Factors affecting the quality and function of the bovine periovulatory follicle

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Animal and Poultry Science

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September 7th, 2018
Blacksburg, VA

Keywords: Estrus, follicle, ovulation, oocyte, blastocyst

Academic Abstract

For many cattle operations, profitability depends on the success of reproductive management programs. Opportunities for improving fertility exist within the numerous challenges related to reproductive management. Non-conventional, creative tools for reproductive management could help producers overcome these challenges. In an effort to produce information that could be used to improve reproductive performance of cattle, the following studies were undertaken. The objectives of these studies were threefold: to determine whether GnRH administered as an epidural injection causes ovulation in healthy cows and heifers, to evaluate whether the follicular environment (specifically, follicle fluid) surrounding the oocyte during the maturation phase affects the ability of the cumulus-oocyte complex to progress through early embryonic development, and to investigate the relative effects of estradiol and progesterone on oocyte maturation and early embryo development.

Ability of GnRH to elicit an ovulatory response when administered as an epidural was evaluated in crossbred angus cows and heifers. The preliminary study evaluated this route of administration in crossbred angus cows. Animals were assigned randomly to either intramuscular or epidural administration, and ovaries were visualized via transrectal ultrasound every 6 h until ovulation of the dominant follicle. Results indicated that epidural administration of GnRH was able to trigger an ovulatory response, but timing of ovulation was not measured. The main experiment evaluated incidence of ovulation, time to ovulation, and ovulatory follicle size in crossbred angus heifers administered GnRH either epidurally or intramuscularly. Heifers were randomly assigned to treatment and ovaries were visualized every 4 h via transrectal ultrasound until ovulation of the dominant follicle. Results indicated that epidural administration of GnRH was able to elicit an ovulatory response in heifers, and the timing of ovulation and ovulatory

follicle size was not different between administration route. Further investigation is needed to determine if characteristics of the ovulatory response (such as the luteinizing hormone surge) and circulating concentrations of GnRH are altered by epidural administration, which may impact fertility.

GnRH administration is standard practice in many estrous synchronization programs. For fixed-time artificial insemination programs, the detection of estrus prior to insemination has been shown to improve conception and decrease early embryonic loss. The impact of behavioral estrus expression on the oocyte and early embryo were evaluated. Oocytes were matured *in vitro* in follicle fluid collected from synchronized cows who were classified as having expressed behavioral estrus or not expressing estrus. Embryo cleavage was not affected by estrus expression, but there was a tendency for improved blastocyst development in embryos matured in follicle fluid from animals who had expressed estrus. Cell number was not affected by estrus expression, but future research is needed as to the effect on oocyte acquisition of competence and early embryonic development.

Despite the progress that has been made in culture conditions for *in vitro* produced embryos, developmental capacity following fertilization is limited at best, with only around one-third of oocytes placed into maturation resulting in viable embryos. During *in vivo* maturation, the oocyte undergoes final maturation within the follicle, surrounded by a changing microenvironment of estradiol and progesterone. Although the effects of steroids on oocyte development *in vitro* have been studied on an individual basis, a direct comparison between the ratio of estrogen and progesterone relative to follicle size has not been investigated Effects of steroid hormones estradiol and progesterone on oocyte maturation and early embryonic development were evaluated. Oocytes were matured *in vitro* in media supplemented with either

estradiol, progesterone, or a combination of estradiol and progesterone. Oocytes were fertilized after maturation and cultured for 7 d until development to blastocyst stage. Addition of estradiol alone did not support oocyte maturation or early embryonic development *in vitro*, and a combination of estradiol and progesterone exhibited an inhibitory effect on oocyte maturation and early embryonic development. Addition of progesterone alone resulted in improved development when compared with estradiol alone or a combination of estradiol and progesterone.

These results indicate that efficiency of reproductive management programs is controlled by multi-faceted factors and opportunities for improvement of reproductive outcomes exist in all of these factors. Although ovulation can be elicited via epidural administration, the impact of this ovulatory trigger on fertility requires further investigation. Display of estrus after synchronization for fixed-time artificial insemination improves conception and decreases early embryonic loss and has a may improve blastocyst development. This effect on early embryo development could be the focus of future research, further improving fertility and possibly the efficacy of *in vitro* embryo production. Steroid hormones play crucial roles in oocyte competency and the addition of progesterone during *in vitro* maturation improves development compared with estradiol alone or a combination of estradiol and progesterone.

General Abstract

Reproductive success is critical for economic sustainability for many cattle operations. Creative tools for fertility management could help cattle producers overcome many challenges to fertility. In an effort to produce information that could be used to improve reproductive performance of cattle, the following studies were undertaken. The objective of these studies was to determine whether hormone administration as an epidural injection causes ovulation in healthy cattle (young and mature cattle assessed). Additionally, the second study evaluated whether the follicle (fluid-filled compartment surrounding the egg on the ovary) environment affects the female egg prior to ovulation, and the early embryo after fertilization. Finally, the third study looked at the impact of follicle fluid and specific hormones on embryo growth.

An experiment was conducted in cows and heifers to determine if administering a hormone as an epidural injection, as opposed to conventional methods, could cause ovulation of the follicle. Animals received either an intramuscular or epidural hormone injection, and the ovaries of the animals were observed on an ultrasound until the follicle ruptured, releasing the egg. Epidural administration of the hormone was indeed able to trigger the rupture of the follicle.

Hormone administration is standard practice in many cattle fertility programs. To maximize fertility, animals need to come into "heat" or estrus (period of sexual receptivity). Coming into heat is important for fertility in the female as it is indicative of impending ovulation and preparation of the egg for fertilization. In some reproductive management systems, reproductive cycles can be controlled in ways that deemphasize the need for behavioral estrus. Recent reports have suggested that animals in these systems that exhibit behavioral estrus are more fertile, as it makes it more likely for the female to conceive and stay pregnant compared to

females who do not come into heat. The impact of heat on the female egg and early embryo of the cow has not been investigated. To evaluate the impact of heat on embryos, eggs were taken from the ovary of the cow and matured in a cell culture lab overnight in media containing fluid taken from the follicles of animals who came into heat, and animals that did not come into heat. The eggs were then fertilized, and embryos developed. There was only a tendency for improvement in embryo development for those matured in fluid from animals in heat compared with animals not in heat.

When growing embryos in a culture lab, success rates are lower than embryos developing in the animal. When the egg is being prepared for release, it goes through important maturation steps to enable fertilization and eventual growth into a calf. Hormones in the follicle fluid facilitate maturation, and the conditions in the follicle are not easily replicated in the lab. The addition of these critical hormones to the lab conditions may help facilitate improved development in lab-produced embryos. Two hormones (estrogen and progesterone) were added to follicle fluid that was used in the lab culture environment to determine their effect on embryo growth. When progesterone was added, embryos grew well, matching the development rate of the control medium. When estrogen was added, embryos experienced poor development. Neither resulted in embryo development that exceeded the control medium.

These results indicate that control of reproduction in cattle is complex, and multiple opportunities exist to improve fertility. Future research on how the oocyte and embryo react to their environment is needed and will facilitate further improvement of reproductive management systems in cattle. Improved reproductive management will enhance efficiency, sustainability and profitability of cattle production systems.

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AI	Artificial insemination
ATP	Adenosine tri-phosphate
BMP4	Bone morphogenic protein 4
BMP6	Bone morphogenic protein 6
BMP15	Bone morphogenic protein 15
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine 3', 5'-monophosphate
CL	Corpus luteum
COC	Cumulus-oocyte complex
CYP17	Cytochrome P450 17A1
DND1	Dead protein homologue 1
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
FTAI	Fixed-time artificial insemination
GDF9	Growth differentiation factor 9
GnRH	Gonadotropin-releasing hormone
HDL	High-density lipoprotein
IM	Intramuscularly
LH	Luteinizing hormone
LLC	Large luteal cell
OCM	Oocyte collection medium
OMM	Oocyte maturation medium
$P450_{scc}$	P450 side-chain cleavage
PDE3A	Phosphodiesterase 3A
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PKA	Protein kinase A
SLC	Small luteal cell
SOHLH1	Spermatogenesis and oogenesis-specific basic helix loop-Helix1
StAR	Steroidogenic regulatory protein
STRA8	Stimulated by retinoic acid gene 8
TAF4B	TATA-box binding protein associated factor 4b
TGF-β	Transforming growth factor β

Acknowledgements

I would like to thank the people who helped make this dream a reality. My wonderful parents provided the loving support and understanding which helped get me through the toughest moments. Thank you so much for believing in me. To my advisor Shelly, I don't know how you put up with me for three years, but I am so grateful you did. Thank you so much for your support and mentorship. To my lab mates Veronica and Jake, thanks for all your help during the field projects which always seemed to fall on the worst weather, and in the lab during the seemingly endless IVF projects. It really does take a strong support group, and I never could have done it without you all.

Dedication

To God. I never give you enough credit. Thanks for never giving up on me.

Chapter 1 - Literature Review

Introduction

Reproductive failure in cattle is a major financial loss in both beef and dairy cattle production, with an annual combined cost to the beef and dairy industry of about $1 billion (Bellows et al., 2002). New methods to improve conception with fewer services and pregnancy retention are essential to the economic sustainability of both beef and dairy industries.

There are numerous challenges related to the reproductive management of both beef and dairy cattle. Some of these include the management of subfertile/infertile cows, efficacy of estrous synchronization protocols and even public perception concerns (relating to food safety) over how often and through what route cattle receive hormones for synchronization. Non-conventional, creative tools for reproductive management could help producers overcome these challenges.

The biological signals responsible for fertility in cattle are complex and have been the topic of intense research. In the past decade, one of the critical factors impacting fertility in animals synchronized for fixed-time artificial insemination (FTAI) has been identified as estrus display (Perry and Perry, 2008b; Richardson et al., 2016). Physiological display of estrus has been demonstrated to improve conception in FTAI protocols, and it may be that these effects of improved estrus may impact not only the reproductive tract, but the follicular microenvironment and acquisition of competence in the oocyte. While improved estrus display is primarily thought to improve reproductive tract function through improved uterine function, it may also be ideal for oocyte maturation and early oocyte development.

The importance of the steroid hormones estradiol and progesterone in oocyte maturation and early embryonic development are well established, but their individual roles in oocyte

maturation and early development is still under investigation. During maturation *in vivo* oocytes undergo critical cytoplasmic and nuclear development milestones under the influence of changing concentrations of estradiol and progesterone, as well as other follicle fluid components such as gonadotropins and growth factors (Moor et al., 1980; Fukui et al., 1982). To maximize the efficiency of *in vitro* embryo production systems, replicating the follicular environment as closely as possible is critical. Previous studies have reported mixed results with the addition of either estradiol or progesterone to maturation media *in vitro*, but since these hormones are a critical component for oocyte maturation, further investigation is warranted (Fukui et al., 1982; Silva and Knight, 2000; Salehnia and Zavareh, 2013).

Before launching new investigations into the effects of reproductive hormones and/or their administration on subsequent fertility, pertinent information from previous work must be reviewed. This review of literature will include broad discussions of factors that regulate the reproductive cycle of the cow, methods of estrous cycle manipulation and how that manipulation can impact fertility. More specifically, practices and circumstances that affect the ovarian follicle, oocyte and/or early embryo (both in vivo and *in vitro*) will be emphasized.

Reproductive Cyclicity in Cattle

Reproductive cyclicity in female mammals, also known as the estrous cycle is a series of events beginning at puberty and continuing throughout the life of the female (Downey, 1980; Crowe, 1999; Forde et al., 2011a). During these cycles, there are repeated opportunities for the female to become pregnant. Changes in hormonal levels during the cycle influence the growth and development of ovarian structures, as well as enacting changes within the reproductive tract (Colazo and Mapletoft, 2014). Hormonal control of reproduction is generally regulated by a series of positive and negative feedback loops (Roche, 1996). Feedback from structures on the

ovary communicate with the hypothalamus, anterior pituitary, and reproductive tract to coordinate the functions of the reproductive cycle and pregnancy (Hansel and Convey, 1983; Forde et al., 2011b).

The hypothalamus is divided into two sections in the female, the surge center located in the anterior, and the tonic center located posterior to the surge center and directly above the pituitary gland. The tonic center of the hypothalamus is responsible for basal secretions of gonadotropin releasing hormone (GnRH), releasing small pulses of GnRH over a period of days or weeks (Moenter et al., 1990; Moenter et al., 1992). These small pulses have varying frequencies and amplitudes which fluctuate during the estrous cycle, but generally occur every 1.5 to 2 h (Moenter et al., 1992; Goodman et al., 2013). The surge center, by contrast, secretes a high amplitude preovulatory "surge" of GnRH in response to hormonal triggers. The gonadotrope cells of the anterior pituitary respond to GnRH by releasing gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). The timing and patterns of release of these hormones is critical to completion of a successful estrous cycle and will be further described throughout this review.

The estrous cycle of the cow averages 21 days and consists of a follicular phase and luteal phase. These can be further broken down into a series of four phases: proestrus (follicular phase), estrus (follicular phase), metestrus (luteal phase), and diestrus (luteal phase). These categories are assigned based upon the relevant levels of reproductive hormones and ovarian structures present at any given time (Downey, 1980; Sartori and Barros, 2011). An overview of the events occurring during these phases will be presented here with more thorough descriptions provided later in the review.

Follicular Phase

The follicular phase of the estrous cycle is defined as the period from regression of the corpus luteum (CL) to ovulation and contains both proestrus and estrus (Forde et al., 2011b). The initiation of the follicular phase of the cycle is luteolysis, which inactivates the CL and causes progesterone concentrations to plummet. During the follicular phase, the primary functional ovarian structure is a developing dominant follicle. During the follicular phase, estrogen controls a cascade of hormone release by acting upon the hypothalamus (Auletta and Flint, 1988; Milvae et al., 1996) .

Proestrus

The period prior to sexual receptivity is classified as proestrus. This phase of the cycle takes place over the course of 2 to 5 days beginning with the destruction of the CL on the ovary and ending with the onset of sexual receptivity or estrus (Forde et al., 2011b). During proestrus, a major endocrine transition is taking place within the animal, from a period of progesterone dominance, to a period of estrogen dominance. This transition is partly mediated by gonadotropins from the anterior pituitary (LH and FSH). Under the direction of rising estrogen concentrations, the hypothalamus releases pulses of GnRH, stimulating the release of LH from the anterior pituitary. The release of LH release stimulates follicular development; particularly the development of the dominant follicle (Schally et al., 1971a; Weck et al., 1998). As the dominant follicle grows and develops, it secretes increasing amounts of estrogen, stimulating the release of hormones from the hypothalamus, pituitary, and ovary. These hormones work to prepare the ovary and uterus for ovulation, conception and pregnancy (Downey, 1980; Forde et al., 2011a). As estrogen levels continue to increase, the animal will begin to exhibit sexual

behavior, ending the proestrous phase and entering into the estrus period of the reproductive cycle (Vailes et al., 1992).

Estrus

Estrus marks the period of sexual receptivity in the female. It is characterized by visible behavioral signs such as lordosis and copulation directed primarily by high relative concentrations of estrogen (Allrich, 1994; Perry et al., 2014). The transition to sexual receptivity is gradual, and behavioral changes exhibited by the animal such as increased vocalization, locomotion, and attempts to mount other animals, can be indicative of her approaching sexual receptivity (Ozturk and Demir, 2010).

During the estrus phase, increasing levels of estrogen stimulate the hypothalamus to produce increasing pulses of GnRH, which then stimulate pulses of LH, increasing them from approximately 6 pulses per day to one per hour (Schally et al., 1971b; Sunderland et al., 1994). The increase in pulse frequency of LH stimulates final growth and development of the dominant follicle, and a threshold peak of estrogen triggers a surge of GnRH from the hypothalamus, which stimulates the release of a surge of LH (Sunderland et al., 1994). This preovulatory surge of LH triggers a cascade of events including increased follicular pressure, weakening of the follicular wall, and increased contraction of ovarian smooth muscle, leading to the ovulation of the dominant follicle on the ovary (Roche, 1996). After ovulation, the animal enters into the luteal phase of the reproductive cycle.

Luteal Phase

The majority of the estrous cycle takes place during luteal phase, beginning with ovulation, ending with luteolysis, and encompassing metestrus and diestrus. The dominant hormone during the luteal phase is progesterone, being secreted by the CL on the ovary. After

ovulation, the theca interna and granulosa cells undergo changes called luteinization, which enable them to begin secretion of progesterone and develop into the CL structure (Alila and Hansel, 1984; Milvae et al., 1996). Progesterone levels in the animal rise as the CL increases in size and steroidogenic ability. The progesterone causes the hypothalamus to reduce GnRH pulse frequency by the tonic center relative to the follicular phase, thereby reducing the release of LH and FSH, but not eliminating it completely (Rahe et al., 1980). Compared to the follicular phase, estrogen secretion remains low during the luteal phase. Relatively low estrogen levels will prevent behavioral estrus and high progesterone levels will prime the brain to enhance responsiveness to estrogen after luteolysis (Gibbs, 1966; Perry and Perry, 2008b).

Metestrus

Metestrus is a transitionary period beginning after ovulation and ending at the formation of a fully-functional CL (Auletta and Flint, 1988). Hormonal transitions are taking place during the structural rearrangements, with estrogen and progesterone in relatively low concentrations. During metestrus, the newly ovulated follicle undergoes major cellular and structural remodeling, to form a functioning endocrine gland on the ovary recognized as the CL (Hansel and Convey, 1983; McCracken et al., 1999a). The transition from ovulated follicle to functional CL is known as luteinization, and begins with the rupture of the follicle wall and blood vessels, leaving a corpus hemorrhagicum observable from the time of ovulation until d 1-3 of the estrous cycle (Hansel and Convey, 1983). From d 3-5 of the estrous cycle the CL begins to increase in size and take on a yellowish color and glandular texture. The mass of the CL continues to increase until mid-cycle, coinciding with maximal progesterone secretion (Forde et al., 2011b).

Diestrus

The longest phase of the estrous cycle is called diestrus and is the period of maximal progesterone secretion by the CL (Milvae et al., 1996). The high progesterone secretion stimulates physiological changes in the uterus to prepare an ideal environment for early embryo development and implantation (Forde et al., 2009). The diestrus period lasts between 10-14 days, with the duration directly influenced by the ability of the CL to produce progesterone (Kimmins and MacLaren, 2001).

The vigor, or ability of the CL to synthesize progesterone is dictated by the number of luteal cells present and the degree to which the CL is vascularized (Milvae et al., 1996). Large luteal cells develop from granulosal cells, and small luteal cells develop from the theca interna, therefore the size of the CL is directly correlated to the size of the ovulatory follicle (Auletta and Flint, 1988; Milvae et al., 1996). Angiogenic factors present in the follicle fluid at ovulation promote the vascularization of the developing CL, and the ability of the CL to become vascularized is most likely related to the concentration of angiogenic factors in the follicle fluid (Forde et al., 2011b).

Oogenesis

Oocytes originate from primordial germ cells which are derived from the primary ectoderm and migrate to the into the wall of the embryonic yolk sac. These primordial germ cells continue to migrate from the yolk sac to the genital ridge of the embryo from the at around 35 days of gestation in cattle (Erickson, 1966; Sanchez and Smitz, 2012). During this time of development, germ cells are classified as oogonia, and develop as clusters of cells connected by intercellular bridges (Pepling and Spradling, 1998). Upon arriving at the genital ridge, the primordial germ cells proliferate via mitosis giving rise to millions of primordial germ cells

(Sanchez and Smitz, 2012). The regulation of primordial germ cell migration and development is due, in part, to members of the TGFβ family including BMP4, BMP8b, and BMP2 (Ying et al., 2000; Ying and Zhao, 2001; Farini et al., 2005). After the PGCs colonize the gonads, they undergo an incomplete phase of mitotic division, failing to complete cytokinesis, which leads to the formation of germ cell nests in the gonad.

As previously mentioned, after mitotic proliferation primordial germ cells are colonized into germ cell nests, which persist into the initial phases of meiosis in the developing oocytes. Germ cell nest maintenance is not well understood, but studies have indicated that estrogen plays a major role in maintaining them (Pepling, 2006). Growth factors from the TGF-β family including GDF9 and BMP15 also appear to be involved in germ cell nest maintenance (Bristol-Gould et al., 2006). Survival of primordial germ cells are dependent on several factors such as factor in the germ line α, a factor which triggers the early expression of glycoproteins which will form the zona pellucida of the oocyte, and RNA binding proteins such as NANOS3 and DND1, which protect the primordial germ cells from apoptosis (Soyal et al., 2000; Tsuda et al., 2003).

Meiotic Arrest

In the fetal ovary, oocytes progress through the meiotic prophase through the diplotene stage. Initiation of the meiotic processes is dependent on retinoic acid and Stra8 (stimulated by retinoic acid gene 8), a cytoplasmic factor expressed by germ cells just prior to initiation of the meiotic prophase (Bowles et al., 2006; Koubova et al., 2006). Oocytes then enter into a prolonged resting phase known as the dictyate stage until the resumption of meiosis is stimulated by the preovulatory LH sure after initiation of puberty (Tripathi et al., 2010a). Cell cycle arrest at the diplotene stage in the oocyte can last for months or years depending on the organism, and

resumption of meiosis is morphologically characterized by germinal vesicle breakdown (GVBD; (Tripathi et al., 2010b).

It has been established that intraoocyte cyclic adenosine 3', 5'-monophosphate (cAMP) plays an important role in the maintenance of meiotic arrest at the diplotene stage of development (Mehlmann, 2005). While the long-standing hypothesis states that continuous transfer of cAMP from the cumulus cells to the oocyte through gap junctions are responsible for maintaining meiotic arrest, recent research has reported that the level of cAMP in the oocyte is critical for maintaining meiotic arrest (Webb et al., 2002; Vaccari et al., 2008). Additionally, cyclic guanosine 3', 5'-monophosphate (cGMP) acts as an inhibitory signal to maintain meiotic arrest in the oocyte (Norris et al., 2009; Sun et al., 2009). The cGMP passes from cumulus cells through gap junctions to the oocyte, where it inhibits the hydrolysis of cAMP by phosphodiesterase 3A, maintaining a high intraoocyte level of cAMP and blocking meiotic resumption (Norris et al., 2009). Cumulus cells surrounding the oocyte are also responsible for maintaining meiotic arrest. Factors such as hypoxanthine produced by the theca cells pass through gap junctions of the cumulus cells, helping to maintain meiotic arrest (Sirard and First, 1988).

Around the time of meiotic arrest, the nests of primordial follicles begin to break down, and oocytes become surrounded by a layer of somatic pre-granulosa cells to form primordial follicles. During nest breakdown oocytes not surrounded by somatic cells are lost via apoptosis (Kezele and Skinner, 2003; Bristol-Gould et al., 2006). Prenatally-formed primordial follicles make up the total reservoir of available oocytes for the entirety of the female's reproductive life (Pepling and Spradling, 1998).

Although now contained within a follicle structure, oocytes have not yet completed their development. As follicles are activated and recruited to the antral stage (discussed subsequently), the oocyte experiences concurrent growth and maturation to a primary oocyte. In the primordial follicle, the oocyte has an inner zona diameter of less than 30 μm and grows to around 120 μm by the tertiary follicle stage (Fair, 2003). During growth of the oocyte, transcripts are needed to synthesize proteins necessary for the metabolism of the cell. Additionally, the buildup of transcripts within the oocyte are needed for meiotic and developmental competence of the oocyte (Hyttel et al., 1997). Transcriptional activity is gradually increased as the nucleus of the oocyte is activated during the growth phase of the oocyte. Near the end of the growth phase, the transcriptional activity of the oocyte is inactivated until final maturation after the LH surge (Hyttel et al., 1997). During the growth of the oocyte, RNA content increases around 300-fold, while protein synthesis increases by 38-fold (Lintern-moore and Moore, 1979).

Folliculogenesis

Follicular growth and degeneration occurs continuously throughout the estrous cycle, with antral follicles developing in response to basal levels of FSH and LH. Antral follicles undergo a series of dynamic changes which include recruitment, selection, dominance, and atresia. In the cow, the major changes that occur in the ovary during the estrous cycle take place in the outer layer of the ovary, known as the ovarian cortex. Within the ovarian cortex, follicles of varying stages will grow and develop throughout the cycle in a process known as folliculogenesis (Sunderland et al., 1994; Roche, 1996; Forde et al., 2011b).

