Strategies for Improving Reproductive Efficiency of Beef Cattle with Assisted Reproductive Technologies

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ABSTRACT

Reproductive efficiency in beef cattle can be improved with reproductive technologies at the herd, individual cow, and embryonic levels. Decreasing the bull:cow ratio for natural service after fixed time artificial insemination (FTAI) can alleviate economic burden associated with FTAI. In experiment 1, the total number of cows exposed per bull was negatively correlated with pregnancy rate to natural service on first return to estrus after FTAI in fall herds. The number of open cows per bull in fall herds using one natural service sire was negatively correlated with pregnancy rate on first return to estrus. There was no correlation between number of cows exposed per bull and pregnancy rates in fall herds with multiple sires or in spring herds. However, bull:cow ratio accounted for only 5–11% of the variation in pregnancy rates, thus we conclude that a reduced bull:cow ratio did not affect natural service return to estrus pregnancy rate. Experiment 2 examined how supplementing calcium salts of soybean oil (CSSO) improves beef cow fertility. Non-pregnant cows received supplement with either saturated fat or omega-6 rich CSSO. There were no changes in dominant follicle diameter, corpus luteum volume, plasma progesterone, or endometrial gene expression (*PTGES* and *AK1B1*, *PPARA*, *PPARA*, *PPARD*) between treatments. Plasma and follicular fluid fatty acid compositions were altered between treatments. Experiment 3 examined if size parameters of zygotes have potential as a non-invasive, objective embryo selection method. The outer diameter, area of ooplasm, and thickness of zona pellucida (ZP) was digitally measured on individual artificially activated oocytes and in-vitro fertilized (IVF) zygotes. Larger outer diameter increased probability of development to the blastocyst stage by days 7 and 8 for activated oocytes and tended to by day 8 for IVF zygotes. Thinner ZP increased probability of development to blastocyst stage on days 7 and 8 for oocytes, and to day 8 for IVF zygotes. Area did not affect development but was positively correlated with blastomere number on day 8. An interaction between diameter and ZP thickness was observed in zygotes, but not activated oocytes, suggesting oocyte activation is not always a suitable replacement for in-vitro fertilization.

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GENERAL AUDIENCE ABSTRACT

We need to improve reproductive efficiency in beef cows if we are to combat the challenges of producing more food while using less resources due to limited land availability and concerns with greenhouse gas emissions from agriculture. In cow-calf production systems, this means producing one healthy calf per cow per year. Cattlemen can implement a variety of assisted reproductive technologies to achieve this goal. Achieving maximal reproductive efficiency will require using technologies that are incorporated into herd management, individual animal care, and *in vitro* embryo production.

Fixed time artificial insemination (FTAI) allows cattlemen to maximize the number of cows becoming pregnant and calving earlier in the season to increase efficiency. Unfortunately, use of FTAI is uncommon in cow-calf production systems because of labor and economic restraints. In order to improve economic feasibility of FTAI, bull-related costs need to be reduced, which can be done through increasing the number of cows serviced per bull (decreasing the bull:cow ratio). This study retrospectively examined correlations between the bull:cow ratio and pregnancy rate on first return to estrus after FTAI. There was little to no correlations between bull:cow ratio and pregnancy rates, and if they were significant, there was much variation in the data. With this we concluded that a reduced bull:cow ratio does not affect pregnancy rate on first return to estrus, allowing producers to increase the number of cows serviced by a single bull and reduce bull related costs.

Supplementing calcium salts of soybean oil (CSSO) that are rich in omega-6 fatty acids can enhance beef cow fertility, but it is unclear why this happens. Using non-pregnant cows as a model, we studied the effects of either saturated fat or CSSO on reproductive parameters such as ovarian structures, hormone concentrations, and uterine gene expression. There were no changes in any of these parameters between treatments, but there were changes in the concentrations of certain plasma and follicular fluid fatty acids. There was also reduced activity of lipid metabolism enzymes. We were unable to pinpoint how CSSO supplementation improves reproduction, but the altered fatty acid content of tissues and altered enzyme activity likely plays a key role, thus ultimately impacting fatty acid utilization and growth of the embryo.

In-vitro embryo production can increase the number of offspring produced from a single female and accelerate the incorporation of animals with high genetic merit into herds. To obtain optimal pregnancy rates with *in vitro* embryos, we should develop non-invasive, objective methods for identifying the most viable embryos. This study examined if size parameters of activated and fertilized oocytes are indicative of successful development. We discovered that oocytes with large diameters and those with thin zona pellucida were most likely to develop to the blastocyst stage, and that the area of the cell was positively correlated with blastocyst total cell number. An interaction between diameter and ZP thickness was observed in zygotes, but not activated oocytes, suggesting oocyte activation is not always a suitable replacement for in-vitro

fertilization. This suggests that digital measurements of fertilized oocytes may have potential as objective selection criteria

Addressing issues of reproductive inefficiency in beef cows requires a comprehensive approach, as there is not one ideal solution. Management techniques can alleviate the cost of FTAI by reducing the number of bulls used without affecting pregnancy rates. Supplementing CSSO can alter tissue fatty acids to enhance fertility. Finally, the efficiency of *in vitro* embryo production can be improved by selecting better embryos for transfer without compromising the embryo. Combinations of all these techniques can create more reproductively efficient animals.

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6-DMAP	6-dimethylaminopurine
AA	Arachidonic acid
AI	Artificial insemination
ALA	Alpha-linolenic acid
CL	Corpus luteum
COC	Cumulus oocyte complex
CSSO	Calcium salts of soybean oil
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EV	Extracellular vesicles
FFAR	Free fatty acid receptor
FSH	Follicle stimulating hormone
FTAI	Fixed time artificial insemination
GnRH	Gonadotropin releasing hormone
ICM	Inner cell mass
IETS	International Embryo Technology Society
IFNT	Interferon-tau
ISG	Interferon stimulated gene
IVF	*In vitro* fertilized
IVP	*In vitro* produced
LA	Linoleic acid
LH	Luteinizing hormone
MPF	Maturation promoting factor
MX1/2	Myxovirus resistance protein 1 and 2
n-6, n-3	Omega-6, omega-3
OAS1	2'-5'-oligoadenylate synthetase 1

OPU	Ovum pick up
PDMS	Polydimethylsiloxane
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
RXR	Retinoid X receptor
SOF	Synthetic Oviductal fluid
VDOC	Virginia Department of Corrections
VEGF	Vascular endothelial growth factor
ZP	Zona Pellucida

CHAPTER 1

Introduction

There is mounting pressure on livestock production systems to produce more while utilizing less, driving the need for increased efficiency as reflected in the Food and Agriculture Organization of the United Nations' report (2017). The world's population continues to grow and is estimated to reach just under 10 billion by 2050. Not only are populations growing, so is the percentage of the population living in urban environments, which is contributing to decreasing, older labor forces in agriculture. Simultaneously, we face growing concerns about climate change and the role of agriculture, especially livestock production (FAO, 2017). Although contributing only 2% of total US greenhouse gas emissions, beef cattle have been targeted as a major source of emissions (EPA, 2018). However, increasing levels of CO_2 also impact nutritional value of essential crops such as soybeans, rice, and wheat: specifically, through reduced amounts of available zinc, iron, and protein (FAO, 2017). This places ever more importance on ensuring access to animal proteins and their incorporation into diets. Combating these challenges requires improving the efficiency of our livestock systems.

