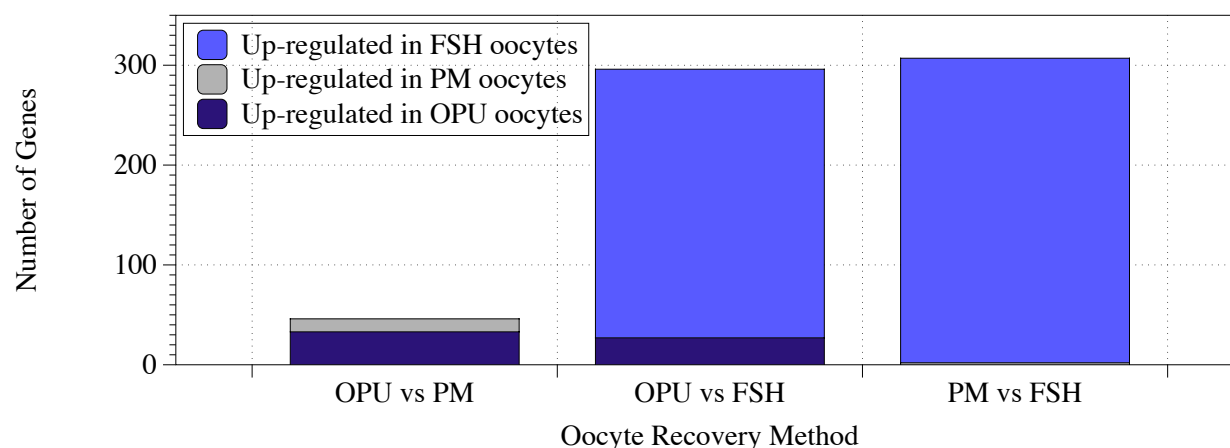


Figure 5.6. Comparison of gene expression in metaphase II bovine oocytes recovered by three different methods.

Embryo cleavage rates were relatively low especially in OPU oocytes, ranging from 11% to 29% of oocytes (Table 5.3). Following cleavage, the majority of embryos in FSH and PM recovery groups continued to develop to the morula stage, while OPU oocytes had decreased developmental rates, with a high proportion being arrested at the 2- and 4-cell stages (5/7 arrested, compared to only 3/20 for FSH and 8/46 PM). There was a significant difference in embryo development rates ( $P < 0.01$ ) at the morula stage, with embryo development being decreased in OPU oocytes, compared to those retrieved by other means. On day-7 when blastocyst rate was recorded, there was a significant effect of recovery method on the blastocyst rate, although development rates were low across the board, ranging from 2% from OPU to 14% from FSH and 17% from PM oocytes ( $P < 0.01$ ). However, when blastocyst development rate was calculated as the proportion of cleavage stage embryos that continued to develop, the recovery method was found to have a more notable effect. With only 14% of cleaved zygotes developing to blastocysts following OPU, but 55% following FSH and 59% following PM retrievals,

conventional OPU was found to have significantly decreased embryo production rates (P=0.0067) than either of the other two retrieval methods.

Table 5.3. Embryo production rates as affected by bovine oocyte retrieval method. Superscripts denote significant differences between values within a column (P=0.0067).

Collection Method	n	Cleavage (%)	Morula (%)	Blastocyst (%)	% Blastocysts by Cleaved
Oocyte Pick-up	66	7 (11%)	2 (3%)	1 (2%)	14% <sup>A</sup>
Super-stimulation	80	20 (25%)	17 (21%)	11 (14%)	55% <sup>B</sup>
Post-mortem	160	46 (29%)	38 (24%)	27 (17%)	59% <sup>B</sup>

## 5.4 Discussion

There have been discrepancies in the literature on the effect of follicle atresia on oocyte competence, with reports suggesting atresia could have anywhere from a positive effect, or no effect to a strongly negative one (Blondin and Sirard, 1995). However, while follicle atresia has not been found to be conclusively correlated with oocytes of decreased competence, oocytes collected during the follicular growth phase, when atresia is not present, have been shown to result in more competent oocytes and production of a greater number of overall high quality blastocysts (Hagemann et al., 1999). One suggestion from previous studies is that collecting oocytes from early atretic follicles results in the highest blastocyst production rate, while collection from late atretic follicles yields oocytes of decreased competence (Moor and Trounson, 1977; Blondin and Sirard, 1995). The premise that early atresia can be beneficial has been seen in collecting both post-mortem oocytes and those following super-stimulation. During the period between cow death, harvesting ovaries and recovering oocytes from the ovaries, physiological processes mimicking atresia occur, having a beneficial effect on oocyte competence for the first four hours post mortem and an increasingly negative effect thereafter

(Blondin et al., 1997). In ovum pick-up following super-stimulation, it is common to allow a coasting period following the last FSH injection prior to oocyte collection. This causes the follicles to undergo FSH starvation and to initiate atresia, improving both oocyte competence and yield for the first 48-60 hours of coasting and resulting in declining developmental competence thereafter.

In the current study, both FSH super-stimulation and post-mortem oocyte collection resulted in increased embryo production compared to oocytes collected following conventional OPU, in agreement with this theory. However, de Witt et al. (2000) show evidence that the reasoning behind this apparent improvement in competence is that a greater proportion of oocytes collected from these follicles had a strong cumulus cell investment, perhaps because the atretic cells of the follicle had more easily loosened connections with the cumulus-oocyte complexes (COCs). Oocytes collected via OPU during the current project had a decreased cumulus investment, resulting in an overall decreased grade compared to oocytes recovered by other methods (77/232, 136/228 and 360/375 “A” grade oocytes from OPU, FSH and PM, respectively). Furthermore, perhaps because of the poor cumulus cell support, oocytes collected by OPU showed the poorest maturation rates. It is possible that this is the primary reason for failed fertilization in the OPU group oocytes presented above, supporting the hypothesis that the ease of removing highly competent COC’s from early atretic follicles may play a major role in improving oocyte developmental competence.

It was found that genes involved in energy production and mitochondrial function were among those most differentially expressed between the different treatments, especially following *in vitro* maturation. It is accepted that oocyte energy production is important to early embryo

survival, and that irregularities in oocyte mitochondrial function can lead to decreased developmental rate. In addition, the genes involved in metabolism have been shown to be amongst those, most differentially expressed between fast and slow developing blastocysts, indicating that there is significant correlation between the ability of an embryo to produce energy and its developmental potential (Ispada et al., 2018). Although by the blastocyst stage, the genes involved in energy production are of the embryonic genome, this is indicative of the importance of energy production to embryo development and the issues of delayed development that may occur as a result of even minor aberrations. The design of this study did not allow for comparison to *in vivo* matured oocytes to determine which had the most normal gene expression patterns but the sheer frequency of differences of metabolic gene expressions between oocytes at the same maturational levels is indicative that the different follicular dynamics and handling methods involved in the different oocyte retrieval methods are causing metabolic stress on oocytes. The fact that these differences are maintained, despite oocytes being matured in similar environments, indicates that the alterations in gene expression are lasting and may have long-term implications in embryonic and fetal development.

One study assessing the differences between immature and *in vitro* matured oocytes found that there were a total of 2117 differentially expressed genes, of which 1528 were down-regulated following maturation, and 589 were up-regulated (Mamo et al., 2011). Of these, the majority of the over-expressed genes were involved in pathways related to cell growth and development, molecular transport, protein synthesis and embryo development (Mamo et al., 2011). Other studies have corroborated the effects of IVM on altering oocyte gene expression, interfering with pathways involved in meiotic and mitotic progression and embryonic

development (Eppig et al., 1994; Trounson et al., 2001; Combelles et al., 2002). In comparison to the present study, in which all oocytes were matured *in vitro* but were recovered through different methods, the most frequently aberrantly expressed gene pathways were primarily related to metabolism, and mitochondrial function. However, few other studies have been published looking at similar effects to act as direct comparisons to this work. Results of the current study are in agreement with past studies in that there is a high degree of change in gene expression between GV and MII oocytes. It is generally expected that the majority of genes should be down-regulated over the course of maturation. The lack of genome silencing that was seen in OPU oocytes in this study may be an indication that either oocytes were of poor developmental competence or that molecular maturation was incomplete. This is in agreement with the poor embryonic development that was seen in oocytes, especially those recovered by conventional OPU.

Initially, in GV oocytes both OPU and FSH oocytes were found to have remarkably similar gene expression to each other, suggesting that the stimulation protocol had no major effects on molecular development within oocytes. However, PM and OPU oocytes had different expression patterns, with post-mortem collected oocytes having more active expression than those collected via OPU. It is possible that the higher degree of metabolic activity in oocytes collected post-mortem allows for increased energy stores, improving embryo competence even if the genes are later silenced. It is believed that some of the proteins required for GVBD are only synthesized *in vivo* just prior to ovulation, and are not present in antral follicles 2-8 mm (Bilodeau-Goeseels, 2012). However, many other proteins and mRNAs required for embryonic development are acquired prior to oocyte maturation, during oocyte growth and folliculogenesis

(Minami et al., 2007; Wrenzycki et al., 2007) and so should not differ in expression levels between highly competent immature and mature oocytes. However, because there was less control in the size of follicles collected by OPU than by either of the other two methods, it is possible that OPU oocytes would have different gene expression patterns by not having completed this stage of oocyte development, and that this difference is not necessarily indicative of competence.

By completion of MII the pattern of gene expression differences between the collection methods had changed notably from that seen in immature oocytes. In mature oocytes, OPU and PM oocytes had similar gene expression levels, while FSH oocytes were significantly more actively transcribing genes than oocytes from either of the other two collection methods, with over 200 genes over-expressed compared to each of OPU and PM recovered oocytes. Since MII oocytes are accepted as being transcriptionally quiet compared to GV oocytes, this high level of gene expression in FSH oocytes could be indicative of failure to complete molecular maturation. However, an alternate theory is possible. RNA sequencing is based on the presence of mRNA transcripts, which are normally short-lived within somatic cells. However, in oocytes mRNA is produced during folliculogenesis and maturation and is then stored for a period of days to support life-sustaining functions in the early embryo, prior to the initiation of transcription of the embryonic genome (Sirard et al., 2006a; Watson, 2007). Based on this premise, it is feasible that oocytes of the highest competence, which have successfully completed meiotic maturation, will have large mRNA stores. It is possible that these oocytes will artificially appear to be actively transcribing genes that instead have mRNA stored from prior transcription. In this case apparent gene up-regulation could be an indication of competence in MII oocytes, a theory that is

corroborated by the fact that FSH oocytes that had the highest gene expression levels at MII also had the highest embryo production rates.

A third possibility to specifically explain the increased abundance of transcripts involved in energy regulation found in oocytes recovered following super-stimulation is the concept that highly competent, and perhaps also highly competent oocytes, undergo a shift in glucose metabolism away from the tricarboxylic acid cycle (TCA) and towards the pentose phosphate pathway (Richard and Sirard, 1996; Krisher and Prather, 2012). This metabolic shift coupled with overall increased glucose metabolism, mimicking the Warburg effect, could increase the production of nucleic acids and fatty acids, both necessary for the rapid cell division and development which occur during early embryogenesis (Wakefield et al., 2011). Previous studies have shown the benefit of cells exhibiting the Warburg effect towards early embryo development, and since blastomeres at this stage of development are transcriptionally quiescent, it is reasonable that increased transcription of genes involved in these processes would be necessary prior to the completion of oocyte maturation. As such, it is possible that the oocytes recovered in this study, following super-stimulation may be exhibiting increased transcript abundance in accordance with the Warburg effect and so may be of higher developmental competence than oocytes recovered by either unstimulated OPU or those retrieved post-mortem. Further studies are warranted to determine the metabolic activity and lasting effects of any differences found in these super-stimulated oocytes.

In all, it is evident that, be it beneficial or detrimental, oocytes recovered following super-stimulation have high levels of RNA transcripts present at the MII stage that are not present in MII oocytes collected post-mortem or by unstimulated OPU. These oocytes, however, have a

very similar gene expression profile at the GV stage to oocytes collected by OPU. This suggests that if stimulation has an effect on oocyte genome regulation, it is one that is more visible later in development though potentially with lasting metabolic effects on the fetus. Meanwhile, oocytes recovered post-mortem have only slightly irregular gene expression at the GV stage, but this effect does not seem to be permanent and oocytes exhibit transcript profiles similar to those in oocytes retrieved by OPU following *in vitro* maturation.

## **CHAPTER VI**

### **THE EFFECT OF BOVINE OOCYTE RECOVERY METHOD ON OOCYTE ULTRASTRUCTURE**

#### **6.1 Introduction**

*In vitro* maturation supports meiotic maturation of oocytes but is often reported as poorly stimulating cytoplasmic maturation, resulting in mature oocytes with poor developmental competence (Eppig et al., 1994; Combelles et al., 2002; Salamone et al., 2003; Schramm et al., 2003; Jimenez-Macedo et al., 2006). In addition to the risk of *in vitro* maturation causing aberrations in cytoplasmic maturation, there is also the possibility that superstimulation can be disruptive to oocyte development, causing abnormal organelle behavior (Lee et al., 2006; Zeng et al., 2009; De los Reyes et al., 2011). The accelerated growth rates imposed on follicles by high doses of follicle stimulating hormone cause aberrations in the external hormonal milieu in which oocytes are developing. This results in accelerated oocyte development at the risk of missed developmental steps and the accrueement of abnormalities, most especially amongst the cytoplasmic changes that occur during development in preparation for oocyte cytoplasmic maturation. This can be largely detrimental to embryo production rates as the reorganization of organelles and storage of mRNA, proteins and transcription factors involved in cytoplasmic maturation ensures the proper distribution and cytoplasmic dynamics necessary to activate the oocyte for fertilization and to support embryonic development (Sirard et al., 2006b; Watson, 2007). Meanwhile, follicular atresia, such as that seen in ovaries collected post mortem, has been shown to be potentially beneficial to oocyte competence, but the mechanism and the effects on oocyte cytoplasmic maturation ability are as of yet undetermined (Blondin and Sirard, 1995; Blondin et al., 1997).

The importance of completed cytoplasmic maturation for oocyte competence has been well established and has been broken down into three distinct aspects; molecular maturation, and the reorganization of cytoskeletal polymers and of the organelles. However, many aspects of cytoplasmic maturation have proven difficult to characterize, and among those that are well characterized the relative importance of each factor is uncertain. Although many of the differences that occur in *in vitro* matured oocytes compared to their *in vivo* matured counterparts are known, the effects that the different oocyte recovery methods have on these differences are as yet unclear. Since it is believed that follicular environment plays a significant role in driving oocyte development, it stands to reason that oocytes collected from under the influence follicles with different, unnatural, dynamics may have notable differences in their own developmental characteristics.

The current study was designed to use transmission electron microscopy to examine the effect of three major bovine oocyte retrieval methods (traditional OPU, super-stimulation followed by OPU, and oocyte recovery post-mortem) on the ultrastructure of immature oocytes. The ability of oocytes recovered from each method to complete cytoplasmic maturation *in vitro* will then also be assessed and compared, assessing the same cytoplasmic parameters. Although this study was designed to be descriptive in nature, it was hypothesized that 1) organelle distribution and reorganization would follow patterns characteristic of bovine oocytes. 2) Super-stimulation would result in irregularities in oocyte ultrastructure compared to other oocyte recover methods.

## **6.2 Methods**

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

### **6.2.1 Animals**

All procedures in this experiment were approved by the Louisiana State University Agricultural Center's Institutional Animal Care and Use Committee and were carried out at the LSU Reproductive Biology Center, St. Gabriel, LA, USA. Thirty mixed breed beef cows, housed on pasture with ad libitum access to mixed native grasses supplemented with hay and full access water were used for oocyte collections through October and November of 2017.

### **6.2.2 Experimental Design**

Oocytes were collected via three different collection methods in an attempt to determine if the oocyte source affects the ultrastructure of oocytes. Because they are the three most commonly employed methods of oocyte retrieval in bovine IVP research, oocytes were collected from live cows via ovum pick-up either at random time points in the estrous cycle (OPU), or following a super-stimulation protocol (FSH), and oocytes were retrieved post-mortem from abattoir derived ovaries (PM). Recovered oocytes from all three treatments were then randomly allocated to one of two treatment groups, where they were either fixed immediately at the germinal vesicle (GV) stage, or matured *in vitro* to the metaphase II (MII) stage and then fixed. All groups of fixed oocytes were subjected to the same protocols and were analyzed by transmission electron microscopy to determine ultrastructural differences between groups. This study was specifically designed to analyze differences in cortical granule location, mitochondrial structure and location, lipidation levels, and vesicle locations and amount.

This project was designed as a preliminary, descriptive study only.