Activation of primordial follicles is not well understood, but the PTEN/PI3K pathway is known to be involved in the regulation of primordial follicle activation and arrest (Cantley, 2002; Reddy et al., 2005). KIT ligands are expressed in both early somatic cells and oocytes and are

thought to control initiation of follicular activation by promoting proliferation of granulosa cells, theca cell recruitment, and oocyte growth (Nilsson and Skinner, 2004). Expression of anti-Müllerian hormone by the granulosa cells is also involved in the regulation of primordial follicles, maintaining inactive primordial follicles in the resting pool (Durlinger et al., 2002; Fortune, 2003). Primordial follicles give rise to primary follicles which are identified by the development of a single layer of cuboidal granulosa cells. Progression from primordial to primary follicle is driven by transcription factors from the oocyte Sohlh1 and Nobox as well as TGFβ factor BMP4 from the early stromal cells (Rajkovic et al., 2004).

Primary to secondary follicle development is driven by intraovarian paracrine factors produced by theca cells, granulosa cells, and oocytes. Growth factors GDF9 and BMP15 secreted by the oocyte are required for the follicle to develop beyond the primary stage. TATA-binding protein 2 is an oocyte-specific transcription factor expressed during folliculogenesis and has been suggested to be a regulator of folliculogenesis in mice (Dong et al., 1996; Gazdag et al., 2009). A TATA box binding protein-associated factor known as TAF4B is produced by the granulosa cells and germ cells (Falender et al., 2005). Granulosa cells proliferate at a high rate during this phase of folliculogenesis, giving rise to a multi-layered secondary preantral follicle (Sanchez and Smitz, 2012).

While the development of early follicle stages takes place independent of gonadotropin signaling, once follicles reach the preantral stage they begin to express functional FSH and LH receptors, allowing them to respond to gonadotropins. Follicular development at this stage is still primarily directed by intraovarian factors, despite the follicle's ability to now respond to gonadotropins (Sanchez and Smitz, 2012).

Follicle antrum

The formation of the antrum in the ovarian follicle is a more complex process than if it was forming in simple epithelium. Instead of cell-cell junctions between a single layer of cells and the lining of a basal lamina, the secondary ovarian follicle has multiple layers of cells, and junctions between layers and surrounding cells. During follicle growth, multiple foci of fluid accumulate throughout the follicle where fewer cell-cell contacts are present, with the cavities expanding as a result of cell death. Although exact mechanisms are unknown, dead granulosa cells have been observed in healthy follicles, and the death of the cells would provide space for fluid to accumulate, and an osmotic force through the DNA left behind by the dead granulosa cells. This mechanism of cell death for fluid accumulation takes place in the early stages of antrum formation, and the secretion of osmotically active molecules toward the foci are needed to draw fluid into the spaces (Rodgers and Irving-Rodgers, 2010).

Development through the antral stages is dependent on gonadotropin secretion from the anterior pituitary. FSH is a critical stimulant for antral follicle development and induces the expression of LH receptors on the mural granulosa cells. The development of LH receptors on the granulosa cells is necessary for the follicle to respond to the developmental and ovulatory signals of LH. Additionally, insulin-like growth factor I and II cooperate with the gonadotropins to regulate follicular selection and development, along with activin and inhibin acting in paracrine roles and regulating LH-induced hormone synthesis in the granulosa and theca interna cells (Nayudu and Osborn, 1992; Zhou et al., 1997; Findlay et al., 2000; Khamsi and Roberge, 2001).

Composition of follicle fluid

Follicle fluid plays a crucial role in nuclear and cytoplasmic maturation of the oocyte as well as final follicle maturation and ovulation. Because of its contact with the oocyte and

surrounding granulosa cells, follicle fluid components can serve as an index for the function of the follicle (Spicer and Zinn, 1987). As the follicle and oocyte grow and develop, the composition of the follicle fluid undergoes dynamic changes (Aller et al., 2013).

Follicle fluid is composed of blood plasma and substrates locally produced by the theca interna and granulosa cells. In the follicle, sodium, chloride, and potassium concentrations are on a gradient suggesting an inward transport from serum to the follicle (Leroy et al., 2004). Glucose, a major energy source for ovarian cells, varies depending on stage of growth, and increases as follicular diameter increases (Landau et al., 2000). Protein in follicle fluid is about 75 % similar to the content of serum and does not appear to differ based upon follicle size or stage of the estrous cycle, suggesting that a substantial portion of the protein content of follicle fluid is derived from serum (Edwards, 1974; Wise, 1987).

Triglycerides are present in relatively high concentrations in small follicles and decrease as the follicle grows. Levels of triglycerides in small follicles are higher than in blood serum, suggesting that this component is differentially regulated in some manner. Furthermore, triglyceride concentrations in the follicle fluid do not change with diet or physiological status as they do in serum (Wehrman et al., 1991; Leroy et al., 2004). In follicle fluid, triglycerides may serve as an alternate energy source as oocytes and embryos cultured with triglycerides will absorb and metabolize triglycerides from the culture medium (Kim et al., 2001).

Cholesterol in follicle fluid is present at approximately 42 % of serum concentration, and as follicle size increases, cholesterol concentrations increase. Cholesterol present in follicle fluid is bound to high-density lipoprotein (HDL), and the increased concentration of cholesterol in large follicles is thought to accumulate due to the increased permeability of the follicle wall,

allowing larger molecules to pass through the follicle wall (Wehrman et al., 1991; Leroy et al., 2004).

Steroidogenesis

Steroid production is critical for normal reproductive function including the reproductive tissues and brain. Local ovarian steroid production is also essential for ovarian, oviductal and uterine functions. Regulation of steroid production is critical for fertility, as excess steroid production can lead to abnormal ovarian pathology. These abnormalities can include cystic follicles, anovulation, and subsequently, infertility (Jamnongjit and Hammes, 2006).

Steroidogenesis is a highly regulated process involving several signaling pathways across multiple cell types. In the ovarian cortex, follicles contain one oocyte surrounded by cumulus cells, a specialized type of granulosa cell, and together comprise the cumulus-oocyte complex (COC; (Webb et al., 1999). Mural granulosa cells surround the COC, which are surrounded by thecal cells (Sanchez and Smitz, 2012). Steroidogenesis in livestock species occurs via a 2-cell, 2-gonadotropin model, during which androgens are synthesized from cholesterol under the direction of gonadotropins LH and FSH (Hillier et al., 1994; Jamnongjit and Hammes, 2006). Androgens are synthesized from cholesterol in LH-stimulated theca interna cells, which are then converted to estrogen in granulosa cells under the direction of FSH. Receptors for LH are present on theca interna cells only prior to dominant follicle selection. At selection, LH receptors can also be found on granulosa cells (Walters et al., 2008). The enzyme responsible for the conversion of pregnenolone and progesterone to dehydroepiandrosterone (DHEA) and androstenedione, respectively, is called CYP17 and is primarily expressed in theca cells. Aromatase (CYP19), an enzyme responsible for the conversion of androgens to estrogens, is expressed primarily in granulosa cells (Jamnongjit and Hammes, 2006).

The primary substrate for steroidogenesis is cholesterol, which exists in two forms in cells and in plasma lipoproteins, specifically free cholesterol and cholesterol esters (Bao and Garverick, 1998; Christenson and Devoto, 2003). Free cholesterol is the precursor for steroidogenesis, and both theca interna and granulosa cells express the necessary enzymes to recruit and convert cholesterol to pregnenolone and/or progesterone. Steroidogenic regulatory protein (StAR) is the primary transporter of cholesterol from the outer membrane of the mitochondria to the inner membrane (Bao and Garverick, 1998). The inner mitochondrial membrane is the location of steroidogenic enzymes, and StAR protein is the rate-limiting factor of steroid production in steroidogenic tissues. The expression and activity of StAR protein is regulated by several factors, beginning with the activation of LH receptors in the theca interna cells. Activation of LH receptor-signaling via G protein induces second messenger activation which regulates StAR activity and steroidogenesis in general (Jamnongjit and Hammes, 2006).

Gonadotropin-induced elevation of cAMP has proven to be an important regulator of steroidogenesis. Gonadotropins bind to the G*s* subunit of the receptor, and upon activation, stimulate the activation of protein kinase A, which in turn elevates cAMP (Wood and Strauss, 2002). Elevation of cAMP in theca interna or granulosa cells promotes steroidogenesis by increasing the expression of StAR and increasing StAR activity via phosphorylation of serine (Strauss et al., 1999). Additionally, cAMP regulates the expression and activity of steroidogenic enzymes CYP19 and CYP17 in granulosa and theca interna cells, respectively, which in turn regulates the production of androgens and estrogens.

Cumulus-oocyte interactions

As follicles grow and develop, granulosa cells separate into 2 distinct sub-types: the cumulus cells, and the mural granulosa cells. The cumulus cells surround the oocyte and are in

direct metabolic contact, while the mural granulosa cells line the follicle wall to form a stratified epithelium within the basal lamina (Gilchrist et al., 2004). Cumulus cells have specialized tans-zonal cytoplasmic processes which penetrate through the zona pellucida of the oocyte and form the COC, (Albertini et al., 2001). Using this intimate connection, the oocyte and the surrounding cells experience a bi-directional communication, which is crucial in driving the development and maturation of both the oocyte and follicle. Gap junctions at the end of the cumulus cells processes facilitate the transfer of small molecular weight molecules between the oocyte and cumulus cell, and in between cumulus cells. Large molecular weight molecules are transported by receptor-mediated endocytosis (Gilchrist et al., 2004).

Molecules that move through gap junctions include ions, metabolites, and amino acids, which are necessary for oocyte growth and development (Simon et al., 1997). Gap junctions are composed of proteins known as connexins. Gap junctions allow for the exchange of metabolites, and removal of the oocyte from the follicle causes the closure of gap junctions, triggering the resumption of meiosis in the oocyte (Fair, 2003).

The oocyte is a pivotal regulator of folliculogenesis, and recent interest has focused on oocyte-secreted factors and the role of the oocyte in the process of ovarian function and fertility. Oocyte-secreted factors regulate folliculogenesis by controlling activities associated with growth and differentiation of granulosa cells (Eppig, 2001). Two members of the TGFβ superfamily, GDF9 and BMP15 are expressed in the oocyte during follicular development and are fundamental to the activation of primordial follicles and the final events of maturation and ovulation (Paulini and Melo, 2011). BMP6, GDF9, and BMP15 are all expressed in the oocyte, while BMP2, and BMP6 are expressed in granulosa cells, and BMP3b, BMP4, BMP6, and BMP7 are expressed in theca cells (Galloway et al., 2000; Gilchrist et al., 2006). These oocyte-

regulated pathways are responsible for differentiation of granulosa cells to the cumulus cell phenotype.

Cumulus cells display a high rate of proliferation, low steroidogenic capacity, and low LH receptor expression when compared with mural granulosa cells. Additionally, cumulus cells have the ability to secrete hyaluronic acid and undergo mucification/expansion, an ability mural granulosa cells do not possess (Armstrong et al., 1996; Eppig et al., 1997). Removal of the oocyte from the COC results in cumulus cells reverting to the mural granulosa phenotype, suggesting the oocyte is responsible for maintaining the cumulus cell phenotype, and that the default phenotype for granulosa cell development is towards the mural granulosa cell (Eppig et al., 1997; Li et al., 2000).

Follicular Dynamics

Physiological changes to the ovary take place in a predictable series of events during the course of the estrous cycle. The characteristic changes involving follicles and are known as follicular dynamics. The initial proposition for two waves of follicular development per cycle in cattle was made first by Rajakoski in 1960. With the development of transrectal ultrasonography, researchers were able to conclude that follicular growth in cattle occurs in a wave-like fashion, and that the majority of cattle experience two or three waves during a single cycle (Rajakoski, 1960; Ginther, 2016). Follicular wave emergence is triggered by FSH and is characterized by the sudden emergence of 18-40 small follicles of approximately 3-4 mm in diameter. Follicles grow at approximately the same rate for around 2 days, when one follicle is selected for dominance and the rest undergo atresia (Ginther, 2016). In both 2 and 3-wave cycles the emergence of the first follicular wave occurs on the day of ovulation, classified as d 0 of the cycle, with the second wave emerging between d 9-10 or 8-9 of the cycle for 2 and 3-wave cycles, respectively (Ginther

et al., 2017). A third wave will emerge on d 15-16 in 3-wave cycles. During non-ovulatory follicle waves, a follicle achieves dominance under the influence of high progesterone concentrations. In these cases, the dominant follicle does not ovulate and instead, will undergo atresia. If, however, the follicle reaches dominance around the time of luteolysis, the associated endocrine signals will trigger ovulation and the recruitment of the next follicular wave (Ginther et al., 1997). The regression of the CL occurs earlier in 2-wave cycles (d 16) when compared with 3-wave cycles (d 19), resulting in correspondingly shorter estrous cycles for 2-wave animals (Ginther et al., 1997; Adams et al., 2008).

Ovulation

Early in the follicular phase, low levels of progesterone stimulate GnRH pulse frequency from the hypothalamus, triggering additional release of LH from the anterior pituitary. The gonadotropin release stimulates the growth of the dominant follicle which secretes increasing amounts of estrogen as it develops (Karsch, 1987; Moenter et al., 1992). As follicles continue to grow and develop they release increasing levels of not only estrogen, but inhibin. Both hormones exhibit a negative feedback effect on the anterior pituitary, selectively inhibiting the secretion of FSH and allowing dominant follicle development. Upon reaching threshold levels, estrogen triggers the release of a GnRH surge from the hypothalamus, which then triggers a preovulatory surge of LH and FSH from the anterior pituitary (Karsch, 1987; Moenter et al., 1990; Webb et al., 2004)

The events of ovulation commence after the LH surge in a cascade ending in the rupture of the dominant follicle. Since the oocyte does not possess the receptors necessary to respond to LH signals, the resumption of meiosis in the oocyte is triggered indirectly via signals from the cumulus cells (Coticchio et al., 2015). Ovulation is the process by which purposeful destruction

of the follicular tissue leads to the release of the oocyte contained within. The LH surge induces a rapid and transient expression of genes in the granulosa cells critical for ovulation (Richards et al., 1998). These genes include progesterone receptor, PGS-2 and COX2, enzymes responsible for prostaglandin secretion (Sirois et al., 1992; Richards, 1994). Histamine, an organic nitrogen compound produced by basophils elicits a local immune-like response. Along with prostaglandins histamines control local elevated blood flow in the follicle known as hyperemia (Espey, 1980). After the LH surge, blood flow to the ovary increases 7-fold, causing an edematous effect on the theca interna due to the increase of vascular permeability from elevated histamine levels. The edema in the theca cells causes increased hydrostatic pressure in the follicle, facilitating its rupture (Gonçalves et al., 2012).

Angiogenic factors in follicle fluid indicate that the dominant follicle may have control over its own blood flow, ensuring the follicle has the necessary hormones and metabolites for final maturation and ovulation (Espey, 1980). Following the LH surge, theca cells of the dominant follicle begin to secrete progesterone instead of androstenedione. Local elevation of progesterone is essential for ovulation. Increased progesterone levels stimulate the secretion of collagenase, an enzyme that breaks down the component of connective tissue called collagen, which makes up the tunica albuginea, the outer covering of the ovary. Combined with the breakdown if the tunica albuginea, follicle fluid volume begins to increase, advancing the weakening of the follicular apex (Richards et al., 1998). Local production of prostaglandin stimulates lysosomes within the granulosa cells to rupture, releasing enzymes which further the degradation of the follicular apex. Prostaglandin also stimulates contractions in the myoid muscles in the ovary, increasing follicular pressure further, and forcing the apex of the follicle to

protrude dramatically from the surface of the ovary. Eventually, the apex of the follicle ruptures, releasing the cumulus-oocyte complex into the oviduct (Caldwell et al., 1973).

Luteinization

Luteinization is a process of tissue remodeling during which the follicle folds around the empty antrum, and the theca and granulosa cells differentiate into luteal cells, forming the CL. The success of a pregnancy is dependent upon the efficient transition from follicle to CL, and the vigor of the CL.

Within 7 h of the LH surge, theca and granulosa cells begin an irreversible transition to luteal cells (Richards et al., 1998). During this terminal differentiation, cells cease to divide and are reprogrammed to express luteal-specific gene patterns. After ovulation, histologic evidence has shown that theca cells transition to small luteal cells (SLC), with granulosa cells becoming large luteal cells (LLC; (Murphy, 2000). Proliferation of LLCs rarely occurs after ovulation, rather, these cells undergo extensive hypertrophy, increasing around 3-fold during the course of luteinization. From d 4-16 of the ovine estrous cycle, the number of SLC increases 5-fold. Proliferation of cells in the CL including fibroblasts and endothelial cells results in a mitotic rate similar to that of rapidly growing tumors (Niswender et al., 2000). In most livestock species, there are extensive amounts of migration and intermixing of follicular-derived cells during the formation of the CL, such that LLC, SLC, fibroblast, and endothelial cells are in close proximity to one another. This contrasts with CL development in primates, where migration and intermixing of cells are less extensive (Grazul-Bilska et al., 1995). Factors regulating cell proliferation during the development of the CL are not well understood, but may involve fibroblast growth factors, growth hormone, and LH, as well vascular endothelial growth factor.

The expression of CYP17 ceases at luteinization, stopping the conversion of cholesterol to androstenedione while allowing the synthesis of progesterone. This disappearance of CYP17 at luteinization is employed as a marker for luteinization in the sheep and cow. Expression of StAR protein is upregulated in granulosa cells during luteinization, as well as expression of p450 side chain cleavage (P45scc) enzyme required to recruit and convert cholesterol into progesterone (Pescador et al., 1996). Biosynthesis of progesterone requires only two enzymatic processes; the conversion of cholesterol to pregnenolone, triggered by P450scc in the inner mitochondrial membrane, and conversion of pregnenolone to progesterone catalyzed by 3β-hydroxysteroid dehydrogenase located in the smooth endoplasmic reticulum (Christenson and Devoto, 2003).

Luteolysis

If pregnancy does not occur following ovulation, The CL on the ovary needs to regress to allow for ovulation of a new follicular wave. This process, known as luteolysis, is induced by the endometrial lining of the uterus secreting prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in a pulsatile manner at 6 to 8 h intervals. Initial pulses of $PGF_{2\alpha}$ begin just prior to the onset of luteolysis, or prior to significant decrease in progesterone, and a series of 5-8 pulses of varying magnitude induce CL regression. Initiation of luteolysis begins with a change in the responsiveness of the uterus to oxytocin. Under the influence of prolonged exposure to progesterone (10-14 d), estradiol loses the ability to suppress the upregulation of oxytocin receptors in the endometrium. Oxytocin binding to its receptors in the endometrium stimulates the secretion of $PGF_{2\alpha}$, and oxytocin may mediate this stimulation through phospholipase C (Caldwell et al., 1973; Peterson et al., 1975; Lamothe et al., 1977; Lafrance and K Goff, 1986; Niswender et al., 2000).

Luteolysis in mammals takes place in two phases: it begins with rapid functional loss of the CL, and is followed by a more gradual structural regression (McCracken et al., 1999b). Functional and structural loss of the CL is mediated by several intraluteal factors which direct the actions of $PGF_{2\alpha}$ and the synthesis of progesterone. Endothelin-1 is a cell product of endothelial cells and a critical component of $PGF_{2\alpha}$-mediated luteal regression in cattle. During luteolysis, expression of the endothelin 1 gene EDN1 is upregulated, causing vasoconstriction of luteal tissue and subsequent decrease in progesterone production (Mamluk et al., 1999; Rosiansky-Sultan et al., 2006). Cytokines also play a role in both functional and structural luteolysis. Around the time of structural luteolysis, leukocyte number infiltration in the CL increases, inducing apoptosis in luteal tissue. Additionally, tumor necrosis factor α is present in the CL during luteolysis, promoting EDN-1 production and cell apoptosis (Sakumoto et al., 2000; Neuvians et al., 2004).

Changes in blood flow to the CL have also been implicated in luteolysis. In both sheep and cattle, blood flow to the CL decreases in response to $PGF_{2\alpha}$, causing a decrease in progesterone secretion. Loss of blood flow to the CL has been demonstrated to induce hypoxic conditions within the gland, causing depravation of substrates and nutrients for steroidogenesis and CL support. (Nett et al., 1976; Niswender et al., 2000). Hypoxic conditions within the CL cause a reduction in progesterone production by suppression of P450scc, an enzyme responsible for the conversion of cholesterol to pregnenolone. (Nishimura et al., 2006).

Estrous Synchronization

Assisted reproductive technologies such as fixed time artificial insemination (FTAI) and estrous synchronization are often employed within a reproductive management program to improve pregnancy success and shorten the calving interval. Designed to assist in the

reproductive management of groups of animals, synchronization programs restrict the interval during which estrus detection needs to be performed, or by eliminating the need to detect estrus altogether.

Presynchronization

Ovulation in response to an initial GnRH injection at a random point in the estrous cycle only induces ovulation in 50-60 % of cows (Moreira et al., 2001). Producers can improve this response rate by ensuring that cows are in the most ideal stage of their estrous cycle prior to beginning a synchronization protocol. This is commonly accomplished through use of a presynchronization program involving two injections of $PGF_{2\alpha}$. The goal of a presynchronization protocol is to have the majority of animals between d 5 and 12 of their cycle, increasing the probability that an LH-sensitive follicle will be present at the time of the first GnRH injection of the synchronization protocol (Pulley and Stevenson, 2015). Typically, two injections of $PGF_{2\alpha}$ 14 d apart are administered in cattle, with variations of 11 and 12 d not uncommon (Colazo and Mapletoft, 2014). For the first AI service postpartum, a presynchronization program is particularly effective in maximizing the response of the ovary to synchronization protocols (Small et al., 2009) and is more commonly used with dairy cattle than with beef cattle (due to ease of animal handling).

Ovsynch

While numerous programs are available to synchronize ovulation for the purpose of timed AI, the use of programs that include GnRH and $PGF_{2\alpha}$ to manipulate follicular development, ovulation, and luteal lifespan have been refined to optimize response to insemination. One of the most popular is the Ovsynch protocol. The first published Ovsynch protocol utilized a d0 GnRH injection, d 7 $PGF_{2\alpha}$, d9 GnRH, and d 10 timed AI (Pursley et al.,

1995a). Ovulation is triggered by the d0 GnRH injection and results in improved synchronization of follicular waves and reduced period of follicular dominance. Combined with a pre-synch protocol, Ovsynch can trigger ovulation after the first GnRH injection in about 70 % of animals (Bisinotto et al., 2014). Luteolysis of the CL with $PGF_{2\alpha}$ and the final injection of GnRH must be adequately timed so that the cow is allowed an optimal proestrus period. Amongst other considerations, optimal timing of these events is important for both preparation of the reproductive tract for pregnancy, and final follicle maturation (Ribeiro et al., 2012). In order for pregnancy to occur, progesterone levels in an animal at the time of insemination should fall below 0.3 ng/ml. A single dose of $PGF_{2\alpha}$ to induce complete luteolysis of the CL on d 7 after initial GnRH results in progesterone levels <0.03 ng/ml on the day of AI in 70-84 % of animals (Giordano et al., 2013). The interval between the induction of ovulation via the second GnRH injection and insemination is critical for fertility and must coincide with the window during which the oocyte and sperm are competent and viable for fertilization (Saacke, 2008).

Estrus Display during Fixed Time Artificial Insemination

During synchronization for FTAI, GnRH is used to induce ovulation of the dominant follicle. Follicle size is not always accurate indicator of estrus display, as small dominant follicles (<12.4 mm) can produce relatively high estrogen levels (≥8.4 pg/ml) at the time of the second GnRH injection (Jinks et al., 2013; Perry et al., 2014). Typically, approximately 50 % of synchronized animals will display estrus within 24 h after administration of $PGF_{2\alpha}$. Recent studies have demonstrated that animals subjected to FTAI that display standing estrus prior to insemination are more likely to become pregnant than animals that do not (Perry et al., 2014; Larimore et al., 2015). As previously discussed, elevated circulating estrogen levels are responsible for many physiological changes in the reproductive tract, including increasing

smooth muscle contractions, decreasing uterine pH, and increasing cervical mucus secretion (Richardson et al., 2016). Estradiol levels for animals exhibiting behavioral estrus after synchronization for FTAI have been reported at 9.9 ± 1.0 pg/ml compared with those not exhibiting estrus at 6.2 ± 0.67 pg/ml (Perry et al., 2014). These changes in the female in response to elevated estrogen are most likely responsible for the improved fertility in animals which display estrus, however the precise mechanisms behind the improvement in fertility remain unknown.

Developmental Competence Following Fertilization

As the oocyte grows and matures, it acquires the ability to undergo meiosis, fertilization, and initiate and sustain development. During oogenesis, oocytes acquire the ability to mature in concordance with follicle growth and development. While around 80 % of oocytes recovered from antral follicles are capable of undergoing maturation, only around 40-60 % of oocytes are able to support development to the blastocyst stage, known as developmental competence (Lonergan and Fair, 2008). Developmental competence of oocytes is affected by multiple factors, including follicle size, stage of follicular growth, and cumulus cell quality (Hendriksen et al., 2000; Lequarre et al., 2005). Overall, oocytes recovered from small (3-4 mm) follicles are less developmentally competent when compared with oocytes from follicles greater than 6 mm (Lonergan et al., 1994; Lequarre et al., 2005).