For cow-calf production systems, efficiency means achieving the goal of producing one healthy calf per cow per year. Unfortunately, infertility is one of the major factors that prevents cattlemen from obtaining this goal, bringing with it weakened reproductive efficiency and economic burden. It's estimated that the cost of infertility in U.S. beef cattle is upwards of $3 billion annually, and each 1% decrease in pregnancy rates costs an individual producer $6.25 per cow exposed (Lamb et al., 2011). The majority of reproductive failure in cattle can be attributed

to embryonic loss, especially prior to day 35 of gestation (Santos et al., 2004). In beef cattle specifically, the greatest periods of embryonic loss occur before day 7, especially before day 4 during the early cleavage stages of the fertilized oocyte, and between days 16 and 32, during the time of maternal recognition of pregnancy and placentation (Reese et al., 2020). By day 100 of gestation after a single insemination, only about 50% of beef cattle will maintain their pregnancy (Reese et al., 2020).

There is an abundance of assisted reproductive technologies available for use in cattle production systems, such as artificial insemination (AI) and *in vitro* fertilization (IVF) that improve production efficiency and circumvent infertility due to anestrus or fertilization failure. Unfortunately, adoption of these technologies in beef cattle systems is far less prevalent than in comparison to other livestock industries. The primary limiting factors to utilizing these technologies are the associated costs and labor or time involved in implementing them (USDA, 2009). In order to improve dissemination of this technology in beef production systems, the efficiency of the technology needs to increase while the costs are reduced to lessen its economic impacts. From there, reproductive technologies can be more readily implemented into production systems and combat the growing demand for sustainable beef.

Review of the literature, concerning both reproductive physiology and reproductive technologies, reveals how complex ensuring reproductive efficiency is and that it is a multifaceted issue; thus, a more comprehensive approach is needed for improvement. This dissertation will examine how reproductive efficiency can be improved through herd management, a focus in the individual cow, and utilization of *in vitro* embryo production system. Different technologies can be applied on each level of intervention to further improve reproductive function and efficiency.

CHAPTER 2

Literature Review

BOVINE REPRODUCTIVE PHYSIOLOGY

The Estrous Cycle

The estrous cycle of cattle, ranging from 17 to 24 days with an average of 21 days, comprises four phases: 1) estrus, 2) metestrus, 3) diestrus and 4) proestrus. Estrus signals the start of the estrous cycle and lasts roughly 20 hours. It is characterized by physical behaviors such as a cow or heifer's receptivity to mounting by bulls and other cows, and increased blood concentrations of estradiol secreted from a large dominant follicle, generating a surge of luteinizing hormone (LH) to stimulate ovulation. Metestrus follows and lasts three to five days. During this time ovulation occurs approximately 20 to 30 hours after the LH peak and onset of estrus (Chenault et al., 1975). Follicular tissue luteinizes to form the corpus luteum (CL) and produce progesterone. The first few days of the12-day diestrus period are characterized by increasing CL growth and progesterone secretion, but CL size peaks by day 8. The CL is responsive to luteolysis by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), naturally occurring around day 17 of the cycle. Proestrus begins and is characterized by declining levels of progesterone, which removes inhibitory feedback on the pituitary and allows increased gonadotropin-releasing hormone (GnRH) and LH pulsatility to stimulate follicular growth and the return to estrus after two to three days (Amstalden and Williams, 2015).

Folliculogenesis

Follicle development occurs continuously throughout the bovine estrous cycle, generating waves of follicle recruitment, selection, and dominance followed by either ovulation or atresia. The first phases of follicular growth are gonadotropin independent. Pre-antral follicle growth begins with the transition from primordial follicle to primary follicle, where the flattened granulosa cells form cuboidal morphology and Kit-ligand binding inhibits Foxo3 transcription factor to permit oocyte and cumulus growth (Braw-Tal and Yossefi, 1997; Saatcioglu et al., 2016). Pre-antral follicle growth continues through proliferation of cumulus cells and increasing oocyte diameter, generating a secondary follicle with two layers of cuboidal granulosa (Braw-Tal and Yossefi, 1997; Fair et al., 1997b). With development of the antrum, the follicle reaches the tertiary stage and contains several layers of granulosa cells surrounding the oocyte (Fair et al., 1997b). Once the tertiary follicle reaches a diameter of 4 mm, follicular growth becomes dependent on gonadotropins secreted by the pituitary gland, and increased levels of follicle stimulating hormone (FSH) recruit small antral follicles to begin a new follicular wave (Campbell et al., 1995).The follicle then acquires the ability to suppress pituitary FSH secretion with its own secretion of inhibitory factors such as estradiol or inhibin and the selection phase begins. Upon reaching an 8 mm diameter, the follicle switches to dependence on LH for continued growth and selection for the dominant follicle begins (Ginther et al., 2001). The dominant follicle will grow to 10 mm where it gains ovulatory capacity, while simultaneously inhibiting growth of the subordinate follicles sending them to atresia (Sartori et al., 2001).

Oocyte Maturation

Simultaneously during follicle growth, oocyte maturation begins in preparation for successful fertilization and conceptus development. The bovine oocyte first enters meiosis in the fetal ovary around 75 days of gestation and around 140 days of gestation arrests in the diplotene

phase of prophase I, from which the primary follicle can form (Erickson, 1966; Yang and Fortune, 2008; Pepling, 2013). Progression to the early antral phases while in meiotic arrest allows time for acquisition of nuclear and cytoplasmic maturation in the oocyte. Zona pellucida (ZP) formation and transcriptional activity of the oocyte begin in the secondary follicle and continues until the oocyte reaches a diameter of 110 μm (Fair et al., 1995; Fair et al., 1996; Fair et al., 1997a). While meiotic competence is reached with formation of the antrum, cytoplasmic maturation is still underway. The oocyte increases the number of Golgi complex and mitochondria, develops cortical granules, and dislocates organelles to the periphery of the oocyte until oocyte growth plateaus at 120-130 μm (Fair et al., 1997b; Mehlmann, 2005). As the follicle approaches it's ovulatory diameter, the oocyte accumulates lipids and reduces the number of Golgi complexes (Assey et al., 1994). Resumption of meiosis is achieved with the ovulatory LH surge. At this stage, cortical granules align to the periphery of the oocyte, more lipids are accumulated, the cell pulls away from the ZP increasing the perivitelline space, and the nuclear membrane breaks down allowing completion of meiosis I and extrusion of the first polar body (Hyttel et al., 1989; Suzuki et al., 1994). The oocyte progresses until arresting in metaphase of meiosis II, only completing meiosis II and extrusion of the second polar body after successful fertilization (Hyttel et al., 1989).

Ovulation

Ovulation of a competent oocyte is necessary for successful reproduction of cattle. Increasing concentrations of estradiol produced by the dominant follicle stimulate increased GnRH pulse frequency in the hypothalamus, likely through Kisspeptin neurons, when progesterone is absent, and increased GnRH pulse frequency stimulates the LH surge (Moenter et al., 1991; Amstalden and Williams, 2015). This LH surge acts on the dominant follicle to

create a cascade of events leading to ovulation. Luteinizing hormone induces expansion of cumulus cells through increased production of hyaluronan and promotes resumption of meiosis II. Luteinizing hormone also acts on the granulosa cells, promoting increased progesterone production, decreases estradiol production, and further stimulating the production of local prostaglandins and pro-inflammatory eicosanoids. These vasoactive substances then move to the theca cells to ultimately increase proteinases that weaken the follicular wall. Local prostaglandins induce ovarian smooth muscle contractions, and the combination of increased follicular pressure with a weakened follicular wall causes ovulation (Richards et al., 2015).