### **6.2.3 Super-stimulation and Oocyte Retrieval**

Cows assigned to super-stimulation were subjected to a dominant follicle removal (DFR) where all follicles greater than 6mm in diameter were ablated, using the same methods described below for transvaginal ultrasound-guided follicle aspiration (TUGA). Super stimulation followed the same protocols as those outlined in section 5.3.3. A CIDR<sup>®</sup> (Zoetis Laboratories, USA) was inserted at the time of DFR and beginning 36 hours later, cows were administered Folltropin<sup>®</sup> (Bioniche Animal Health, Athens, GA) im every twelve hours for a total of six doses (200mG). Twenty-four hours after the last FSH injection CIDRs were removed, and oocyte retrieval was performed 24 hours after CIDR removal, allowing a 48-hour coasting period between the final FSH injection and oocyte recovery.

Oocytes collected via either stimulated OPU or traditional OPU were recovered as cumulus-oocyte complexes (COCs), from live cattle using TUGA, using methods described above in section 3.3.3. Cows were restrained in a manual squeeze chute, administered 30 mg xylazine intramuscularly and given a 5mL, 2% lidocaine epidural. Ovaries were palpated rectally and held against the vaginal wall so follicles could be visualized on ultrasound and aspirated using the aide of an ultrasound probe that had been inserted vaginally. Once visualized, all follicles greater than three mm in diameter were aspirated. Follicular fluid was aspirated directly into a 50mL Falcon<sup>™</sup> tube that had been primed with collection medium. The aspiration system was rinsed with collection medium after each ovary to prevent oocytes from being held in the aspiration lines. Once recovered, oocytes were placed in HEPES-TALP at 38.5°C until further

processing. The total number of oocytes recovered was recorded as well as their visible grade, based on cumulus cell enclosure and cytoplasm appearance (Appendix).

For post-mortem collection of oocytes, ovaries were harvested at a local abattoir following slaughter of cows. They were stored in an insulated cooler to maintain temperature for a maximum of one hour before being transported 15 minutes to the laboratory. On arrival at the laboratory, ovaries were trimmed and rinsed three times with warmed saline. All visible follicles were then aspirated by inserting an 18-gauge, 1.5 inch needle connected to a vacuum line. Lines containing follicular fluid were rinsed with Dulbecco's phosphate buffered saline to ensure oocytes were recovered from the collection lines. Once recovered oocytes were rinsed and moved directly to treatment.

#### **6.2.4 Oocyte Maturation**

Oocytes from both OPU and super-stimulation collection methods (that were assigned to treatment requiring maturation) were rinsed three times in IVM medium (Appendix) before being transferred in groups of 10-12 to fresh 35 $\mu$ L droplets of IVM medium under oil. They were then cultured for 22 hours in a humidified incubator at 38.5°C and 5% CO<sub>2</sub>.

Post-mortem recovered oocytes were purchased from a commercial source and shipped for further processing. Those assigned to treatments requiring maturation were rinsed three times before being transferred in groups of 50 to a Falcon<sup>TM</sup> tube containing 2mL of IVM medium, that had been gassed and equilibrated under 5% CO<sub>2</sub>. They were then transported overnight in a portable incubator (MiniTube) and maintained at a constant temperature of 37°C. On arrival in the laboratory, they were transferred to a humidified incubator at 38.5°C, under 5% CO<sub>2</sub> for the remainder of the 22 hour maturation period.

Following the completion of maturation, oocytes from all treatment groups were washed in BO-Wash (IVF Biosciences product #61008) before being vortexed in 200 $\mu$ L of BO-wash to remove cumulus cells. Oocytes were rinsed two times in fresh BO-wash and two times in 0.1M phosphate buffer (Appendix) before beginning fixation for electron microscopy.

### **6.2.5 Fixation and Electron Microscopy**

Oocytes were fixed and prepared for transmission electron microscopy following minimally modified methods previously published (Appendix) (Hyttel et al., 1986; Hyttel and Madsen, 1987). Oocytes were initially fixed in a 3% glutaraldehyde solution before being embedded individually in 2 mm cubic blocks of 4% agar. They were then post-fixed 1% osmium tetroxide for one hour at room temperature, before being washed and stained for one hour in 0.5% uranyle acetate in the dark, at room temperature. Oocytes were washed twice in water before being dehydrated in five step-wise increasing concentrations of ethanol from 50% to 100%. Alcohol was removed from blocks by incubation in propylene oxide prior to embedding in epon-araldite resin. Epon polymerization was gradual over three days with a 24-hour incubation at each of 24°C, 37°C and 60°C. Following polymerization, resin blocks containing the oocytes were trimmed and oocytes were cut into ultra-thin sections that were stained with uranyle acetate and lead citrate. Sections were mounted on copper grids and examined on a Transmission Electron Microscope (JEOL 2011 TEM, JEOL USA, Peabody, MA, USA, equipped with a Gatan SC10000 CCD camera, Gatan Inc, Warrendale, PA, USA).

## **6.3 Results**

A minimum of two oocytes were analyzed from each treatment group, and a minimum of three sections of each oocyte was assessed at each two different levels within the ooplasm.

Results within an oocyte did not appear to show marked differences between imaging sites with regard to organelle and oolemma characteristics of note. However there were some apparent differences between both collection methods and maturational status (Table. 6.1).

In the majority of oocytes, microvilli densely covered the extracellular surface of the oolemma, projecting towards the zona pellucida. In oocytes collected via OPU, both immature and mature oocytes had a large number of long, thin microvilli, relatively evenly distributed over the oolemma. Oocytes collected from post mortem ovaries and from OPU following super-stimulation exhibited similar patterns among the microvilli of matured oocytes as that seen in OPU oocytes. However, immature oocytes collected via these methods both had a decreased density of microvilli, and those microvilli that were present appeared to be shorter and found only in clusters on the oolemma.

Cortical granules were found more frequently in *in vitro* matured oocytes than in immature oocytes, regardless of retrieval method employed. However, in both methods of recovery from live cattle, even immature oocytes had sparse cortical granules present. In matured oocytes recovered from abattoir-derived ovaries, or following traditional OPU, cortical granules were found in a single, relatively continuous row directly below the oolemma (Figure 6.1). However, oocytes collected following stimulation exhibited an aberrant pattern, whereby immature oocytes presented a single sub-oolemmal layer of cortical granules, but mature oocytes were found instead to have peripheral clusters of cortical granules (Figure 6.2).

Table 6.1. Ultrastructural differences observed in immature and mature bovine oocytes collected through different retrieval methods.

Characteristic	Immature Oocytes			Mature Oocytes		
	OPU	PM	Stim	OPU	PM	Stim
<b>CG Number</b>	Rare	None	Rare	Few	Many	Rare
<b>CG Distribution</b>	Peripheral clusters	—	Suboolemma layer	Suboolemma layer	Suboolemma layer	Peripheral clusters
<b>Lipid Density</b>	Rare	Rare	Some	Few	Few	Some
<b>Mitochondrial Density</b>	Low	High	Moderate	Moderate	Moderate	High
<b>Mitochondrial Distribution</b>	Peripheral or diffuse	Peripheral or diffuse	Clusters	Diffuse or clusters	Clusters	Peripheral or clusters
<b>Mitochondrial Association</b>	Strong	Strong	Strong	Strong	Medium	Strong
<b>Oolemma Microvilli</b>	Long, thin	Few and short	Few, variable length	Long, thin	Long, thin	Long, thin
<b>Vesicle Density</b>	Moderate	High	High	Low	Moderate	Moderate

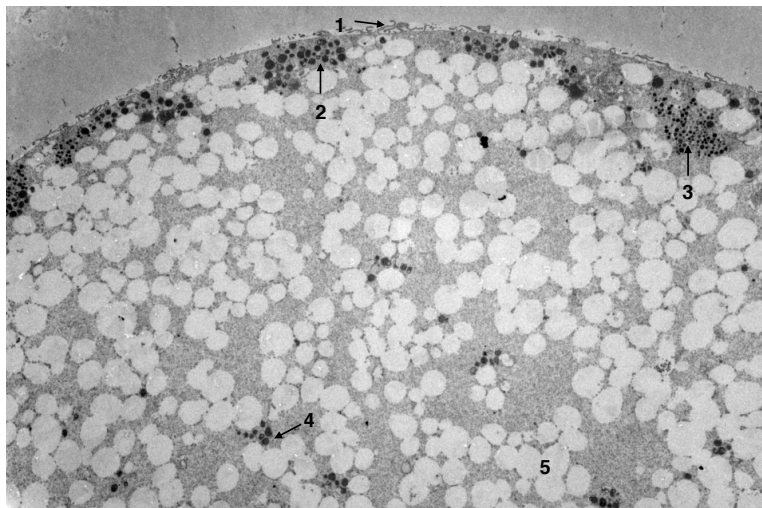


Figure 6.1. Transmission electron microscope image of an immature bovine oocyte. 1. Micovilli are long and thin but are relatively sparse. 2. Mitochondria are found primarily in the peripheral region of the ooplasm. 3. Cortical granules are present in large clusters in the peripheral region of the ooplasm. 4. Few mitochondria are scattered in small clusters throughout the cytoplasm. 5. Vesicles occupy the vast majority of the oocyte volume.

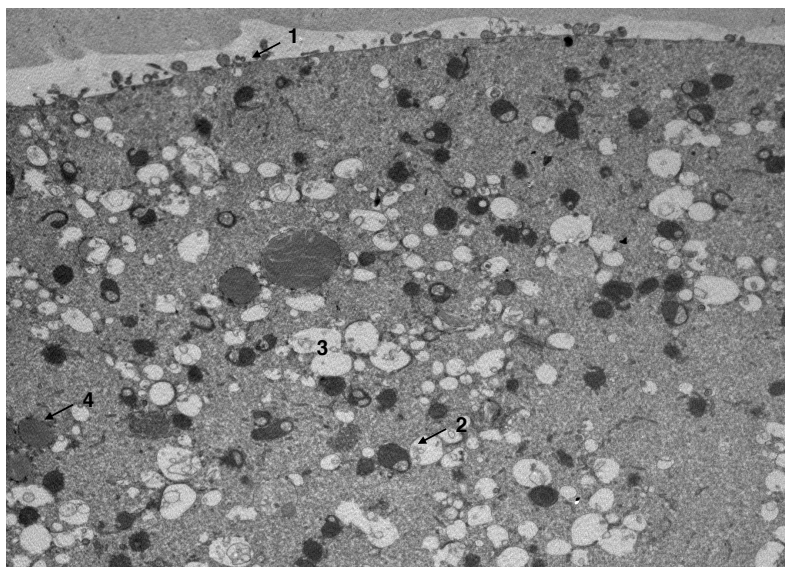


Figure 6.2. Transmission electron microscope image of an immature bovine oocyte collected post mortem. 1. Microvilli are sparse and those present are short. 2. Mitochondria are distributed evenly throughout the ooplasm. 3. Vesicles are present and many are in close proximity to mitochondria. 4. Lipids are present but do not occupy a large volume.

While lipid concentrations were low in most oocytes, they were slightly more abundant in matured oocytes. Where present, lipids were found in close association with mitochondria, often as mitochondria-lipid clusters (Figure 6.3). Vesicles were also frequently found in close proximity with mitochondria. However, unlike lipids, vesicles were abundant in oocytes, occupying the vast majority of the ooplasm. This was especially true in immature oocytes, regardless of recovery method.

Mitochondria were less abundant than expected across all recovery methods, and this did not change with maturation. Overall, mitochondrial numbers appeared to be the lowest in oocytes collected by unstimulated OPU. In addition to frequently being found in association with lipids and vacuoles, there were three primary mitochondrial distribution patterns found among the oocytes. In immature oocytes collected from live cattle and matured oocytes collected post

mortem, mitochondria were frequently found to be either primarily or exclusively in the peripheral region (Figure 6.1). Immature post-mortem collected oocytes and *in vitro* matured oocytes recovered by all three methods frequently had mitochondria found in large clusters throughout the cytoplasm, most often in association with vacuoles, lipids and endoplasmic reticulum (Figure 6.3). Finally, select few immature oocytes collected via OPU, both with and without stimulation, exhibited mitochondria in a diffuse distribution with even distribution throughout the cytoplasm (Figure 6.2). This diffuse pattern was also found in two of the oocytes collected by unstimulated OPU following IVM.

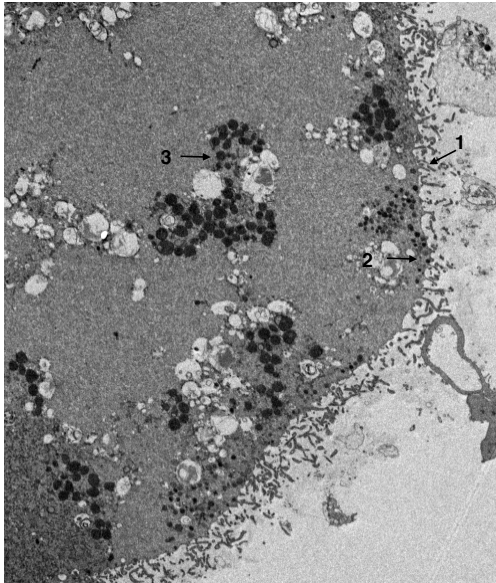


Figure 6.3. Transmission electron microscope image of an *in vitro* matured bovine oocyte. 1. Microvilli are long and thin, evenly distributed over the oolemma. 2. Cortical granules are present as a single sub-olemmal layer. 3. Mitochondria are found in large clusters within the ooplasm, primarily in association with vacuoles and lipids.

In oocytes of all collection methods, and both maturation levels mitochondria were primarily found to be in the hooded configuration. However, mitochondrial populations within each individual oocyte were highly heterogenous (Figure 6.4). As anticipated, there were a

combination of hooded mitochondria, and those more typical, spherical mitochondria. Spherical mitochondria were found with transverse cristae and also with only peripheral mitochondria. Similar in structure to the hooded mitochondria were the visualized vacuolated mitochondria and ring- or cup-shaped mitochondria. Finally, a portion of the mitochondria found were of irregular shapes, primarily with peripheral cristae only but otherwise lacking vacuoles to explain their misshapenness. There was no apparent pattern discovered for any one mitochondrial shape to be more prevalent in one treatment group. Of the irregularly shaped mitochondria were a small subset that appeared to be budding or undergoing asymmetrical division (Figure 6.5). These mitochondria were found in immature oocytes collected via both stimulated and unstimulated OPU and in *in vitro* matured oocytes that had been collected by OPU, but not in oocytes collected post mortem.

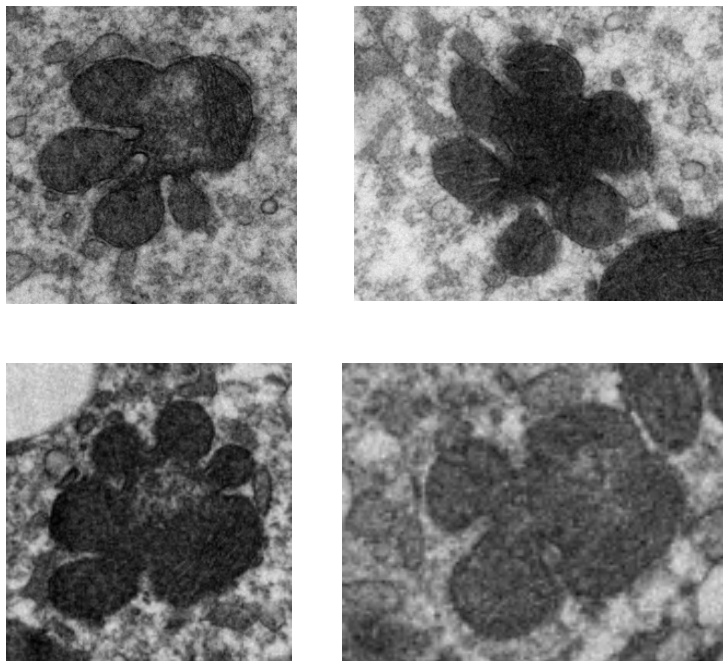


Figure 6.4. Mitochondrial populations within oocytes were highly heterogenous. 1. Ring-shaped mitochondria. 2. Hooded mitochondria. 3. Irregularly-shaped mitochondria. 4. Spherical mitochondria with peripheral cristae only. 5. Vacuolated mitochondria. 6. Spherical mitochondria with transverse cristae.