Transcriptional activity in oocytes also plays a role in developmental competence. Oocytes found in secondary follicles are transcriptionally active, storing mRNA needed for the eventual completion of meiosis after ovulation (Fair et al., 1997). With the onset of germinal vesicle breakdown following ovulation, transcriptional activity is reduced, and by the time of MII development transcriptional activity is no longer detectable (Tomek et al., 2002)

The oocyte undergoes dynamic cytoplasmic changes during the course of development and maturation, which directly impacts development potential. Cortical granules in the cytoplasm of the oocyte relocate from even distribution in the cytosol to the periphery of the cytosol membrane (Liu, 2011). Cortical granules are responsible in part for preventing polyspermy, and placement of the cortical granules is critical for developmental success after fertilization. Mitochondria also experience relocation concurrent with follicle/oocyte growth and development. During the growing phase, the mitochondria are located at the periphery of the oocyte, alongside lipid droplets (Dadarwal et al., 2015). As the follicle reaches preovulatory size, the mitochondria remain at the periphery of the oocyte, with increased contact of lipid droplets and mitochondria. Post-ovulation, as the oocyte undergoes fertilization, the mitochondria redistribute, resulting in even numbers of mitochondria and lipid droplets throughout the cytoplasm (Dadarwal et al., 2015).

Embryo Metabolism

During in vivo embryo development, the early preimplantation embryo develops in a constantly changing environment containing a complex variety of nutrients. In preparation for implantation, nutrients such as pyruvate, lactate, and glucose, all help prepare the embryo for implantation as it travels from the oviduct to the uterus (Leese, 1995). In addition to changes in the uterine environment, the embryo undergoes dramatic changes during the preimplantation phase. The development of the preimplantation embryo can be discussed in two phases: precompaction and postcompaction (Zander-Fox and Lane, 2012). These phases roughly coincide with the transport of the embryo through the oviduct and its transition to the uterus.

Precompaction embryos experience undifferentiated cell growth, but no net growth, and genetic control relies on maternal mRNA prior to embryonic genome activation around the time

of 4- to 8- cell stage (Gad et al., 2012). During the early stages of development, metabolic activity in the embryo is relatively low in terms of DNA/RNA synthesis, oxygen consumption, and protein synthesis (Leese, 1991; Gardner et al., 1996). By contrast, the later stages of development become regulated by the embryonic genome and the early embryo experiences a dramatic increase in metabolism similar to brain tissue, with a blastocyst experiencing a metabolic rate similar to tumor cells (Leese, 1991, 1995).

In vitro Culture of Embryos

In vitro embryo production (IVP) has been practiced in domestic animals for more than two decades. There are many applications for *in vitro* culture, but one of the main uses of the technology is to better understand normal *in vivo* embryo development. Additionally, IVP can be a useful model in evaluating pitfalls for *in vivo* production (Lonergan and Fair, 2014). More recently, since the advent of ovum-pick up, IVP has been used to generate genetically superior offspring from valuable females. There are unique challenges associated with *in vitro* production of embryos, and many of these challenges originate from the oocytes used in culture (Lonergan and Fair, 2014). Embryos produced from *in vitro* culture have been known to experience deficits such as abortion, increased birth weights, dystocia, and increased incidence of neonatal death (Kruip and Den Daas, 1997; Farin et al., 2006). To minimize the detrimental effects of *in vitro* culture, a quality embryo culture system must be instigated. Many variations of culture protocols exist, and quality of these systems are equally variable (Lonergan and Fair, 2014).

In vitro culture media

Culture media for *in vitro* embryo production generally consist of a combination of salts, energy sources, amino acids, proteins, buffers, and growth factors (Baltz, 2012; Houghton, 2012; Swain, 2012; Zander-Fox and Lane, 2012). The majority of solutes present in embryo culture

media consist of inorganic ions from salts. The ions Na^+ and Cl^- are the greatest concentration in every culture medium, with other inorganic ions such as K^+, Ca^{2+}, Mg^{2+}, and SO_4^{2-} present in lesser concentrations (Baltz, 2012). Sources of inorganic ions in culture media largely include salts used in media formulation. Ionic compounds introduced into media dissociate into component ions in aqueous solutions, therefore, once a medium has been made, all ions of a given species are interchangeable, and only the total concentration of each type of ion are important for healthy embryo development (Baltz, 2012).

Inorganic ions serve a variety of cellular functions and are critical for cellular metabolism. One of the main functions of ions is the establishment of intracellular gradients in which K^+ ion concentration is high, and Na^+ and Cl^- concentrations are low (Alexander and Grinstein, 2006). Cellular ion gradients are required for the maintenance of cell membrane potential, as well as serving as an energy source for metabolic transporters, and transporters responsible for regulating cellular pH (Hediger et al., 2004). Inorganic ions also contribute to the total osmolality of culture media.

The osmolality of a solution is a measure of total osmotic pressure, and media osmolality plays a major role in the ability of an embryo to develop (Waymouth, 1970; Waring, 1976). Cell volume is regulated osmotically, by adjusting the concentrations of osmolytes to control intracellular pressure, and the main defense mechanism against unwanted cell volume changes is the activation of Na^+/H^+ exchangers, which mediate the uptake of Na^+ into the cell (Alexander and Grinstein, 2006). One of the primary functions of inorganic ions in the extracellular space of culture media is to provide Na^+ and Cl^- during cell volume regulation. While the oviduct itself has proven to exist in a relatively high osmotic concentration (300-330 mosmoles/kg), lower osmolality (260-280 mosmoles/kg) in culture media appears necessary for normal embryo

development (Waymouth, 1970). One possibility for the difference might be explained by a missing component present in vivo which is critical for normal embryo development in higher osmolality, leading to a necessary lower osmolality *in vitro* to compensate for the absence of this component (Baltz, 2012).

To accommodate the relatively low energy requirements of the early precompaction embryo and the increased metabolic rate of the postcompation embryo and blastocyst, energy substrates are provided during *in vitro* culture. Energy metabolism in the early embryo is a balance of different metabolic pathways and shifts in metabolism by one pathway can impact pathways downstream (Zander-Fox and Lane, 2012). Embryos metabolize four main substrates for the purpose of generating energy: pyruvate, glucose, amino acids, and fatty acids. Pyruvate is oxidized directly by the mitochondria via oxidative phosphorylation to yield adenosine tri-phosphate (ATP), and during early embryo development, pyruvate metabolism is the only source of energy production available to the embryo (Biggers and Stern, 1973). Pyruvate is a critical component of culture media, particularly in cleavage-stage embryos where pyruvate concentrations are higher in concentration than media designed for blastocysts (Biggers and Stern, 1973).

Glucose is used by almost all mammalian cells as the primary energy source for ATP production, being taken up by cells and converted to pyruvate via the Embden-Meyerhof pathway in the cytosol (Zander-Fox and Lane, 2012). As previously discussed, early embryos (prior to the 8-cell stage) cannot utilize glucose for energy metabolism, but instead rely on pyruvate. In blastocyst-stage embryos, however, glucose alone is the preferred energy substrate (Brinster and Thomson, 1966). In mouse embryos, it has been suggested that glucose is not only a necessary substrate for energy metabolism, but that exposure to glucose is essential for healthy

embryo development (Martin and Leese, 1995). Studies have indicated that glucose uptake and glucose metabolism affect embryo viability. In cattle, it has been shown that blastocysts taking up glucose during development were more likely to produce pregnancies upon transfer when compared with embryos which did not take up glucose (Renard et al., 1980).

The oviduct and uterus contain significant levels of amino acids (AA). Amino acids serve multiple functions in the embryo, including substrates for energy, pH regulation, and protein and nucleotide synthesis (Epstein and Smith, 1973; Takahashi and First, 1992; Edwards et al., 1998). Amino acids play a critical role in embryo metabolism and development, and addition of AA to culture media significantly improves embryo development *in vitro* (Takahashi and First, 1992; Steeves and Gardner, 1999). Composition of AA required by the developing embryo differs based on stage of development. During precompaction, the AA content in culture media is similar to that of Eagle's non-essential AA, while postcompation embryo culture media contains a more complete range of AA similar to those used for the culture of somatic cells (Eagle, 1959).

Follicle fluid in culture media

During in vivo development and maturation of the oocyte, final maturation and resumption of meiosis occurs in the presence of ovarian follicle fluid. Follicle fluid is composed of a complex mixture of follicle cell secretions and blood serum, and the metabolites present in follicle fluid are crucial for the regulation of oocyte development (Coleman et al., 2007). In mammals, oocytes mature in proximity to follicular somatic cells which affect their development (van den Hurk and Zhao, 2005). During oocyte growth, metabolic control is directed by both the oocyte as well as the theca and granulosa cells of the follicle. Follicular cells produce large quantities of steroids and a variety of proteins, including growth factors. Granulosa and theca

cells produce paracrine factors which regulate many aspects of oocyte development, and oocyte-secreted factors also help regulate oocyte maturation (Coleman et al., 2007).

During *in vitro* culture, oocytes are removed from the follicle prior to final maturation which would otherwise be initiated by the LH surge in the female. Maturation *in vitro* is conducted to compensate for the discrepancy, and the use of follicle fluid in maturation media has been investigated as a way to improve maturation of the oocyte. Multiple studies have claimed beneficial effects of maturing oocytes in follicle fluid during *in vitro* culture (Kim et al., 1996; Choi et al., 1998a), however results are variable and are dependent on follicle size and quality.

Size of the follicle used in supplementation during *in vitro* culture influences maturation and development in oocytes and early embryos. In a previous study, supplementing fluid from large (>8 mm) follicles during maturation resulted in improved cleavage and blastocyst development when compared with fluid from small (2-5 mm) follicles (Ali et al., 2004). Results have also shown an inhibitory effect of supplementation with large follicle fluid. Supplementation with large (10-20 mm) follicle fluid at a rate of 100 % resulted in an inhibitory effect on oocyte maturation and embryo development when compared with supplementation of small (2-5 mm) follicle fluid (Choi et al., 1998b). Supplementation with 100 % follicle fluid has been previously shown to have an inhibitory effect on maturation and early embryonic development, and complete supplementation of maturation media with follicle fluid *in vitro* will not adequately support oocyte maturation (Ayoub and Hunter, 1993).

The success of embryo development *in vitro* after supplementation with follicle fluid during maturation is also dependent upon the concentration of fluid supplemented, and relative status of the LH surge. Follicle fluid supplemented prior to the LH surge at the time of GnRH

injection had a negative impact on oocyte maturation when supplemented at either 10 % or 40 %, while follicle fluid supplemented 20 h post-GnRH injection improved meiotic maturation and early embryonic development (Romero-Arredondo and Seidel, 1996). Since the composition of follicle fluid changes rapidly after the LH surge and final maturation takes place during this time, supplementation with fluid that was collected after the LH surge should better support the final maturation of the oocyte when compared with fluid taken from follicles prior to the LH surge.

Conclusions

To maximize the efficiency of reproductive management in cattle, it is important to consider the physiology behind the estrous cycle before manipulating it for production programs. It is clear that control of the estrous cycle is complex and multifaceted, with aspects of the cycle still not well understood. Of critical importance is the fact that follicular and luteal communication with the hypothalamus control the reproductive cycle via hormonal interactions. The importance of these interactions are illustrated by previous research showing that follicle size at ovulation is correlated with high peak estradiol, and display of estrus corresponding with peak estradiol is associated with conception and early pregnancy establishment (Jinks et al., 2013; Perry et al., 2014). Progesterone is equally as important for its involvement in maturation of the oocyte and early pregnancy establishment, with higher post-LH surge progesterone improving early pregnancy establishment (Henricks et al., 1971; Ozturk and Demir, 2010).

Further understanding of how estrus display influences fertility is critical in maximizing effectiveness of estrous synchronization. Fixed-time artificial insemination in particular relies on timing of injections for insemination rather than display of estrus, especially in the dairy industry, but an increasing amount of data are reporting the importance of behavioral estrus to fertility in synchronization programs in both beef and dairy animals (Perry and Perry, 2008a, c;

Perry et al., 2014; Pereira et al., 2016; Richardson et al., 2016). The effect of behavioral estrus has been shown to positively influence the reproductive tract, but the effects of estrus on the oocyte and early embryo have not been investigated. Furthering the understanding of *in vivo* influences on the oocyte and early embryo could be important for the fertility of animals that conceive after exogenous hormone administration.

The administration of exogenous hormones and antibiotics to animals that enter the human food supply has been a topic of debate, especially in the past decade. Frequency of administration of hormones and the route by which they are administered are part of the public concern and include those hormones used for estrous synchronization programs. Development of effective, alternative techniques for reproductive management that do not use as many hormones or use them more efficiently without sacrificing animal welfare and productivity are crucial for the continued sustainability of the industry.

It has been well established that steroid hormones influence fertility in cattle. Estradiol and progesterone influence the hypothalamus, pituitary, and reproductive tract, as well as playing a role in oocyte maturation and early embryo development. The production of embryos *in vitro* regularly includes estradiol in maturation media but does not include progesterone. The influence of progesterone on the oocyte post-LH surge, and the early embryo post-fertilization is still under investigation, but there have been reports suggesting that progesterone may be an important regulator of oocyte maturation and early embryo development (Girmus and Wise, 1992; Clemente et al., 2009; Salehnia and Zavareh, 2013). Understanding the effects of both estradiol and progesterone individually on *in vitro* maturation and development, as well as the interaction between these hormones will elucidate the hormonal needs of the oocyte and early embryo

during *in vitro* development, which may lead to improvement for *in vitro* embryo production systems.

Chapter 2 - Epidural administration of gonadotropin releasing hormone during a fixed-time artificial insemination protocol in beef cows and heifers

Abstract

Current challenges facing cattle producers necessitate the development of non-conventional, creative tools for reproductive management. Some of these challenges include improving the response rate and efficiency of estrus synchronization prior to insemination while simultaneously meeting demands of the consumer to reduce exogenous hormone administration. With these challenges in mind, two experiments were conducted to determine the efficacy of an alternative route of synchronization hormone administration, namely, epidural administration of gonadotropin releasing hormone (GnRH). The first experiment was preliminary proof-of-concept with a singular objective, which was to determine whether epidural injection of GnRH would cause ovulation in mature beef cows in conjunction with a fixed-time artificial insemination protocol. Fourteen crossbred non-lactating beef cows randomly assigned to one of two treatments where the final injection of GnRH the CoSynch estrus synchronization protocol was either administered intramuscularly (IM; n=7) or as an epidural (n=7). Beginning 12 h after GnRH injection, cows were ultrasounded transrectally every 6 h until ovulation of the dominant follicle. In this preliminary experiment, epidural injection of GnRH did indeed cause ovulation of the dominant follicle. Incidence of ovulation did not differ (P>0.10) between IM (7/7; 100 %) or epidural (5/7; 71.4 %) treated beef cows. Based upon the results of the preliminary experiment, the main experiment was designed to determine whether epidural injection of GnRH was equally effective in cyclic beef heifers, and whether synchrony of ovulation would differ between administration routes. Fourteen crossbred heifers (348.45 ± 8.38 kg body weight; 14.57 ± 0.57 months of age) were randomly allocated to one of the two treatments that had been

administered during the preliminary experiment: either IM (n=12 across both replicates) or epidural (n=16 across both replicates) injection of the final GnRH of the CoSynch protocol. Beginning 12 h after treatment, ovaries were ultrasounded every 4 h until ovulation of the dominant follicle. No differences in incidence or timing of ovulation were detected. Overall mean time to ovulation was 26.1 ± 1.06 h. These two experiments prove that epidural administration of GnRH causes ovulation of the dominant follicle in mature beef cows and heifers. No advantages or disadvantages in incidence or time to ovulation were detected. Thus, although it is effective, it does not appear that this alternate route of GnRH administration is capable of improving estrus synchronization response or frequency. Additional research would be needed to determine this method's usefulness in meeting consumer demands to reduce exogenous hormone administration.

Introduction

Reproductive failure is a source of major financial loss in both beef and dairy cattle production, with an annual cost of about $1 billion (Bellows et al., 2002). With production costs rising, the economic losses associated with poor reproductive performance are projected to continue rising. New methods to improve fertility and pregnancy retention with fewer services are essential to the economic sustainability of both beef and dairy industries. Opportunities for improving fertility exist within the numerous challenges related to reproductive management. Some of these include the management of subfertile/infertile cows, efficacy of estrous synchronization protocols and even public perception concerns (relating to food safety) over how often and through what route cattle receive hormones for synchronization. New methods of reproductive management are needed to help producers overcome these challenges.

Problem breeder or repeat breeder cows are a challenge that can be found in beef and dairy herds, and are defined as those animals that fail to become pregnant during a 60-90-d breeding season, or after 3 or more services via artificial insemination (AI; (Ferguson et al., 2012). Previous studies have investigated reproductive profiles of fertile and infertile matings and shown that infertile cattle exhibit a prolonged estrus period and delayed pre-ovulatory luteinizing hormone (LH) surge when compared with fertile animals (Erb et al., 1976; Gustafsson et al., 1986; Albihn, 1991). Infertile cattle also experience a delay in post-ovulatory rise in progesterone, due to the developing corpus luteum producing less progesterone per weight of luteal tissue. This developing luteal tissue may be producing less progesterone because the cells are less responsive to luteotropins such as LH and potentially PGE_2 (Shelton et al., 1990).

Problems with fertility can also stem from follicular cysts. Follicular cysts are most often encountered in dairy cattle and are associated with abnormal estrus behavior and infertility. Cystic follicles are defined as having a diameter of at least 17 mm, a thin follicular wall, and persist on the ovary for at least 6 days (Rizzo et al., 2011). The occurrence of cystic follicles in the dairy industry is varied, ranging from 5-30 %, and contributes to economic loss through more days open, increased veterinary costs, and culling after reproductive failure (Peter, 2004; Vanholder et al., 2006). While the etiopathogenesis of follicular cysts are not well understood, current research indicates a multifaceted cause which includes dysfunction of the hypothalamic-pituitary-ovarian axis, insufficient gonadotropin releasing hormone (GnRH) release, and reduced follicular receptor responsiveness to gonadotripic stimuli (Silvia et al., 2002; Peter, 2004).

Regardless of the cause, these subfertile/infertile cows reduce reproductive success within any beef or dairy herd and are difficult to identify prospectively. But even cows that are considered fertile sometimes fail to become pregnant after their first insemination. Many of

these animals, whether fertile, subfertile or infertile, are inseminated following a fixed-time artificial insemination (FTAI) protocol. Conception rates from these protocols are widely variable depending on the health/nutritional status of the herd, weather conditions, lactation demands, or estrus display prior to insemination (Stevenson et al., 2015; Richardson et al., 2016; Garcia-Ispierto et al., 2018). Even under optimal circumstances, conception rates do not reach 100 %, indicating that there is opportunity for improvement.

One way to improve the response to FTAI would be to reduce the variability in the cows' response to the injections (including incidence of ovulation and timing of ovulation). In some studies, ovulation following GnRH injection has varied from 63 to 87 % (Stevenson et al., 2008; Stevenson and Pulley, 2016). Although the time from GnRH injection to LH surge is relatively short and consistent, the time from GnRH injection to ovulation ranges from 24 to 32 h (Pursley et al., 1995b; Wiltbank and Pursley, 2014). Thus, it is likely that conception rates to FTAI could be improved by increasing the percentage of cows that ovulate following an injection of GnRH and/or reducing the variability in the time from GnRH injection to ovulation.

Variability in response to GnRH injection could possibly be reduced by changing the route of GnRH administration. Many pharmacological agents such as local anesthesia and analgesics are administered in the epidural space in the cow, and this method of administration can also be used to elicit a more direct pharmacological response. Bovine ovaries are innervated by sympathetic neurons stemming from the ovarian plexus and hypogastric nerves, and the epidural administration of GnRH has been shown to influence the ovaries, as it is effective in treating follicular cysts. This method of administration appears to be effective because follicular cysts develop GnRH receptors, and as a result, epidural administration of GnRH analogue can

trigger an ovulatory response in cystic cattle (Gourlay et al., 1985; Hogan, 2002; Rizzo et al., 2011).

Although epidural administration of GnRH has been demonstrated to be an effective treatment for follicular cysts when compared with intramuscular injection, it has not been investigated as a method for generally improving fertility in beef and dairy herds. Epidural administration of GnRH may prove to be a useful management tool, especially when facing selected challenges. Epidural administration of GnRH may elicit a local trigger for the LH surge, could shorten and/or lessen variability in the time from injection to ovulation and may increase the percentage of females that eventually ovulate in response to the injection. It also could prove to be a more effective route of GnRH administration for problem breeders and may be deemed a more acceptable injection site in response to the public's food safety concerns. Prior to investigating the efficacy of epidural administration of GnRH as reproductive management tool, however, it was imperative to prove that this method of administering GnRH does indeed causes ovulation in healthy females subjected to a FTAI protocol. Therefore, the objective of this preliminary trial was to determine whether GnRH administered as an epidural injection causes ovulation in healthy cows and heifers.

Materials and Methods

These studies were conducted at the Virginia Tech beef cattle facilities with the approval of the Institutional Animal Care and Use Committee in December 2016 and March 2018.

Preliminary Experiment

Animals, Housing, and Diet

The objective of this preliminary experiment was to determine whether epidural administration of GnRH was capable of causing ovulation in mature, cyclic beef cattle subjected to a FTAI protocol. Fourteen crossbred non-lactating beef cows were housed on mixed grass

pasture with ad libitum access to grazing and water. The 14 cows were split into two replicates (n=7 for each replicate) that were conducted consecutively.

Estrous synchronization

Animals were randomly allocated to one of two treatments: final injection of GnRH administered intramuscularly; or administered as an epidural injection. All cows were subjected to a CoSynch estrous synchronization protocol and were treated the same for the first two injections of the CoSynch protocol. For this protocol, an intramuscular injection of GnRH (100μg of gonadorelin diacetate tetrahydrate; 2mL of Cystorelin, Merial Ltd., Iselin, NJ) was administered on d 0 to all cows. On d 7, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 25 mg of dinoprost tromethamine; 5 ml of Lutalyse, Pfizer Animal Health) was administered intramuscularly to all cows. Approximately 60 h after $PGF_{2\alpha}$ administration, cattle were given GnRH (100 μg of gonadorelin diacetate tetrahydrate as 2 ml of Cystorelin) either intramuscularly (IM; n=7) or in the sacrococcygeal epidural space (n=7).

Ovarian ultrasonography

Ovaries for each animal were examined via transrectal ultrasound (Ibex ®Pro, E.I Medical Imaging, Loveland, CO) using a 7.5 MHz transrectal transducer. Ovaries were localized on the ultrasound screen and complete ovarian maps were drawn for each ovary at each ultrasound event. Size and position of follicles >5 mm in diameter and corpora lutea were recorded to determine cyclic status prior to administration of $PGF_{2\alpha}$. Serial ultrasounds to verify the incidence of ovulation began 12 h after secondary GnRH administration and were conducted every 6 h until ovulation of the dominant follicle.

Main Experiment

Animals, housing, and diet

The objective of this experiment was two-fold: 1) to determine whether epidural injection of GnRH could cause ovulation in cyclic beef heifers subjected to a FTAI protocol, and 2) to determine whether synchrony of ovulation would differ between GnRH administration routes. Fourteen crossbred heifers with mean weight of 348.45 ± 8.38 kg and age of 14.57 ± 0.57 months were housed on mixed grass pasture with ad libitum access to grazing and water. Individual animals were subjected to this protocol twice, in two different replicates with 7 days in between replicates.

Estrous synchronization

As for the preliminary experiment, animals were randomly allocated to one of two treatments: final injection of GnRH administered IM; or administered as an epidural injection. Briefly, all heifers were subjected to a CoSynch estrous synchronization protocol and were treated the same for the first two injections of the CoSynch protocol with intramuscular injections of GnRH on d 0 (100 μg of gonadorelin diacetate tetrahydrate; 2 ml of Cystorelin, Merial Ltd.) and $PGF_{2\alpha}$ on d 7 (25 mg of dinoprost tromethamine; 5 ml of Lutalyse). Approximately 30 h after $PGF_{2\alpha}$ administration, cattle were given GnRH (100 μg of gonadorelin diacetate tetrahydrate as 2 ml of Cystorelin) either IM (n=12) or in the sacrococcygeal epidural space (n=16).