Fertilization

Sperm travel a great distance from either the site of natural semen deposition (the vagina) or the site of AI (the uterine body) to the ampullary-isthmus junction for fertilization. Sperm first travel in a rapid transport phase, with mostly dead sperm appearing near the ovary within 30 minutes of insemination, and a sustained transport phase that occurs over several hours (Vandemark and Moeller, 1951; Dobrowolski and Hafez, 1970). Once in the oviduct, sperm bind to the oviductal epithelium, mediated by binder of sperm proteins (BSPs), for storage and capacitation (Suarez, 2016). Oviductal epithelial cells facilitate the removal of BSPs and cholesterol to increase sperm membrane fluidity and intracellular calcium to increase hyperactivity in motile sperm (Parrish, 2014). Successfully capacitated sperm then are released, bind to the ZP, and undergo acrosome exocytosis which will expose the inner membrane and several proteasomes necessary for degradation of ZP proteins (Zimmerman et al., 2011; Suarez, 2016).

After successful ZP penetration, the sperm and oocyte bind and fuse together, activating the oocyte. Phospholipase C zeta on the sperm membrane hydrolyzes DAG from PIP_2 on the

oocyte membrane to stimulate calcium release from the endoplasmic reticulum in a series of calcium oscillations that are necessary to degrade maturation promoting factor (MPF) and promote the completion of meiosis (Fissore et al., 1992; Macháty and Prather, 1998; Ross et al., 2008). Sperm fusion to the oocyte also stimulates the exocytosis of cortical granules to prevent binding of and fertilization from multiple sperm (Soloy et al., 1997; Macháty and Prather, 1998). The ZP undergoes ultrastructural changes after fertilization such as tightening of the ZP protein filaments/mesh structure and changes in biomechanical properties like ZP stiffness (Suzuki et al., 1994; Papi et al., 2012)

Luteinization

Formation of the CL requires rapid cellular proliferation, differentiation, and angiogenesis. The surge of LH during estrus will induce ovulation, and the remaining tissue of the ruptured follicle collapses into the cavity while ruptured vessels form blood clots, generating the corpus hemorrhagicum during the first 1 to 3 days post-ovulation (Jennings et al., 2017). Luteinization of theca and granulosa cells begin in response to the LH surge, and CL begins to grow rapidly for 8 days until plateauing in size (Donaldson et al., 1965; Amstalden and Williams, 2015). Luteinizing hormone stimulates the differentiation of granulosa and theca cells into large and small luteal cells, respectively, which are responsible for the production of progesterone (Alila and Hansel, 1984). The large luteal cells, constituting only about 3% of the total luteal cell population, generate the majority of luteal progesterone and some oxytocin, but are not responsive to LH to stimulate more progesterone (Fields and Fields, 1996). Small luteal cells on the other hand, while having little steroidogenic capacity, can be induced to produce progesterone when LH is administered (Fields and Fields, 1996).

The CL is an immensely vascular structure, thus extensive proliferation of endothelial cells and development of new vasculature is necessary to support progesterone production. Vascular endothelial growth factor (VEGF) is a potent driver of angiogenesis and stimulates the necessary proliferation, migration, and differentiation of endothelial cells needed for formation and stabilization of new vasculature (Chen and Zheng, 2014). Berisha's group has demonstrated that VEGF is greatly expressed in the bovine CL during luteinization and can be stimulated in the granulosa derived luteal cells, and suggests that the combination of fibroblast growth factor 2 and VEGF promote angiogenesis during CL formation (Berisha et al., 2000). A variety of other factors such as cytokines, prostaglandin E_2 (PGE_2), and leukotrienes also contribute to the formation and function of the CL (Skarzynski et al., 2013).

Luteolysis

In the absence of a conceptus, the bovine uterus will initiate the process of luteolysis around days 16 and 17. Functional regression of the CL will occur first where progesterone production ceases and circulating levels decline, followed by apoptosis and involution of the CL (Skarzynski et al., 2013). Luteolysis is initiated in the bovine much the same way it occurs in the sheep, with uterine $PGF_{2\alpha}$ acting as the luteolytic agent (Schramm et al., 1983). As described by McCracken (1999), towards the end of the cycle, downregulation of progesterone receptors allows for action of estrogen through estrogen receptors in the hypothalamus and endometrium. In the endometrium, binding of estrogen to estradiol receptor quickly upregulates production of oxytocin receptors. In the hypothalamus, estrogen activity stimulates release of oxytocin from the pituitary gland, which then binds to the oxytocin receptors in the endometrium to initiate low level pulses of $PGF_{2\alpha}$. The low levels of pulsatile $PGF_{2\alpha}$ travel to the CL via countercurrent exchange between the uterine vein and ovarian artery to stimulate oxytocin release from large

luteal cells, which will act directly on the uterus to further stimulate $PGF_{2\alpha}$ secretions and cease progesterone secretion and induce apoptosis (McCracken et al., 1999). Full regression of the CL results in the formation of the corpus albicans, the non-functional, white scar tissue left on the ovarian surface (Jennings et al., 2017).

Early Embryonic Development

After fertilization, syngamy of sperm and oocyte pronuclei requires structural changes in both paternal and maternal genomes. The paternal genome undergoes rapid changes involving chromosome decondensation, replacement of sperm protamines with histones, and rapid demethylation of sperm DNA by conversion of 5-methylcytoseine to 5-hydroxymethylcytoseine followed by some de novo re-methylation in the bovine (Park et al., 2007; Wossidlo et al., 2011; Ramathal et al., 2015). After successful paternal epigenetic reprogramming and fusion of the pronuclei, the first mitotic division occurs within 24 to 30 hours post-fertilization (Barnes and Eyestone, 1990). From there the embryo will continue to undergo mitotic divisions from 2 to 4 to 8 cells, at which stage the embryo transitions from relying on maternally provided transcripts to activation of the embryonic genome (Camous et al., 1986; Frei et al., 1989). However, it's been noted that decreased levels of transcription are occurring before then, beginning as early as the 2-cell stage, and that these genes may play important roles in embryonic development (Memili and First, 1998; Graf et al., 2014). Meanwhile, the maternal genome becomes passively demethylated during the early cell divisions (Ramathal et al., 2015).

The embryo reaches the morula stage around the 16 and 32 cell stage. Compaction begins and neighboring cells begin to form tight adhesions with each other. The typical pattern of symmetric cell divisions seen up to this point are lost and individual cells begin to polarize, creating distinction between the apical and basal domains of cells (Ramathal et al., 2015). These

events of compaction prepare the embryo for cavitation and formation of the blastocyst around days 7 or 8. At this time, the fluid filled blastocoel cavity forms and the first developmental cell lineage specification occurs, separating cells of the trophectoderm and the inner cell mass (ICM). The trophectoderm is the outermost layer of cells, gives rise to the placenta during development, and can be differentiated from the ICM by the presence of CDX2 protein (Kuijk et al., 2008). The formation of tight junctions during compaction allow for retention of the blastocoel, which is formed by the movement of sodium ions by the Na/K-ATPase channel and the subsequent movement of water to retain osmolarity (Watson et al., 2004). The ICM can be distinguished in the bovine by presence of SOX2 protein and undergoes further cell lineage specifications to generate extra-embryonic membranes and the fetus (Goissis and Cibelli, 2014). The ICM will further differentiate into the epiblast and hypoblast, that latter which will line along the trophectoderm and form the yolk sack (Maddox-Hyttel et al., 2003).

Conceptus Development In-Utero

The embryo's development beyond the blastocyst stage is dependent on uterine secretions. Between days 8 and 10, the bovine blastocyst hatches from the ZP and trophectoderm begins to proliferate exponentially, transforming the conceptus from a spherical shape, to ovoid, and finally to an elongated filamentous shape more than 20 cm long by day 19 (Betteridge et al., 1980; Brooks et al., 2014). As early as day 7, the blastocyst secretes bioactive factors such as prostaglandins and low levels of interferon-tau (IFNT). These begin to alter uterine gene expression in preparation for supporting elongation and implantation, such as reducing stimulate production of PGE_2 and reduce $PGF_{2\alpha}$ secretion (Hernandez-Ledezma et al., 1993; Nadeau et al., 1994; Sponchiado et al., 2017). While the bovine embryo can develop to the blastocyst stage *in vitro*, elongation cannot occur outside of the uterine environment (Lonergan and Fair, 2014).