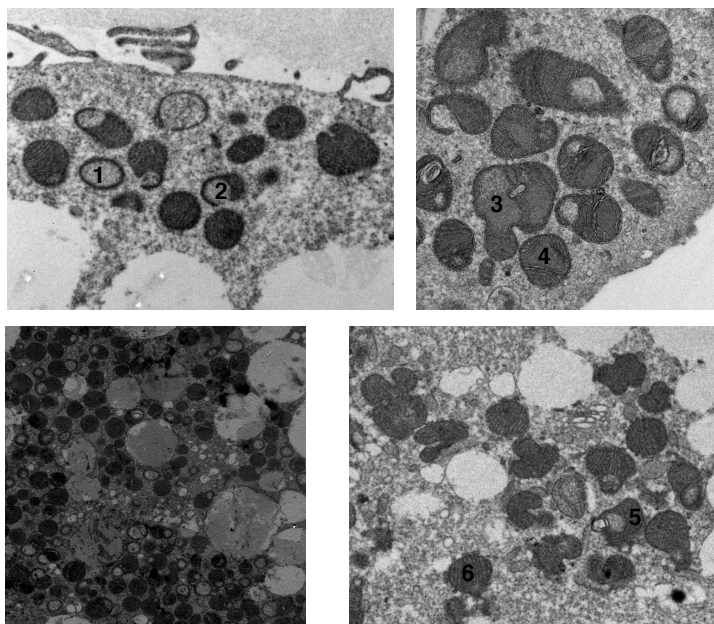


Figure 6.5. Transmission electron images of budding-type mitochondria found in bovine oocytes collected by both stimulated (GV) and unstimulated (GV and MII) OPU.

Cumulus cells had a very different ultrastructure from that of the oocytes (Figure 6.6). Mitochondria were found in high concentrations and were all oblong with transverse cristae. A high proportion of the cumulus cell cytoplasm was occupied by lipid, though it was not particularly associated with mitochondria. Finally, though Golgi networks occupied a minor portion of the ooplasm, they constituted a large portion of the volume of the cumulus cells. Golgi networks were particularly prevalent in the periphery of the cumulus cells.

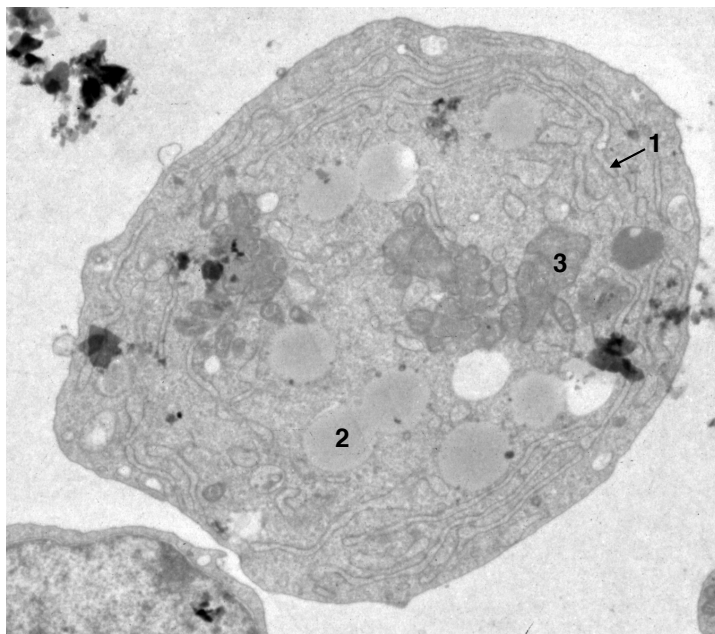


Figure 6.6. Transmission electron microscope image of a cumulus cell found in conjunction with a mature bovine oocyte. 1. Golgi networks are extensive and found peripherally. 2. Lipids occupy a relatively large proportion of the cell volume. 3. Mitochondria are found clustered, are ovoid with transverse cristae.

#### 6.4 Discussion

Transmission electron microscopy allows the benefit of defining multiple aspects of the oocyte's ultrastructure simultaneously, without the limitation of interfering wavelengths from multiple stains. This allows us to gain the most possible information on the organelles and how their interactions with each other may be mediated by different treatments imposed on the oocytes. Analyses in this regard found that while oocytes collected from all three recovery methods had similarities to each other and to the expected ultrastructural organization, there were some key differences observed.

Although immature PM oocytes were the only ones in which cortical granules (CGs) were not present, with regards to CG parameters FSH oocytes were discordant with oocytes of other treatment groups. Cortical granules develop from the Golgi network during oocyte

development and maturation and are normally initially found diffusely throughout the cytoplasm but in MII oocytes CGs should be found in one or two rows immediately below the oolemma and form a near-continuous layer due to their density (Hosoe and Shioya, 1997; Shahedi et al., 2013). This pattern held for oocytes recovered by OPU and post-mortem with density increasing over maturation concurrent to peripheral migration. However FSH oocytes were discordant in both regards. Mature oocytes assessed had no more CG visible than their immature counterparts and while CG were found peripherally in immature FSH oocytes, they were diffuse in the cytoplasm at MII.

As an oocyte matures, mitochondria migrate from peripheral distribution to a general dispersed distribution (Hyttel et al., 1986) before becoming granulated and finally clustered in areas requiring high ATP levels (Wang et al., 2009; Matchatkova et al., 2012). One characteristic of oocyte maturation is that mitochondria migrate to areas of high energy consumption to improve efficiency (Mau et al., 2014). Oocytes in this project, both within and between groups, were highly variable in regards to mitochondrial location. The general pattern exhibited, with the exception of FSH oocytes, was for peripherally located mitochondria to be most frequently seen in immature oocytes and for mitochondrial clusters to be more common following maturation. Oocytes recovered following super-stimulation displayed mitochondrial clusters in both GV and MII stage oocytes, suggesting a higher degree of mitochondrial maturation was possible during follicular growth. However, the fact that oocytes with peripherally located mitochondria were only found in MII oocytes of the FSH group indicates that this advanced development is not pervasive, and in fact it is likely that mitochondrial development is highly variable within these oocytes.

It is anticipated that both lipid and vesicle concentrations increase over the course of maturation in healthy oocytes, and both should be found in complexes with mitochondria (Thibault et al., 1987; Shahedi et al., 2013). Vesicle numbers, in particular, are high in *in vitro* matured oocytes when compared to *in vivo* matured oocytes (Thibault et al., 1987). Although lipid levels were low in all oocytes assessed in this project, this pattern did hold true, though less notably for FSH oocytes. Vesicles occupied the vast majority of the oocyte volume in most oocytes. However, oocytes from all retrieval methods had the highest vesicle volume prior to maturation. This suggests that the Golgi network had already been largely dissociated by the time oocyte retrieval was performed, which was corroborated by the fact that few Golgi apparatuses were noted. Lipid was exclusively found in mitochondria-lipid aggregates, suggesting metabolically active oocytes. High metabolic activity may be one explanation for low lipid levels visible, if oocytes had already metabolized lipid stores. However, low lipid levels before fertilization indicate poor competence in bovine oocytes since lipid stores are heavily used for energy production of embryos. Mitochondria-vesicle aggregates were also commonly found, especially in oocytes with clustered mitochondria, where the clusters would often form around vesicles.

As mitochondria aggregate into larger clusters, especially those surrounding lipids, ATP production is increased (Yu et al., 2010). This was commonly seen in oocytes here, especially following maturation, indicating high ATP production of MII oocytes from all recovery methods. However, it is believed that stimulation results in oocytes with impaired mitochondrial function (Mau et al., 2014). While mitochondrial activity was not measured in this study, both the quantity of mitochondria and the mitochondrial structures were in line with those seen in oocytes from

other collection methods. However, mitochondria in this project did show some structural differences that have not been described in the bovine before, which may be indicative of irregular function. Previous work has reported that hooded, vacuolated and ring-shaped mitochondria, as were seen in the current work, are frequent in bovine oocytes, in addition to the more normal spherical mitochondria (Hyttel and Madsen, 1987; Reader et al., 2017). It is probable that the ring-shaped, vacuolated, and some of the irregularly shaped oocytes are in fact hooded mitochondria that have been transected at different angles and planes of the mitochondrion (Senger and Saake, 1970). As such, it is likely that most of the mitochondria viewed in the current study, were actually hooded mitochondria. The hood is believed to be a method of increasing mitochondrial surface area to increase energy production (Senger and Saake, 1970). This would suggest that oocytes containing increased proportions of hooded or irregularly shaped mitochondria may be of greater competence than those containing primarily spheroid mitochondria. Oocytes in all treatments in the current study were found to contain a large proportion of hooded mitochondria, but the irregularly shaped mitochondria were most frequent in super-stimulated oocytes. This may be a factor of bisecting hooded mitochondria at different planes, or it may be a function of the exogenous hormones disrupting the oocyte growth phase and disturbing mitochondrial development and structure.

Some of those irregularly shaped mitochondria, found in both immature and mature oocytes collected from live cattle only, appeared to be in the process of budding. As mitochondria do undergo a massive increase in quantity during oocyte growth and early development, it is possible that these “budding” mitochondria are a sign of oocytes being recovered from small follicles prior to the completion of the growth phase. It is possible that

presence of these mitochondria are a sign that mitochondrial division has not been completed and that these oocytes are less likely to have the ability to acquire competence, a possible downfall of collecting oocytes from live cattle where the ability to see and selectively aspirate antral follicles is limited. It is possible that, since the process of recovering oocytes post mortem allows greater control of follicle selection than OPU does, this allows for a pool of oocytes with greater competence. Irregularities in mitochondrial cristae and altered morphology are associated with poor mitochondrial function and decreased ATP production. Given the importance of mitochondria in energy production, it is reasonable that oocytes with a greater number of irregularly shaped, budding mitochondria, are likely to have an overall decreased energy production.

One reason that the success of IVM is limited is believed to be due to the fact that the oocytes being matured are from a heterogenous population of developmental phases at the initiation of culture. Although, super-stimulation provides a more homogenous follicle size at the time of maturation, there was no notable improvement in oocyte morphology to indicate greater competence. It has been proposed that one reason for this poor competence of oocytes following super-stimulation is that the rapid follicular growth supported by high doses of FSH causes asynchronous follicular growth and oocyte development (Hyttel et al., 1986; Dieleman and Bevers, 1993). This would result in oocytes of variable developmental levels and qualities, potentially including those pre-programmed for apoptosis, being exposed to a hormonal environment, which while stimulatory, may not enhance developmental competence. It is likely that the combined, abnormal hormonal environment and the stimulation of oocytes with poor developmental competence, combine to cause the cytoplasmic irregularities that were seen in

FSH oocytes in this study. It is possible that this difference has the potential to have significant results on the developmental competence and that further comparative analysis is warranted.

## CHAPTER VII

### DISCUSSION

The goal of any *in vitro* embryo production facility is to maximize the number of transferable embryos produced from each cohort of oocytes recovered. Current research in the field aims to maximize embryo production rates through altering the *in vitro* culture conditions, aiming to find the optimal media to increase developmental competence of oocytes and the resultant embryos (O'Doherty et al., 1997; Hussein et al., 2006; Balboula et al., 2010). The current study, instead, focuses on the innate competence of the oocyte and on attempting to determine the best methods of recovery to ensure that those oocytes retrieved are of the utmost competence at the start of the *in vitro* procedures. This is based on the belief that while embryo quality and conditions during early development are integral to pregnancy establishment, embryo production rates rely foremost on the intrinsic oocyte competence and oocyte handling and maturation conditions (Merton et al., 2003; Lonergan et al., 2006).

As with everything else in oocyte development, the acquisition of cytoplasmic competence is acquired in a stepwise manner. It is understood that the first such step is the multiplication of organelles and the completion of mRNA and protein synthesis and their storage followed by the redistribution of the cytoskeleton polymers and organelles (Sirard et al., 2006a; Watson, 2007; Ferreira et al., 2009). Studies presented here assessed three molecular markers of competence; mRNA transcript abundance (section 5), IP<sub>3</sub>R1 expression (section 4), and cytoplasmic Ca<sup>2+</sup> levels (section 4), the cytoskeletal marker F-actin aggregation (section 3), and multiple organelle markers including; mitochondria morphology and location (section 3, 4, 6), cortical granule location (section 6), and lipid and vesicle quantity (section 6). While there was a

degree in heterogeneity among all variables measured, some changes appeared to occur more reliably over the course of *in vitro* maturation than others. Similarly, some factors were easily affected by oocyte handling and treatment while others appeared to be resilient.

Throughout this series of experiments, the effects of pre-maturation protocols on oocyte cytoplasmic maturation and competence were analyzed. In the first study, the success of the expected permissive effect of holding oocytes in meiotic inhibitors on cytoplasmic maturation completion were tested. While the meiotic inhibitors used in this study, IBMX and forskolin, were successful at maintaining gap junction communication between oocytes and the surrounding cumulus cells, this did not appear to improve cytoplasmic maturation, as measured by mitochondrial relocation and F-actin aggregation. Further, a decreased maturation rate was observed in oocytes that had been held in inhibitors prior to completing maturation. Results combine to suggest that rather than improving oocyte competence, this combination of meiotic inhibitors would be more likely to decrease total embryo production rates.

In the second project, the aim was to assess the effects of follicular environment on oocyte competence. To do this, oocytes were recovered during three different follicular phases, (growth, peak, and atresia) with the expectation that the follicular and ovarian effects exerted on the oocyte population prior to collection would have significant effects on oocyte competence. While the follicular phase did not impact oocyte recovery rates, or mitochondrial maturation parameters, it was found that oocytes collected during follicle growth showed higher levels of IP<sub>3</sub>R1 expression than those collected from atretic follicles. Meanwhile, while embryo production was unaffected by follicle growth or atresia, cytoplasmic calcium levels were negatively correlated with embryo production. Results indicate that there is only a slight benefit

to collecting oocytes from growing follicles, but that oocyte competence is correlated to calcium being sequestered within the endoplasmic reticulum and not free within the cytoplasm.

In the third and fourth projects, oocytes were collected using three primary methods, super-stimulation, ovum pick-up and aspiration from ovaries post-mortem. Since the previous project failed to show a significant effect of follicular wave phase, unstimulated ovum pick-up was performed at random time points. Oocytes were then subjected to RNA Seq (in both GV and MII oocytes), transmission electron microscopy (in both GV and MII oocytes) and *in vitro* fertilization to determine the effect of oocyte retrieval method on oocyte competence dynamics. Overall oocytes collected following super-stimulation were most different in both ultrastructure and gene expression in MII oocytes. There was no significant difference in embryo development rates between oocytes collected following super-stimulation and those recovered post-mortem, but embryo development from OPU oocytes was significantly lower than that of oocytes from the other two recovery methods. Because of the potential lasting effects of the irregular gene expression and organelle dynamics in super-stimulated oocytes, it is suggested that post-mortem retrieved oocytes may be the most probable to be highly developmentally competent.

Results across all three studies indicated that mitochondria in bovine oocytes do not undergo normal maturational parameters *in vitro*. Immature oocytes were recovered in all three experiments, and regardless of treatment, with mitochondria found in any of the described locations from peripheral, diffuse or clustered. While some significant interaction between meiotic maturation and mitochondrial progression was observed, this was incomplete and unaffected by attempts at delaying meiotic maturation, or by oocyte source. Mitochondria were also found to exhibit anomalous shapes, when viewed on transmission electron microscopy,

suggesting that oocytes collected and matured *in vitro*, regardless of collection method, may have incomplete mitochondrial division, resulting in impaired energy metabolism. If this effect can be confirmed, it may be possible that this is one of the leading reasons that *in vitro* embryo development rates are not higher, in which case further research should be aimed specifically at improving mitochondrial dynamics in *in vitro* matured oocytes.

Overall results suggest that use of calcium indicators is a potentially strong method of detecting competent oocytes, with an expectation that highly competent mature oocytes possess high levels of sequestered calcium, low levels of cytoplasmic calcium and high expression of calcium signaling receptors (such as IP<sub>3</sub>R1). Mitochondrial dynamics, on the other hand, are poor indicators of maturation as aberrations are frequent. Oocytes collected post-slaughter appear to be of the highest competence, but when performing OPU timing appears to have little effect on oocyte developmental ability.