Ovarian ultrasonography

Transrectal ovarian ultrasonography was conducted using the same methods described for the preliminary experiment. Ovaries were localized on the ultrasound screen (7.5 MHz transrectal transducer; Ibex ®Pro, E.I Medical Imaging) and complete ovarian maps were drawn for each ovary, which included all visible luteal structures and follicular structures >5 mm in diameter. Ovarian structures were observed and recorded immediately prior to $PGF_{2\alpha}$

administration of the first replicate in order to determine cyclicity status. Beginning 12 h after secondary GnRH administration, ovaries were ultrasounded every 4 h until the dominant follicle had ovulated.

Statistical analysis

Data for main effects of GnRH administration method were analyzed using the GLMMIX procedure in SAS. Where applicable, dependent variables were incidence of ovulation, time to ovulation initial follicle size, ovulatory follicle size, and ovulation side. Class variables were animal ID, replicate, and treatment. Replicate was also considered a random variable in the model. Time to ovulation was recorded as the time of the ultrasound event during which the dominant follicle was no longer present. Data for the preliminary experiment and main experiment were analyzed separately and the results from those analyses are, likewise, reported separately. Results from both experiments are reported as the least squared means ± the standard error of the mean. Statistical significance was declared at $P<0.05$.

Results

Preliminary Experiment

Results for both the preliminary experiment and main experiment are summarized in table 2-1. Overall there was no difference in incidence of ovulation between treatments in mature, non-lactating beef cows. The incidence of ovulation for the epidural animals was 71.4 ± 0.000019 % (5/7) in the epidural animals and 100 ± 0.000031 % (7/7) for the IM animals. There was no difference in initial follicle size (13.10 ± 1.14 mm epidural, 13.0 ± 0.97 mm IM), ovulatory follicle size (14.70 ± 0.92 mm epidural, 14.36 ± 0.78 mm IM; Fig. 2-1), or side of ovulation between treatments. Six out of 12 ovulations occurred on the right ovary (50 %) while the other 6 ovulations occurred on the left ovary (50 %).

Main Experiment

There was no difference in incidence of ovulation or time to ovulation between heifers receiving epidural administration of GnRH and heifers receiving IM (Fig. 2-2). Incidence of ovulation did not differ between treatments. In the epidural treatment 75 % (12/16) heifers ovulated, while 75 % (9/12) IM heifers ovulated. Time to ovulation was 28.0 ± 3.58 h in the epidural treatment, and 29.6 ± 3.8 h in the IM treatment. Of the 21 total ovulations across both replicates, 71.4 % occurred on the right ovary (15/21), while 28.5 % occurred on the left ovary (6/21). The mean follicle size at initial ultrasound (conducted at secondary GnRH administration) was not different between treatments: 9.63 ± 0.63 mm for epidural-treated animals, and 11.44 ± 0.77 mm for IM. Likewise, mean follicle diameter at ovulation did not differ between treatments, measuring 12.91 ± 1.26 mm for epidural and 12.0 ± 1.39 mm for IM (Fig. 2-3).

Discussion

Previous research has shown that epidural administration of GnRH can elicit a response in cystic dairy animals (Rizzo et al., 2011), but has not been studied in induction of ovulation in normally cycling animals. Thus, the current work was undertaken to determine whether epidural administration of GnRH would induce ovulation in cyclic cattle and was conducted in conjunction with a FTAI synchronization protocol.

Incidence of ovulation following epidural administration of GnRH was recorded initially in mature, non-lactating cyclic beef cows. Since this first experiment was a preliminary experiment that was prerequisite for additional work, it was designed to address a single question: does epidural administration of GnRH cause ovulation in cyclic cows? During this preliminary study, the time from injection of GnRH to ovulation was not precisely timed.

Anecdotal information, however, suggested there could be differences in the timing of ovulation based on method of GnRH administration. Therefore, as the subsequent main experiment was being designed, the time between ultrasound events was shortened from 6 h intervals to 4 h intervals in order to provide a better indication as to whether route of administration of GnRH affected time to ovulation. Of course, even more frequent ultrasound examinations (<4 h intervals) would have been preferable as they would have provided a more precise measurement of time to ovulation. Because the main experiment was being conducted in young heifers, however, concerns over animal welfare prevented more frequent examinations of the ovarian structures via transrectal ultrasonography. Furthermore, we felt the biological significance of putative difference in time to ovulation could be adequately captured using 4 h intervals.

The characteristics and timing of the LH surge following GnRH injection was not evaluated in these studies as it was beyond the scope of the objectives (although related). Previous work conducted in cattle indicates that the LH surge occurs within a quick, fairly consistent timeframe after GnRH administration. The timing of the LH surge following intramuscular injection of GnRH is 1-2 h, with little variation (Pulley et al., 2015; Rantala and Taponen, 2015; Stevenson and Pulley, 2016). This suggests the effect of GnRH when administered intramuscularly is fairly consistent despite differences in blood volume and metabolism between animals. For these reasons, it seems unlikely that route of administration of GnRH would significantly affect the timing of the LH surge.

Other characteristics of the LH surge may affect ovulation, however, and could have differed between intramuscular and epidural administration routes. The presence of progesterone, for example, is known to affect the GnRH-induced LH surge. A study in crossbred Angus heifers found that high progesterone levels at the time of GnRH administration negatively impacted the

magnitude and efficiency of the LH surge. When GnRH was administered 48 h after the vaginal insertion of a progesterone-infused controlled internal drug release device the magnitude of the GnRH-induced LH surge was reduced when compared with heifers receiving GnRH either 6 h prior to or immediately upon device insertion. Additionally, dominant follicle size and ovulatory response was diminished in heifers receiving GnRH 48 h after device insertion (Perry and Perry, 2009).

Characteristics of the LH surge can also differ between the different analogues of GnRH. In a study measuring the efficacy of four GnRH analogues (Cystorelin, Factrel, Fertagyl, and Ovacyst), Factrel produced a lower peak LH concentration and decreased ovulatory response when compared with the other products and despite differences in follicle size, body condition score, and parity within animals (Souza et al., 2009). Another study compared peak LH surge concentration and duration in spontaneous and gonadorelin-induced LH release in dairy cattle. In animals administered 100 μg of gonadorelin 72 h after $PGF_{2\alpha}$ treatment, the duration of LH release was shorter (6.1 ± 0.8 h) in gonadorelin-treated animals compared to spontaneous (11.0 ± 0.7 h) LH release. Interestingly, maximal LH release was similar between spontaneous and gonadorelin-induced groups (Lucy and Stevenson, 1986). Overall these factors indicate it might be worthwhile to further examine the characteristics of the LH surge in future experiments where multiple analogues of GnRH are administered epidurally.

During the first replicate of the main experiment involving heifers, 7 heifers failed to respond to the synchronization protocol and never developed a dominant follicle. Although the ovaries of the heifers were examined immediately prior to $PGF_{2\alpha}$ administration of the first replicate to check for cyclicity status, it is possible that these heifers were not actively cycling prior to and/or during the synchronization protocol. Unfortunately, a single transrectal ultrasound

observation is not always informative of cyclicity status. During the second replicate, however, all animals responded to the synchronization protocol, even animals that failed to respond during the first replicate. Previous research has shown that the ability of a heifer to respond to GnRH changes as a prepubertal heifer grows, with the greatest response ability occurring around puberty, although animals as young as 1 month are able to generate an LH surge in response to GnRH administration (Schams et al., 1981; Nakada et al., 2002).

In the present studies, epidural administration of GnRH successfully induced ovulation of the dominant follicle in association with FTAI synchronization of normally cycling cows and heifers. Previously, this type of response had only been demonstrated in cystic dairy cattle. In a study conducted by Rizzo and co-workers (2011), Lecirelin (a GnRH analogue) was administered as either an IM or epidural injection to evaluate the effect of administration route on regression of follicular cysts and return to estrus in lactating multiparous Friesian cattle. In cows receiving an epidural dose, regression of the follicular cyst and return to estrus were detected in 75 % of animals (45/60) with 93 % of those recovered animals pregnant after 2 artificial-insemination services (42/45). In animals receiving IM GnRH, regression of the follicular cyst and return to estrus was detected in 57 % of cows (34/60), with 76 % of those recovered animals pregnant after 2 artificial-insemination services (26/34). While animals in the current study were normally cycling beef cattle, their ability to respond to epidural GnRH administration by ovulating the dominant follicle is in agreement with this previous research.

As previously mentioned, data initially collected from beef cows during the preliminary experiment suggested that timing of ovulation might be affected by the method of GnRH administration. Unfortunately, the anecdotal evidence was not borne out in the more frequent observations that were conducted in heifers during the main experiment. We cannot rule out the

possibility that a difference existed in the mature cows but failed to be detected as a result of the less frequent ultrasound events. If true, it would certainly not be the first aspect of estrus synchronization that differed between cows and heifers. When observing naturally occurring estrous cycles in both beef and dairy breeds, it was found that heifers ovulated 3.04 h earlier than multiparous cows (Brewster and Cole, 1941). In cows and heifers synchronized using a Presynch+OvSynch protocol, heifers had a higher conception to timed-artificial insemination, but lower synchronization rate overall compared with cows (Gordon et al., 2010). Thus, in the current study, inherent differences in the physiology of heifers compared to mature cows could have been a basis for differences in time to ovulation in one group but not in the other. Additional research would be needed to determine if such differences exist.

The epidural route of hormone administration could have benefits beyond possible improvements in physiological response. By injecting the dose directly into the epidural space, the hormone can be distributed through the dura to the cerebral spinal fluid (CSF) into the brain, possibly reducing the concentration of hormones entering the systemic circulation and consumable products. In the case of GnRH, to our knowledge, no research has evaluated the concentration of GnRH in the circulation after epidural administration in cattle, but research conducted in humans it suggests is not unreasonable to hypothesize that a peptide hormone such as GnRH would be mostly confined to the CSF during epidural administration (Gourlay et al., 1985; Hogan, 2002). Hormone administration in beef and dairy cattle has been the topic of intense scrutiny by consumers in recent years, and epidural administration of hormones, when possible, may reduce the amount of hormone residue reaching the muscle and milk (Fajt et al., 2011). There is also a rising concern over the hormonal residues present in animal manure and environmental implications of incorporating manure application to fields as a waste management

strategy (Khan and Lee, 2012). While this concern has risen primarily from large-scale confinement operations such as feedlots, large dairies and cow-calf operations also contribute to animal fertilizer and minimizing hormonal residues in fertilizer is a critical part of ensuring future sustainability of animal production.

Conclusions

In summary these data indicate that epidural administration of GnRH will induce an ovulatory response when administered as part of an estrous synchronization protocol in both beef heifers and cows. Time to ovulation was not measured in cows and was not altered with epidural administration in heifers. Despite this, epidural administration of GnRH could be advantageous for other reasons and future studies should focus on these questions. Administration of GnRH (and potentially other hormones) through epidural injection presents opportunities to elicit a response with little or no exogenous hormone entering the systemic circulation. This might allow producers to administer a lower pharmaceutical dose while still attaining the desired response. Furthermore, consumer perceptions of food safety have demanded a re-evaluation of conventional reproductive management practices. Development of methods that reduce the amount of exogenous hormones entering food products will improve sustainability and profitability of food animal production.

Table 2-1: Effects of Epidural vs. Intramuscular (IM) Administration of Gonadotropin Releasing Hormone on Reproductive Parameters in Crossbred Cows and Heifers. A summary of reproductive parameters measured after epidural or IM injection of gonadotropin releasing hormone analogue during a CoSynch estrous synchronization protocol in crossbred cows and heifers.

	IM	**Epidural**	P-value
Incidence of Ovulation: Cows	7/7 animals	5/7 animals	P=0.99
Incidence of Ovulation: Heifers	3/4 animals	3/4 animals	P=0.99
Time to Ovulation: Heifers	29.6 ± 3.8 h	28.0 ± 3.58 h	P=0.78
Initial Follicle Diameter: Cows	13.0 ± 0.97 mm	13.0 ± 1.14 mm	P=83
Initial Follicle Diameter: Heifers	11.44 ± 0.77 mm	9.63 ± 0.63 mm	P=0.1
Ovulatory Follicle Diameter: Cows	14.36 ± 0.78 mm	14.70 ± 0.92 mm	P=0.78
Ovulatory Follicle Diameter: Heifers	12.0 ± 1.39 mm	12.91 ± 1.26 mm	P=0.88

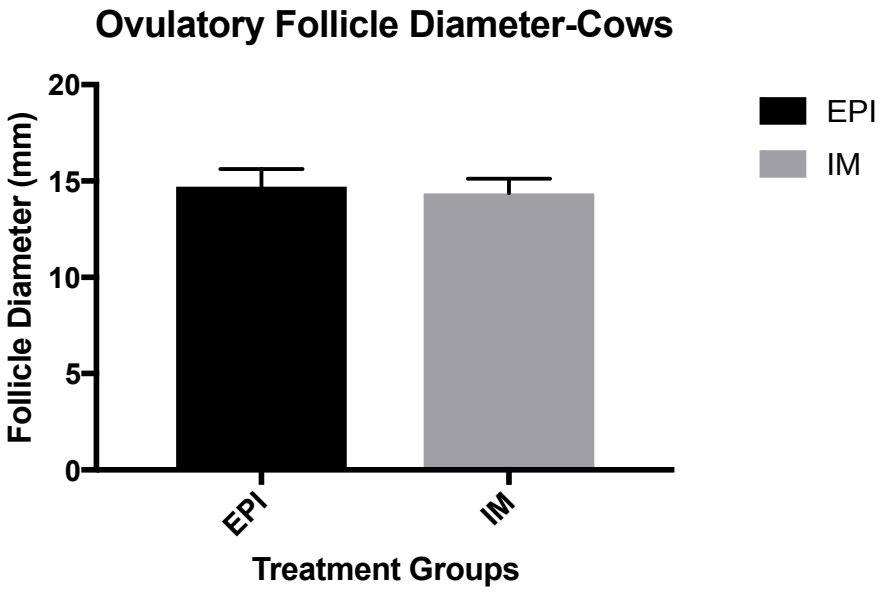

Figure 2-1: Effect of intramuscular (IM) vs. epidural (EPI) administration route on ovulatory follicle size in cows. Animals received 100μg of gonadorelin diacetate tetrahydrate; 2mL of Cystorelin by either IM or EPI to induce ovulation of the dominant follicle, and ovaries were observed every 6 h via transrectal ultrasound until the dominant follicle had ovulated. Ovulatory follicle size was recorded as the size (mm) of the follicle at last observation prior to ovulation.

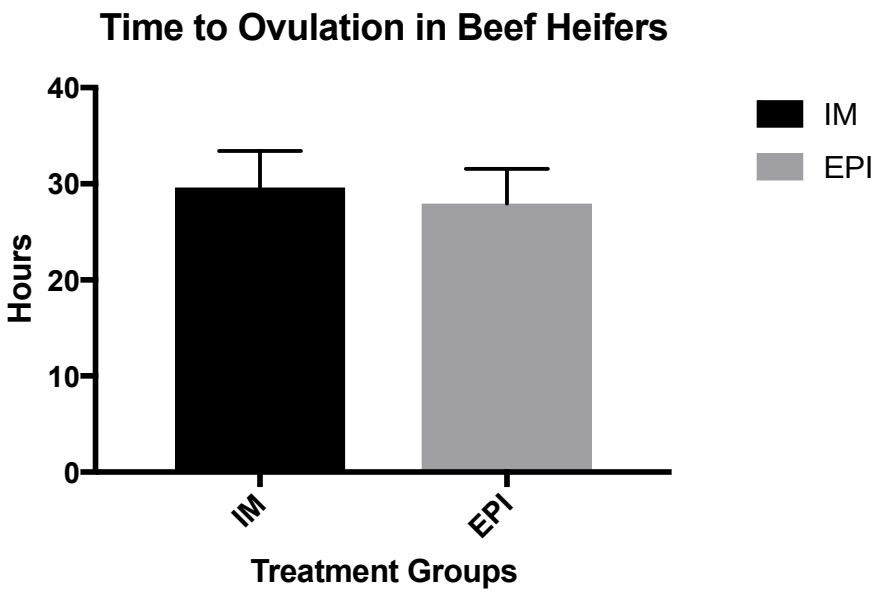

Figure 2-2: Effect of intramuscular (IM) vs. epidural (EPI) administration route on timing of ovulation (h) in heifers. Animals received 100μg of gonadorelin diacetate tetrahydrate; 2mL of Cystorelin by either IM or EPI to induce ovulation of the dominant follicle, and ovaries were observed every 4 h via transrectal ultrasound until the dominant follicle had ovulated. Time to ovulation was recorded as the time of ultrasound during which the disappearance of the dominant follicle was confirmed.

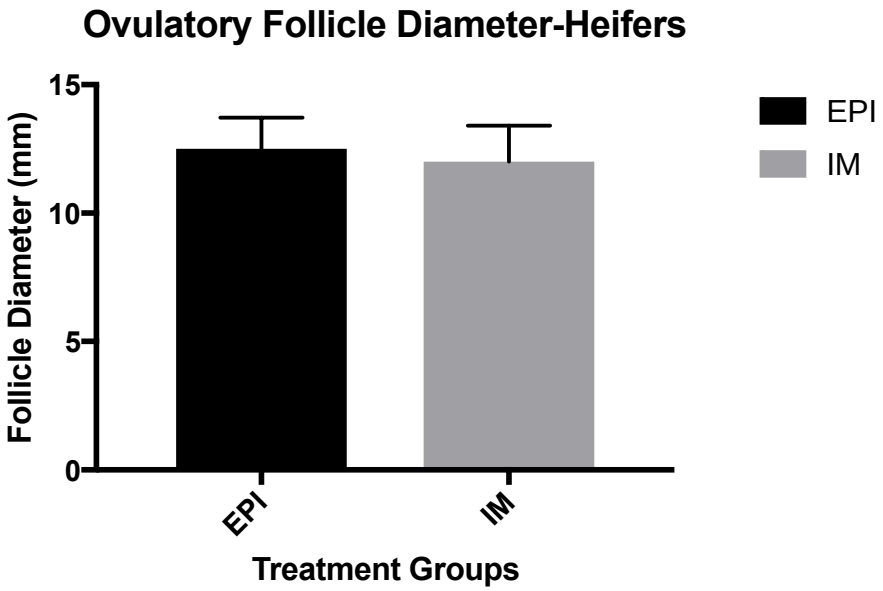

Figure 2-3: Effect of intramuscular (IM) vs. epidural (EPI) administration route on ovulatory follicle size in heifers. Animals received 100μg of gonadorelin diacetate tetrahydrate; 2mL of Cystorelin by either IM or EPI to induce ovulation of the dominant follicle, and ovaries were observed every 4 h via transrectal ultrasound until the dominant follicle had ovulated. Ovulatory follicle size was recorded as the size (mm) of the follicle at last observation prior to ovulation.

Chapter 3 - Effects of addition of bovine follicle fluid collected after luteolysis of a fixed-time artificial insemination protocol to maturation medium on *in vitro* embryo production

Abstract

Successful reproductive management programs are critical for the profitability of any cattle operation. Although development and implementation of fixed-time artificial insemination (FTAI) programs have largely eliminated the need for estrus detection, display of behavioral estrus has been shown to increase fertility, even when non-responding animals are removed from calculations. The reasons for this effect are likely multi-faceted and currently under investigation. The oocyte undergoes critical developmental milestones during the preovulatory estrus period, yet no research has yet been conducted on the impact of behavioral estrus during FTAI protocols on development of the oocyte and early embryo. To investigate these impacts, two experiments were conducted to determine whether the follicular characteristics of cows experiencing behavioral estrus affect oocyte maturation and early embryonic development. The first experiment was conducted in collaboration with South Dakota State University and using the follicle fluid of 9 crossbred Angus cows that were divided into high and low follicle fluid estradiol groups (H; 1,565 ±196.0 pg/ml; n=4, L; 398 ±175.0 pg/ml; n=5). Follicle fluid estradiol concentrations were used for grouping as a proxy for estrus because the two have been tightly correlated in previous work. Follicle fluid from H and L cows was added to maturation media at a rate of 20 % in preparation for *in vitro* embryo production. There were no differences in embryo cleavage or development to blastocyst between H or L estradiol follicle fluid. Cleavage was 73.6 % for H, 70.0 % for L, 69.0 % for P, 72.6 % for C-, and 75.3 % for C. Day 8 blastocyst rate was 15.2 % for H, 14.9 % for L, 22.2 % for P, 20.5 % for C- and 16.7 % for C. These results showed no indication of an effect of H estradiol follicle fluid on the cumulus-oocyte complexes

(COC). Because follicle fluid samples were categorized based only upon follicle fluid estradiol concentrations, there may have been physiological differences that cause cows to express estrus during FTAI which were not accounted for. Because of this, Experiment 2 was designed so that fluid from the dominant follicle could be collected and categorized based upon display of behavioral estrus and additional analyses could be conducted. Thirty lactating Angus crossbred cows (mean BCS of 5.56 ± 0.14 and mean weight of 603.36 ± 0.24 kg) were subjected to a CoSynch estrous synchronization protocol. Estrus behavior was recorded beginning 12 h after administration of $PGF_{2\alpha}$. Detection consisted of watching animals at least 30 min twice daily and visually observing paint remaining on the tailhead. Animals being ridden by other animals resulted in gradual removal of tail paint, and animals with ≤25 % of tail paint remaining just prior to aspiration were considered to have exhibited standing estrus. Dominant follicles were aspirated approximately 60 h following $PGF_{2\alpha}$ administration. Contents of the dominant follicle were collected via transvaginal aspiration, then follicle fluid (approximately 1.5 ml/animal) from estrus animals (n=9) and non-estrus animals (n=7) was randomly assigned to replicate (n=3 estrus, n=2 non-estrus per replicate) and supplemented at a rate of 25 % into an *in vitro* maturation medium. Total COC for each treatment was 210 C-, 289 C+, 1,200 non-estrus, and 1,238 estrus. Cleavage for the estrus group was 62.11 %, and 63.33 % for the non-estrus group. There was a tendency for a difference in development to blastocyst between COC treated with follicle fluid from estrus and non-estrus animals (P=0.087). Day 8 blastocyst rate for the estrus group was 19.62 %, and 13.08 % for the non-estrus group. Cell numbers (inner cell mass, trophoblast and total) did not differ between estrus and non-estrus animals. This study demonstrated that when estrus display is accompanied by increased follicle diameter, there is a

trend for the follicle fluid to cause an improvement in *in vitro* development to the blastocyst stage. Cleavage and blastocyst cell numbers, however, were unaffected by estrus display.

Introduction

For any cattle operation, profitability depends on the success of reproductive management programs. The development and implementation of fixed-time artificial insemination (FTAI) protocols have largely eliminated the need for estrus detection, reducing labor costs while increasing the number of animals inseminated via AI (Richardson et al., 2016). While FTAI protocols increase efficiency, estrus detection remains one of the most straightforward indicators of correct response to a synchronization protocol. The purpose of a FTAI protocol, however, is to eliminate the need for estrus detection by inducing ovulation of the dominant follicle with an injection of gonadotropin releasing hormone (GnRH), regardless of estrus expression (Bridges et al., 2014).

Although it was thought to be unnecessary, there is opportunity for cows to exhibit estrus during a FTAI protocol, prior to the final injection of GnRH. It has been well documented that animals who display estrus behavior have greater fertility when compared with animals not displaying estrus(Richardson et al., 2016). Logically, one's first inclination is to assume that a higher proportion of cows displaying estrus have properly responded to the injections administered during the FTAI protocol, which would explain the improvement in fertility. Removal of non-responders from the calculations does not negate the effect, however, indicating that there is something about the display of estrus that provides a reproductive advantage. Meta-analysis of five FTAI protocols reported that animals displaying estrus experienced a 27 % increase in conception compared with animals that did not show estrus (Richardson et al., 2016). Circulating estradiol and follicle size were also greater in concentration and diameter,

respectively, in animals that displayed estrus after FTAI protocols (Perry et al., 2014). Even two months after AI, those cows that had displayed estrus prior to FTAI continued to outperform their counterparts as they had lower pregnancy loss when compared with animals not displaying estrus (Pereira et al., 2016). Up to this point, only limited research investigating the mechanisms responsible for the observed improvement in fertility has been conducted. None, however, have examined the effects of the follicular environment (estrus follicular environment vs. non-estrus follicular environment) preceding ovulation on the cumulus-oocyte complex.