Lipids, amino acids, growth factors are secreted from the endometrial glands to support conceptus growth, and ablation of these glands results in conceptuses that are unable to elongate and establish pregnancy (Gray et al., 2002). Activation of the peroxisome proliferator activated receptor gamma (PPARG), driven by histotrophic lipids, is also essential for conceptus elongation in ruminants (Brooks et al., 2015b; Ribeiro et al., 2016a). While elongation is occurring the epiblast will further differentiate into the major cell lineages for development (mesoderm, ectoderm, and endoderm) during the processes of neurulation and gastrulation to set the stage for fetal development (Maddox-Hyttel et al., 2003).

Maternal Recognition of Pregnancy

To maintain pregnancy, the conceptus must signal to the mother its presence and ensure continued luteal function before luteolysis occurs, typically between days 14 and 16 post-ovulation. Drs. Bazer, Roberts and Thatcher's research groups have identified IFNT as the anti-luteolytic protein responsible for maternal recognition of pregnancy in ruminants and have demonstrated IFNT production by the conceptus dramatically increases as the trophectoderm rapidly proliferates during elongation between days 12 and 15 of the cycle (Godkin et al., 1982; Godkin et al., 1984; Vallet et al., 1988). Interferon-tau silences the expression of the estrogen receptor alpha (*ER1*) gene, which in turn prevents the estradiol-induced expression of oxytocin receptors (*OXTR*) needed to stimulate luteolytic pulses of $PGF_{2\alpha}$ from the endometrium, ultimately preventing luteolysis and ensuring continual progesterone for conceptus development (Spencer et al., 1995b, a; Spencer and Bazer, 1996). Interferon-tau secretion by the conceptus also upregulates prostaglandin synthesis to increase PGE_2 in the luminal epithelium (Charpigny et al., 1997).

The actions of IFNT also extend beyond the uterus to modulate pregnancy establishment. There is evidence that IFNT can act directly on the CL to preserve CL function and lifespan. Interferon-tau has antiapoptotic effects on large luteal cells and stimulates angiogenic factors *in vitro* (Basavaraja et al., 2019). These pro-angiogenic genes, along with a variety of interferon stimulated genes (ISGs), are also upregulated in the CL of pregnant cows on day 18 of gestation (Basavaraja et al., 2019). Furthermore, direct infusion of recombinant IFNT into the uterine vein of ewes can maintain progesterone secretion by the CL after luteolytic challenge by an injection of $PGF_{2\alpha}$, demonstrating IFNT can directly impact luteal lifespan (Shirasuna et al., 2015). Interferon-tau also acts on the peripheral blood leukocytes in the maternal circulation and induces expression of ISGs. In cattle, the genes 2′5′ oligoadenylate synthetase (OAS1), ISG-15, and myxovirus resistance protein 1 (MX1) and 2 (MX2) are significantly upregulated in pregnant animals by day 18 of gestation, before a noticeable decline in progesterone (Gifford et al., 2007; Green et al., 2010). Detecting ISGs could serve as an early pregnancy detection marker, well before the time pregnancy can be detected by ultrasonography or palpation, but ISG response in older cows is not as robust as in heifers and may be difficult to accurately assess (Green et al., 2010).

Postpartum Return to Cyclicity

After parturition, cattle experience a period of anestrus which further reduces their reproductive efficiency. Cows must resume cyclicity and establish pregnancy within an 85-day window to achieve the cow-calf operation's goal of producing one calf per cow per year. During the postpartum period, cows need to first undergo uterine involution in which vasoconstriction and contractions reduce uterine size and remove fetal membranes, and endometrial tissue repairs itself (Kiracofe, 1980). Although uterine involution does influence the length of the postpartum

period, it can impair fertilization in the first 20 days postpartum by hindering sperm transport through the reproductive tract (Short et al., 1990). Meanwhile, ovarian activity has resumed almost immediately after parturition, but ovulation does not occur until around the third follicular wave in beef cattle due to insufficient LH pulsatility in response to the dominant follicle's estradiol (Murphy et al., 1990; Crowe, 2008).

The two main factors preventing sufficient LH pulsatility and lengthening postpartum anestrus are the presence of a nursing calf and poor maternal body condition (Short et al., 1990). The bond between a mother cow and her own calf is necessary for reducing LH pulse frequency while lactation alone and nursing unrelated calves does not reduce pulse frequency (Griffith and Williams, 1994; Williams and Griffith, 1995). The nutrition of cows prepartum and their body condition at parturition is more important than postpartum, as cows of adequate condition (body condition score ≥ 5) have shorter postpartum intervals (Richards et al., 1986; Short et al., 1990). When LH pulse frequency increases enough to produce an ovulatory LH surge, the first postpartum estrus in cattle typically occurs without the behavioral signs of estrus. A shortened luteal phase follows ovulation due to premature $PGF_{2\alpha}$ release by the uterus (Cooper et al., 1991; Crowe, 2008).

TECHNOLOGIES FOR MAXIMIZING REPRODUCTIVE EFFICIENCY IN CATTLE

Estrus Synchronization and Artificial Insemination

Artificial insemination was one of the first reproductive technologies created in cattle, and its development also opened the door for a variety of non-surgical reproductive technologies like embryo flushing and transfer (Ivanoff, 1922; Moore and Hasler, 2017). Estrus synchronization in conjunction with AI maximizes its efficiency and efficacy by ensuring

properly timed fertilization and servicing multiple cows at once. Early synchronization protocols only involved inducing or delaying estrus. Estrus expression can be induced with injections of $PGF_{2\alpha}$ to induce luteolysis, or it can be delayed with synthetic progestins such as norgestomet implants (Lauderdale et al., 1974; Miksch et al., 1978; Spitzer et al., 1978a; Spitzer et al., 1978b). Later, it was discovered that an injection of GnRH could induce ovulation, through stimulation of LH production from the pituitary, and begin a new follicular wave in cattle with large dominant follicles (Garverick et al., 1980). Unfortunately, these techniques will not always work at any random point in a cow's cycle. Prostaglandin $F_{2\alpha}$ can only induce estrus if there is a CL older than 5 days present to be able to respond to $PGF_{2\alpha}$ (Rowson et al., 1972; Braun et al., 1988). Gonadotropin releasing hormone requires the presence of follicles larger than 10 mm in diameter, and the provided dose may not generate sufficient LH to ovulate follicles less than 12 mm (Sartori et al., 2001). This poses challenges to synchronization protocols for anovulatory or pre-pubertal animals.

Combining these tools generated protocols to achieve acceptable pregnancy rates without estrus detection. The first such protocol, Ovsynch, involves an initial injection of 100 mg of GnRH to ovulate dominant follicles and begin a new follicular wave, 35 mg of $PGF_{2\alpha}$ 7 days later to regress the newly formed CL, another injection of GnRH 48 hours later to ovulate the generated follicle, and finally insemination without estrus detection 16-24 hours later (Pursley et al., 1995). This protocol, typically used in dairy cattle, reduced the number of days open post-partum while achieving pregnancy rates similar to traditional breeding off estrus detection (Pursley et al., 1997a). Unfortunately, the Ovsynch protocol was not effective in heifers and required major modifications for use in beef cattle systems (Pursley et al., 1995; Pursley et al., 1997b). First, the number of handling events was reduced to three by administering the second

GnRH injection at the time of insemination, creating the co-synch protocol and salvaging economic loses from additional labor (Geary et al., 1998; Geary et al., 2001). Progesterone was then added for seven days via an intervaginal device (CIDR), alleviating problems with premature ovulation (Macmillan and Peterson, 1993; Lamb et al., 2001; Sakase et al., 2007). This new CO-synch + CIDR protocol increased pregnancy rates overall as well as in non-cycling cows due to tightened ovulation synchrony between animals (Lamb et al., 2001).