## REFERENCES

- Abbott,A.L. and T. Ducibella. 2001. Calcium and the control of mammalian corticle granule exocytosis. *Frontiers in Bioscience*. 6:D792-D806.
- Abdoon,A.S.S., O.M. Kandil, S.M. Zeng, and M. Cui. 2011. Mitochondrial distribution, ATP-GSH contents, calcium oscillation during in vitro maturation of dromedary camel oocytes. *Theriogenology*. 76:1207-1214.
- Adona,P.R., P.R.L. Pires, M.D. Quetglas, K.R.L. Schwarz, and C.L.V. Leal. 2008. Prematuration of bovine oocytes with butyrolactone I: Effects on meiosis progression, cytoskeleton, organelle distribution and embryo development. *Animal Reproduction Science*. 108:49-65.
- Agung,B., P. Wongriskeao, M. Murakami, H. Watari, and T. Otoi. 2006. The effects of maturation culture period on the sex ratio of bovine IVF embryos. *Reproduction Fertility and Development*. 52:123-127.
- Albuz,F.K., M. Sasseville, M. Lane, D.T. Armstrong, J.G. Thompson, and R.B. Gilchrist. 2010. Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Human Reproduction*. 25:2999-3011.
- Armstrong,D.T. 2001. Effects of maternal age on oocyte developmental competence. *Theriogenology*. 55:1303-1322.
- Auclair,S., R. Uzbekov, S. Elis, L. Sanchez, I. Kireev, L. Lardic, R. Dalbies-Tran, and S. Uzbekova. 2013. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. *American Journal of Physiology, Endocrinology and Metabolism*. 304:E599-E613.
- Avery,B., A. Hay-Schmidt, P. Hyttel, and T. Greve. 1998. Embryo development, oocyte morphology, and kinetics of meiotic maturation in bovine oocytes exposed to 6-dimethylaminopurine prior to in vitro maturation. *Molecular Reproduction and Development*. 50:334-344.
- Bagg,M.A., M.B. Nottle, D.T. Armstrong, and C.G. Grupen. 2007. Relationship between follicle size and oocyte developmental competence in prepubertal and adult pigs. *Reproduction Fertility and Development*. 19:797-803.
- Bagg,M.A., M.B. Nottle, C.G. Grupen, and D.T. Armstrong. 2006. Effect of dibutyl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes. *Molecular Reproduction and Development*. 73:1326-1332.

Bailey,C.L. Effects of dietary conjugated linoleic acid supplementaion on bovine oocyte lipid metabolist, lipid composition and embryo cryotolerance. Doctoral Dissertation. Baton Rouge: Louisiana State University, School of Animal Science; 2014

Balboula,A.Z., K. Yamanaka, M. Sakatani, A.O. Hegab, S.M. Zaabel, and M. Takahashi. 2010. Cathepsin B activity is related to the quality of bovine cumulus oocyte complexes and its inhibition can improve their developmental competence. *Molecular Reproduction and Development*. 77:439-448.

Barritt,J.A., M. Kokot, J. Cohen, N. Steuerwald, and C.A. Brenner. 2002. Quantification of human ooplasmic mitochondria. *Reproductive Biomedicine Online*. 4:243-247.

Bianchi,V., G. Macchiarelli, A. Borini, M. Lappi, S. Cecconi, S. Miglietta, G. Familiari, and S.A. Nottola. 2014. Fine morphological assessment of quality of human mature oocytes after slow freezing or vitrification with a closed device: a comparative analysis. *Reproductive Biology and Endocrinology*. 12:110-122.

Bilodeau-Goeseels,S. 2003a. Effects of phosphodiesterase inhibitors on spontaneous nuclear maturation and cAMP concentrations in bovine oocytes. *Theriogenology*. 60:1679-1690.

Bilodeau-Goeseels,S. 2003b. Manganese inhibits spontaneous nuclear maturation via cAMP/protein kinase A pathway in bovine cumulus-enclosed oocytes. *Journal of Animal Veterinary Advances*. 2:12-21.

Bilodeau-Goeseels,S. 2011. Cows are not mice: The role of cyclic AMP, phosphodiesterases, and adenosine monophosphate-activated protein kinase in the maintenance of the meiotic arrest in bovine oocytes. *Molecular Reproduction and Development*. 78:734-743.

Bilodeau-Goeseels,S. 2012. Bovine oocyte meiotic inhibition before *in vitro* maturation and its value to *in vitro* embryo production: does it improve developmental competence? *Reproduction in Domestic Animals*. 47:687-693.

Bleach,E.C., R.G. Glencross, S.A. Feist, N.P. Groome, and P.G. Knight. 2001. Plasma inhibin A in heifers: relationship with follicle dynamics, gonadotropins, and steroids during the estrous cycle and after treatment with bovine follicular fluid. *Biology of Reproduction*. 64:743-752.

Blondin,P., K. Coenen, L.A. Guilbault, and M.A. Sirard. 1997. In vitro production of bovine embryos: developmental competence is acquired before maturation. *Theriogenology*. 47:1061-1075.

Blondin,P. and M.A. Sirard. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular Reproduction and Development*. 41:54-62.

Brackett, B.G., D. Bousquet, M.L. Boice, W.J. Donawick, J.F. Evans, and M.A. Dressel. 1982. Normal development following *in vitro* fertilization in cow. *Biology of Reproduction*. 27:147-158.

Brevini Gandolfi, T.A.L. and F. Gandolfi. 2001. The maternal legacy to the embryo: cytoplasmic components and their effects on early development. *Theriogenology*. 55:1255-1276.

Brevini, T.A., F. Cillo, S. Antonini, V. Tosetti, and F. Gandolfi. 2007. Temporal and spatial control of gene expression in early embryos of farm animals. *Reproduction Fertility and Development*. 19:35-42.

Broadbent, P.J., D.F. Dolman, R.G. Watt, A.K. Smith, and M.F. Franklin. 1997. Effect of the frequency of follicle aspiration on oocyte yield and subsequent superovulatory response in cattle. *Theriogenology*. 47:1027-1040.

Brown, H.M., K.R. Dunning, M.L. Sutton-McDowall, R.B. Gilchrist, J.G. Thompson, and D.L. Russell. 2017. Failure to launch: aberrant cumulus gene expression during oocyte *in vitro* maturation. *Reproduction*. 153:R109-R120.

Byskov, A.G. 1986. Differentiation of mammalian embryonic gonad. *Physiology Review*. 66:71-117.

Campello, S., R.A. Lacelle, M. Batella, S. Manes, L. Scorrano, and A. Viola. 2006. Orchestration of lymphocyte chemotaxis by mitochondrial dynamics. *Journal of Experimental Medicine*. 203:2879-2886.

Cavalieri, F.L.B., F. Morotti, M.M. Seneda, A.H.B. Colombo, M.A. Andreazzi, I.P. Emanuelli, and L.P. Rigolon. 2017. Improvement of bovine *in vitro* embryo production by ovarian follicular wave synchronization prior to ovum pick-up. *Theriogenology*. 1-4.

Chaubal, S.A., L.B. Ferre, J.A. Molina, D.C. Faber, P.E.J. Bols, P. Rezamand, X. Tian, and X. Yang. 2007. Hormonal treatments for increasing the oocyte and embryo production in an OPU-IVP system. *Theriogenology*. 67:719-728.

Chian, R.C. and M.A. Sirard. 1995. Effects of cumulus cells and follicle-stimulating hormone during *in vitro* maturation on parthenogenetic activation of bovine oocytes. *Molecular Reproduction and Development*. 42:425-431.

Clarke, H.J. 2012. Regulation of mitochondrial DNA accumulation during oocyte growth and meiotic maturation in the mouse. *Reproduction*. 144:177-185.

Combelles, C.M., N.A. Cekleniak, C. Racowsky, and D.F. Albertini. 2002. Assessment of nuclear and cytoplasmic maturation in *in-vitro* matured human oocytes. *Human Reproduction*. 17:1006-1016.

Connors, S.A., M. Kanatsu-Shinohara, R.M. Schultz, and G.S. Kopf. 1998. Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation and cortical granule anchoring in mouse eggs. *Developmental Biology*. 200:103-115.

Coticchio, G., A. Borini, V. Distratis, M. Maione, G. Scaravelli, V. Bianchi, G. Macchiarelli, and S.A. Nottola. 2010. Qualitative and morphometric analysis of the ultrastructure of human oocytes cryopreserved by two alternative slow cooling protocols. *Journal of Assisted Reproduction and Genetics*. 27:131-140.

Coticchio, G., M. Dal Canto, R. Fadini, M. Mignini Rezini, M.C. Guglielmo, S. Miglietta, M.G. Palmerini, G. Macchiarelli, and S.A. Nottola. 2016. Ultrastructure of human oocytes after *in vitro* maturation. *Molecular Human Reproduction*. 22:110-118.

Coticchio, G., M. Dal Canto, M. Mignini Rezini, M.C. Guglielmo, F. Brambillasca, D. Turchi, P.V. Novara, and R. Fadini. 2015. Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics, and cytoplasmic reorganization. *Human Reproduction Update*. 21:427-454.

Cotterill, M., S.E. Harris, E. Collado Fernandez, J. Lu, J.D. Huntriss, B.K. Campbell, and H.M. Picton. 2013. The activity and copy number of mitochondrial DNA in ovine oocytes throughout oogenesis *in vivo* and during oocyte maturation *in vitro*. *Molecular Human Reproduction*. 19:444-450.

Cran, D.G. 1985. Qualitative and quantitative structural changes during pig oocyte maturation. *Journal of Reproduction and Fertility*. 74:237-245.

Cran, D.G., R.M. Moor, and M.F. Hay. 1980. Fine structure of the sheep oocyte during antral follicle development. *Journal of Reproduction and Fertility*. 59:125-132.

Cran, D.G., R.M. Moor, and R.F. Irvine. 1988. Initiation of the cortical reaction in hamster and sheep oocytes in response to inositol trisphosphate. *Journal of Cell Science*. 91:139-144.

Critser, E.S., M.L. Leibfried-Rutledge, W.H. Eyestone, D.L. Northey, and N.L. First. 1986. Acquisition of developmental competence during maturation *in vitro*. *Theriogenology*. 25:150.

De los Reyes, M., J. Palomino, V.H. Parraguez, M. Hidalgo, and P. Saffie. 2011. Mitochondrial distribution and meiotic progression in canine oocytes during *in vivo* and *in vitro* maturation. *Theriogenology*. 75:346-353.

de Wit, A.A.C., Y.A. Wurth, and T. Kruip. 2000. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. *Journal of Animal Sciences*. 78:1277-1283.

- Dieleman,S.J. and M.M. Bevers. 1993. Folliculogenesis and oocyte maturation in superovulated cattle. *Molecular Reproduction and Development*. 36:271-273.
- Dielman,S.J., P.J. Hendriksen, D. Viuff, P.D. Thomsen, P. Hyttel, and H.M. Knijn. 2002. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology*. 57:5-20.
- Dode,M.A. and C. Graves. 2002. Involvement of steroid hormones on in vitro maturation of pig oocytes. *Theriogenology*. 57:811-821.
- Dumollard,R., M. Duchen, and J. Carroll. 2007. The role of mitochondrial function in the oocyte and embryo. *Current Topics in Developmental Biology*. 77:21-49.
- Dunning,K.R., K. Cashman, D.L. Russell, J.G. Thompson, R.J. Norman, and R.L. Robker. 2010. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biology of Reproduction*. 83:909-918.
- Dunning,K.R., L.N. Watson, D.J. Sharkey, H.M. Brown, R.J. Norman, J.G. Thompson, R.L. Robker, and D.L. Russell. 2012. Molecular filtration properties of the mouse expanded cumulus matrix: controlled supply of metabolites and extracellular signals to cumulus cells and the oocyte. *Biology of Reproduction*. 87:89.
- Dunning,K.R., L.N. Watson, V.J. Zhang, H.M. Brown, A.M. Kaczmarek, R.L. Robker, and D.L. Russell. 2015. Activation of mouse cumulus-oocyte complex maturation in vitro through EGF-like activity of versican. *Biology of Reproduction*. 92:116.
- Edwards,R. 1965. Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*. 208:349-351.
- El Shourbagy,S.H., E.C. Spikings, M. Freitas, and J.C. St John. 2006. Mitochondria directly influence fertilisation outcome in the pig. *Reproduction*. 131:233-245.
- Eppig,J.J., R.M. Schultz, M. O'Brien, and F. Chesnel. 1994. Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. *Developmental Biology*. 164:1-9.
- Fair,T., F. Carter, S. Park, A.C. Evans, and P. Lonergan. 2007. Global gene expression analysis during bovine oocyte in vitro maturation. *Theriogenology*. 68:S91-S97.
- Fair,T., S.C. Hulshof, P. Hyttel, T. Greve, and M. Boland. 1997. Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anatomy and Embryology*. 195:327-336.
- Fair,T., P. Hyttel, and T. Greve. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Molecular Reproduction and Development*. 42:437-442.

Fair,T., P. Hyttel, J. Motlik, M. Boland, and P. Lonergan. 2002. Maintenance of meiotic arrest in bovine oocytes in vitro using butyrolactone I: effects on oocyte ultrastructure and nucleolus formation. *Molecular Reproduction and Development*. 62:375-386.

Farmer,S.E. Effect of cAMP modulators on in vitro maturation of bovine oocytes. Master thesis. Baton Rouge. Louisiana State University, Animal and Dairy Sciences; 2014

Fatehi,A.N., E.C. Zeinstra, R.V. Kooija, B. Colenbrander, and M.M. Bevers. 2002. Effect of cumulus cell removal of in vitro matured bovine oocytes prior to in vitro fertilization on subsequent cleavage rate. *Theriogenology*. 57:1347-1355.

Ferreira,E.M., A.A. Vireque, P.R. Adona, M. FV, R.A. Ferriani, and P.A. Navarro. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology*. 71:836-848.

Fukui,Y. and Y. Sakuma. 1980. Maturation of bovine oocytes cultures *in vitro*: relation to ovarian activity, follicular size and presence or absence of cumulus cells. *Biology of Reproduction*. 22:669-673.

Gasparrini,B., L. Boccia, L. Marchandise, R.D. Palo, F. George, I. Donnay, and L. Zicarelli. 2006. Enrichment of in vitro maturation medium for buffalo (*Bubalus bubalis*) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development. *Theriogenology*. 65:275-287.

Gharibi,S.H., M. Hajian, S. Ostadhosseini, S.M. Hosseini, M. Forouzanfar, and M.H. Nasr-Esfahani. 2013. Effect of phosphodiesterase type 3 inhibitor o nuclear maturation and *in vitro* development of ovine oocytes. *Theriogenology*. 80:302-312.

Gibbons,J.R., W.E. Beal, R.L. Krisher, E.G. Faber, R.E. Pearson, and F.C. Gwazdauskas. 1994. Effects of once versus twice weekly transvaginal aspirations on bovine oocyte recovery and embryo development. *Theriogenology*. 42:405-419.

Gilchrist,R.B., P.L. Nayudu, Nowshari, MA, and J.K. Hodges. 1995. Meiotic competence of marmoset monkeys is related to follicle size and oocyte-somatic cell associations. *Biology of Reproduction*. 52:1234-1243.

Gilchrist,R.B., L.J. Ritter, and D.T. Armstrong. 2004. Oocyte-somatic cell interactions during follicle development in mammals. *Animal Reproduction Science*. 82:431-446.

Gilchrist,R.B. and J.G. Thompson. 2007. Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology*. 67:6-15.

Gilula,N.B., M.L. Epstein, and W.H. Beers. 1978. Cell-to-cell communication and oclution. A study of the cumulus-oocyte complex. *Journal of Cell Biology*. 78:58-75.

Ginther,O.J., D.R. Bergfelt, L.J. Kulick, and K. Kot. 2000. Selection of the dominant follicle in cattle: role of estradiol. *Biology of Reproduction*. 63:383-389.

Godke,R.A., Sansinena,M., and Youngs,C.R. 2014 Assisted reproductive technologies and embryo culture methods for farm animals. In *Transgenic animal technology*, C.A. Pinkert,Ed Academic Press.

Goodhand,K.L., R.G. Watt, M.E. Stained, J.S.M. Hutchinson, and P.J. Broadbent. 1999. In vivo oocyte recovery and in vitro embryo production from bovine donors aspirated at different frequencies or following FSH treatment. *Theriogenology*. 51:951-961.

Greve,T., K.P. Xu, H. Callesen, and P. Hyttel. 1987. In vivo development of in vitro fertilized bovine oocytes matured in vivo versus in vitro. *Journal of In Vitro Fertilization and Embryo Transfer*. 4:281-285.

Gutnisky,C., S. Morado, G.C. Dalvit, J.G. Thompson, and P.D. Cetica. 2013. Glycolytic pathway activity: effect on IVM and oxidative metabolism of bovine oocytes. *Reproduction Fertility and Development*. 25:1026-1035.

Hagemann,L.J. 1999. Influence of the dominant follicle on oocytes from subordinate follicles. *Theriogenology*. 51:449-459.

Hagemann,L.J., S.E. Beaumont, M. Berg, M.J. Donnison, A. Ledgard, A.J. Peterson, A. Schurmann, and H.R. Tervit. 1999. Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. *Molecular Reproduction and Development*. 53:451-458.