In vivo, the time in question (estrus vs. non-estrus of FTAI) coincides with oocyte maturation. The oocyte must complete critical maturation milestones prior to ovulation in order to prepare for fertilization and subsequent embryonic development. Preovulatory nuclear and cytoplasmic maturation of the oocyte take place simultaneously in response to a specific physiological signal: the preovulatory luteinizing hormone (LH) surge. Cytoplasmic maturation requires redistribution of cytoplasmic organelles. Mitochondria migrate to areas of high energy consumption in the oocyte during maturation, moving from the periphery of the cell, to a more even distribution throughout the cytoplasm (Ferreira et al., 2009). Cortical granules in the cytoplasm also experience dynamic changes during maturation, moving from clustered groups throughout the cytoplasm to an even distribution near the plasma membrane to await sperm entry and egg activation (Hosoe and Shioya, 1997). The genetic material contained within the nucleus of the oocyte must also be modified during the maturation phase. Immature bovine oocytes are arrested in the G2/M phase of the first meiotic division and will remain in dictyate until the LH surge preceding ovulation (Sun et al., 2001). If completed successfully, the nuclear and cytoplasmic changes occurring during oocyte maturation will prepare the oocyte for fertilization.

Artificial manipulation of follicular development for FTAI may affect the ability of the oocyte to successfully complete maturation and later develop into a competent embryo. Many factors influence the rate and efficiency of final maturation of the oocyte and may involve the differences in the follicular environment of cows that experience estrus compared to those that do not. If true, these differences could contribute to the observed improvement in pregnancy rates for those cows exhibiting estrus during a FTAI protocol. Therefore, the purpose of this study was to evaluate whether the follicular environment (specifically, follicle fluid) surrounding the oocyte during the maturation phase affects the ability of the cumulus-oocyte complex to progress through early embryonic development.

Materials and Methods

Experiment 1

Follicle fluid collection and preparation

This experiment was conducted in collaboration with researchers from South Dakota State University. Their methods were previously described (Larimore et al., 2016). Briefly, 9 crossbred Angus cows were synchronized using the Co-Synch FTAI protocol, and follicle fluid was collected after sacrifice at 36 to 42 h post-$PGF_{2\alpha}$ administration. Fluid was collected via needle aspiration from follicles between ≥10 mm in diameter. Concentration of estradiol was determined by validated radioimmunoassay (Perry and Perry, 2008b) with an inter-assay CV of 4.4 % and an intra-assay CV of 6.9 % Follicle fluid samples were grouped based on estradiol concentration into either a high (H; 1,565 ±196.0 pg/ml; n=4) or low (L; 398 ±175.0 pg/ml; n=5) treatment group.

Remaining follicle fluid was then frozen and shipped to Virginia Tech on dry ice. This follicle fluid was then added to *in vitro* maturation media that was used to mature oocytes

collected from slaughterhouse ovaries (procedures described below). Although it would have been preferable to have conducted these experiments on the cumulus-oocyte complexes (COC) that were collected from the 9 cows included in the experiment, those oocytes were not available and there were not enough of them to conduct meaningful experiments investigating developmental competence. Therefore, maturation of slaughterhouse COC in follicle fluid from those 9 cows was used as a proxy, with the important advantage of being able to collect data on hundreds of COC.

Oocyte collection

Bovine ovaries were collected from an abattoir (Brown Packing, Gaffney, SC) and transported to the laboratory in 0.9% saline supplemented with penicillin (100 units/ml) and streptomycin (100 units/ml). Follicular content was collected from antral follicles (2-7 mm diameter) of abattoir-derived ovaries via slashing into approximately150 ml of oocyte collection medium (OCM). Medium was filtered through a 0.2 μm cell strainer to concentrate cumulus-oocyte complexes (COC), then the filter material was rinsed onto a gridded plate and examined under a dissecting microscope in order to visualize and collect COCs. The oocytes with healthy, multiple layers of cumulus cells were collected and washed twice in fresh OCM and randomly assigned to treatment maturation media (formulation described below). Oocytes were matured in groups of 15 in 50 μl oocyte maturation medium (OMM) drops overlaid with mineral oil (Origio, Målov Denmark) for 21 h at 38.5 °C under 5 % CO_2.

Media preparation

Maturation media were prepared as experimental treatments. Positive control OMM was the medium formulation that our laboratory normally uses for *in vitro* maturation. It was included in the experiment (and run in each replicate) to ensure that the *in vitro* system was

working properly and so that the quality of each replicate could be assessed. This OMM (C+) consisted of TCM-199+ Earls Salts (LifeTech 11150-059), 10 % FBS (ThermoFisher 10437010), 1.14 % Glutamax 100X (Gibco 35050-061), 1.14 % sodium pyruvate (LifeTech 11360-070), 40 μg/ml follicle-stimulating hormone (Folltropin®, AgTech INC), estradiol (Sigma-Aldrich E2758-1G), 50 μg/ml gentamycin (Sigma-Aldrich), and EGF (Sigma-Aldrich, E9644-.5MG) and was not supplemented with follicle fluid. Negative control (C-) medium contained no undefined ingredients and consisted of TCM-199+ Earls Salts (LifeTech 11150-059), 1.14 % Glutamax 100X (Gibco 35050-061), 0.3 % PVP (Sigma-Aldrich PVP40-50G), and 1.14 % sodium pyruvate (LifeTech 11360-070). This C- OMM served as the base OMM for all experimental treatments and was supplemented with 20 % follicle fluid from individual cows. Follicle fluid (approximately 0.5 ml/ animal) from H animals (n=4) and L animals (n=5) were randomly assigned to replicate (3 cows per replicate). Additionally, follicle fluid was collected from slaughterhouse ovaries over the course of 3 weeks, pooled and added to maturation medium at a rate of 20% to serve as a control (P). One replicate was performed each week, and 4 total replicates were conducted for each animal. Oocytes were matured for 21 h at 38.5 °C in 5 % CO_2. A total of 1,112 COC were included in the H treatment, and 954 in the L treatment. There was a total of 266 COC in the C+ treatment, 224 in the C- treatment, and 268 COC in the P treatment.

In-vitro fertilization (IVF) and in-vitro culture (IVC) media were both prepared as previously described (Rivera and Hansen, 2001; Negron-Perez et al., 2017). Formulations of these media did not differ between treatment groups.

Experiment 2

Animals, housing, and diet

This study was conducted at the Virginia Tech beef cattle facilities at Kentland Farm with the approval of the Institutional Animal Care and Use Committee in April 2018. Thirty lactating Angus crossbred cows were housed on mixed grass pasture with ad libitum access to grazing and water. All cows were evaluated for BCS with a mean of 5.56 ± 0.14. Animals were weighed prior to the beginning of the study, with the average for all animals being 603.36 ± 0.24 kg.

Ovarian Ultrasonography

Ovaries for each animal were examined via transrectal ultrasound (Ibex ®Pro, E.I Medical Imaging, Loveland, CO) using a 7.5 MHz linear rectal transducer. The first ovarian ultrasound was conducted to detect the presence of a CL and determine cyclic status prior to administration of $PGF_{2\alpha}$. Maps of the ovaries were drawn for each individual animal, and size and position of follicles ≥5 mm were recorded. Additionally, the position and diameter of the CL was recorded, as well as the presence or absence of a hollow center in the CL. The dominant follicle diameter for each animal was measured immediately prior to follicle aspiration

Estrous synchronization and detection

Cows were subjected to a CoSynch estrus synchronization protocol. Briefly, cattle were administered GnRH (100 μg of gonadorelin diacetate tetrahydrate; 2 ml of Cystorelin, Merial Ltd., Iselin, NJ) on d 0. On d 7 $PGF_{2\alpha}$ was administered (25 mg of dinoprost tromethamine; 10 mg of Lutalyse, Pfizer Animal Health) and tail paint (DetectHer, H&W Products, Salem, OH) was applied liberally to the tailhead of each animal. Animals were observed for estrus behavior beginning 12 h after administration of $PGF_{2\alpha}$.

Estrus detection consisted of watching the animals for at least 30 min every morning and every evening as well as visually observing the amount of paint on the tailhead. Animals allowing themselves to be ridden by other animals resulted in the progressive removal of tail

paint, and animals with ≤25 % of their tail paint remaining prior to aspiration were considered to have exhibited standing estrus (Fig. 3-1).

Blood and follicle fluid collection

Approximately 7 ml of blood was collected via venipuncture of the median coccygeal vein into vacutainer tubes (Fisher Scientific) with heparin. Blood samples were collected immediately prior to follicle aspiration and placed on ice for transport to the laboratory. Samples were centrifuged within 5 h of collection at 513 RCF (g) for 15 minutes for plasma separation. Plasma was stored at -20 °C until analysis.

The dominant follicle was aspirated approximately 60 h following $PGF_{2\alpha}$ administration. Contents of the dominant follicle were collected via transvaginal aspiration using a 5.0 MHz convex-array transducer (ALOKA 500, Aloka Co. Ltd., Wallingford, CT). The 17-gauge single lumen needle and transducer were mounted on a plastic handle and introduced into the fornix vagina after the animal had received an epidural anesthetic with 5 ml of 2 % Lidocaine (Lidocaine HCl 2 %, 20 mg/ml, Vedco INC, St. Joseph, MO). The dominant follicle for aspiration was localized on the ultrasound screen, and the aspiration needle was inserted into the follicle. Correct placement of the needle was verified on the ultrasound screen, and follicle fluid was aspirated using a 12 ml syringe attached to the needle. Follicle fluid was centrifuged at 513 RCF (g) for 10 minutes, then the fluid was placed on ice until transport to the laboratory where it was frozen and stored at -20 °C until analyses.

Maturation media preparation

In vitro maturation, fertilization and culture media were prepared as described for experiment 1. Follicle fluid (approximately 1.5 ml/animal) from estrus animals (n=9) and non-estrus animals (n=7) was randomly assigned to replicate (n=3 estrus, n=2 non-estrus per

replicate). Each replicate contained either 30 or 60 COC per treatment and varied based on ovary availability. Total COC for each treatment were 210 C-, 289 C+, 1200 non-estrus, and 1238 estrus. Two replicates were performed each week, and 4 total replicates were conducted for each animal.

Immunofluorescence

After 8 days of culture, blastocysts were collected for cell counting. Immunolabeling protocols were conducted as previously described (Negron-Perez et al., 2017). All immunolabeling procedures were performed at room temperature unless otherwise noted. Briefly, for labeling with CDX2, embryos were placed in permeabilization solution (Dulbecco's phosphate-buffered saline (DPBS) + polyvinylpyrrolidone (PVP) containing 0.25 % (v/v) Triton X-100) and incubated for 30 minutes, then transferred to blocking solution (5 % (w/v) bovine serum albumin (BSA) in DPBS) for 1h. Embryos were then transferred to mouse monoclonal anti-human antibody against CDX2 (0.4 μg/mL; CDX2-88, Biogenex) and incubated for 1 h in the dark. Embryos were then washed three times in washing buffer (DPBS + 0.1 % BSA (w/v) and 0.1 % (v/v) Tween-20) and transferred to the secondary antibody: conjugated goat polyclonal anti-mouse IgG (1 μg/ml fluorescein isothiocyanate, Abcam, Cambridge, MA, USA) for an incubation period of 1 h in the dark. Embryos were washed three times and counterstained with 1 μg/ml Hoechst 33342 in DPBS-PVP for 15 min, after which they were washed once in DPBS-PVP. Embryos were then transferred to a 10 μl drop of SlowFade Gold antifade reagent (S36936, Life Technologies) on a glass microscope slide and covered with a coverslip. To determine non-specific labeling, primary antibodies were replaced with rabbit or mouse IgG (1 μg/ml).

Images of embryos were captured with a 40X objective using a Nikon Eclipse Ti fluorescence microscope (Melville, NY). ImageJ v 1.51n (National Institutes of Health, Bethesda, MD, USA) was used to measure images and count the number of cells.

Statistical analyses for experiments 1 & 2

Development data were analyzed for the main effect of treatment using SAS statistical software (SAS Institute Inc., Cary, NC.). Treatment effect was assessed using PROC GLIMMIX, with the dependent variables including cleavage of embryos, development to blastocyst, inner cell mass cell number, trophoblast cell number, and total cell number. Class variables included replicate, treatment, initial follicle diameter, ovulatory follicle diameter, and body condition score. Replicate was considered a random variable. Day 3 cleavage rate was calculated as the number of cleaved embryos divided by the total number of oocytes and d 8 blastocyst rate was calculated as the total number of blastocysts divided by the total number cleaved. Random effect of replicate was included in the model statement. For experiment 2, body weight, days postpartum, and body condition score were also compared between estrus and non-estrus cows using the GLIMMIX procedure of SAS. Separation of means was conducted with the LSMEANS statement in SAS with the Tukey adjustment. Results are reported as least squares means ± standard error of the mean. Statistical significance was declared at $P<0.05$.

Results

Experiment 1

During Experiment 1, follicle fluid was collected from cattle at sacrifice and those animals were categorized as H or L based upon estradiol concentrations within their follicle fluid. When follicle fluid from these cows was added to the *in vitro* maturation medium, there were no differences in embryo cleavage (Fig. 3-2) or development to blastocyst (Fig. 3-3)

between H or L estradiol follicle fluid. Cleavage for H was 73.6%, 70.0% for L, 69.0% for P, 72.6% for C-, and 75.3% for C. Day 8 blastocyst for H was 15.2 %, 14.9% for L, 22.2% for P, 20.5% for C- and 16.7 % for C. These results yielded no indication of an effect of H estradiol follicle fluid on the COC. Since these samples were categorized solely based upon follicle fluid estradiol concentrations, however, this may not take into account all of the physiological differences that cause cows to express estrus during FTAI. Furthermore, it was unusual that in this experiment the diameter of the dominant follicles from the H vs. L cows did not differ (Larimore et al., 2016). For these reasons, Experiment 2 was designed so that fluid from the dominant follicle could be collected and categorized based upon the presence or absence of behavioral estrus and additional analyses could be conducted.

Experiment 2

During Experiment 2, cows were closely observed between the $PGF_{2\alpha}$ injection and the time of follicle aspiration. This period coincides with the time during which cows that are being prepared for FTAI may express estrus. Based upon behavioral observations and changes in tail paint, 50 % of the 30 cows originally synchronized exhibited estrus. Images of representative tail paint scores are shown in Fig. 3-1. Of the 30 cows that were subjected to the experimental protocol, usable follicle fluid samples were collected from 17 of them (n= 10 estrus, n=7 non-estrus).

Initial follicle diameter was larger in animals that displayed heat and those that did not ($P=0.04$). Initial follicular diameter was 9.04 ± 1.82 mm for estrus animals and 4.71 ± 0.81 mm in non-estrus animals. Follicle diameter at time of aspiration was different between animals that displayed heat and animals that did not ($P<0.01$). Mean follicle diameter at aspiration was 16.34 ± 0.07 mm for the estrus group and 12.75 ± 0.07 mm for the non-estrus group. There was no

difference in embryo cleavage (Fig. 3-4) between animals displaying estrus when compared with animals not displaying estrus. Cleavage for the estrus group was 62.11 %, and 63.33 % for the non-estrus group (76.31% C+, 71.28% C-). There was a tendency for a difference in development to blastocyst (Fig. 3-5) between those COC treated with follicle fluid from estrus and non-estrus follicle fluid (P=0.087). Day 8 blastocyst rate for the estrus group was 19.62 %, and 13.08 % for the non-estrus group (43.84% C+, 29.85% C-). Cleavage was highest in cows 77 days postpartum when compared with all other days (P=0.03). This effect of days postpartum on cleavage did not carry over to blastocyst development. There was no effect of body weight, days postpartum, or body condition score (BCS) on blastocyst development nor were there differences in these parameters between estrus and non-estrus cows.

Blastocyst cell count yielded no differences between estrus and non-estrus groups. Mean cell number for inner cell mass was 27.46 ± 1.58 for estrus embryos and 24.85 ± 2.06 for non-estrus embryos (Fig.3-6). Mean cell number for the trophoblast was 84.84 ± 3.37 for estrus embryos and 83.40 ± 4.41 for non-estrus embryos (Fig. 3-7). Mean total cell number was 112.30 ± 4.39 for estrus embryos and 108.25 ± 5.74 in non-estrus embryos (Fig. 3-8).

Discussion

Previous research has demonstrated the strong correlation between follicle size and estradiol concentration. In beef cattle synchronized using Co-Synch, cows exhibiting estrus were found to have a greater follicular diameter (14.8 ± 0.7 mm) when compared with cows not displaying estrus (11.8 ± 0.4 mm). Additionally, cattle displaying estrus had higher peak estradiol concentrations (9.9 ± 1.0 pg/ml) when compared with non-estrus animals (6.2 ± 0.67 pg/ml; (Perry et al., 2014). Similarly, a report of follicles dissected from the stroma and categorized according to size and stage of the cycle also demonstrated a correlation between size

of the follicle and concentration of estradiol, with large (8-11 mm) follicles having an higher estradiol concentrations compared with intermediate (5-8 mm) and small (2-5 mm) follicles in the same stage of the estrous cycle (Kruip and Dieleman, 1985). The implications of the observed differences relate directly to fertility. Cattle who ovulated small (≤10 mm diameter) follicles in response to GnRH administration exhibited lower circulating estradiol concentration, fertilization, and pregnancy establishment when compared with animals that ovulated larger (>10 mm diameter) follicles. It is important to note that, animals that ovulated in response to the first GnRH injection of a Co-Synch protocol were more likely to have larger follicles and greater circulating concentrations of estradiol when compared with animals that did not ovulate in response to the first GnRH injection (Jinks et al., 2013). Taken together, the results of these studies emphasize the importance of generating a relatively large, highly functional dominant follicle during an estrous synchronization protocol, even if that protocol is designed for FTAI.

The role of estradiol in synchrony between the oviductal/uterine environment and the embryo has been well established. Estradiol influences receptivity to the embryo by regulating the secretion of glycoproteins from the oviduct and the biological regulation of the uterus (Buhi, 2002; Nakamura et al., 2005). Peak estradiol concentrations occur around 36 h prior to ovulation and elevated peak estradiol concentrations have been associated with improved fertilization, embryo quality, and pregnancy establishment (Chenault et al., 1975; Atkins et al., 2013). In experiment 2 of the current study, dominant follicles were aspirated prior to ovulation of the dominant follicle after the $PGF_{2\alpha}$ injection. Cows that displayed estrus prior to aspiration developed a larger follicle when compared with animals that did not show estrus, corroborating previously reported data.

Interestingly, this was not the case during experiment 1 as follicle size did not differ even though estradiol concentrations did differ (Larimore et al., 2016). The reason for the lack of difference is unknown but may have contributed to the discrepancy in results between the two experiments.

For experiment 1, when follicle fluid was supplemented into the *in vitro* maturation medium, embryonic development was not different between H or L treatment groups. In experiment 2, however, development to blastocyst tended to differ between those COC treated with follicle fluid from estrus compared to non-estrus follicles. Unfortunately, follicle fluid estradiol concentrations for the second experiment were not measured, but based upon previously discussed literature, they were undoubtedly higher in cows displaying estrus than those that did not display estrus. Taken together, these results suggest there may be other inherent characteristics of the dominant follicle besides estradiol concentration that affects the ability of the COC to develop to the blastocyst stage.

Previous studies have shown that stage of the estrus cycle alters follicular microenvironments, impacting oocyte maturation and early embryo development. During the luteal phase of the estrus cycle, concentrations of estradiol in the follicle are lower than those of the follicular phase, resulting in a decreased ability of the female reproductive tract to ovulate and maintain a pregnancy, and for the oocyte to undergo critical maturation milestones necessary for successful fertilization and embryo development (Kruip and Dieleman, 1985). Consideration of the stage of the estrus cycle is of particular interest for FTAI synchronization protocols because, in most cases, cattle begin these protocols at random, different stages of estrous cycle. For example, in the case of the Co-Synch protocol, cattle might already have a corpus luteum (CL) at the time of the first GnRH injection and, therefore, either not respond to the injection or

ovulate and produce a secondary CL. Cattle without a CL at the time of first GnRH injection may be cyclic and naturally in proestrus or estrus or could be anestrus. These differences alone could subsequently produce dominant follicles with different microenvironments and, likewise, different likelihoods of success.

The LH surge also affects the follicular microenvironment, altering concentrations of steroids in a relatively short period. These differences can be illustrated when follicle fluid is supplemented during maturation *in vitro*. Bovine oocytes matured in 20 or 40 % follicle fluid that was collected either 0 or 20 h after the LH surge demonstrated the possible presence of a meiotic inhibitor in the follicle fluid collected at 0 h post-LH. Maturation and development were reduced in the 0 h treatment when compared with the 20 h treatment, and the reduction in development was greater in the 40 % fluid supplementation when compared with the 20 % medium (Romero-Arredondo and Seidel, 1996). This indicates that the contents and attributes of the follicle fluid changed so much within those 20 h that it was detectable as a difference in subsequent embryo development.

The current study collected follicle fluid prior to ovulation, with 50 % of animals exhibiting estrus prior to follicular aspiration. Previous studies have shown that estrus display prior to insemination results in improved fertility and delaying insemination for cows that fail to exhibit estrus by 60 h post $PGF_{2\alpha}$ administration allows delayed cows to exhibit estrus and improve overall fertility. Using split-time artificial insemination (STAI) protocols instead of FTAI has been shown to improve fertility by allowing non-estrus animals a longer interval in which to exhibit estrus (Bishop et al., 2017). Split-time artificial insemination is a breeding system which manages animals separately based on estrus expression prior to insemination in order to maximize conception rates (Bishop et al., 2016). While this aspect of the approach

increases the amount of labor involved, the benefit often outweighs the costs. Reports have shown that estrus expression prior to insemination in STAI systems is about 86 % compared with FTAI at 60 %. Likewise, pregnancy rates were improved in STAI (56 %) when compared with FTAI (49 %) protocols (Bishop et al., 2017).

It is possible in the current study that animals not displaying estrus at aspiration may have benefited from a delay of 24 h. Recall that follicle diameter in cows that failed to display estrus was lower than those that did. Thus, the non-estrus animals might have developed a comparable-sized follicle if allowed a 24 h delay, leading to an increase in circulating estradiol, and subsequent estrus display. Ultimately, it is likely that the additional time would have made follicle fluid from the two types of cows (estrus and delayed estrus) very similar and negated the observed effects of the follicle fluid on *in vitro* embryo development.

A trend for improved blastocyst development for the estrus group in experiment 2 suggests that there may be an effect on embryonic development, but lack of differences in cleavage and cell number between the estrus and non-estrus groups suggest that the main effect of behavioral estrus requires further investigation as to the impact on both oocyte acquisition of competence and early embryonic development.

Conclusion

This study demonstrated that when estrus display is accompanied by increased follicle diameter, there is a trend for the follicle fluid to cause an improvement in *in vitro* development to the blastocyst stage. Cleavage and blastocyst cell numbers, however, were unaffected by estrus display. Future research investigating the microenvironment of the peri-ovulatory follicle, and its impact on oocyte competence could be critical in improving our understanding of the mechanisms impacting oocyte maturation and acquisition of developmental competence

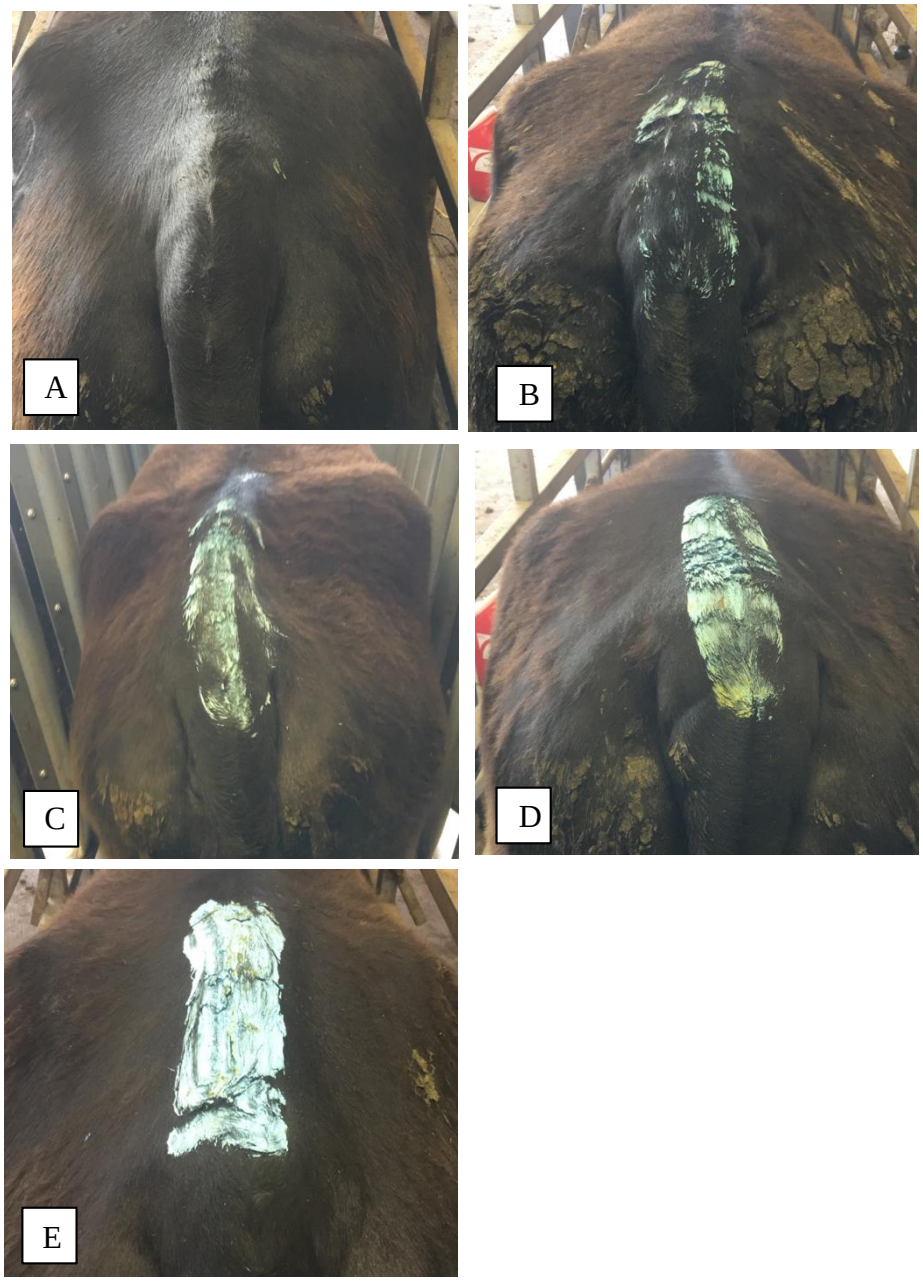

Figure 3-1: Tail paint scores in cows synchronized for estrus. Tail paint remaining was classified as either 0% (A), 25% (B), 50% (C), 75% (D), and 100% (E). Cows were considered in estrus if 25% or less tail paint remained immediately prior to follicle aspiration.