A variety of synchronization and fixed time artificial insemination (FTAI) protocols have since been developed for use in cows and heifers with the ability to induce cyclicity in anestrous and prepubertal cattle (Lamb et al., 2010). Despite the variety of protocols to suit a cattleman's needs, adoption of estrus synchronization and AI into beef operations is lagging. Only about 8% of U.S. beef cattle operations actively use synchronization and AI, compared to nearly 90% of dairy operations (USDA, 2009, 2018). The lack of utilization leaves beef operations behind in terms of improving reproductive efficiency. While implementing FTAI protocols does require economic investment, the economic benefits likely outweigh the costs. It is estimated that there is a $49 advantage per cow enrolled in FTAI compared to those exposed to only natural service (Rodgers et al., 2012). Much of this economic gain can be attributed to heavier weaning weights, primarily due to FTAI cows calving earlier in the season, but can also tighten the breeding season to increase calf and herd value over time (Rodgers et al., 2012; Lamb and Mercadante, 2016). Estrus synchronization and FTAI can help improve reproductive efficiency and economic value of herds.

In Vitro Embryo Production

In vitro embryo production involves *in vitro* maturation of cumulus oocyte complexes (COC), IVF of those COCs with sperm, and *in vitro* culture of the presumptive zygotes to the

blastocyst stage. Each stage of the *in vitro* production process for bovine embryos occurs at 38.5°C, 5% CO_2, 5% O_2 for *in vitro* culture, and in the absence of light. Successful *in vitro* embryo production in cattle lagged behind other species, such as rabbit and human, and it was not until 1987 that an embryo produced entirely from *in vitro* techniques delivered the first live calf (Chang, 1959; Steptoe and Edwards, 1978; Lu et al., 1987; Moore and Hasler, 2017). Although *in vitro* produced (IVP) bovine embryos were slow to commercial use, improvements in oocyte collection and *in vitro* culture methods has resulted in today's IVP embryo production doubling that of *in vivo* derived embryos (Viana, 2019).

Oocyte Collection

Cumulus oocyte complexes can be collected either from living donors through ovum pick-up (OPU) or from post-mortem ovaries. Ovum pick-up, first studied in the bovine by Pieterse and colleagues, involves ultrasound guided, transvaginal aspiration of follicles for COC collection and can be performed repeatedly with no adverse effects on the cow or her cyclicity (Pieterse et al., 1988). This technique allows for continual oocyte collection of superior genetic merit animals, even during pregnancy, and in conjunction with IVF, generates more than one offspring per year from a single animal. Cumulus oocyte complexes can also be collected from follicles on post-mortem ovaries. Slicing up the entire ovary can yield greater numbers of COCs, but it also produces more oocytes of reduced quality out of the total collection (Saleh, 2017). In contrast, Wang and colleagues suggest that slicing ovaries can yield greater quality oocytes compared to aspiration techniques (Wang et al., 2007). Aspiration of follicles, either *in vivo* or post-mortem, can produce variable COC yield and quality depending on the length and gauge of needle used, and amount of pressure exerted for extraction (Bols et al., 1996; Fry et al., 1997). The follicle slashing technique is the most user friendly, as it generates less debris than slicing

allowing for easier collection and yields more quality COCs and more blastocysts per ovary than aspiration and slicing techniques (Carolan et al., 1994; Saleh, 2017).

In Vitro Maturation

Regardless of collection method, COCs must first undergo a period of *in vitro* maturation to achieve the cytoplasmic and nuclear maturity they naturally would *in vivo*. *In vitro* maturation is a critical step to the success of blastocyst formation, and *in vitro* culture systems are sub-optimal resulting in poor blastocyst development rates for IVP embryos (Lonergan and Fair, 2016). Development rates can be improved to an extent with stringent COC selection for those with brown, homogenous or slightly granulated ooplasm and 5 layers of compact cumulus cells (de Wit and Kruip, 2001; Nagano, 2019; Walker and Biase, 2020). Typical maturation media consists of a tissue culture medium like M-199 supplemented with serum or bovine serum albumin (BSA), growth factors, antibiotics, and gonadotropin or steroid hormones; however, alterations to maturation media recipes have not moved blastocyst development rates beyond 50% (Sirard et al., 1988; Lonergan and Fair, 2016). In contrast, cumulus oocyte complexes collected via OPU and stimulated with LH prior to collection were able to generate 80% blastocyst rates (Blondin et al., 2002). It has also been noted that COCs from larger follicles have increased blastocyst development (Lonergan et al., 1994; Hagemann et al., 1999; Sartori et al., 2001). While nuclear maturation of *in vitro* matured COCs is comparable *in vivo* matured COCs, cytoplasmic maturation is abnormal and delayed (Hyttel et al., 1986; Luciano and Sirard, 2018). There is likely a necessity for prolonged development in the follicle and stimulation with LH to achieve the greatest development rates that is not being mimicked correctly during *in vitro* maturation.

In Vitro Fertilization

After up to 24 hours of maturation, IVF takes place. First, sperm must undergo capacitation. The addition of heparin to fertilization media is vital to sperm capacitation and preparing the sperm for successful acrosome reaction (Parrish et al., 1985). Heparin binds to the sperm membrane, facilitating removal of bovine seminal plasma proteins, increased intracellular levels of calcium, increased intracellular cAMP, and changes to the intracellular pH (Parrish, 2014). Fertilization media must be absent of glucose to prevent sperm glycolysis and reduced intracellular pH, as well as contain at least 10 mM bicarbonate to stimulating sperm adenylate cyclase and increase cAMP production (Parrish et al., 1989a; Parrish, 2014). Bovine serum albumin is also found in most all fertilization media, and while it can induce capacitation without heparin, capacitation of sperm is increased when both BSA and heparin are present (Parrish et al., 1989b). Most bovine IVF is performed using frozen-thawed bull semen, which requires shorter capacitation time compared to fresh semen and reduced doses of heparin (Parrish, 2014). Fertilization success can be altered by changing the concentration of heparin, the concentration of sperm used for co-culture with oocytes, or even the individual sire used (Leibfried-Rutledge et al., 1989; Ward et al., 2002; Parrish, 2014). Co-culture of sperm and COCs for up to 19 hours results in the formation of presumptive zygotes.

In Vitro Culture

The presumptive zygotes are denuded and placed into culture for development to the blastocyst stage. There are several factors that can influence the development of the early embryo *in vitro*, such as pH and osmolarity of media, exposure to increased levels of oxygen, or even the types of metabolic substrates provided (Thompson, 1996). With so many factors needed for successful *in vitro* culture and a lack of understanding of what happens *in vivo*, there is much discrepancy between the IVP embryo and its *in vivo* counterpart. The IVP embryo is of much

lesser quality, as seen by decreased total cell numbers and decreased proportion of ICM cells relative to trophectoderm compared *in vivo* derived embryos (Iwasaki et al., 1990; Knijn et al., 2003). *In vitro* produced embryos also have increased apoptosis, altered metabolomic profiles, and transcriptional profiles (Bertolini et al., 2002; Gjorret et al., 2003; de Souza et al., 2015). As mentioned, a lot of development problems may arise from issues in maturation; however, the variety of factors that influence development increase the probability the *in vitro* culture system can exert more unnatural and unnecessary stressors.