Hamano,S. and M. Kuwayama. 1993. In vitro fertilization and development of bovine oocytes recovered from the ovaries of individual donors: a comparison between the cutting and aspiration method. *Theriogenology*. 39:703-712.

Hanada,A., Y. Enya, and T. Suzuki. 1986. Birth of calves by non-surgical transfer of *in vitro* fertilized embryos obtained from oocytes matured *in vitro*. *Japanese Journal of Animal Reproduction*. 32:208.

Hashimoto,S., N. Minami, R. Takakura, and H. Imai. 2002. Bovine immature oocytes acquire developmental competence during meiotic arrest *in vitro*. *Biology of Reproduction*. 66:1696-1701.

Hasler,J.F., W.B. Henderson, P.J. Hurtgen, Z.Q. Jin, A.D. McCauley, S.A. Mower, B. Neely, L.S. Shuey, J.E. Stokes, and S.A. Trimmer. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology*. 43:141-151.

Homa,S.T. 1988. Effects of cyclic AMP on the spontaneous meiotic maturation of cumulus-free bovine oocytes cultured in chemically defined medium. *Journal of Experimental Zoology*. 248:222-231.

Hosoe,M. and Y. Shioya. 1997. Distribution of corticle granules in bovine oocytes classified by cumulus complex. *Zygote*. 5:371-376.

Hunter,R.H.F., R.A.S. Lawson, and R. LEA. 1972. Maturation, transplantation and fertilization of oovarian oocytes in cattle. *Journal of Reproduction and Fertility*. 30:325-328.

Hussein,T.S., J.G. Thompson, and R.B. Gilchrist. 2006. Oocyte-secreted factors enhance oocyte developmental competence. *Developmental Biology*. 296:514-521.

Hyttel,P. 1987. Bovine cumulus-oocyte disconnection *in vitro*. *Anatomy and Embryology*. 176:41-44.

Hyttel,P. 2011 Electron microscopy of mammalian oocyte development, maturation and fertilization. In *Oocyte maturation and fertilization: A long history for a short event*, E. Tosti and R. Boni,Eds Bentham Science Publishers.

Hyttel,P., H. Callesen, and T. Greve. 1986. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. *Journal of Reproduction and Fertility*. 76:645-656.

Hyttel,P., T. Fair, H. Callesen, and T. Greve. 1997. Oocyte growth capacitation, and final maturation in cattle. *Theriogenology*. 47:23-32.

Hyttel,P., T. Greve, and H. Callesen. 1989a. Ultrastructural aspects of oocyte maturation and fertilization in cattle. *Journal of Reproduction and Fertility Supplemental*. 38:35-47.

Hyttel,P., T. Greve, and H. Callesen. 1989b. Ultrastructure of oocyte maturation and fertilization in superovulated cattle. *Progress in Clinical Biology Research*. 296:287-297.

Hyttel,P. and I. Madsen. 1987. Rapid method to prepare mammalian oocytes and embryos for transmission electron microscopy. *Acta Anatomica*. 129:12-14.

Hyttel,P., K.P. Xu, S. Smith, and T. Greve. 1986. Ultrastructure of *in vitro* oocyte maturation in cattle. *Journal of Reproduction and Fertility*. 78:615-625.

Ireland,J.J., R.L. Fogwell, W.D. Oxender, K. Ames, and J.L. Cowley. 1984. Production of estradiol by each ovary during the estrous cycle of cows. *Journal of Animal Sciences*. 59:764-771.

Iritani,A. and K. Niwa. 1977. Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. *Journal of Reproduction and Fertility*. 50:119-121.

Ispada,J., C.B. de Lima, M.A. Sirard, P.K. Fontes, M.F.G. Nogueira, K. Annes, and M.P. Milazzotto. 2018. Genome-wide screening of DNA methylation in bovine blastocysts with different kinetics of development. *Epigenetics and Chromatin*. 11

Jeong,W.J., S.J. Cho, H.S. Lee, G.K. Deb, Y.S. Lee, and I.K. Kwon. 2009. Effect of cytoplasmic lipid content on in vitro developmental efficiency of bovine IVP embryos. *Theriogenology*. 72:584-589.

Jimenez-Macedo,A.R., D. Izquierdo, A. Urdaneta, B. Anguita, and M.T. Paramio. 2006. Effect of roscovitine on nuclear maturation, MPF and MAP kinase activity and embryo development of prepubertal goat oocytes. *Theriogenology*. 65:1769-1782.

Jurema,M.W. and D. Nogueira. 2006. *In vitro* maturation of human oocytes for assisted reproduction. *Fertility and Sterility*. 4:281-285.

Kafi,M., F. Mesbah, H. Nili, and A. Khalili. 2005. Chronological and ultrastructural changed in camel (*Camelus dromedarius*) oocytes during in vitro maturation. *Theriogenology*. 63:2458-2470.

Kaneko,H., K. Taya, G. Watanabe, J. Noguchi, K. Kikuchi, A. Shimada, and Y. Hasegawa. 1997. Inhibin is involved in the suppression of FSH secretion in the growth phase of the dominant follicle during the early luteal phase in cows. *Domestic Animal Endocrinology*. 14:263-271.

Kastrop,P.M., S.C. Hulshof, M.M. Bevers, O.H. Destree, and T.A. Kruip. 1991. The effects of alpha-amanitin and cycloheximide on nuclear progression, protein synthesis, and phosphorylation during bovine oocyte maturation in vitro. *Molecular Reproduction and Development*. 28:249-254.

Kline,D. and J.T. Kline. 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Developmental Biology*. 149:80-89.

Krisher,R.L. 2004. The effect of oocyte quality on development. *Journal of Animal Sciences*. 82:E14-E23.

Krisher,R.L. and R.S. Prather. 2012. A role for the Warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. *Molecular Reproduction and Development*. 79:311-320.

Kulbeka,M., J. Motlik, R.M. Schultz, and A. Pavlok. 2000. Butyrolactone I reversibility inhibits maturation of bovine oocytes without influencing chromosome condensation activity. *Biology of Reproduction*. 62:292-302.

Labrecque,R. and M.A. Sirard. 2014. The study of mammalian oocyte competence by transcriptome analysis: progress and challenges. *Molecular Human Reproduction*. 20:103-116.

Larsen,W.J., S.E. Wert, and G.D. Brunner. 1986. Differential modulation rate of follicle cell gap junction population at ovulation. *Developmental Biology*. 112:517-521.

Leal,C.L.V., S. Mamo, T. Fair, and P. Lonergan. 2012. Gene expression in bovine oocytes and cumulus cells after meiotic inhibition with the cyclin-dependent kinase inhibitor butyrolactone I. *Reproduction in Domestic Animals*. 47:615-624.

Lee,S.T., H.J. Han, S.J. Oh, E.J. Lee, J.Y. Han, and J.M. Lim. 2006. Influence of ovarian hyperstimulation and ovulation induction on the cytoskeletal dynamics and developmental competence of oocytes. *Molecular Reproduction and Development*. 73:1022-1033.

Leibfried-Rutledge,M.L., E.S. Critser, W.H. Eyestone, D.L. Northey, and N.L. First. 1987. Development potential of bovine oocytes matured in vitro or in vivo. *Biology of Reproduction*. 36:376-383.

Lequarre,A.-S., C. Vigneron, F. Ribaucour, P. Holm, I. Donnay, R. Dalbies-Tran, H. Callesen, and P. Mermillod. 2005. Influence of antral follicle size on oocyte characteristics and embryo development in the bovine. *Theriogenology*. 63:841-859.

Liu,S., Y. Li, H.L. Feng, J.H. Yan, M. Li, S.Y. Ma, and Z.J. Chen. 2010. Dynamic modulation of cytoskeleton during in vitro maturation in human oocytes. *American Journal of Obstetrics and Gynecology*. 203:151.e1-151.e7.

Lonergan,P., A. Dinnyes, T. Fair, X. Yang, and M. Boland. 2000. Bovine oocyte and embryo development following meiotic inhibition with butyrolactone I. *Molecular Reproduction and Development*. 57:204-209.

Lonergan,P., I. Faerge, P. Maddox Hyttel, M. Boland, and T. Fair. 2003. Ultrastructural modifications in bovine oocytes maintained in meiotic arrest in vitro using roscovitine or butyrolactone. *Molecular Reproduction and Development*. 64:369-378.

Lonergan,P., T. Fair, D. Concoran, and A.C.O. Evans. 2006. Effect of culture environment on gene expression and development on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology*. 65:137-152.

Lonergan,P., H. Khatir, C. Carolan, and P. Mermillod. 1997. Bovine blastocyst production in vitro after inhibition of oocyte meiotic resumption for 24h. *Journal of Reproduction and Fertility*. 109:355-365.

Looney,C.R., B.R. Lindsey, C.L. Gonseth, and D.L. Johnson. 1994. Commercial aspects of oocyte retrieval and in vitro fertilization(IVF) for embryo production in problem cows. *Theriogenology*. 41:67-72.

Lu,K.H., I. Gordon, M. Gallagher, and H. McGovern. 1987. Pregnancy established in cattle by transfer of embryos derived from *in vitro* fertilization of oocytes matured *in vitro*. Veterinary Record. 121:259-260.

Luberda,Z. 2005. The role of glutathione in mammalian gametes. Reproductive Biology. 5:5-17.

Luciano,A.M., F. Franciosi, S.C. Modina, and V. Lodde. 2011. Gap junction-mediated communications regulate chromatin remodeling during bovine growth and differentiation through cAMP-dependent mechanism(s). Biology of Reproduction. 85:1252-1259.

Luciano,A.M., P. Pocar, E. Milanese, S. Modina, D. Rieger, A. Lauria, and F. Gandolfi. 1999. Effect of different levels of intracellular cAMP on the *in vitro* maturation of cattle oocytes and their subsequent development following *in vitro* fertilization. Molecular Reproduction and Development. 54:86-91.

Machatkova,M., K. Krausova, E. Jokesova, and M. Tomanek. 2004. Developmental competence of bovine oocytes: effects of follicle size and the phase of follicular wave on *in vitro* embryo production. Theriogenology. 61:329-335.

Malcutt,C., M. Kurokawa, and R.A. Fissore. 2006. Calcium oscillations and mammalian egg activation. Journal of Cellular Physiology. 206:565-573.

Mamo,S., F. Carter, P. Lonergan, C.L.V. Leal, A. Al Naib, P. McGettigan, J.P. Mehta, A.C.O. Evans, and T. Fair. 2011. Sequential analysis of global gene expression profiles in immature and *in vitro* matured bovine oocytes: potential molecular markers of oocyte maturation. BMC Genomics. 12:151-164.

Mao,L., H. Lou, Y. Lou, N. Wang, and F. Jin. 2014. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. Reproductive Biomedicine Online. 28:284-299.

Matchatkova,M., M. Jeseta, P. Hulinska, L. Knitlova, L. Nemcova, and J. Kanka. 2012. Characteristics of bovine oocytes with different meiotic competence in terms of thier mitochondrial status and expression and nuclear-encoded factors. Reproduction in Domestic Animals. 47:806-814.

Mau,L., H. Lou, Y. Lou, N. Wang, and F. Jin. 2014. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. Reproductive BioMedicine Online. 28:284-299.

May-Panloup,P., M.F. Chretien, C. Jacques, C. Vasseur, Y. Malthiery, and P. Reynier. 2005. Low oocyte mitochondrial DNA content in ovarian insufficiency. Human Reproduction. 20:593-597.

Mermillod,P., B. Oussaid, and Y. Cognie. 1999. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *Journal of Reproduction and Fertility Supplement*. 54:449-460.

Merton,J.S., A.P.W. de Roos, E.P.C. Koenen, B.A.J. Roelen, P.L.A.M. Vos, E. Mullaart, and H.M. Knijn. 2012. Bovine OPU-derived oocytes can be matured *in vitro* for 16-28 h with similar developmental capacity. *Reproduction in Domestic Animals*. 47:1037-1042.

Merton,J.S., A.P.W. de Roos, E. Mullaart, L. de Ruigh, L. Kaal, P.L.A.M. Vos, and S.J. Dieleman. 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology*. 59:651-674.

Minami,N., T. Suzuki, and S. Tsukamoto. 2007. Zygotic gene activation and maternal factors in mammals. *Journal of Reproduction and Development*. 53:707-715.

Moor,R.M. and A.O. Trounson. 1977. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. *Journal of Reproduction and Fertility*. 49:101-109.

Morotti,F., B.V. Sanches, J.H. Pontes, A.C. Basso, E.R. Siqueira, L.A. Lisboa, and M.M. Seneda. 2014. Pregnancy rate and birth rate of calves from a large-scale IVF program using reverse-sorted semen in *Bos indicus*, *Bos indicus-taurus*, and *Bos taurus* cattle. *Theriogenology*. 81:696-701.

Nagano,M., S. Katagiri, and Y. Takahashi. 2006. ATP content and maturational/developmental ability of bovine oocytes with various cytoplasmic morphologies. *Zygote*. 14:299-304.

Nottala,S.A., C. G, L. De Santis, G. Macchiarelli, M. Maione, S. Bianchi, M. Iaccarino, C. Flamigni, and B. A. 2008. Ultrastructure of human mature oocytes after slow cooling cryopreservation with ethylene glycol. *Reproductive Biomedicine Online*. 17:368-377.

Nottala,S.A., C. G, R. Sciajno, A. Gambardella, M. Maione, S. G, S. Bianchi, G. Macchiarelli, and B. A. 2009. Ultrastructural markers of quality in human mature oocytes vitrified using cryoleaf and cryoloop. *Reproductive Biomedicine Online*. 19:17-27.

Nottala,S.A., C.H. Macabelli, C. G, S. Bianchi, S. Cecconi, L. De Santis, S. G, C. Flamigni, and B. A. 2007. Ultrastructure of human mature oocytes after slow cooling cryopreservation using different sucrose concentrations. *Human Reproduction*. 22:1123-1133.

Nottola,S.A., A. E, C. G, P. MG, C. Lorenzo, S. G, B. A, L.-S. PE, and G. Macchiarelli. 2016. Freeze/thaw stress induces organelle remodeling and membrane recycling in cryopreserved human mature oocytes. *Journal of Assisted Reproduction and Genetics*. 33:1559-1570.

O'Doherty,E.M., M.G. Wade, J.L. Hill, and M.P. Boland. 1997. Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology*. 48:161-169.

O'Brien,J.K., D. Dwarte, J.P. Ryan, W.M. Maxwell, and G. Evans. 1996. Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. *Reproduction Fertility and Development*. 8:1029-1037.

Oliveira,L.H., C.P. Sanches, A.S. Seddon, M.B. Veras, F.A. Lima, P.L.J.J. Monteiro, M.C. Wiltbank, and R. Sartori. 2016. Short Communication: follicle superstimulation before oocyte pick-up for in vitro embryo production in Holstein cows. *Journal of Dairy Science*. 99:9307-9312.

Pavlok,A., A. Lucas-Hahn, and H. Niemann. 1992. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Molecular Reproduction and Development*. 31:63-67.

Pederson,H.S., H. Callesen, P. Lovendahl, F. Chen, J.R. Nyengaard, N.K. Nikolaisen, P. Holm, and P. Hyttel. 2016. Ultrastructure and mitochondrial numbers in postpubertal pig oocytes. *Reproduction Fertility and Development*. 28:586-598.

Petr,J., J. Rozinek, V. Hruban, F. Jilek, M. Sedmikova, Z. Vanourkova, and Z. Nemecek. 2001. Ultrastructural localization of calcium deposits during in vitro culture of pig oocytes. *Molecular Reproduction and Development*. 58:196-204.

Petropoulos,S., D. Edsgard, B. Reinius, Q. Deng, S.P. Panula, S. Codeluppi, A.P. Reyes, S. Linnarsson, R. Sandberg, and F. Lanner. 2016. Single-cell RNA-Seq reveals lineage and X chromosome dynamics in human preimplantation embryos. *Cell*. 165:1012-1026.

Phongnimitr,T., Y. Liang, K. Srirattana, K. Panyawai, N. Sripunya, C. Treetampinich, and R. Parnpai. 2013. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. *Animal Science Journal*. 84:719-725.

Pieterse,M.C., K.A. Kappen, T.A.M. Kruip, and M.A.M. Taverne. 1988. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. *Theriogenology*. 30:751-762.

Piko,L. and K.D. Taylor. 1987. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Developmental Biology*. 123:364-374.

Pincus,G. and E.V. Enzmann. 1935. The comparative behavior of mammalian eggs *in vivo* and *in vitro*. *Journal of Experimental Medicine*. 62:665-675.