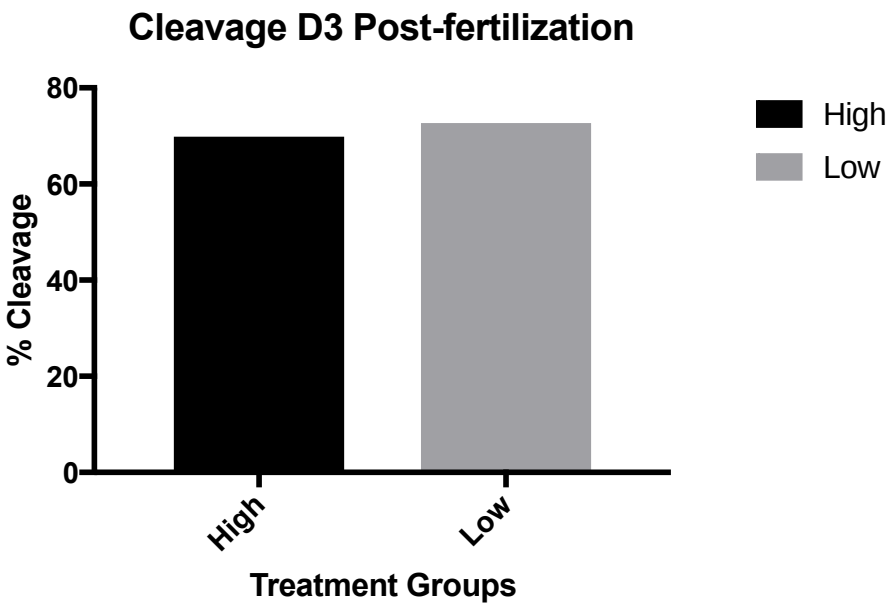

Figure 3-2: Effect of estradiol concentration of follicle fluid (High; 1,565 ±196.0 pg/ml; n=4 vs. Low; 398 ±175.0 pg/ml; n=5) on embryo cleavage (%) for embryos supplemented with 25 % follicle fluid during 21 h maturation. Embryos evaluated on d 3 post-fertilization.

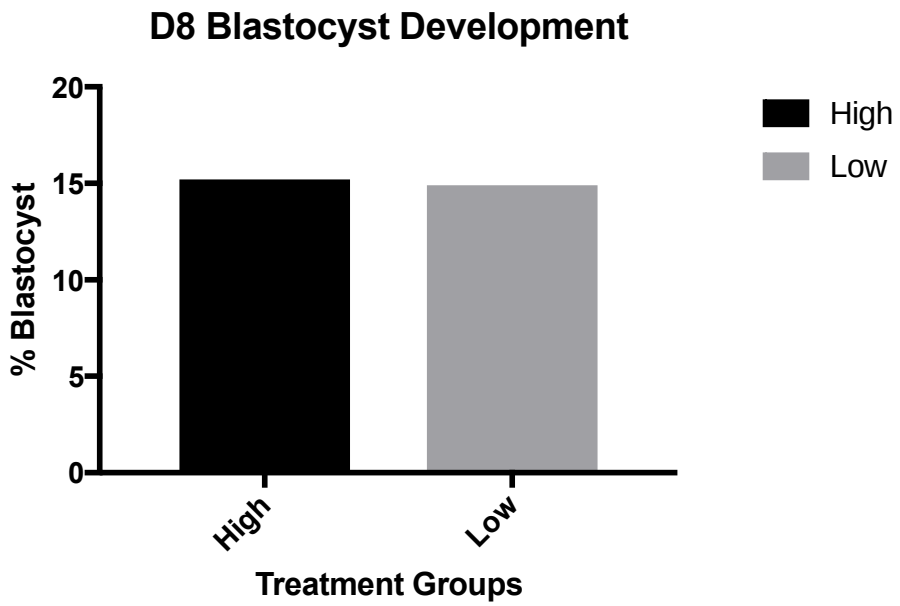

Figure 3-3: Effect of estradiol concentration of follicle fluid (High; 1,565 ±196.0 pg/ml; n=4 vs. Low; 398 ±175.0 pg/ml; n=5) on development to blastocyst (%) for embryos supplemented with 25 % follicle fluid during 21 h maturation. Embryos evaluated on d 8 post-fertilization.

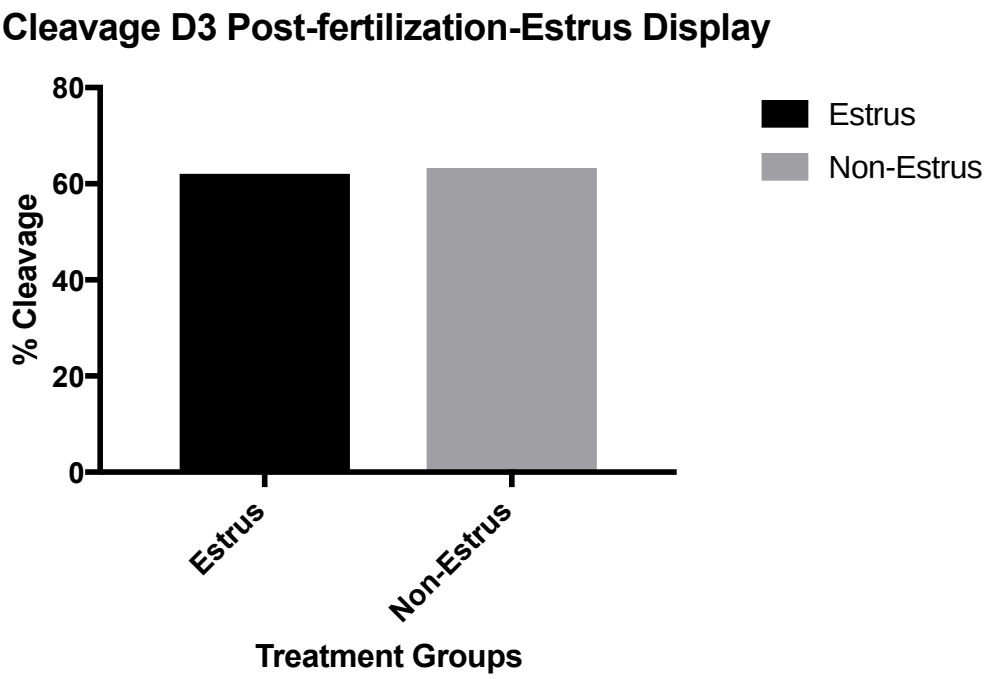

Figure 3-4: Effect of follicle fluid collected from cows that did or did not display behavioral estrus on embryo cleavage (%). Media was supplemented with 25% follicle fluid during 21 h maturation. Embryos evaluated on d 3 post-fertilization.

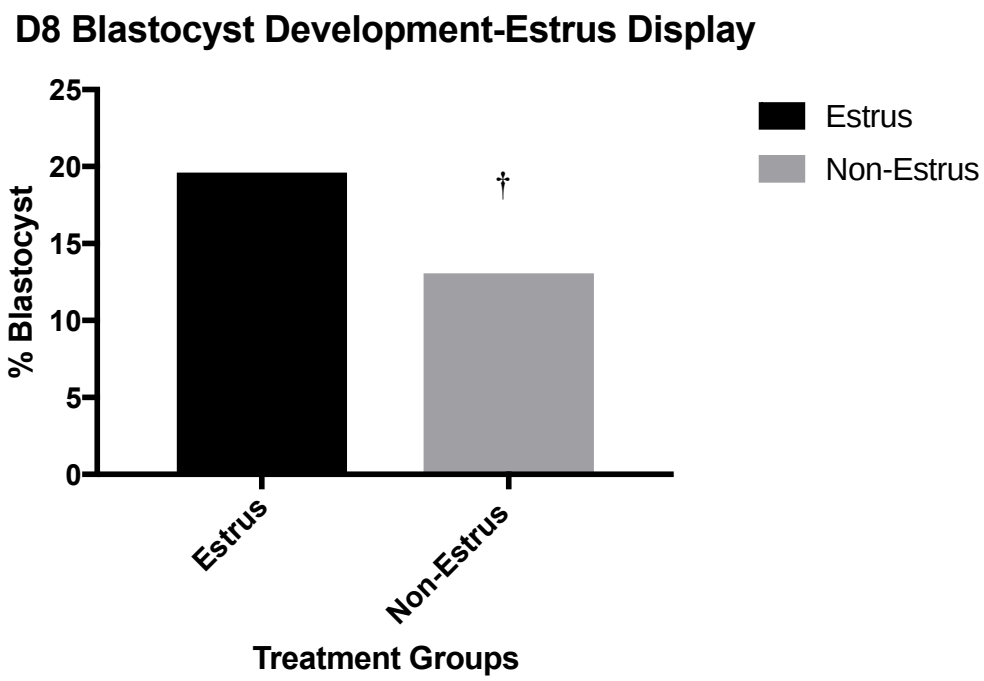

Figure 3-5: Effect of follicle fluid collected from cows that did or did not display behavioral estrus on embryo cleavage (%). Media was supplemented with 25% follicle fluid during 21 h maturation. Embryos evaluated on d 8 post-fertilization. Columns with † indicate a tendency for a difference at P=0.09

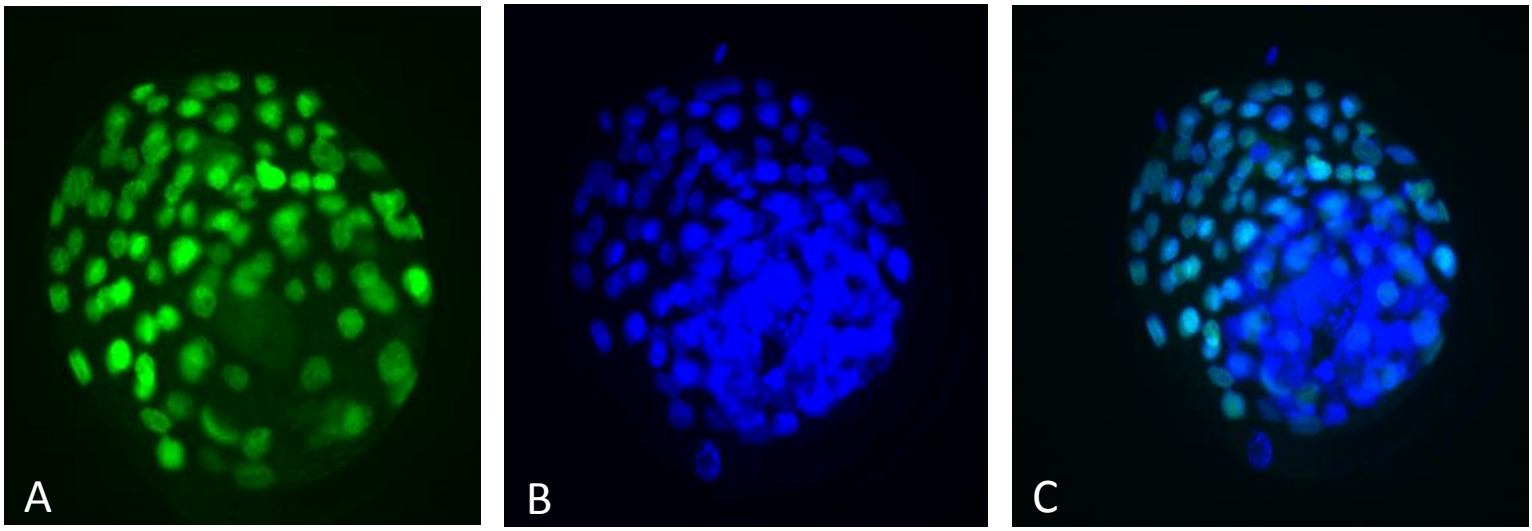

Figure 3-6: Immunofluorescent staining in embryos matured in follicle fluid collected from animals that did or did not display behavioral estrus. Embryos stained for CDX2/trophoblast cells (A) and Hoechst/inner-cell mass (B). Image channels combined in panel C. Inner-cell mass, trophoblast, and total cell number did not differ, so image provided is representative of all stained embryos.

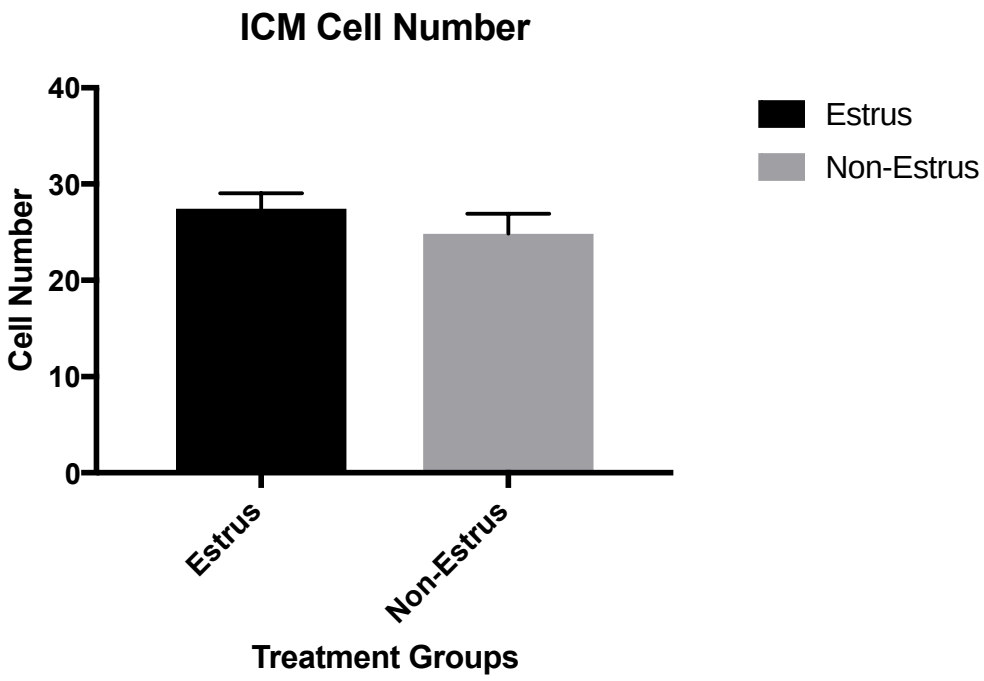

Figure 3-7: Effect of follicle fluid collected from cows that did or did not display behavioral estrus on blastocyst inner-cell mass (ICM) number in embryos supplemented with 25% follicle fluid during 21 h maturation. Blastocysts evaluated and fixed on d 8 post-fertilization.

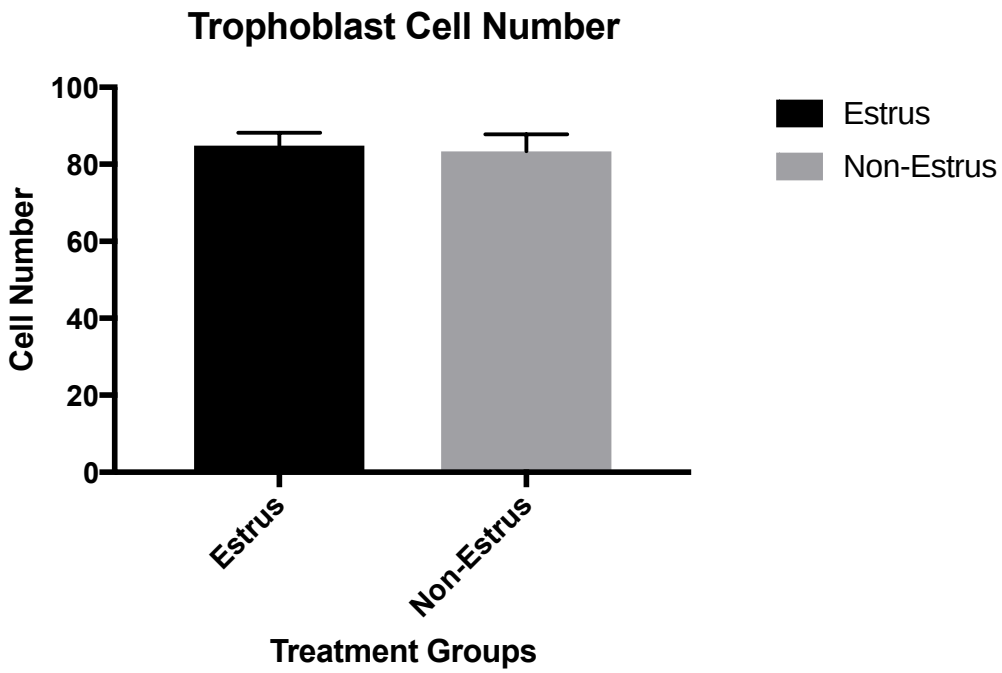

Figure 3-8: Effect of follicle fluid collected from cows that did or did not display behavioral estrus on trophoblast cell number in embryos supplemented with 25% follicle fluid for 21 h maturation. Blastocysts evaluated and fixed on d 8 post-fertilization.

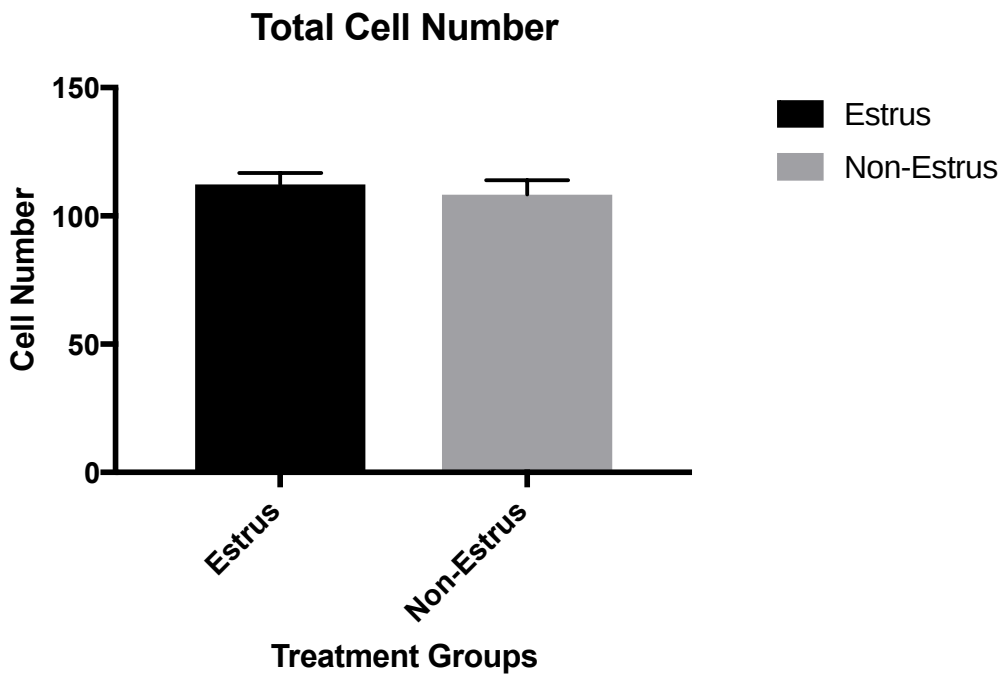

Figure 3-9: Effect of follicle fluid collected from cows that did or did not display behavioral estrus on total cell number in embryos supplemented with 25% follicle fluid during 21 h maturation. Blastocysts evaluated and fixed on d 8 post-fertilization.

Chapter 4 - Effects of altered estradiol and progesterone concentrations in follicle fluid added to maturation medium used during *in vitro* embryo production

Abstract

Although laboratory procedures for *in vitro* bovine embryo production have improved immensely, developmental capacity following fertilization is still limited. *In vivo*, the maturing oocyte would be exposed to dynamic changes in concentrations of steroid hormones as well as other follicle fluid components that naturally occur during follicular growth. Closer replication of the *in vivo* environment in *in vitro* maturation systems may improve embryo production while providing insight into the relative importance of the follicle fluid components for *in vivo* oocyte competence (especially steroid hormones). Therefore, the objective of this study was to investigate the impact of follicle size and relative estradiol and progesterone concentrations on oocyte maturation and early embryo development following follicle fluid exposure during maturation *in vitro*. Follicle fluid was collected via needle aspiration from small (2-5 mm diameter) and large (10-20 mm diameter) follicles and pooled according to size. The large follicle fluid (LFF) treatment medium consisted of a base medium (C- OMM) supplemented with 75 % LFF. Likewise, small follicle fluid (SFF) treatment medium consisted of C- OMM supplemented with 75 % SFF. Charcoal-stripped LFF (CSL) and charcoal-stripped SFF (CSS) treatment media were C- OMM with 75 % charcoal-stripped LFF or 75 % charcoal-stripped SFF, respectively. Progesterone and/or estradiol were added to the charcoal-stripped follicle fluid treatments based on average concentrations found in fluid from pooled large or pooled small follicles that had been collected in the same manner during a previous experiment (Harl et al., 2017). These six treatment media were formulated using C- OMM as a base with the following designations and additions: charcoal-stripped LFF + progesterone (CSLP) had 75 % LFF and

160 ng/ml progesterone; charcoal-stripped SFF + progesterone (CSSP) was 75 % charcoal-stripped SFF and 140 ng/ml progesterone; charcoal-stripped LFF + estradiol (CSLE) had 75 % charcoal-stripped LFF and 37 ng/ml estradiol; charcoal-stripped SFF + estradiol (CSSE) was 75 % charcoal-stripped SFF and 23 ng/ml estradiol; charcoal-stripped LFF + progesterone + estradiol (CSLPE) consisted of 75 % charcoal-stripped LFF, 160 ng/ml progesterone, and 37 ng/ml estradiol; charcoal-stripped SFF + progesterone + estradiol (CSLPE) was 75 % charcoal-stripped SFF, 140 ng/ml progesterone, and 23 ng/ml estradiol. Cumulus-oocyte complexes (n=4,004) were matured in groups of 15 in 50 μl OMM drops for 21 h. After 21 h maturation, oocytes were subjected to *in vitro* fertilization and *in vitro* culture. Cleavage was evaluated at d 3 post-fertilization, and blastocysts were evaluated at d 8 post-fertilization. Cleavage rates were affected by treatment ($P<0.01$). The C+ treatment yielded the highest cleavage rate, and this rate exceeded the C- and all follicle fluid treatments ($P<0.05$). Blastocyst rates were also affected by treatment ($P<0.01$). As with cleavage rates, the C+ treatment yielded the numerically highest blastocyst rate. The addition of estradiol alone or with progesterone did not support early embryonic development, while the addition of progesterone alone improved development and returned blastocyst development rates to the same level as the control treatment. The cause of the inhibitory effect of the estradiol-progesterone combination is still unknown, and future research investigating these mechanisms would further the understanding of the follicular microenvironment in which the oocyte undergoes final maturation.