Exposure to these culture-based stressors can lead to problems with development farther into gestation. *In vitro* production systems predispose the ruminant embryo to potentially developing large offspring syndrome. This disease is characterized by heavier birth weights, longer gestation, placental abnormalities, and in some cases congenital defects (Hasler et al., 1995; Schmidt et al., 1996; van Wagtendonk-de Leeuw et al., 2000; Farin et al., 2001). While several faults in the *in vitro* production system may be contributing to this, one targeted cause is undefined culture media, which contains a biologic fluid of unknown or variable composition. Incomplete understandings of an early embryo's metabolic requirements meant that early culture systems needed to be undefined; however, the use of serum and co-culture cells had unintended consequences for early embryonic development. *In vitro* culture media supplemented with serum seems to accelerate blastocyst formation early on, alters cellular ultrastructure of the embryo, and alters gene expression in comparison to defined synthetic oviductal fluid (SOF) media (Van Langendonckt et al., 1997; Lonergan et al., 1999; Crosier et al., 2001; Rizos et al., 2003). Removing serum and co-culture cells from SOF culture media has been able to ameliorate some of the negative calf outcomes in IVP embryos, such as reducing gestation length, birth weight, chest circumference, and increasing heart rate (van Wagtendonk-de Leeuw et al., 2000).

Unfortunately, it has not been able to fully normalize IVP embryos to *in vivo* embryos, as IVP embryos, regardless of serum use, are still heavier than AI or multiple ovulation embryo transfer embryos, have longer gestations, and increased calving difficulty (van Wagtendonk-de Leeuw et al., 2000). Refinement in the *in vitro* embryo production process is still needed to better normalize the developmental effects induced by stressors in the *in vitro* production system.

Artificial Oocyte Activation

An oocyte could potentially undergo the physiologic process of activation without fertilization from sperm to generate parthenogenic embryos. The occurrence of spontaneous cleavage in *in vitro* bovine oocytes is rare, between 7 and 10%, they develop at much a slower pace than fertilized oocytes, and very few will reach blastocyst stage (Plante and King, 1996; Lechniak et al., 1998). Oocytes can be activated electrically or chemically with ethanol, thimerosal, and calcium ionophores (like ionomycin and A23187) (Kono et al., 1989; Behalova et al., 1993; Collas et al., 1993; Macháty and Prather, 1998). Artificial activation is most successful in aged bovine oocytes, requiring at least 24 hours of *in vitro* maturation to achieve the necessary cytoplasmic maturation (Susko-Parrish et al., 1994). With the exception of thimerosal, chemical agents can only produce a single rise in intracellular calcium, unlike the fertilized oocyte which experiences several oscillations (Fissore et al., 1992; Nakada and Mizuno, 1998). While thimerosal produces multiple calcium oscillations, it will oxidize the meiotic spindle unless countered with a reducing agent like DTT (Cheek et al., 1993; Machaty et al., 1997). Calcium ionophores are able to stimulate the release of intracellular calcium stores instead of inducing uptake of extracellular calcium into the cell, thus allowing for activation in calcium free media (Macháty and Prather, 1998). When these agents are used alone, very poor blastocyst rates are achieved (Wang et al., 2008).

Development rates can be greatly improved when these chemicals are used in conjunction with inhibitors of protein synthesis, like cycloheximide, or kinase inhibitors, like 6-DMAP (Presicce and Yang, 1994; Susko-Parrish et al., 1994; Wang et al., 2008). The protein synthesis inhibitor blocks the synthesis of new cyclin-B proteins, especially in younger oocytes, which is a component of MPF and thus prevents re-activation of MPF and allows resumption of meiosis (Presicce and Yang, 1994; Alberio et al., 2001). Protein kinase inhibitors will inhibit the kinase activity of MPF, rendering it useless in preventing the resumption of meiosis (Motlik et al., 1998; Alberio et al., 2001). A technique has been developed to generate parthenogenetic embryos from bovine oocytes that can induce multiple oscillations of intracellular calcium by micro-injecting bovine phospholipase C zeta cRNA into mature oocytes (Ross et al., 2008). This method does require micromanipulation equipment and a skilled technician, but reduces problems with the increased rates of aneuploidy seen in chemically activated oocytes which may be more beneficial for applications to intracytoplasmic sperm injection of bovine oocytes where additional activation is required.

Activated oocytes can form into diploid or haploid parthenotes dependent on the activation protocol used and whether the second polar body is extruded with the resumption of meiosis, but development rates of haploid parthenotes are greatly reduced (Henery and Kaufman, 1992; Wang et al., 2008). Activation using ionomycin alone will generate haploid embryos, but adding 6-DMAP will inhibit second polar body extrusion and reducing the time interval between ionomycin and 6-DMAP treatment further reduces the prevalence of haploid embryos (Susko-Parrish et al., 1994). Immediate exposure to 6-DMAP results in almost no haploid parthenotes, as the kinase inhibitor inhibits spindle formation, preventing polar body extrusion and advancing oocytes with a single diploid pronucleus directly into mitotic divisions (Navara et al., 1994;

Susko-Parrish et al., 1994). Polar body extrusion can also be inhibited with the addition of cytochalasin-B, which inhibits microfilament formation and produces parthenotes with two pronuclei and one polar body (Kono et al., 1989; Theodoropoulos et al., 1994).

Parthenogenesis may have potential as a model for studying early embryo development when *in vitro* fertilization is not feasible. Research on human embryos or even fertilized oocytes is controversial, and so parthenogenesis may be used as a model to circumvent ethical or legal issues (Paffoni et al., 2008). Parthenogenetic embryos have already been used in place of *in vitro* fertilization in studies concerning porcine embryo development (Gupta et al., 2007; Hoyos-Marulanda et al., 2019). Paffoni argues that since development rates are similar between human parthenotes and traditional IVP embryos, they can be used as good models in place of IVP embryos (Paffoni et al., 2008). However, it's important to consider that while development rates may be similar, there are physiologic differences. Oocytes activated by chemicals exhibit only one rise in intracellular calcium, while fertilized embryos experience many (Nakada and Mizuno, 1998). Activated oocytes also exhibit incomplete cortical granule release when compared to fertilized oocytes (Gulyas, 1976; Soloy et al., 1997). Because of these subtle differences in physiology, parthenogenetic embryos may not be an appropriate substitute for *in vitro* fertilized embryos.

Microfluidics in Embryo Production

The use of microfluidics to study early embryonic development has grown within the past 20 years as scientists try to transform the unnaturally static embryo culture into a dynamic one that would better resemble the *in vivo* microenvironment. Traditional embryo culture involves culturing groups of embryos in microdroplets of media under oil, and they are left untouched for several days. In vivo, the embryo is moving through the oviduct and experiencing a variety of

chemical, thermal, and physical changes in its environment. Microfluidics can provide researchers and clinicians the necessary tools to better mimic the *in vivo* environment and cab be applied to all areas of the IVP system. *In vitro* maturation in microchannels achieves similar levels of nuclear maturation as traditional static culture and can support maturation of individual oocytes without negatively impacting development (Yuan et al., 2014; Wheeler and Rubessa, 2017). Microfluidic channels can be used for *in vitro* fertilization to reduce polyspermy and increase fertilization rates with reduced sperm concentration; something with application for human clinics or sexed semen in cattle IVF (Clark et al., 2005; Sadani et al., 2005; Suh et al., 2006). Microfluidics can even separate motile sperm from dead cells and seminal plasma or filter out sperm with greater motility and mitochondrial activity to improve blastocyst rates (Schuster et al., 2003; Sano et al., 2010; Li et al., 2016a; Li et al., 2016b).