Ponderato,N., G. Crotti, P. Turini, R. Duchi, C. Galli, and G. Lazzari. 2002. Embryonic and foetal development of bovine oocytes treated with a combination of butyrolactone I and

roscovitine in an enriched medium prior to IVM and IVF. *Molecular Reproduction and Development*. 62:513-518.

Ponderato,N., I. Lagutina, G. Crotti, P. Turini, C. Galli, and G. Lazzari. 2001. Bovine oocytes treated prior to *in vitro* maturation with a combination of butyrolactone I and roscovitine at low doses maintain a normal developmental capacity. *Molecular Reproduction and Development*. 60:579-585.

Razza,E., H. Pederson, L. Stroebach, M. Machado, M.F.G. Nogueira, H. Kadarmideen, H. Callesen, and P. Hyttel. 2015. Prematuration of bovine cumulus-oocyte complexes with cyclic adenosine monophosphate modulators affects both oocyte and blastocyst ultrastructure. *Reproduction Fertility and Development*. 28:192.

Reader,K.L., N.R. Cox, J.-A.L. Stanton, and J.L. Juengel. 2015. Mitochondria and vesicles differ between adult and prepubertal sheep oocytes during IVM. *Reproduction Fertility and Development*. 27:513-522.

Reader,K.L., J.A.L. Stanton, and J.L. Juengel. 2017. The role of oocyte organelles in determining developmental competence. *Biology*. 6

Richard,F.J. and M.A. Sirard. 1996. Effects of follicular cells on oocyte maturation 1: effects of follicular hemisections on bovine oocyte maturation. *Biology of Reproduction*. 54:16-21.

Rizos,D., F. Ward, P. Duffy, M.P. Boland, and P. Lonergan. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development*. 61:234-248.

Robertson,S.A., G. Mayhofer, and R.F. Seamark. 1996. Ovarian steroid hormones regulate granulocyte-macrophage colony-stimulating factor synthesis by uterine epithelial cells in the mouse. *Biology of Reproduction*. 54:183-196.

Rosenkrans,C.F.J., G.Q. Zeng, G.T. Mcnamara, P.K. Schoff, and N.L. First. 1993. Development of bovine embryos in vitro as affected by energy substrates. *Biology of Reproduction*. 49:459-462.

Team,R.S. 2016. RStudio: Integrated Development for R.

Saeki,K., Y. Nagao, M. Kishi, and M. Nagai. 1997. Developmental capacity of bovine oocytes following inhibition of meiotic resumption by cycloheximide or 6-dimethylaminopurine. *Theriogenology*. 48:1161-1172.

Salamone,D.F., P. Damiani, R.A. Fissore, J.M. Robl, and R.T. Duby. 2003. Biochemical and developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. *Biology of Reproduction*. 64:826-833.

Salhab,M., S. Dhorne-Pollet, S. Auclair, C. Guyader-Joly, D. Brisard, R. Dalbies-Tran, J. Dupont, C. Ponsart, P. Mermillod, and S. Uzbekova. 2013. In vitro maturation of oocytes alters gene expression and signaling pathways in bovine cumulus cells. *Molecular Reproduction and Development*. 80:166-182.

Sathananthan,A.H., A. Trounson, and L. Freeman. 1987. Morphology and fertilizability of frozen human oocytes. *Gamete Research*. 16:343-354.

Schramm,R.D., A.M. Paprocki, and C.A. VandeVoort. 2003. Causes of developmental failure of in vitro matured rhesus monkey oocytes: impairments in embryonic genome activation. *Human Reproduction*. 18:826-833.

Schroeder,A.C. and J.J. Eppig. 1984. The developmental capacity of mouse oocytes that matured spontaneously in vitro is normal. *Developmental Biology*. 102:493-497.

Seneda,M.M., C.R. Esper, J.M. Garcia, J.A. Oliveira, and R. Vantini. 2001. Relationship between follicle size and ultrasound-guided transvaginal oocyte recovery. *Animal Reproduction Science*. 67:37-43.

Senger,P.L. and R.G. Saake. 1970. Unusual mitochondria of the bovine oocyte. *The Journal of Cell Biology*. 46:405-408.

Shahedi,A., K. MA, M. Soleimani, and S. Morshedizad. 2013. Ultrastructure of in vitro matured human oocytes. *Iranian Red Crescent Medical Journal*. 15:e7379.

Shioya,Y., M. Kuwayama, M. Fukushima, S. Iwaski, and A. Hanada. 1988. *In vitro* fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cell matured *in vitro*. *Theriogenology*. 30:489-496.

Sirard,M.A. and N.L. First. 1988. In vitro inhibition of oocyte nuclear maturation in the bovine. *Biology of Reproduction*. 39:229-234.

Sirard,M.A., H.M. Florman, M.L. Leibfried-Rutledge, F.L. Barnes, M.L. Sims, and N.L. First. 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biology of Reproduction*. 40:1257-1263.

Sirard,M.A., L. Picard, M. Dery, K. Coenen, and P. Blondin. 1999. The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. *Theriogenology*. 51:699-708.

Sirard,M.A., F. Richard, P. Blondin, and C. Robert. 2006a. Contribution of the oocyte to embryo quality. *Theriogenology*. 65:126-136.

Sirard,M.A., F. Rishard, P. Blondin, and C. Robert. 2006b. Contribution of the oocyte to embryo quality. *Theriogenology*. 65:126-136.

Souza-Fabjan,J.M., Y. Locatelli, N. Duffard, E. Corbin, J.-L. Touze, C. Perreau, J.F. Beckers, V.J.F. Freitas, and P. Mermillod. 2014. In vitro embryo production in goats: slaughterhouse and laparoscopic ovum pick up-derived oocytes have different kinetics and requirements regarding maturation media. *Theriogenology*. 81:1021-1031.

Sreenan,J.M. 1970. *In vitro* maturation and attempted fertilization of cattle follicular oocytes. *Journal of Agricultural Science*. 75:393-396.

Stojkovic,M., S.A. Machado, P. Sotojkovic, V. Zakhartchenko, P. Hutzler, P.B. Goncalves, and E. Wolf. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* fertilization and culture. *Biology of Reproduction*. 64:904-909.

Sun,Q.Y. and S. H. 2006. Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction*. 131:193-205.

Sunderland,S.J., M.A. Crowe, M.P. Boland, J.F. Roche, and J.J. Ireland. 1994. Selection, dominance and atresia of follicles during the oestrus cycle of heifers. *Journal of Reproduction and Fertility*. 101:547-555.

Suss,U., K. Wuthrich, and G. Strangzinger. 1988. Chromosome configuration and time sequence of the first meiotic division in bovine oocyte matured *in vitro*. *Biology of Reproduction*. 38:871-880.

Sutton-McDowall,M.L., R.B. Gilchrist, and J.G. Thompson. 2005. Effect of hexoses and gonadotropin supplementation on bovine oocyte nuclear maturation during in vitro maturation in a synthetic follicle medium. *Reproduction Fertility and Development*. 17:407-415.

Sutton-McDowall,M.L., M. Mitchell, P. Cetica, G. Delvit, M. Pantaleon, M. Lane, R.B. Gilchrist, and J.G. Thompson. 2006. Glucosamine supplementation during in vitro maturationinhibits subsequent embryo development: possible role of the hexosamine pathway as a regulator of developmental competence. *Biology of Reproduction*. 74:881-888.

Sutton,M.L., R.B. Gilchrist, and J.G. Thompson. 2003. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Human Reproduction Update*. 9:35-48.

Swain,J.E. and T.B. Pool. 2008. ART failure: oocyte contributions to unsuccessful fertilization. *Human Reproduction Update*. 14:431-446.

Szollosi,D., V. Desmet, N. Crozet, and C. Brender. 1988. In vitro maturation of sheep ovarian oocytes. *Reproduction Nutrition and Development*. 28:1047-1080.

Tarazona,A.M., J.I. zrodriguez, L.F. Restrepo, and M. Olivera-Angel. 2006. Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. *Reproduction in Domestic Animals*. 41:5-11.

Thibault,C., D. Szollozi, and M. Gerard. 1987. Mammalian oocyte maturation. *Reproduction, Nutrition and Development*. 27:865-896.

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

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

Thomas,R.E., J.G. Thompson, D.T. Armstrong, and R.B. Gilchrist. 2004b. Effect of specific phosphodiesterase isoenzyme inhibitors during in vitro maturation of bovine oocytes on meiotic and developmental capacity. *Biology of Reproduction*. 71:1142-1149.

Thompson,J.G. 2006. The impact of nutrition of the cumulus oocyte complex and embryo on subsequent development in ruminants. *Journal of Reproduction and Development*. 52:169-175.

Thompson,J.G., C. McNaughton, B. Gasparrini, L.T. McGowan, and H.R. Tervit. 2000. Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos in vitro. *Journal of Reproduction and Fertility*. 118:47-55.

Thompson,J.G., A.C. Simpson, P.A. Pugh, O.E. Donnelly, and H.R. Tervit. 1990. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *Journal of Reproduction and Fertility*. 89:573-578.

Thundathil,J., F. Filion, and L.C. Smith. 2005. Molecular control of mitochondrial function in preimplantation mouse embryos. *Molecular Reproduction and Development*. 71:405-413.

Trounson,R., C. Anderiesz, and G. Jones. 2001. Maturation of human oocytes *in vitro* and their developmental competence. *Reproduction*. 121:51-75.

Van Blerkom,J. 2009. Mitochondria in early mammalian development. *Seminars in Cell and Developmental Biology*. 20:354-364.

Van Wagtendonk-de Leeuw,A.M. 2006. Ovum pick up and in vitro production in the bovine after use in several generations: a 2005 status. *Theriogenology*. 65:914-925.

Verlhac,M.H. 2011. Spindle positioning: going against the actin flow. *Nature Cell Biology*. 13:1183-1185.

Wakai,T., V. Vanderheyden, S.Y. Yoon, B. Cheon, N. Zhang, J.B. Parys, and R.A. Fissore. 2012. Regulation of inositol 1,4,5-trisphosphate receptor function during mouse oocyte maturation. *Journal of Cellular Physiology*. 227:705-717.

Wakefield,S.L., M. Lane, and M. Mitchell. 2011. Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. *Biology of Reproduction*. 84:572-580.

Walls,M., S. Junk, J.P. Ryan, and R. Hart. 2012. IVF versus ICSI for the fertilization of in-vitro matured human oocytes. *Reproductive BioMedicine Online*. 25:603-607.

Walls,M.L., T. Hunter, J.P. Ryan, J.A. Keelan, E. Nathan, and R.J. Hart. 2015. In vitro maturation as an alternative to standard in vitro fertilization for patients diagnosed with polycystic ovaries: a comparative analysis of fresh, frozen and cumulative cycle outcomes. *Human Reproduction*. 330:88-96.

Wang,L.Y., D.H. Wang, X.Y. Zou, and C.M. Xu. 2009. Mitochondrial functions on oocytes and preimplantation embryos. *Journal of Zhejiang University Science*. 10:483-492.

Ward,F., B. Enright, D. Rizos, M. Boland, and P. Lonergan. 2002. Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology*. 57:2105-2117.

Warzych,E., P. Pawlak, M. Pszczola, A. Cieslak, and D. Lechniak. 2017. Prepubertal heifers versus cows- the differences in the follicular environment. *Theriogenology*. 87:36-47.

Watson,A.J. 2007. Oocyte cytoplasmic maturation: a key mediator of oocytes and embryo developmental competence. *Journal of Animal Sciences*. 85:E1-E3.

Whitaker,M. 2006. Calcium at fertilization and in early development. *Physiology Review*. 86:25-88.

Wrenzycki,C., D. Herrmann, and H. Niemann. 2007. Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology*. 68:S77-S83.

Wrenzycki,C. and H. Stinshoff. 2013. Maturation environment and impact on subsequent developmental competence of bovine oocytes. *Reproduction in Domestic Animals*. 48:38-43.

Wright,R.W.J. and K.R. Bondioli. 1981. Aspects of in vitro fertilization and embryo culture in domestic animals. *Journal of Animal Sciences*. 53:702-729.

Wu,D., Q.C.K. Cheung, L. Wen, and J. Li. 2006. A growth-maturation system that enhances the meiotic and developmental competence of porcine oocytes isolated from small follicles. *Biology of Reproduction*. 75:547-554.

Yu,Y., R. Dumollard, A. Rossbach, F.A. Lai, and K. Swann. 2010. Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation. *Journal of Cellular Physiology*. 224:672-680.

Zeng,H.T., W.S. Yeung, M.P. Cheung, P.C. Ho, C.K. Lee, G.L. Zhuang, X.Y. Liang, and W.S. O. 2009. In vitro-matured rat oocytes have low mitochondrial deoxyribonucleic acid and adenosine triphosphate contents and have abnormal mitochondrial redistribution. *Fertility and Sterility*. 91:900-907.

Zhang,L., S. Jiang, P.J. Wozniak, X. Yang, and R.A. Godke. 1995. Cumulus cell function during bovine oocyte maturation, fertilization, and embryo development in vitro. *Molecular Reproduction and Development*. 40:338-344.

## APPENDIX. SUPPLEMENTAL MATERIALS

### A.1 Oocyte Grading Scheme

Oocyte Grade	Morphological Characteristics
A	Complete enclosure with 3+ layers of cumulus cells, homogenous, dark cytoplasm with no granularity, small perivitelline space
B	Complete enclosure with 1-2 layers of cumulus cells, dark cytoplasm perhaps with slight presence of granularity or vacuoles
C	Partial enclosure with cumulus cells, cytoplasm may be light, irregular or containing a moderate degree of granularity, moderate perivitelline space
D	Fragmented oocyte, or clear degeneration with with high degrees of cytoplasmic irregularities or discontinuous oolemma, Few to no cumulus cells associated

### A.2 Hepes-TALP

Component	Concentration	Source
Hepes TL	0.977	Caisson (IVL01)
Sodium Pyruvate	0.01	Sigma (P-4562) 2.2mg/mL stock solution
Penicillin/Streptomycin	0.01	Gibco (15140)
Bovine Serum Albumin Fraction V	0.003	Sigma (A4503)

### A.3 Pre-IVM medium with FSH and IBMX

Component	Concentration	Source
Hepes TALP	0.99	Stock solution described above
Forskolin	0.001	Sigma F-6886 (100 $\mu$ M stock solution)
IBMX	0.001	Sigma I-7018 (500 $\mu$ M stock solution)

### A.4 Maturation Medium Composition

Component	Concentration	Source
TCM-199	0.855	Sigma M4530
Fetal Bovine Serum	0.1	Cellgro 35-010-CV
Sodium Pyruvate	0.01	Sigma P-4562 (stock solution)
L-Glutamine	0.01	Sigma G-8540 (29.2mg/mL stock solution)
FSH	0.005	Bioniche (Folltropin)
EGF	0.01	(50ng/mL)

Penicillin/Streptomycin	0.01	Gibco 15140
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<b>A.5 IVF-TALP Medium</b>		
<b>Component</b>	<b>Concentration</b>	<b>Source</b>
TL-Fert	0.894	Caisson IVL02
Penicillamine/ Hypotaurine	0.04	Sigma P-4875 and H-1384 (stock solution)
Heparin	0.04	Sigma H-3149 (stock solution)
Pen/Strep	0.01	Gibco 15130
Sodium pyruvate	0.01	Sigma P-4562 (stock solution)
BSA	0.006	Sigma A-6003

<b>A.6 SOF- IVC Medium</b>		
<b>Component</b>	<b>Concentration</b>	<b>Source</b>
SOF	0.932	Caisson IVL05
BSA- Fatty acid free	0.003	Sigma A-6003
BME (essential amino acids)	0.02	Gibco 11140-050 100X
MEM (non-essential amino acids)	0.01	Gibco 11130-051 50X
Penicillin/streptomycin	0.01	Gibco 15140
Sodium pyruvate	0.02	Sigma P-4562 (stock solution)
L-Glutamine	0.005	Sigma G-8540 (stock solution)

<b>A.7 Ionomycin Activation Medium</b>		
<b>Component</b>	<b>Concentration</b>	<b>Source</b>
<b>Step 1 (0.1mM stock);</b>		
Hepes-TALP	0.98	See above AppendixB
Ionomycin stock	0.02	Sigma I-0634 (5mM stock)
<b>Step 2 (working solution);</b>		
Hepes-TALP	0.95	See above AppendixB
0.1mM Ionomycin	0.05	Ionomycin working solution from step 1

#### A.8 DMAP activation Medium

Component	Concentration	Source
SOF	0.99	See above Appendix F
DMAP stock	0.01	Sigma D-2629 (200mM stock)

#### A.9 Hoechst Working Solution

Component	Concentration	Source
Hoechst stock	0.0035	Stock solution
Hepes-TALP	0.9965	Appendix B

#### A.10 PBST-BSA

Component	Concentration	Source
PBS	0.9955	Corning Cellgro 21-030-CV
BSA Fraction V	0.001	Sigma A7906
Tween 20	0.001	Sigma P1379
Phenol red	0.001	Sigma 0290
NaOH 1M	~0.15%	Sigma S2770

Add NaOH in 5 $\mu$ L increments, mixing and testing pH after each addition to get a final pH of ~7.4

#### A.11 0.1M Phosphate Buffer pH 7.4

Component	Concentration	Source
<b>Step 1: 0.2M sodium phosphate dibasic</b>		
Sodium phosphate dibasic	0.0276	Sigma S3264
Water	0.9724	
<b>Step 2: 0.2M sodium phosphate monobasic</b>		
Sodium Phosphate monobasic	0.0536	Sigma Sigma S3139
Water	0.9464	
<b>Step 3: 0.1M phosphate buffer</b>		
0.2M sodium phosphate dibasic solution	0.1	Step 1 above
0.2M sodium phosphate monobasic solution	0.4	Ste 2 above
Water	0.5	

#### A.12 Epon Resin

Component	Concentration	Source
Epon 812	12.33g	
Araldite M 502	9.98g	
DDSA	29.93g	
DMP 30*	1mL/30mL	

Kit Supplied by EMS, product # RT 13940 contains individual bottles of each ingredient.