Introduction

Over the decades, laboratory procedures and conditions for *in vitro* fertilization and embryo culture have evolved in order to maximize embryo production. Despite the progress that has been made, developmental capacity following fertilization is limited at best, with only

around one-third of bovine oocytes placed into maturation resulting in viable embryos (Lonergan et al., 1994). The low efficiency of bovine embryo production *in vitro* is a multifaceted problem, with factors like oocyte quality, media composition, and culture environment all playing a major role in the successful (or unsuccessful) production of embryos (Fukui et al., 1982).

In an effort to improve *in vitro* embryo production, researchers have closely examined and attempted to recreate aspects of the natural *in vivo* environment. During *in vivo* development, final maturation of the bovine oocyte takes place within the dominant follicle under the influence of gonadotropins, steroids, and growth factors. (Fukui et al., 1982; Silva and Knight, 2000). In the preovulatory stage, follicular components change drastically, and it is likely that many important components and interactions have yet to be identified. Considering the complexity of the environment in which oocytes and early embryos develop *in vivo*, it is in some ways astounding that any viable embryos can be produced *in vitro*.

In vitro production of bovine embryos begins with collection of a follicle-enclosed cumulus-oocyte complex (COC), making this the first stage at which laboratory-based conditions affect the process. In mammalian preovulatory follicles, luteinizing hormone (LH) secretion and its eventual surge in secretion would normally be responsible for stimulating the production of androgens and estrogens, triggering the resumption of meiosis and final maturation of the oocyte. Interestingly, the LH surge also stimulates luteinization of the theca and granulosa, causing an increase in progesterone production before the follicle even ruptures (Osborn and Moor, 1983; Lonergan et al., 1994; Silva and Knight, 2000). About 6 h after the LH surge, estradiol concentrations decrease, followed by an increase in progesterone. By 18 h post-LH surge, the follicular steroid concentration is composed of about 90 % progesterone (Osborn and Moor, 1983).

Fortunately, the events stimulated by LH *in vivo* can be replicated *in vitro* reasonably well. Manual removal of the bovine oocyte from the follicle results in spontaneous meiotic maturation, thus facilitating *in vitro* maturation of oocytes in preparation for fertilization (Lonergan et al., 1994). The spontaneous resumption of meiosis following removal of the bovine oocyte from the follicle was first demonstrated in 1935, although the mechanism(s) responsible remains unclear (Pincus and Enzmann, 1935). Irrespective of the mechanism(s) however, *in vitro* bovine oocyte nuclear maturation rates are high, frequently exceeding 80 % (Rizos et al., 2002; Cebrian-Serrano et al., 2013; Sen and Kuran, 2018).

Even though the nuclear maturation of the bovine oocyte is generally successful under standard *in vitro* conditions, usually less than half of successfully matured oocytes will go on to develop to the blastocyst stage. This indicates there are other developmental hurdles that must be overcome for eventual production of a viable embryo. *In vivo*, the majority, if not all of these hurdles are overcome prior to follicle rupture while the COC is contained within the follicular microenvironment. An important component of this microenvironment is the follicle fluid, and it has been suggested that the addition of follicle fluid to *in vitro* media during maturation could improve developmental results (Osborn and Moor, 1983).

The addition of follicle fluid to maturation media has produced mixed results in the bovine, with positive (Romero-Arredondo and Seidel, 1996) or negative (Ayoub and Hunter, 1993) effects being reported. Meiotic inhibitors have been documented in porcine and murine follicular fluid, and in the bovine are produced by the theca interna and theca externa cells (Richard and Sirard, 1996). As previously stated, the microenvironment of the follicle changes rapidly after the LH surge, and the oocyte is able to resume meiosis and undergo final maturation. It is possible that the effects of the meiotic inhibitor are mediated by the decrease in

the number of gap junctions between the granulosa cells after the LH surge, or that a meiotic stimulator is generated after the LH surge which overcomes the effects of the inhibitor (Romero-Arredondo and Seidel, 1996). In oocytes matured in either 20, 40 % bovine follicle fluid collected before (0 h), or 20 h after the LH surge, cleavage was increased in the 20 h fluid (72 ± 7.8 %) regardless of % supplementation when compared with 0 h (45 ± 18.9 %) fluid (although not statistically different from the control; 85 ± 5.1 %). Additionally, blastocyst development was increased (45 ± 18.1 %) in embryos matured in 20 h follicle fluid when compared with 0 h fluid (36 ± 16.5 %) and the control (14 ± 7.3 %). Similarly, inhibitory effects (defined as the prevention of germinal vesicle breakdown in the oocyte) of follicle fluid were observed when oocytes were matured in fluid collected from small and medium follicles when compared with large. This inhibitory effect was found to be reversible when follicle fluid was removed from the culture media by the 24 h maturation mark, allowing the resumption of meiosis and maturation of the oocyte. Fluid from large follicles had lowered ability to inhibit maturation of the oocyte regardless of the stage of the cycle from which they were collected (Ayoub and Hunter, 1993). Both studies have shown that follicle fluid can have an inhibitory effect on oocyte maturation, but also illustrates that this effect is limited to immature follicles, or follicles that have not yet experienced the LH surge.

Although not ultimately beneficial for embryo development, the addition of follicle fluid to maturation media does affect cumulus cell expansion. Our laboratory previously reported that that when COCs are matured in media supplemented with 0, 50, 75, or 100 % follicle fluid, they experience maximal expansion with 75 % follicle fluid, regardless of the size of the follicle from which the fluid was taken (Harl et al., 2017). Despite having a positive effect on cumulus expansion however, embryo cleavage and development to blastocyst are inhibited when oocytes

are matured in 75 % fluid from small (3-5 mm) follicles in comparison to fluid from large (8-10 mm) follicles (Al Naib et al., unpublished). Our laboratory was not the first to observe this difference. Others have reported similar outcomes in porcine and bovine when follicle fluid from small and large follicles was added to maturation media at rates between 10 and 20 % (Lonergan et al., 1994; Qian et al., 2001). The components within the follicle fluid that are responsible for these effects on subsequent development have not been isolated. We hypothesize that the inherent differences in estradiol and progesterone concentrations between the small and large follicle fluid are a major factor contributing to observed differences in embryo development following exposure to those fluids during maturation.

The effects of steroid hormones to the maturing mammalian oocyte have been under investigation for decades, with mixed results being reported *in vitro*. In preovulatory follicles, the LH surge stimulates the initial release of androgens and estrogens, with estrogens beginning to decrease in concentration 6 h after the LH surge. As estrogen levels fall, progesterone levels begin a steady increase until about 18 h post-LH surge. At this time, progesterone constitutes about 90 % of the intrafollicular steroid concentration, indicating a careful balance and sequence of steroid concentrations may be necessary for complete maturation of the oocyte (Osborn and Moor, 1983; Silva and Knight, 2000). In bovine oocytes supplemented *in vitro* with testosterone (100 $nmol1^{-1}$), a significant improvement was made in cleavage compared with the control, although no difference in blastocyst development was observed. The addition of progesterone (300 $nmol1^{-1}$) to maturation media did not significantly improve either cleavage or blastocyst development when compared with the control (Silva and Knight, 2000). Positive effects of estrogen in bovine maturation media have been reported when added along with FSH. When added at a rate of 5 μg/ml, FSH along with estradiol at 1 μg/ml yielded improved development to

blastocyst when compared with maturation media not supplemented with hormones. Additionally, the addition of estradiol at 1 μg/ml with 10 μg/ml of LH also showed an improvement over non-supplemented media. All media in this study contained either fetal calf serum, or cow serum from animals on either D0, D1, D10 or D20 of the estrous cycle (Younis et al., 1989).

The biological influences of estradiol and progesterone maturation and early embryo are not well understood. Dynamic changes in concentrations of steroid hormones during follicular growth and after the LH surge are major factors that could explain the variation in development results for produced embryos. To maximize the efficiency of *in vitro* embryo production, it is necessary to investigate the effects of these hormones at physiologically relevant level for stage of follicular development. Therefore, the objective of this study was to investigate the impact of follicle size and relative estradiol and progesterone concentrations on oocyte maturation and early embryo development following follicle fluid exposure during maturation *in vitro*.

Materials and methods

All bovine IVF and IVC protocols were based on previously described procedures (Negron-Perez et al., 2017).

Collection and preparation of follicle fluid

Bovine ovaries were collected from an abattoir (Brown Packing, Gaffney, SC) and transported to the laboratory in 0.9 % saline supplemented with penicillin (100 units/ml) and streptomycin (100 units/ml). Follicle fluid was collected via needle aspiration from small (2-5 mm diameter) and large (10-20 mm diameter) follicles and pooled according to size into a 15 ml conical tube. Fluid was filtered through a 0.2 μm cell strainer into a 15 ml conical tube, then stored at -20 °C. After several replicates of follicle fluid collections, follicle fluid was thawed,

brought to room temperature and pooled. Half of each type of follicle fluid (from large and small follicles) was separated into a 15 ml conical tube for charcoal stripping in order to remove the majority of the steroid hormones as previously described (McNeilly, 1984; Redmer et al., 1985). Briefly, follicle fluid was filtered through a 0.2 μm cell strainer to remove debris. Activated charcoal was added to follicle fluid at a rate of 10 mg/ml, then incubated on a shaker at room temperature for 1 h. The fluid-charcoal mixture was then centrifuged at 4 °C for 20 minutes at 513 RCF (g). Fluid was then decanted into aliquots of 1 ml and frozen at -20 °C until use. Fetal bovine serum (FBS) was charcoal-stripped according to the above protocol and stored in 5 ml aliquots at -20 °C until use.

Estradiol and Progesterone preparation

β-Estradiol (Sigma-Aldrich E2758-1G) was diluted in pure ethanol at a rate of 1 mg/ml as per manufacturer recommendations, then diluted again into cell culture water (Sigma-Aldrich W3500-500 ml) at a rate of 1 ng/μl and frozen in 40 μl aliquots at -20 °C until use. Progesterone (FisherScientific AC225650050-5G) was diluted in pure ethanol at a rate of 1 mg/ml as per manufacturer recommendations, then diluted again into cell culture water at a rate of 1 ng/μl. Aliquots of 65 μl were stored at -20 °C until use.

Maturation media preparation

Table 4-1 contains a summary of all media treatments and abbreviations. Two control media were used in this experiment. One was designated the positive control oocyte maturation medium OMM (C+; n=504 COC) because it was the usual formulation that is used in our laboratory and included untreated fetal bovine serum (FBS; not charcoal stripped). Specifically, this medium consisted of TCM-199+ Earls Salts (LifeTech 11150-059), 10 % FBS (ThermoFisher 10437010), 1.14 % Glutamax 100X (Gibco 35050-061), 1.14 % sodium pyruvate

(LifeTech 11360-070), 40 μg/ml follicle-stimulating hormone (Folltropin®, AgTech INC), estradiol (Sigma-Aldrich E2758-1G), 50 μg/ml gentamycin (Sigma-Aldrich), and EGF (Sigma-Aldrich, E9644-.5MG) and was not supplemented with any FF. Negative control (C-) medium (n=392 COC) consisted of TCM-199+ Earls Salts, 10 % charcoal-stripped FBS, 1.14 % Glutamax 100X, 50 μg/ml gentamicin (Gibco, Grand Island, NY, USA), and 1.14 % sodium pyruvate and was not supplemented with any hormones or FF. The C- OMM was a stand-alone treatment and also served as the base OMM for all experimental treatments. The large follicle fluid (LFF; n=240 COC) treatment medium consisted of C- OMM supplemented with 75 % LFF. Likewise, small follicle fluid (SFF; n=372 COC) treatment medium consisted of C- OMM supplemented with 75 % SFF. Charcoal-stripped LFF (CSL; n=282 COC) and charcoal-stripped SFF (CSS; n=364 COC) treatment media were C- OMM with 75 % charcoal-stripped LFF or 75 % charcoal-stripped SFF, respectively. Progesterone and/or estradiol were added to the charcoal-stripped follicle fluid treatments based on average concentrations found in fluid from pooled large or pooled small follicles that had been collected in the same manner during a previous experiment (Harl et al., 2017). These six treatment media were formulated using C- OMM as a base with the following designations and additions: charcoal-stripped LFF + progesterone (progesterone; CSLP; n=319 COC) had 75 % LFF and 160 ng/ml progesterone; charcoal-stripped SFF + progesterone (CSSP; n=342 COC) was 75 % charcoal-stripped SFF and 140 ng/ml progesterone; charcoal-stripped LFF + estradiol (estradiol; CSLE; n=262) had 75 % charcoal-stripped LFF and 37 ng/ml estradiol; charcoal-stripped SFF + estradiol (CSSE; n= 345 COC) was 75 % charcoal-stripped SFF and 23 ng/ml estradiol; charcoal-stripped LFF + progesterone + estrogen (CSLPE; n=295 COC) consisted of 75 % charcoal-stripped LFF, 160 ng/ml progesterone, and 37 ng/ml estradiol; charcoal-stripped SFF + progesterone + estradiol

(CSSPE; n=287 COC) was 75 % charcoal-stripped SFF, 140 ng/ml progesterone, and 23 ng/ml estradiol.

Oocyte collection and maturation

Oocytes were collected from antral follicles (2-7 mm diameter) of abattoir-derived ovaries via slashing into ~150 ml of oocyte collection medium (OCM). The medium was then filtered through a 0.2 µm cell strainer to collect COC. The material collected by the filter was rinsed onto a gridded plate for search and collection of COC. The oocytes with healthy layers of cumulus cells were collected and washed twice in fresh OCM and randomly assigned to treatment maturation media. Cumulus-oocyte complexes were matured in groups of 15 in 50 µl OMM drops overlaid with mineral oil (Origio, Målov Denmark) for 21 h at 38.5 °C under 5 % CO_2.

After being subjected to treatments during the maturation period, all COC were treated the same for the remainder of the procedures. All *in vitro* fertilization and *in vitro* culture procedures were based on previously described protocols (Al Naib et al., 2011; Negron-Perez et al., 2017). Briefly, after 21 h maturation, COCs from the same treatment were pooled, washed three times in HEPES- Tyrode's albumin lactate pyruvate [HEPES-TALP; HEPES-TL (Caisson Laboratories, Inc; North Logan, UT, USA) supplemented with 3 mg/ml BSA (Fraction V), 22 µg/ml sodium pyruvate and 75 µg/ml gentamicin] and fertilized in plates containing 500 ml of IVF-TALP [IVF-TL (Caisson Laboratories) supplemented with 6 mg/ml BSA (essentially fatty acid free), 22 µg/ml sodium pyruvate, 10 µg/ml heparin and 50 µg/ml gentamicin]. Two frozen-thawed semen straws from two *B. taurus* bulls were pooled, purified with BoviPure-BoviDilute 40 % [v/v and 80 % (v/v)], and diluted to a final concentration in the fertilization dishes of 1 x 10^6/ml. Fertilization time was 18-22 h in a humidified gas atmosphere of 5 % (v/v) CO_2 and 19

% (v/v) O_2 at 38.5 °C for all groups. Putative zygotes were collected, exposed to hyaluronidase (1000 U/ml in ~0.5 ml HEPES-TALP) and vortexed for 5 minutes to remove cumulus cells. Putative zygotes were then washed three times in HEPES-TALP and placed in groups of 15 zygotes per 25 µl drop of synthetic oviductal fluid – bovine embryo 2 (SOF-BE2) covered with mineral oil in a humidified gas atmosphere of 5 % (v/v) CO_2, 5% (v/v) O_2 and the balance nitrogen, at 38.5 °C. Cleavage rates were assessed on d 3 and blastocyst rates at d 8 post-fertilization. A replicate was defined as the COC collected in one day for *in vitro* fertilization procedures. A replicate consisted of either 30 or 60 oocytes per treatment and fertilization with semen from a pool of two bulls. Only replicates with a cleavage rate of ≥65 % in C+ embryos were included in data analysis.

Immunofluorescence

After 8 days of culture, blastocysts from large follicle treatments were collected for cell counting. Large follicle treatments were selected because this environment is, theoretically, the best representation of *in vivo* conditions. Immunolabeling protocols were conducted as previously described (Negron-Perez et al., 2017). All immunolabeling procedures were performed at room temperature unless otherwise noted. Briefly, for labeling with CDX2, embryos were placed in permeabilization solution (Dulbecco's phosphate-buffered saline (DPBS) +polyvinylpyrrolidone (PVP) containing 0.25 % (v/v) Triton X-100) and incubated for 30 minutes, then transferred to blocking solution (5 % (w/v) bovine serum albumin (BSA) in DPBS) for 1 h. Embryos were then transferred to mouse monoclonal anti-human antibody against CDX2 (0.4 µg/ml; CDX2-88, Biogenex) and incubated for 1 h in the dark. Embryos were then washed three times in washing buffer (DPBS + 0.1 % BSA (w/v) and 0.1 % (v/v) Tween-20) and transferred to the secondary antibody: conjugated goat polyclonal anti-mouse IgG (1

μg/ml fluorescein isothiocyanate (FITC) Abcam, Cambridge, MA, USA) for an incubation period of 1 h in the dark. Embryos were washed three times and counterstained with 1 μg/ml Hoechst 33342 in DPBS-PVP for 15 minutes, after which they were washed once in DPBS-PVP. Embryos were then transferred to a 10 μl drop of SlowFade Gold antifade reagent (S36936, Life Technologies) on a glass microscope slide and covered with a coverslip. To determine non-specific labeling, primary antibodies were replaced with rabbit or mouse IgG (1 μg/ml).

Images of embryos were captured with a 40X objective using a Nikon Eclipse Ti fluorescence microscope (Melville, NY). ImageJ v 1.51n (National InstituteZ of Health, Bethesda, MD, USA) was used to measure images and count the number of cells.

Statistical analysis

Development data were analyzed for the main effect of treatment using SAS statistical software (SAS Institute Inc., Cary, NC.). Development data were analyzed for treatment effect using PROC GLIMMIX, with the dependent variables including cleavage of embryos, development to blastocyst, inner cell mass number trophoblast cell number, and total cell number. Class variables included treatment and replicate, and replicate was considered a random variable. Day 3 cleavage rate was calculated as the number of cleaved embryos divided by the total number of oocytes and d 8 blastocyst rate was calculated as the total number of blastocysts divided by the total number cleaved. Separation of means was conducted with the LSMEANS statement in SAS with the Tukey adjustment. Results are reported as least squares means ± standard error of the mean.

Results

Two control maturation media were used in this experiment, a positive and negative control. The C+ treatment was used as an indicator for a successful IVF replicate, while the C-

medium served as a basis for comparison with the experimental treatment media (since it was the base medium for all treatment media). For these reasons, the C+ and C- treatments were important to the experiment as they served two roles: 1) production of experimental data for treatment comparison, and 2) quality controls. The overall cleavage and blastocyst rates for C+ were 81.3 ± 0.2 and 29.8 ± 0.02 %, respectively, while the overall cleavage and blastocyst rates for C- were 69.3 ± 0.2 % and 24.2 ± 0.02 %, respectively (Fig. 4-1, 4-2).

Cleavage rates were affected by treatment ($P<0.01$). The C+ treatment yielded the highest cleavage rate, and this rate exceeded the C- and all follicle fluid treatments ($P<0.05$) except for one: the CSSP treatment. The CSSP treatment was statistically similar to all treatments except for SFF and CSSE, which were lower ($P<0.05$). Thus, of all the treatments involving follicle fluid, the only differences in cleavage rate that existed were between CSSP compared to SFF and CSSE.

Blastocyst rates were also affected by treatment ($P<0.01$). As with cleavage rates, the C+ treatment yielded the numerically highest blastocyst rate. Unlike with cleavage rates, however, there were several treatments that were statistically similar to C+, including C-, LFF, CSL, CSLP and CSSP. Blastocyst rates for C+ were greater than all other treatments (SFF $P<0.01$; CSS $P<0.01$; CSLE $P<0.05$; CSSE $P<0.01$; CSLPE $P<0.05$; and CSSPE $P<0.05$). The SFF treatment was statistically similar to seven of the other eleven treatments but differed from C+ ($P<0.01$), C- ($P<0.05$), CSLP ($P<0.01$) and CSSP ($P<0.05$).

Inner-cell mass number did differ between treatments, with CSLE having significantly fewer cells than all other treatments ($P<0.05$; Fig. 4-3). Trophoblast (84.06 ± 3.42) and total cell number (128.38 ± 4.38) was not different between treatment groups (Fig. 4-3).

Discussion

To our knowledge this is the first study examining follicle size and the direct effects of estradiol and progesterone on early embryonic development for *in vitro* produced embryos. In most cases, the addition of follicle fluid to the maturation media reduced cleavage and blastocyst rates. Perhaps the most interesting finding, however, is that addition of progesterone to charcoal-stripped follicle fluid rescues embryo development, in that it returns blastocyst rates to the same level as C+. Comparatively, adding estradiol alone to maturation media resulted in negative developmental effects on the embryo, and those negative effects carried over when both progesterone and estradiol were added to maturation media.

During *in vivo* development, the oocyte undergoes maturation in follicle fluid under the influence of decreasing estradiol and increasing progesterone (Moor et al., 1980; Salehnia and Zavareh, 2013). Our lab has previously reported positive effects of follicle fluid during maturation on cumulus cell expansion when added at a rate of 75 % (Harl et al., 2017). These effects did not carry over to improved embryo development, as the addition of 75 % untreated follicle fluid to the maturation medium during this study resulted in reduced cleavage and development to blastocyst in most cases (compared to C+). This result reflects previously reported results in which addition of follicle fluid to maturation media at a concentration of 10 % resulted in reduced development (Ayoub and Hunter, 1993). Follicle size did not influence development in the current study, which contradicted previous reports in which fluid from follicles 2-6 mm resulted in reduced cleavage and blastocyst development when compared to fluid from >6 mm follicles (Lonergan et al., 1994). While oocytes obtained from slaughterhouse ovaries are generally from follicles 2-6 mm in diameter, final nuclear and cytoplasmic maturation of the oocyte occurs during 4-5 days of dominant follicle growth. Since *in vitro*

oocyte maturation is 21 h and dominant follicle growth takes place over the course of several days, it is possible that fluid from follicles not in the dominant stage may be lacking critical components for supporting oocyte maturation (Lonergan et al., 1994). Additionally, follicle fluid was collected from slaughterhouse ovaries in which with stage of the estrus cycle and follicle wave were unknown. Even if fluid was collected from a pool of large and small follicles, it is impossible to know if the follicle was atretic at the time of collection, which could negatively impact embryo development.

It has been well established that the secretion of steroids by follicular cells is critical in oocyte maturation. In mammalian preovulatory follicles, estrogen and progesterone are the primary steroids secreted by the granulosa cells under the stimulation of gonadotropins LH and FSH (Moor et al., 1980; Osborn and Moor, 1983; Zhang and Armstrong, 1989). Although resumption of meiosis in the oocyte can be spontaneous, the presence of follicular steroids is required for proper maturation to take place. In the present study, adding exogenous estradiol to maturation media did not improve cleavage or blastocyst rates over untreated follicle fluid or charcoal-stripped follicle fluid. In fact, treatments involving addition of estradiol resulted in a numerical reduction of cleavage and blastocyst development in comparison to their charcoal-stripped counterparts, regardless of follicle size. In porcine oocytes where estradiol was added to maturation media without exogenous proteins at either 0, 1, or 10 μg/ml, estradiol created an inhibitory effect on development after metaphase I in a dose-dependent manner (Racowsky and McGaughey, 1982). Addition of estradiol alone at a rate of 1 μg/ml did not have an impact on maturation compared with the control, but addition of estradiol along with 1.5 μg/ml of FSH and 2 μg/ml LH caused a significant decrease in germinal vesicle breakdown, a necessary step in nuclear maturation of the oocyte (Singh et al., 1993).

Positive effects of estrogen in bovine maturation media have been reported when added along with FSH. When FSH was supplemented in the follicle fluid along with estradiol, there was an improved rate of development to blastocyst when compared with maturation media not supplemented with hormones. The addition of estradiol at 1 μg/ml with 10 μg/ml of LH also showed an improvement over non-supplemented media. All media in that study contained either fetal calf serum, or cow serum from animals on either D 0, D 1, D 10 or D 20 of the estrous cycle (Younis et al., 1989). The current study had media containing either 37 or 23 μg/ml of estradiol for L or S follicle fluid supplementation, respectively. Although the concentrations were based on physiological levels present in pooled follicle fluid from both large and small follicles, it is possible that the changes in estradiol concentration that take place within the follicle after the LH surge were not accurately reflected and caused a detrimental effect on oocyte development.