The greatest application for microfluidics will be during *in vitro* culture to better mimic the *in vivo* environment. Some studies have shown improved development rates in microfluidic culture systems for bovine and murine embryos with low embryonic vigor or sub-optimal culture conditions; however more recent studies using improved culture conditions and quality gametes do not observe increases in blastocyst development (Beebe et al., 2002; Bormann et al., 2007; Han et al., 2010; Ferraz et al., 2017a). Microfluidic devices can support the fertilization and culture of individual murine embryos (Han et al., 2010). Another device used a porous membrane to support both bovine oviduct epithelial cells and bovine embryos in a single microfluidic device, which helped decreased polyspermy, eliminated spontaneous parthenogenesis, and reduced global DNA methylation to levels more similar to *in vivo* derived embryos (Ferraz et al., 2017a; Ferraz et al., 2017b). Finally, a microfluidic chip developed to mimic the peristaltic contractions occurring in the oviduct during the early cleavage stages

increased the percentage of 8-cell stage zygotes, although there were no differences in blastocyst development or quality (Kim et al., 2009). Microfluidics may eventually be able to better mimic the *in vivo* environment for all stages of *in vitro* embryo production as well as incorporate several steps of the IVP process to generate embryos that better resemble their *in vivo* counterparts.

Embryo Selection

Vital to the success of IVF and embryo transfer systems is the ability to identify the best quality embryo with the best chance of successfully establish pregnancy. A variety of methods and technologies have been developed in attempts to identify the most viable embryo, but translation from research to application in livestock and human IVF has been difficult due to inconsistent results and issues with technology implementation (Gutierrez-Adan et al., 2015; Sanchez et al., 2017). To further add to the issue, many of the techniques for determining embryo quality cannot be performed non-invasively, thus requiring sacrifice of the embryo.

Morphologic evaluation of quality and stage of development is the current method for embryo selection. The International Embryo Technology Society (IETS) outlines the numeric scoring system for embryo quality and stage of development (Stringfellow and Givens, 2009). Stage of development is scored on a scale of 1 to 9. Embryos recovered from cattle on days 6 or 7 post-insemination will most be stage 4 (compact morula), 5 (early blastocyst), 6 (full, non-expanded blastocyst), or stage 7 (expanded blastocyst). Quality score is assigned from a scale of 1 to 4, with 1 being an embryo of good or excellent quality and 4 being degenerate. While embryo stage does not influence pregnancy outcome, embryos receiving better quality scores yield better pregnancy rates for both fresh and frozen transfers (Hasler et al., 1987; Hasler, 2001). These classifications are greatly subjective and, while evaluations from a single embryologist are fairly consistent, evaluations between embryologists vary greatly (Farin et al.,

1995; Baxter Bendus et al., 2006; Paternot et al., 2009). Embryologists tend to agree most upon scores for embryos of extreme quality, either excellent or degenerated, while most disagreement occurs when grading embryos of fair or poor quality (Farin et al., 1995). In addition, the amount of experience an embryologist has factors into the amount of subjectivity in the evaluation. Those working in human clinics that run more than 500 IVF cycles a year have less variation in their quality assessments compared to embryologists that perform less IVF cycles (Baxter Bendus et al., 2006).

The subjectivity in traditional embryo evaluation is driving a search for more objective evaluation techniques. One proposed indicator of embryo quality has been the number of ICM cells in a blastocyst. Embryos with reduced ICM cell numbers exhibit reduced viability, providing a potential explanation as to why IVP embryos are less viable post-transfer (Willadsen and Polge, 1981; Ealy et al., 2019). The advent of differential fluorescent staining allows for quantification of both trophectoderm and ICM cells and can be used to quantitatively assess embryo quality (Iwasaki et al., 1990). Since IVP embryos exhibit reduced total cell numbers than the gold standard *in vivo* derived embryos, selecting for embryos with increased ICM numbers would improve post-transfer outcomes for IVP embryos (Iwasaki et al., 1990; Knijn et al., 2003). However, differential staining techniques are terminal, making cell number evaluation in live embryos extremely difficult. Recently a group in Italy was able to digitally quantify trophectoderm cell numbers using digital image analysis software, allowing for assessment of live embryos (Lagalla et al., 2015). They noted that increased ICM size in expanded blastocysts resulted in greater implantation rates; however, this system could only assess ICM area, not ICM number with the methods used to quantify trophectoderm number (Lagalla et al., 2015).

Genetic and chromosomal characteristics affect embryo quality and viability and can be assessed without sacrificing the desired embryo. While all embryos experience some level of polyploidy and are still viable with up to 25% polyploidy, there is a significant elevation in chromosomal aberrations in IVP embryos (Hare et al., 1980; Viuff et al., 1999; Viuff et al., 2000). Selection of embryos with minimal chromosomal aberrations will have a better chance at establishing pregnancy. It is feasible to test only a portion of cells for chromosomal and genetic abnormalities with a trophectoderm biopsy taken using micromanipulation equipment, which is commonly performed in human embryos with no adverse effects (Forman et al., 2012; Zacchini et al., 2017). Bovine embryos can undergo trophectoderm biopsy to perform karyotyping and assess chromosomal abnormalities and successfully produced live calves after transfer (Turner et al., 2019). Embryo biopsies and microarray analysis have been used successfully in cattle to reveal a variety of embryonic genes that are correlated with successful pregnancy establishment, such as *COX2*, *CDX2*, *PLAC8*, and *BMP15* (El-Sayed et al., 2006). While the implications of this research are promising, the application to production is lagging due to a lack of skilled technicians familiar with micromanipulation techniques in the livestock reproduction industry and the cost of purchasing and installing such equipment into IVF labs.

In efforts to create entirely non-invasive indicators of embryo quality, researchers have examined markers of embryo metabolism and selected metabolites in spent media. Glucose has been identified in several species as a marker of embryonic viability. Bovine embryos with elevated glucose consumption yield greater pregnancy rates after transfer even though they appear to grow at the same rate as embryos that do not utilize supplemented glucose (Renard et al., 1980). Elevated glucose consumption is also associated with viability of bovine embryos after freezing and thawing (Gardner et al., 1996). Gardner and colleagues later demonstrated that

human embryos that successfully establish pregnancy had elevated levels of glucose consumption on days 4 and 5 of culture (Gardner et al., 2011). Embryos utilize a variety of other nutrients in addition to glucose, and so broader metabolomic analysis are becoming of interest. Bovine embryos that exhibit fast or slow growth, determined by timing of first cleavage, have different metabolomic profiles, and it may be possible to determine post-transfer viability in spent culture media assessed (Munoz et al., 2014; Perkel and Madan, 2017). The problem with metabolomic analysis is that there are several factors that may alter an embryo's metabolome which have not been analyzed yet, such as different culture media, stage of embryo, or how the metabolome changes over time. If metabolomic analysis has provided no benefit when used with traditional morphologic assessment in human IVF clinics, there will likely be little benefit for implementing metabolomic analyses into livestock embryo production (Hardarson et al., 2012).

Physical properties of oocytes or embryos may also provide insight into their viability. Assessment of some parameters would be much more feasible and replicable compared to metabolomic analysis. Early work demonstrated that the oocyte diameter is reflective of developmental competence. As ooplasm diameter increases, fertilization rates increase, rates of polyspermy decrease, and blastocyst development rates increase (Otoi et al., 1997). Fair and colleagues further examined the role of size in oocyte development and noted that a greater proportion of oocytes with large diameters reach the metaphase II stage (Fair et al., 1995). Furthermore, oocytes with diameters less than 110 μm display increased levels of active transcription in comparison oocytes larger than 110 μm, indicating smaller oocytes have not fully acquired the necessary transcripts needed for nuclear or cytoplasmic maturation (Farin et al., 1995). Otoi and colleagues also noted that oocytes with diameters greater than 130 μm did not develop to the blastocyst stage (Otoi et al., 1997). In their study, only 7 oocytes were

classified into this category, so a reduced sample size greatly impacted the data. However, it has been noted that when oocytes are injected with cytoplasm to generate a large diameter, there is a reduction in spindle integrity and more error-prone chromosome segregation, which may result in chromosomal aberrations inhibiting proper zygotic genome activation and impairing embryo development (Kyogoku and Kitajima, 2017).