\*Combine first 3 ingredients by careful weight of viscous liquids. Mix well by inverting and store in fridge. Add DMP 30 at time of final embedding only. Always warm to room temperature and mix well by inverting before use.

### A.13 Stock Solutions

1. Dulbecco's Phosphate Buffered Saline: Add 100 mL of 10X D-PBS solution (Sigma D-1283) to 900 mL of autoclaved DI water to make 1 L of D-PBS. Store at room temperature. Add 10 mL of Bovine Calf Serum (HyClone) and 1 mL of Heparin (Sagent Pharmaceuticals, Schaumburg, IL, USA) to make the D-PBS oocyte collection medium.
2. Penicillin/Streptomycin: Gibco 15140. Aliquot  $\approx 200$   $\mu$ L of new pen/strep solution into sterile centrifuge tubes. Store at  $-20^{\circ}\text{C}$  until use.
3. Sodium Pyruvate: Sigma P-4562. Dissolve 22 mg of sodium pyruvate in 10 mL of sterile Millipore-Q water to make 20mM stock solution. Sterile filter into an aluminum foil-wrapped 15 mL conical tube and store at  $4^{\circ}\text{C}$  for up to a month.
4. L-Glutamine: Sigma G-8540. Make a 100X stock solution with a concentration of 20 mM by dissolving 2.92 g of glutamine in 100 mL of DI water. Aliquot 1.0 mL into sterile centrifuge tubes and store at  $-20^{\circ}\text{C}$ .
5. FSH: Folltropin-V (Bioniche). Make a 1000X stock solution (5 mg/mL) by diluting a 400 mg vial of folltropin in 80 mL of DI water. Store at  $-20^{\circ}\text{C}$  in 100  $\mu$ L aliquots.
6. Forskolin: Forskolin, Sigma F-6886. Make a 10 mM stock solution by adding 244  $\mu$ L of DMSO (Sigma D-2650) to 10 mg of FSK. Aliquot 5  $\mu$ L into small centrifuge tube and store at  $-20^{\circ}\text{C}$  until use.
7. IBMX: 3-Isobutyl-1-methylxanthine, Sigma I-7018. Prepare a 500 mM stock solution by adding 899  $\mu$ L of DMSO (Sigma D-2650) to 100 mg of IBMX. Aliquot 5  $\mu$ L into small centrifuge tubes and store at  $-20^{\circ}\text{C}$  until use.
8. Hyaluronidase: Sigma H-3506. Prepare a 1 mg/mL solution by dissolving 10 mg hyaluronidase into 10 mL of HEPES-TALP (see media formulations above). Aliquot 100  $\mu$ L into 1.5 mL sterile centrifuge tubes. Store at  $-80^{\circ}\text{C}$  indefinitely.
9. Penicillamine/Hypotaurine: Combine 5 mL of 1 mM of Hypotaurine and 5 mL of 2 mM Penicillamine to 8 mL of 0.9% saline. Aliquot into 100  $\mu$ L, store at  $-20^{\circ}\text{C}$ .
10. Heparin: Dissolve H-3149 1 mg Heparin into 20 mL of 0.9% saline and filter. Store at  $-20^{\circ}\text{C}$
11. BME essential amino acids: Purchased as 100X stock. Aliquot 100 $\mu$ L and store at  $-20^{\circ}\text{C}$ .
12. MEM non-essential amino acids: Purchased as 50X stock. Aliquot 200 $\mu$ L and store at  $-20^{\circ}\text{C}$ .

13. DMAP: Add 32.63mg 6-DMAP (Sigma D-2629) to 1mL of water to make 200mM stock solution. Heat to boiling to completely dissolve DMSO. Make aliquots of 15 $\mu$ L and store at -20°C. Aliquots will need to be heated again on thaw to re-suspend DMSO.

14. Ionomycin: Add 1mg of Ionomycin (Sigma I-0634) to 268 $\mu$ L DMSO (Sigma D-2650) to make 5mM stock. Make 5 $\mu$ L aliquots and store at -20°C.

15. Hoechst 33342: Dissolve 25 mg of Hoechst in 25 mL distilled water (1mg/mL stock concentration) in a 50 mL centrifuge tube. Aliquot in 1 mL microcentrifuge tubes (1 mL / tube). Store at -20°C for several months. For working solution in embryo culture or holding media dilute to 1  $\mu$ g/mL.

16. Mitotracker deep red: Solubilize mitotracker powder (Molecular Probes M22426) by dissolving entire vial into 50 $\mu$ L of DMSO (Sigma D2650). Mix 4 $\mu$ L of Mitotracker solution with 200 $\mu$ L of PBS. Let sit for 5 minutes in dark before use.

17. PBS+1% BSA: Add 0.1g of BSA Fraction V (Sigma A7906) to 10mL of PBS (Corning Cellgro 21-030-CV). Vortex to mix and sterile filter. (Increase to 0.2g BSA/10mL PBS for PBS + 2%BSA).

18. 0.1% Triton X100: Add 5 $\mu$ L of Triton X100 (Sigma T9284) to 5mL of PBS (Corning Cellgro 21-030-CV). Triton X100 is viscous and needs to be pipetted slowly for precision.

19. 4% Paraformaldehyde: Mix 2mL of 8% paraformaldehyde (Electron Microscopy Sciences product #157-8) with 2mL of PBS (Corning cellgro 21-030-CV). For 1% paraformaldehyde, mix 1mL of 8% paraformaldehyde with 7mL of PBS.

20:Fluo3-AM: Dissolve entire tube of stain (50 $\mu$ g, Molecular Probes F1242) into 44 $\mu$ L of DMSO (Sigma D2650). Mix 1 $\mu$ L of Fluo3-AM suspension in 49 $\mu$ L calcium free PBS (Corning cellgro 21-030-CV) to make 5 $\mu$ M working solutions. Make fresh at time of use.

21: 3% Glutaraldehyde: Mix 120 $\mu$ L 25% glutaraldehyde (Sigma G5882) in 880 $\mu$ L 0.1M sodium phosphate buffer (appendix). Make fresh, day of use.

22: 1% Osmium tetroxide: Dilute 4% Osmium tetroxide (Electron Microscopy Sciences product #19160) by adding 1mL to 3mL of 0.1M sodium phosphate buffer (appendix). Store at 4°C. Warm fully to room temperature before use

23: 0.5% Uranyl acetate: Dilute 1% uranyl acetate (Electron Microscopy Sciences product #22400-1) by adding 2mL to 2mL of sterile water. Store protected from light at 4°C. Warm to room temperature before use, always keeping protected from light.

#### **A.14 Fixation and Staining Protocol for Detecting Mitochondria and IP<sub>3</sub>R1**

Denude oocytes by vortexing for 2 minutes in 200 $\mu$ L of 1mg/mL hyaluronidase within a 1.7mL microcentrifuge tube

Recover oocytes and rinse 3 times in Hepes-TALP

Incubate for 30 minutes at 38.5°C in 400nM Mitotracker deep red, in a humidified chamber

Wash 3 times for 5 minutes each in Hepes-TALP

Incubate oocytes for 1 hour at room temperature in 4% paraformaldehyde (PFA)

Move oocytes to 300 $\mu$ L fresh 1% PFA in a 1.7mL microcentrifuge tube and store at 4°C for up to 4 weeks

\*All procedures after this point at room temperature unless otherwise noted

\*\* All washes should be performed on a rocking platform

Remove oocytes from PFA, wash 3 times for 5 minutes each in PBS+1%BSA

Incubate 30 minutes in 0.1% Triton X100

Wash 3 times, for 5 minutes each in PBS+1%BSA

Incubate for 2 minutes in PBST

Wash 3 times, for 5 minutes each in PBS+1%BSA

Block for 1 hour in PBS+2%BSA

Incubate for 1 hour in 1° Antibody (Rabbit anti-IP<sub>3</sub>R1, 1:500)

Wash 3 times, for 5 minutes each in PBS+1%BSA

Incubate for 1 hours in 2° Antibody (Alexafluor 488 conjugates goat anti-rabbit IgG, 1:500)

Wash 3 times, for 5 minutes each in PBS+1%BSA

Mount on a clean, dry slide in minimal media under 10 $\mu$ L Vectashield+DAPI

Seal slides with clear, non-fluorescent nail polish

## A.15 RNA-Seq Protocol

### SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing

Oocytes were snap frozen in groups of 4 in a total volume of 2 $\mu$ L of PBS in an 0.5mL centrifuge tube

Store samples at -80°C until time of use

Follow protocol for Smart Seq v4 Ultra Low Input RNA Kit for Sequencing (ClonTech, Takara Bio USA, Mountain View CA, USA)

#### A. First strand cDNA synthesis

Clean workspace and pipettes with alcohol followed by RNase Away

1. Thaw 5x Ultra low first strand buffer at RT

Thaw remaining reagents on ice

Thaw samples on ice

Vortex each reagent and spin down for briefly

2. Prepare stock solution of 10X reaction buffer by mixing 10X Lysis Buffer with RNase Inhibitor

Component	Volume
10X Lysis Buffer	19 $\mu$ L
RNase Inhibitor	1 $\mu$ L
Total	20 $\mu$ L

Mix Briefly and Spin down (avoid creating bubbles)

3. Prepare samples to a total volume of 10.5 $\mu$ L in individual RNase free 0.2 $\mu$ L PCR tubes

Component	Negative Control	Positive Control	Test Sample
10X Reaction Buffer	1 $\mu$ L	1 $\mu$ L	1 $\mu$ L
Nuclease-Free Water	9.5 $\mu$ L	7.5 $\mu$ L	7.5 $\mu$ L
Diluted Control RNA*	-	2 $\mu$ L	-
Sample	-	-	2 $\mu$ L
Total	10.5 $\mu$ L	10.5 $\mu$ L	10.5 $\mu$ L

\* Control RNA concentration is 1 $\mu$ g/ $\mu$ L and needs to be diluted in nuclease-free water with RNase inhibitor to match the concentration of the test sample.

Vortex gently to mix and incubate at room temperature for 5 minutes

4. Place Samples on ice

Add 2µL 3' SMART-Seq CDS Primer II A (12µM) to each tube  
Mix well by gently vortexing and spin tubes briefly

Component	Volume
Oocytes/ Total RNA in Reaction Buffer (Table 1)	10.5µL
3' SMART-Seq CDS Primer II A (12 µM)	2µL
<b>Total</b>	12.5µL

5. Incubate tubes at 72°C in a pre-heated hot-lid thermal cycler for 3 minutes

6. Prepare enough Master Mix for all reactions plus 10% of total reaction mix volume, at room temperature  
(This should be completed while samples are incubating in step 5)

Component	Volume
5X Ultra Low First-Strand Buffer	4 µL
SMART-Seq v4 Oligonucleotide (48µM)	1 µL
RNase Inhibitor (40 U/µL)	0.5 µL
SMARTScribe Reverse Transcriptase*	2µL
<b>Total volume per reaction</b>	5.5 µL

\*SMARTScribe Reverse Transcriptase should only be added to the master mix IMMEDIATELY prior to use. Make master mix without SMARTScribe Reverse Transcriptase first and add this later when samples are ready.

Mix well by pipetting up and down, then spin briefly

7. Immediately after 3 minute incubation (Step 5), place samples on ice for 2 minutes

8. Preheat thermal cycler to 42°C

9. Add 7.5µL Master Mix to each reaction tube  
Mix contents by gently pipetting and spin briefly

10. Place tubes on preheated thermal cycler

Program: 42°C 90 min

70°C 10 min

4°C forever

Stopping point: Samples can be stored at 4°C overnight

## B. cDNA amplification

1. Thaw PCR reagents on ice (except for enzyme)  
Gently vortex and spin down each tube. Store on ice
2. Prepare enough PCR Master Mix for all reactions plus 10%

Component	Volume
2X SeqAMP PCR Buffer	25µL
PCR Primer II A (12µM)	1µL
SeqAMP DNA Polymerase*	1µL
Nuclease-Free Water	3µL
Total volume per reaction	30µL

\* Vortex to mix before adding DNA Polymerase. Thaw and add DNA Polymerase just prior to use, mix gently by pipetting.

3. Add 30µL PCR Master Mix to each tube containing 20µL of first-strand cDNA product from section A

Mix well by pipetting and spin down

4. Place tubes in a preheated thermal cycler

Program:

16 cycles;

98°C 10s

65°C 30s

68°C 3 min

72°C 10 min

4°C forever      \*Stopping point, tubes can be held at 4°C overnight

## C. Amplified cDNA purification

Bring AMPure XP bead aliquots to room temperature for at least 30 minutes, mix well to disperse

Prepare fresh 80% ethanol for each experiment (400µL per sample)

1. Add 1µL 10X Lysis buffer to each PCR product from section B.
2. Vortex AMPure XP beads until evenly mixed, then add 50µL of AMPure XP beads to each sample  
Mix thoroughly by pipetting entire volume up and down at least 10 times  
(Beads are viscous, pipette slowly)  
Incubate at room temperature for at 10-15 minutes to allow cDNA to bind beads  
Briefly spin samples

3. Place samples on magnetic separation device for at least 5 minutes, until liquid appears completely clear with no beads left in supernatant  
Keeping samples on magnetic separation device pipette supernatant and discard
4. Keeping samples on magnetic separation device, wash beads by adding 200  $\mu$ L of 80% ethanol to each sample without disturbing beads  
Wait 30s and carefully remove and discard supernatant  
Repeat ethanol wash once more
5. Briefly spin samples to collect liquid from sides of tube  
Place samples on magnetic separation device for 30s then remove all remaining ethanol with a pipette
6. Place samples at room temperature for ~5 minutes to dry until pellet is no longer shiny (but before a crack appears)
7. Add 17 $\mu$ L Elution Buffer to cover the bead pellet  
Remove samples from magnetic separation device and mix thoroughly to re-suspend the beads  
Incubate at room temperature for 5 minutes to rehydrate  
Spin briefly to collect liquid from sides of tube
8. Place samples on magnet for 5 minutes until liquid is completely clear
9. Transfer clear supernatant that contains cDNA from each tube to a nuclease-free, low-adhesion tube  
Label each tube with sample information  
Store at -20°C \*Stopping point. Samples can be stored at -20°C indefinitely

**D. Validation Using Agilent D5000 TapeStation**

1. Equilibrate D5000 reagents at room temperature for 30 minutes  
Launch the 4200 TapeStation Controller Software  
Flick D5000 screentape device to remove bubbles and load single device and loading tips into the 4200 TapeStation  
Vortex reagents and spin down
2. Prepare ladder in a tube strip;  
Mix 2 $\mu$ L D5000 Sample Buffer and 2 $\mu$ L D5000 Ladder
3. Prepare samples;  
Mix 2 $\mu$ L D5000 Sample Buffer and 2 $\mu$ L DNA sample (for each sample)  
Load samples in specific 0.2mL 8-well strip

Spin down then vortex using IKA vortexer (2000 rpm for 1 min)  
Spin down to position samples at bottom of tube

4. Load samples into Agilent 4200 TapeStation Instrument  
Carefully remove caps  
Select sample positions on software  
Click Start and specify filename to save results