The addition of progesterone to media during maturation has also resulted in mixed results. In porcine oocytes, the addition of progesterone to maturation media resulted in a negative impact on nuclear and cytoplasmic maturation (Dode and Graves, 2002). Similarly, in bovine oocytes negative impacts on development were reported when progesterone was added to maturation media (Silva and Knight, 2000). The current study reported a numerically positive effect on blastocyst development when compared with no progesterone or estradiol alone regardless of follicle size, which agrees with previously reported data in which progesterone was added and the medium was overlaid with paraffin oil (Sirotkin, 1992). Progesterone concentration in early pregnancy has been associated with increased embryo success and pregnancy retention *in vivo*. Previous research has shown a correlation between pregnancy failure after insemination and lower progesterone concentration when compared with animals who conceive following insemination (Stronge et al., 2005). Additionally, in the days

immediately following ovulation, progesterone levels have been shown to be significantly lower in animals that return to estrus 18-20 days after mating when compared with animals that become pregnant (Henricks et al., 1971).

In the current study, follicle fluid was charcoal stripped to remove steroids present in the follicle fluid. Charcoal-stripping procedures are done in cell culture media to deplete molecules of interest in biological sera such as fetal bovine serum (FBS), bovine calf serum (BCS), or follicle fluid. Although steroids were the main interest in this study, charcoal-stripping removes other components such as thyroid hormones, peptide hormones, and lipids. Additionally, the charcoal-stripping procedure removes folic acid, vitamins, glucose, and phosphorous (Zhimin et al., 2009). Charcoal-stripping protocols remove small molecules from sera via absorption, and because the absorption is not specifically targeted, absorption of multiple components cannot be ruled out when analyzing effects.

In the current study, steroid secretions by the cumulus cells were not enough to support oocyte competence in treatments with no hormones added. Addition of estradiol alone also failed to support oocyte competence, possibly due to the transition from estradiol to progesterone production by the cumulus cells. The addition of progesterone alone resulted in comparable development to standard maturation medium (C+) but did not result in an enhanced maturation system. These results agree with previously published research in which progesterone added to maturation media at rates of 10, 38, 50, or 100 μm failed to improve development in mouse oocytes. Additionally, when progesterone concentration was raised to 100 μm a decrease in development was reported (Salehnia and Zavareh, 2013). It is possible in the current study, that the combined addition of progesterone alongside the progesterone secretions of the cumulus cells supported oocyte maturation and developmental competence. When both progesterone and

estradiol were added to the maturation medium, cleavage and development to blastocyst were both reduced when compared with standard maturation and progesterone only media.

Conclusions

This study demonstrated the individual and combined effects of estradiol and progesterone on oocyte maturation and early embryonic development of bovine oocytes produced *in vitro*. The addition of estradiol alone did not support early embryonic development, while the addition of progesterone alone showed improved development compared with estradiol alone. The combined addition of estradiol and progesterone demonstrated an inhibitory effect on early embryonic development. The cause of the inhibitory effect of the estradiol-progesterone combination is still unknown, and future research investigating these mechanisms would further the understanding of the follicular microenvironment in which the oocyte undergoes final maturation.

Table 4-1: Description of all maturation media used in the study, including rate of supplementation, hormone concentration, and type of follicle fluid. Treatment abbreviations are listed on the left, with contents on the right. Maturation media final volume was 50 µl. Drops were overlaid with paraffin oil and oocytes were matured in media for 21 h.

Treatment	Contents
C+	Normal OMM
C-	OMM with no estradiol or FSH added that has been charcoal stripped.
LFF	C- + 75% large FF
SFF	C- + 75% small FF
CSLFF	C- + 75% charcoal stripped large FF
CSSFF	C- + 75% charcoal stripped small FF
CSLE	C- + 75% cs lg FF + 37 ng/ml ESTRADIOL
CSSE	C- + 75% cs sm FF + 23 ng/ml ESTRADIOL
CSLP	C- + 75% cs lg FF + 160 ng/ml PROGESTERONE
CSSP	C- + 75% cs sm FF + 140 ng/ml PROGESTERONE
CSLPE	C- + 75% csLFF + 37 ng/ml ESTRADIOL + 160 ng/mL PROGESTERONE
CSSPE	C- + 75% csSFF + 23 ng/ml ESTRADIOL + 140 ng/ml PROGESTERONE

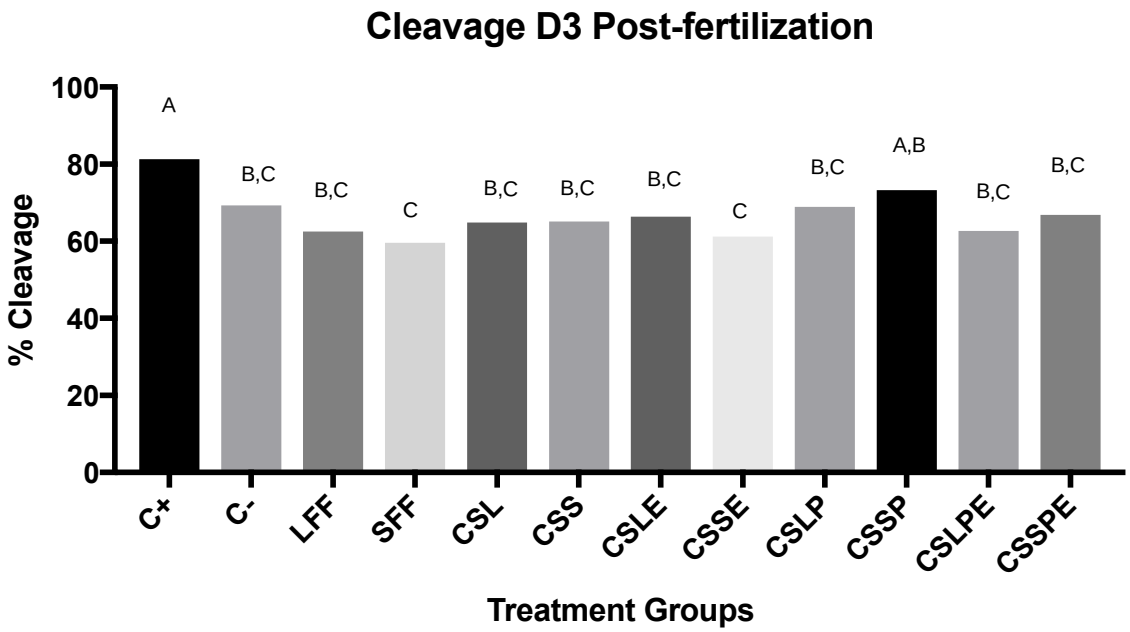

Figure 4-1: Effect of maturation medium treatment on embryo cleavage in embryos from oocytes matured in 75% follicle fluid treatments for 21 h at 38.5 ^{o}C and 5% CO_2. Cleavage evaluated on D 3 post-fertilization. Treatment groups are defined in Table 1. Columns with different superscripts significant at $P<0.05$.

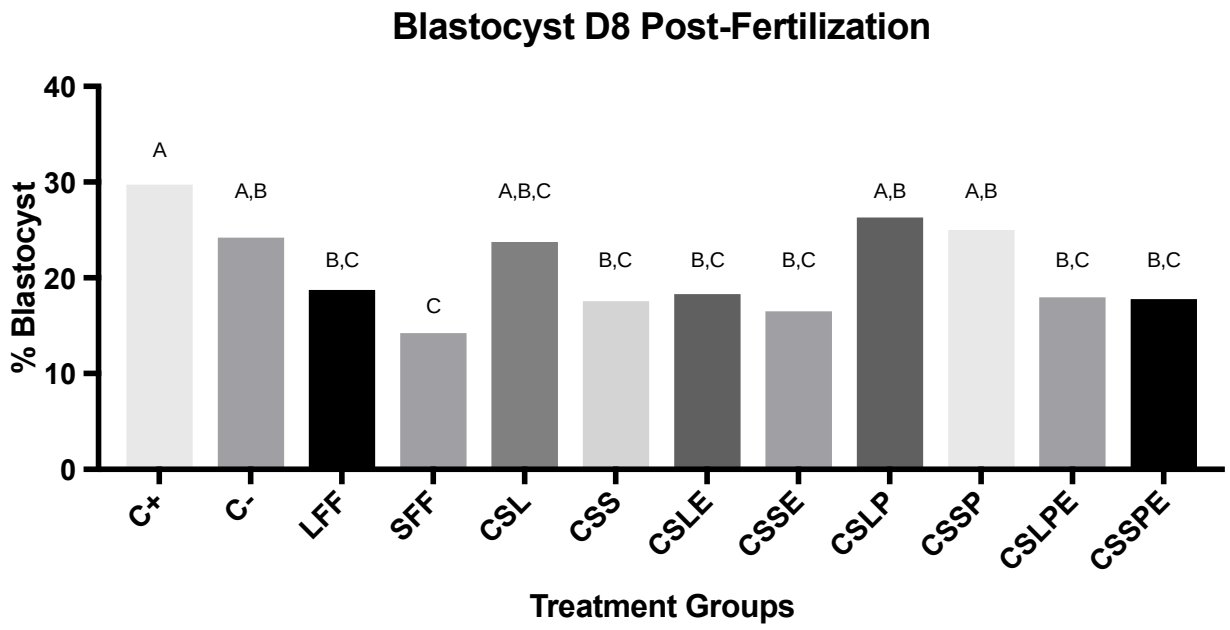

Figure 4-2: Effect of maturation medium treatment on blastocyst development in embryos from oocytes matured in 75% follicle fluid treatments for 21 h at 38.5 ^{o}C and 5% CO_2. Blastocyst development evaluated on D 8 post-fertilization. Treatment groups are defined in Table 1. Columns with different superscripts significant at $P<0.05$.

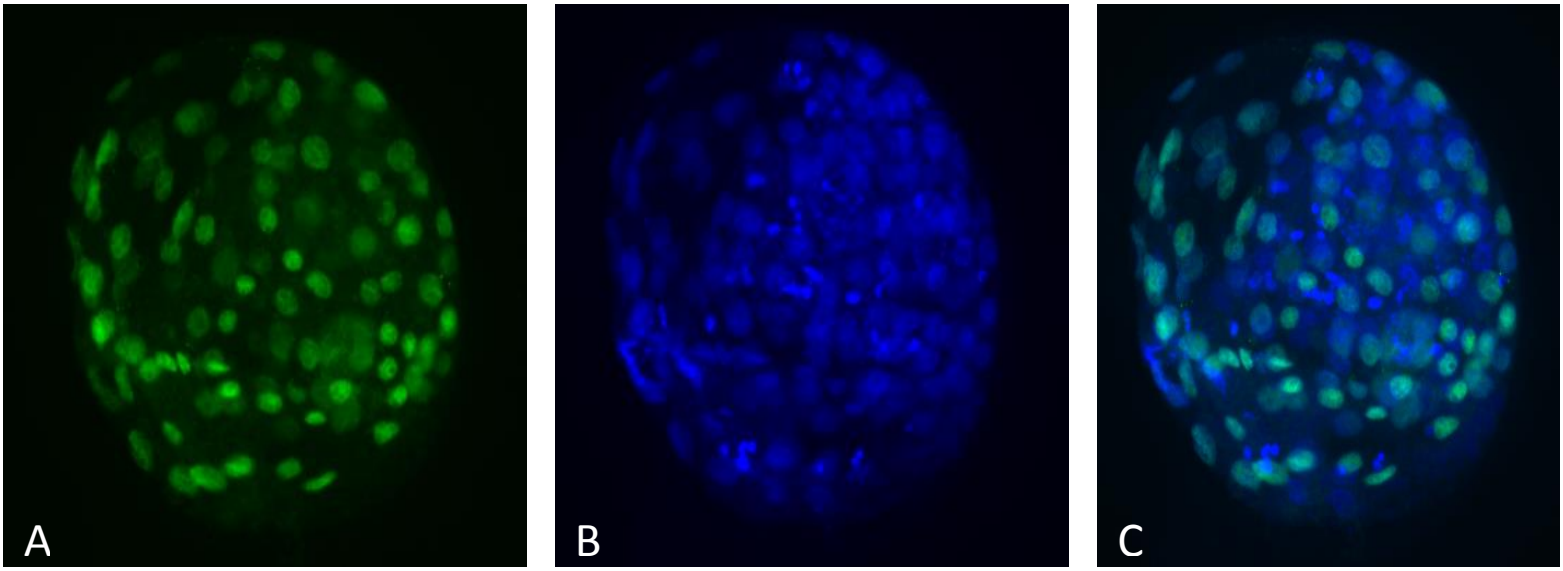

Figure 4-3: Immunofluorescent staining in embryos matured for 21 h in follicle fluid collected from large (10 mm) follicles. Embryos stained for CDX2/trophoblast cells (A) and Hoechst/inner-cell mass (B). Channels merged in panel C. Trophoblast and total cell numbers did not differ, so image provided is representative of all stained embryos.

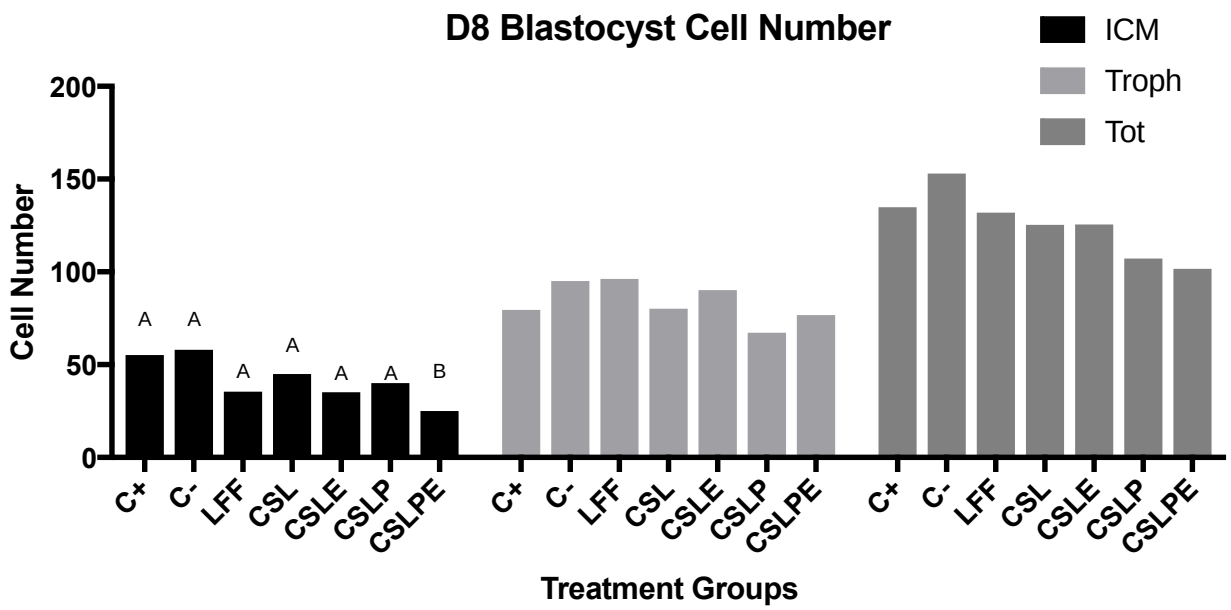

Figure 4-4: Effect of large follicle maturation medium treatments on embryo cell number. Blastocysts fixed for immunofluorescence on D8 post-fertilization. Treatment groups are defined in Table 1. Columns with different superscripts significant at $P<0.05$.

Chapter 5 - Increased utilization of tactile activities in reproductive physiology laboratory sections in order to improve cognitive learning

Abstract

Aspects of the laboratory course environment influence student knowledge retention during laboratory sessions and improves understanding and retention of corresponding lecture course material. Traditional laboratory sessions generally involve a brief overview of pertinent information, followed by a hands-on activity (Forcino, 2013). Sessions are designed to compliment a corresponding lecture series and students are presented with a large volume of information each session. Courses build upon themselves during the term, so retention of knowledge over time is critical for student success. Because students have different styles of learning, it is important to employ diverse teaching strategies in order to engage as many students as possible (Chen et al., 2016). Tactile learning techniques emphasize hands-on and visual learning aids to improve retention through "doing". By expanding laboratory activities to include more tactile learning techniques, a broader range of learning styles will be reached during the session, and students will retain information more efficiently. In this study 4 laboratory sections were randomly divided into one of 2 treatments: control or tactile. Information was delivered to the control sections primarily in written form with minimal tactile learning opportunities. The laboratory sections assigned to the tactile treatment received the same information as controls, but were assigned distinct tactile activities focused on critical concepts. Tactile activities included building models, drawing, and visual recognition. By increasing the amount of hands-on activity during the lab, students gained a better understanding of the concepts and performed better on assessments. Overall, students participating in the tactile

labs experienced increased scores on exam 1. These results indicate that increased hands-on learning in laboratory experiences helps students retain information.

Introduction

One of the goals of higher education is to make learning meaningful and impactful for students by not only increasing their engagement during their education but by preparing them for the workforce (Yates et al., 2015). Studies have shown that when a person experinces a new concept, working through it with hands-on experiences will help the learner retain an average of 80% of the information, compared to 20% retention with hearing information alone (Kolb and Kolb, 2005). Experiential learning is the process of learning by doing. Experiential learning first engages the student in the material with a hands-on experience, then encourages reflection on the experience to develop skills (Lewis & Williams, 1994). The experiental learning model proposed by Kolb emphasizes the need for students to learn through observation and interaction with their environment, as opposed to relying on other's experiences to draw conclusions (Kolb, 2015).

The laboratory environment influences student knowledge retention not only during laboratory sessions, but during the corresponding lecture course (Forcino, 2013). While the concept of a laboratory course is inherently hand-on based, abstract concepts are difficult to include in hands-on activities. Traditional laboratory settings in animal reproductive physiology courses at Virginia Tech generally involve laboratory sessions beginning with a brief concept lecture, with hands-on activites for gross anatomy. There is limited hands-on activity for more abstract concepts such as hormone function and reproductive cyclicity. The corresponding lecture series within the laboratory time period is designed to compliment the lab experience itself and students are presented with a large volume of information each session. Reproductive

physiology courses build upon themselves throughout the semester, so retention of knowledge over time is critical for student success in the course.

A student's preferred method of applying ability is known as their learning style (Herman et al., 2006; Hatami, 2013; Kadhim and Cameron, 2016). Each student has a unique set of preferences, which can include visual, auditory, reading/writing, and tactile information (Kolb and Kolb, 2005; Blevins, 2014). Previous research has indicated that tactile-based activities in higher education helps with cognative learning in mathmatics and physics courses (Clair, 1991; Barnes and Libertini, 2013; Cone, 2013). To our knowledge, application of tactile-based activities has not been tested on more abstract concepts presented during reproductive physiology laboratory sessions.

The purpose of this study is to increase the use of tactile-based learning techniques for abstract concepts in the laboratory sessions to improve student knowledge retention on complex concepts. Techniques involve increasing hands-on and visual learning aids to improve retention through tactile involvement. By increasing the amount of tactile activity during the lab, students will be able to better understand abstract concepts and experience increased success. By implementing active learning techniques in laboratory sessions, we hope to improve student knowledge retention, which should in turn improve student grades for the course.

Materials and Methods

Physiology of Reproduction Lab (ALS 3314) is a required course for all Animal Science majors in the Animal and Poultry Science (APSC) and Dairy Science (DASC) at Virginia Tech and is predominantly taken during the students' junior year, although seniors are also often enrolled. The course is also open to non-APSC/DASC majors. The same instructor has taught the required separately graded lecture Physiology of Reproduction (ALS 3304) course since 2013. A

team of 2-4 graduate students teaches lab sections. During the semester of this experiment (Fall 2016), lab activities were primarily coordinated and executed by one graduate teaching assistant (TA) with the support of three additional graduate TA's. All students enrolled in a lab section were also enrolled in the corresponding lecture section. There were four weekly lab sections occurring between Tuesday and Thursday (T: 14:00-16:50, W: 14:00-16:50, Th: 08:00-10:50, Th: 14:00-16:50). Students were enrolled in one of four sections and teaching responsibility between graduate TAs was divided by week, such that TAs were assigned to teach all four sections of a week. Students across all sections received lectures from the same TA for a given topic. Students enrolled in their lab section based on their schedule but did not have a choice about the lab instructors or type of course (with regards to treatment).

To meet the objectives, the lab sections were randomly assigned to either the control (C) or tactile (T) treatment group. A total of 88 students were enrolled, with an average of 22 students in each section (Tu: n=20, W: n=24, ThAM: n=21, ThPM: n=23). Students in both treatment groups began lab with a 20-30-minute concept lecture taught by a graduate TA and this concept lecture was identical in all labs. After the lecture period, students would break into activity groups. Students in the tactile groups participated in activities that emphasized hands-on participation such as creating models or 3D charts and assigning hands-on activity to abstract concepts including hormone and target tissues, reproductive cyclicity, sperm cell mutations, and ovary mapping. Students in the control groups covered the same concept material as the tactile group but were asked to participate using essay or video formats, with limited hands-on activities such as gross anatomy tissue dissection which were inherent to the labs. Topics in lab were directly related to subjects covered in lecture and in the textbook (Pathways to Pregnancy and Parturition, 3rd ed., Current Conceptions, Inc.) and a new topic was covered in lab each week.

Students were assigned a course grade based on 3 lab practical exams and a presentation (with a partner). Topics during the first half of the term consisted of gross anatomy, spermatogenesis and the estrus cycle, while the second half of the term consisted of pregnancy and applied reproductive technologies. Exam grades from all exams were compared to evaluate differences in knowledge retention based on treatment. Statistical significance was declared at $P < 0.05$. This study was conducted with the approval of the Virginia Tech Internal Review Board.

Results and Discussion

Students in T labs overall achieved higher scores on exam one when compared with C labs ($P = 0.027$). The average score on exam one for the C labs was 76.06% while the average score for the T labs was 79.04%. There was no difference between treatments in the scores from exam 2 ($P=0.357$). The average score for all labs on exam two was 72.3%. There was no difference between treatments in the scores from exam three ($P=0.707$). The average score on exam three for C labs was 77.97% while the average for T labs was 78.82%. Overall students in T labs did not achieve either a higher average letter grade or number of points when compared with C labs. The lack of significance during the second and third exam could be explained by the lab content. The majority of the tactile activity during the course takes place during the gross anatomy and hormone labs which occur during the first five weeks of the semester. This material was assessed on the first practical exam. The second exam did contain some tactical lab activities, but not to the extent of the first exam. While there was a numerical difference between C and T labs on the second exam (73.4% vs 71%) the results were not significant. During the final four weeks of the semester, labs were identical between treatments and were field-trip style reproductive management labs. These labs as well as the case studies were the subject of the third exam. There was no difference in exam scores between treatments on the third (last) exam

(average 78.41%). Since material was presented to the C and T labs using only one teaching style during this portion of the course, the lack of difference between treatments on this last exam indicates that academic ability/effort was evenly distributed between C and T labs. Differences between the first exam and the other exams may also be a result of students withdrawing and dropping from the course. A total of six students dropped the course, 5 from the C sections, and 1 from a T section. Differences between treatment groups are summarized in Table 5-1.

Student feedback regarding the content of the tactile labs was very positive overall. Students enjoyed the content of the activities and stated that performing the activities helped to clarify concepts presented during the concept lecture presented at the beginning of lab. The increased hands-on activity in the lab helped solidify concepts that, prior to the activity, were much more fluid. Future improvements to the course could include the utilization of more activities to incorporate concepts such as hormonal feedback loops, and early embryonic development, which generally do not have a hands-on component, but rely on memorization of charts or diagrams. Additionally, standardizing the lesson plan for each lab will help minimize variation, and make the lab more consistent from term to term.

Conclusion

In conclusion, students participating in lab with increased tactile activity performed better on the first exam, but not subsequent exams in the course. Additionally, students did not experience a difference in overall points or letter grade between labs. Future studies on the benefits of tactile-based activities in reproductive physiology are needed to discern more possible benefits to students. Based on the results of this study, investment of resources and time in the development of hands-on activities was important to knowledge retention for pertinent topics, but not overall course performance. Student responses to the activities were overwhelmingly

positive, and continued development of these activities could prove to be an effective strategy for helping students retain information not only during the course, but throughout their education and careers.

Assessment	Control (pts out of 100)	Tactile (pts out of 100)	-value	
Exam 1	76.23 ± 11.72	79.01 ± 12.55	.027*	(
Exam 2	71.12 ± 12.75	73.42 ± 10.58	.36	(
Exam 3	77.98 ± 10.97	78.82 ± 10.11	.71	(

Table 5-1: Student performance on laboratory exams in Physiology of Reproduction laboratory course between treatments. Results with * are significant at $P<0.05$.

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