**E. Determine cDNA quantity using Qubit dsDNA HS Assay**

Use only Qubit assay tubes (thin-walled 0.5mL PCR tubes)  
Label on tube lids

1. Prepare working solution by adding to each sample tube;  
198 $\mu$ L Qubit working solution  
2 $\mu$ L Sample (1 $\mu$ L sample/ 199 $\mu$ L Qubit solution if high concentration is anticipated)
2. Make 2 standards  
190 $\mu$ L Qubit working solution  
10 $\mu$ L Qubit standard
3. Mix both working solutions and standards by gentle pipetting  
Allow all tubes to incubate at room temperature for 2 minutes
4. Follow instructions on Qubit Fluorometer to read standards and samples  
From home screen select DNA, dsDNA high sensitivity, read standards  
Insert tube containing Standard #1 into sample chamber close lid and press read standard  
When reading is complete remove Standard #1  
Repeat for Standard #2
5. Press Run Samples  
On assay screen select sample volume and units  
Read each individual sample, following prompts on screen  
Top value displayed in large font on the screen is the concentration of the original sample  
Bottom value is the dilution concentration

**F. Nextera XT DNA library preparation**

**Tagment Genomic DNA**

1. Based on Qubit results dilute each sample in DNase-free water to a total concentration of 2.5ng/ $\mu$ L
2. Thaw Tagment DNA Buffer, Tagment DNA enzyme and Genomic DNA on ice  
Gently mix by inverting then spin down  
Label a new 96-well TCY plate

3. Save the following program on a thermal cycler lid  
Preheat lid to 55°C  
hold 55°C for 5 min  
for 10°C
4. Add 5 µL genomic DNA (total 50ng) to each sample well on the labelled TCY  
plate  
Add 10µL of Tagment DNA Buffer to each well containing DNA  
Add 5µL of Tagment DNA Enzyme to each well containing DNA and buffer  
Pipette up and down 10x to mix  
Centrifuge at 280G for 1 minute
5. Place on pre-programmed thermal cycler and run the program (from 3)
6. Add 5µL of Zymo DNA binding buffer to each sample  
Incubate at room temperature for 5 minutes

#### **Amplify Tagmented DNA**

1. Use 2 sets of index primers so that each sample has a unique set  
Add 5µL of each index 1 down each column (A unique index #1 per column)  
Add 5µL of each index 2 across each row (A unique index #2 per row)  
Add 15µL Nextera PCR Master Mix to each sample  
Centrifuge 280G for 1 minute

1. Place of pre-heated thermal cycler and run program;  
72°C for 3 minutes  
98°C for 30 seconds  
12 cycles;  
    95°C for 10 seconds  
    55°C for 30 seconds  
    72°C for 30 seconds  
72°C for 5 minutes  
10°C hold

#### **Clean up libraries**

Bring AMPure XP beads to room temperature prior to use  
Vortex AMPure XP beads

1. Remove plate from thermal cycler and vortex 280G for 1 minute  
Transfer 50µL of PCR product from each sample to new 1.5mL tubes
2. Add 30µL of AMPure XP beads to each sample

Shake at 1800 rpm for 2 minutes  
Incubate at room temperature for 5 minutes

3. Place on magnetic stand ~2 minutes until fluid is clear  
Remove and discard supernatant

4. Wash 2x with 200µL of fresh 80% ethanol  
Incubate for 30 seconds each time  
Remove and discard supernatant without disturbing bead pellet  
Remove residual ethanol from each well

5. Air dry pellets on magnet for 15 minutes  
Remove from stand

6. Add 30µL of resuspension buffer to each tube  
Shake at 1800 rpm for 2 minutes  
Incubate at room temperature for 2 minutes  
Place on magnetic stand for 2 minutes until clear

7. Transfer 50µL of supernatant from each sample to new tubes  
Run Qubit and Agilent to verify quality  
Store at -80°C

#### **Pool indexed libraries**

4. Transfer 5µL from each well in column 1 of dilution plate to column 1 of pooled plate

Repeat for each column of dilution plate until all samples are pooled in column 1 of pooled plate

Ensure to track which samples go into which well to avoid pooling any 2 samples with the same index

5. Combine the contents of each well of column 1 into well A2 of pooled plate  
Shake at 1800 rpm for 2 minutes

6. Denature and dilute pooled libraries to a concentration of 4nM

7. Seal plate and store at -20°C

8. Sequence on NextSeq 500 Illumina Platform

## A.16 Supplemental Information for RNA Seq Results

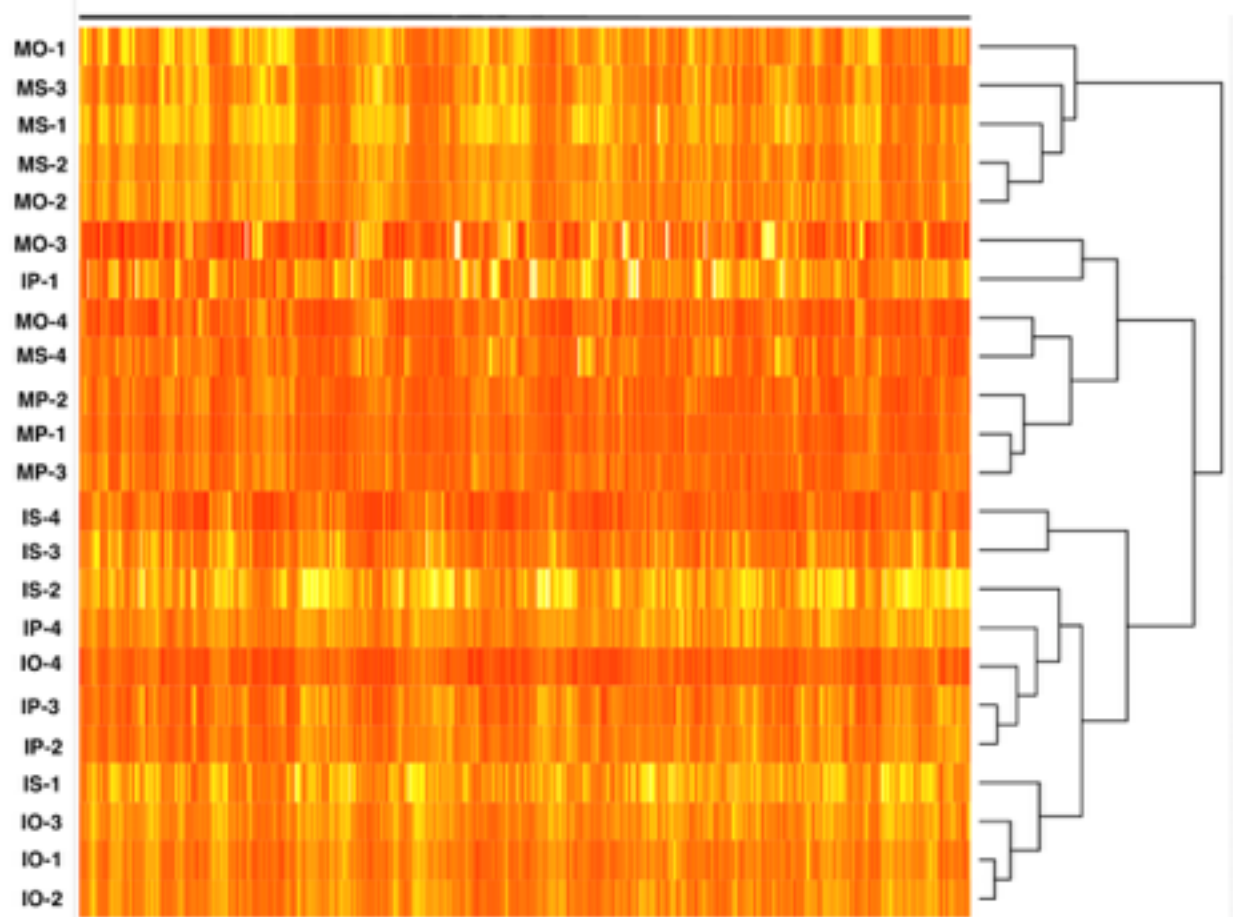
### A. Genome Alignment Reads by Treatment

RNA-Seq results from individual samples showing the number or genes read for each sample and the sample genome qualities. With the exception of sample MO-3, samples were of high quality with a high proportion of reads aligning with bovine genes.

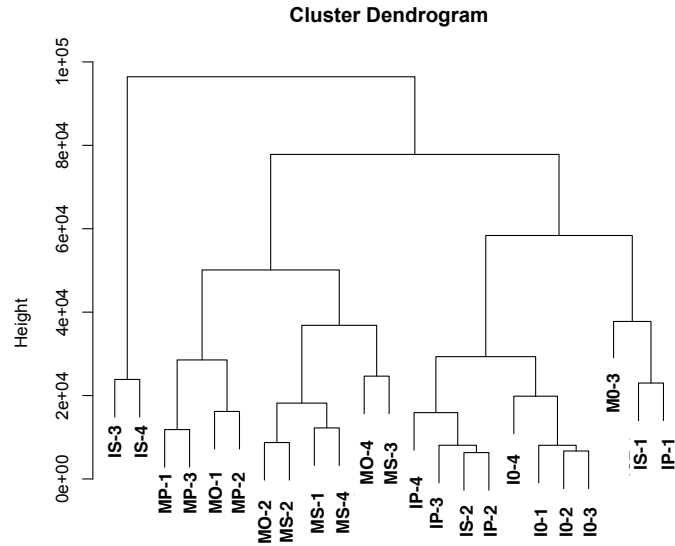
Sample Name	Maturation	Recovery Method	Total Reads	Percent Aligned	Average Length	Average Quality
IO-1	Immature	OPU	27877658	92.64	73.35	34.1
IO-2	Immature	OPU	28924359	93	73.2	34.15
IO-3	Immature	OPU	31806987	93	73.19	34.1
IO-4	Immature	OPU	16668129	93.7	73.66	33.72
IS-1	Immature	FSH	35107502	92.69	73.34	34.13
IS-2	Immature	FSH	33053662	95.14	73.47	34.15
IS-3	Immature	FSH	28132172	95.17	73.32	34.12
IS-4	Immature	FSH	18712801	94.13	73.51	33.75
IP-1	Immature	PM	24110691	91.79	73.26	33.96
IP-2	Immature	PM	19172649	93.89	73.98	33.85
IP-3	Immature	PM	19104861	94.64	73.99	33.75
IP-4	Immature	PM	26948064	92.09	73.07	33.85
MO-1	Mature	OPU	35195046	92.8	73.18	34.19
MO-2	Mature	OPU	30762336	92.52	73.09	34.19
MO-3	Mature	OPU	29538552	51.31	70.88	33.53
MO-4	Mature	OPU	19365086	83.76	73.27	33.86
MS-1	Mature	FSH	37221506	92.37	73.06	34.25
MS-2	Mature	FSH	29714638	93.67	73.24	34.22
MS-3	Mature	FSH	30843614	90.62	72.94	34.2
MS-4	Mature	FSH	22689288	90.73	73.41	33.93
MP-1	Mature	PM	18234951	93.58	73.97	33.92
MP-2	Mature	PM	21268781	93.86	73.98	33.99
MP-3	Mature	PM	21408228	93.98	74	33.92

## B. Heatmap of the 500 Most Variably Expressed Genes

Figure A1. Using the hundred genes with the overall most variable expression between the six

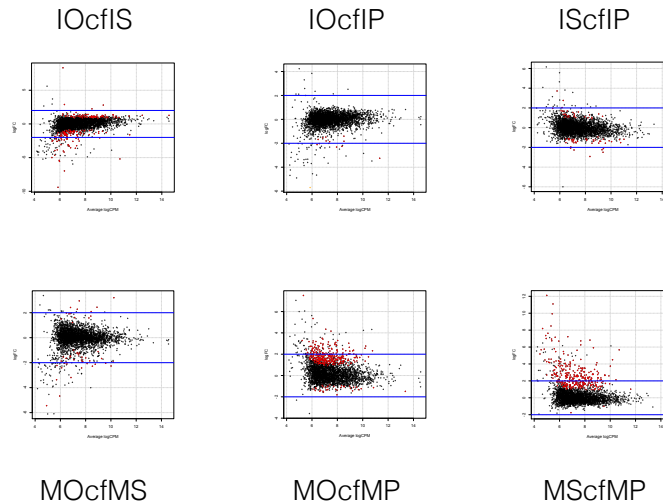


treatment groups, oocyte are categorized by expression on a yellow-to-orange scale with yellow indicating low expression and dark indicating high expression. Based on the overall pattern of expression for these 500 genes, oocytes are given a hierarchical ordering, seen on the right, indicating their similarity in gene expression patterns to each other.



### C. Cluster Dendrogram of Bovine Oocytes Based on Global Gene Expression

Figure A2. Hierarchical relationship between bovine oocytes from different collection sources and maturation levels based on the global differential gene expression levels.



### D. Smear Plots Comparing Oocyte Gene Expression

Figure A3. Smear plots comparing gene expression of oocytes between oocyte recovery methods, within each maturation level. Each dot on the plot indicates one gene, those in red having significantly different expression levels (at FDR  $P < 0.05$ ). Points falling outside of the blue indicator lines are those genes that have an expression level difference in excess of two-fold between the two treatments in question (values  $> 0$  favour the first treatment listed, those  $< 0$  favour the second treatment).

## **A.17 Fixation Protocol for Transmission Electron Microscopy**

### **Primary Fixation**

1. Fix oocytes for 1.5 hours in freshly prepared 3% glutaraldehyde at room temperature
2. Transfer oocytes to a cryovial containing 0.5mL of phosphate buffer and store at 4°C until further processing

### **Agar embedding**

3. Embed oocytes in 4% agar using a stereomicroscope.  
Put oocyte in a minimal drop of phosphate buffer on a clean slide and put a drop of agar at 45-55°C on top of oocytes. Build up with more agar. Cool and solidify agar by placing directly on a dry ice pack.
4. Cut out a small block 2x2x2mm containing each oocyte, using a razor blade
5. Wash 2x for 5 min each in phosphate buffer, at room temperature

### **Secondary, post-fixation**

6. Post-fix in 1% osmium tetroxide for 1 hour at room temperature
7. Wash 2x for 5 min in phosphate buffer at room temperature

### **Uranyl block staining**

8. Wash 2x for 5 min each in double distilled water
9. Stain for 1 hour in 0.5% Uranyl acetate at room temperature, in the dark
10. Wash 2x for 5 min each in water

### **Dehydration and embedding (perform incubations on a shaker plate at room temperature)**

11. Incubate 2x for 5 min each in 50% ethanol
12. Incubate 2x for 5 min each in 70% ethanol \*leave in 70% ethanol overnight
13. Incubate 2x for 5 min each in 80% ethanol
14. Incubate 2x for 5 min each in 90% ethanol
15. Incubate 2x for 10 min each in 100% ethanol
16. Incubate 2x for 5 min each in propylene oxide
17. Incubate 1 hour in 2:1 propylene oxide: epon araldite
18. Incubate 1 hour in 1:1 propylene oxide: epon araldite
19. Incubate 1 hour in 1:2 propylene oxide: epon araldite
20. Overturn tubes and dry for 30s to remove as much propylene oxide as possible
21. Incubate overnight in epon araldite
21. Trim away excess agar from blocks and dry blocks briefly to remove excess Epon and propylene oxide
22. Embed in freshly prepared Epon+DMP 30 for 2 hours in moulds room temperature
23. Replace media in moulds with fresh Epon+DMP 30, being careful to avoid bubbles, incubate in moulds at room temperature for 24 hours
24. Move moulds to 37°C, incubate for 24 hours

25. Move moulds to 60°C, incubate for 24 hours
26. Remove blocks from moulds

Ultra-thin sectioning;

27. Cut a sequence 70nm sections from each oocyte at the periphery and the approxiamate equator
28. Stain sections with uranyle acetate for 15 minutes
29. Rinse 6x for 30s each by placing sections on water droplets
30. Stain with lead citrate for 5 min
31. Rinse 6x for 30s each by placing sections on water droplets
32. Gently dip sections in sterile water and blot dry
33. Transfer sections to a copper grid for microscopy

## VITA

Brittany Alanna Foster is the daughter of Denise Rose Foster and Glen Robert Foster and older sister of Kelsey. She was born in 1988 in Sault Ste Marie, ON where she was raised and attended high school. Following high school, Brittany moved to Guelph, ON where she completed her Bachelor of Science in Animal Biology in 2010, and later a Master of Science in Population Medicine in 2012. On completion of her MSc, Brittany moved to New Orleans, LA where she worked with Dr S.P. Leibo in reproductive technologies, before joining Dr Ken Bondioli's lab in 2014 to pursue her doctoral degree. She plans to graduate from Louisiana State University with a PhD in Animal and Dairy Sciences summer 2018.