The specific gravity of embryos is a novel physical parameter being estimated for embryo selection and seems to be applicable to a variety of species. This method estimates an embryos weight based off its descent time through an up-right tube containing culture medium and ideally a heavier embryo should have a faster descent time through the medium. Initial studies in murine oocytes showed that this device can determine which zygotes are more likely to develop in a population as well as which embryos are more viable after freezing and thawing (Prien et al., 2015; Wessels et al., 2016). Preliminary studies using the specific gravity device have also shown a correlation between the estimated weight of ovine embryos and pregnancy success, as well as claim that this device can also predict the sex of bovine embryos with 65 - 78% accuracy (Wessels et al., 2015, 2016; Schaubhut et al., 2019). The validity of this research needs to be confirmed with other culture conditions and species. Osmolarity of different embryo media will impact the embryos speed of decent and it is unclear if the same trends in pregnancy success in sheep are directly applicable to other species. Even within species, there are going to be differences from both donors and breeds. The fat content of embryos is largely attributed to maternal fat and body condition, where increased maternal body composition results in zygotes with decreased lipid content. Zygotes with decreased lipid content will travel through the specific gravity device at a faster rate, thus be deemed heavier and more viable (Weathers et al., 2013; Weathers and Prien, 2014; Prien et al., 2015). This would be problematic when looking at a

heterogenous population of zygotes and viable embryos from one donor may be mislabeled as non-viable.

Microfluidic devices have great potential for adaptation for quality embryo or gamete selection. Microfluidics has already been adapted for micro-polymerase chain reaction and glucose sensors (Bienvenue et al., 2010; Pu et al., 2016). The benefit to using microfluidics for these assays is that much less reagent is required, and sometimes reactions can happen much faster. Embryo culture already produces minimal amounts of by-product in the small volumes of media, sometimes making it difficult to analyze. Microfluidics may help overcome this issue by reducing the necessary amount of sample needed or by performing the assay on a more appropriate scale. Microfluidics also makes particle sorting possible with its laminar flow and parallel streamlines not seen at the macrolevel (Beebe et al., 2002). Designs have already achieved particle and cell sorting, even successfully separating red and white blood cells from whole blood for diagnostic purposes (Autebert et al., 2012; Li et al., 2014). Microfluidic chips have also already demonstrated successful selection of quality oocytes and motile sperm. These systems took advantage of a quality oocyte or motile sperm's ability to cross the laminar streamlines and separate from the poor-quality gametes (Li et al., 2016b; Iwasaki et al., 2018). As previously mentioned, cell size may play a role in embryo quality, and a microfluidic device may be feasible for selection of quality embryos.

The progression of embryo selection methods has made large strides in developing non-invasive and objective methods for selection. However, adoption of these technologies into clinical and commercial use are proving difficult, not because of flaws in the tested hypothesis, but due to inconsistency and variability in the technology used to execute these tests. For example, a machine was developed to assign embryos a "Viability Score" based of metabolomic

profiling, but testing was discontinued due to large variability and poor repeatability within instrument diagnostics, along with poor reproducibility between the individual instruments (Sanchez et al., 2017). Advancements in artificial intelligence and machine learning will make it possible to identify good quality embryos through morphologic assessment with greater accuracy and no human bias. In addition, this technology also has potential for developing systems to take into account several parameters and generate an assessment of embryo viability on metabolomic, visual, and morphokinetic parameters (Santos Filho et al., 2012; Khosravi et al., 2019; Wang et al., 2019) Certainly, with time, the available technology will further advance and allow for more affordable, more accurate instruments to use for non-invasive embryo selection. In the meantime, cost-effective methods that are easy to implement are needed to improve the efficiency of embryo transfer programs.

Influence of Dietary Fat on Reproduction

Reproductive efficiency is first and foremost controlled by nutrition, as inadequate body condition or energy status impedes cyclicity and pregnancy establishment. Animals in negative energy balance experience prolonged post-partum anestrus, likely due to decreased LH pulsatility affecting follicular growth and ovulation (Randel, 1990; Schillo, 1992). Beef cows with decreased body condition scores yield reduced pregnancy rates compared to those of adequate body condition (Stevenson et al., 2015). Fat supplements can be used to increase the energy content of cattle diets to prevent issues with the nutritional restrictions on cyclicity. Supplementing fat in dairy cattle consuming low energy diets increases LH secretion from the pituitary and can restore normal cyclicity (Hightshoe et al., 1991; Sklan et al., 1991). Fat supplementation also increases the circulating cholesterol and lipid deposition in the CL, necessary for steroid hormone production (Staples et al., 1998b). Therefore, supplemental fat can

increase progesterone production from the CL and maintain high circulating levels of progesterone by decreasing hormone clearance by the liver (Hawkins et al., 1995).

Important considerations need to be made when supplementing fat to ruminants maintained on primarily forage-based diets such as those in cow-calf production systems. First, rumen microbial populations contain lipolytic enzymes that will alter the structure and functional properties of fatty acids through biohydrogenation (Jenkins, 1993). This will reduce the amount of long-chain fatty acids available for absorption in the small intestine. Second, diets with high fat content (greater than 4%) can impair organic matter digestibility and reduce forage intake (Hess et al., 2008). To avoid negative effects on forage intake, Hess et al. (2008) recommends that inclusion of fat to the diet not exceed 2% of total dry matter intake. Problems associated with biohydrogenation of fat can be circumvented by providing rumen-inert fat supplements instead of raw oils or fatty feedstuffs. Calcium salts of fatty acids or prilled fatty acids have been shown to maintain digestibility and do not affect concentrations of acetate, propionate, or butyrate volatile fatty acids in the rumen when compared to diets without fat supplement (Grummer, 1988; Schauff and Clark, 1989).

Interestingly, there are several reports of improved reproductive function in response to feeding fat supplement that are independent from energy content of the diet (Staples et al., 1998b). Not all fats are created equal, so supplementing more or less of a certain type of fat may evoke different biologic and reproductive responses. The fatty acids found in mammalian systems contain a carboxyl group (with the alpha carbon immediately following) and an even numbered chain of carbon atoms (ending with the omega carbon). A fatty acid that does not contain any double bonds between carbon atoms is a saturated fat, and these tend to be more stable and reduce membrane fluidity (Gibbs, 2015). Unsaturated fatty acids contain double bonds

between the carbon atoms, which generates a kink to the normally straight structure and increases membrane fluidity (Rustan and Drevon, 2005). Unsaturated fatty acids can be either monosaturated (containing one double bond) or polyunsaturated (containing two or more double bonds). The polyunsaturated fatty acids (PUFAs) are further classified by the location of their last double bond. Omega-6 (n-6) fatty acids contain the last double bond at the 6^{th} to last carbon and omega-3 (n-3) fatty acids contain the last double bond at the 3^{rd} from last carbon. The n-6 linoleic acid (LA) and n-3 alpha-linolenic acid (ALA) PUFAs are essential fatty acids and must be taken up through the diet. From there they can be desaturated and elongated to produce the n-6 PUFA arachidonic acid (AA) and the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Conversion cannot occur between n-6 and n-3 PUFAs during desaturation and elongation (Rustan and Drevon, 2005). It has been demonstrated that by altering the types of fatty acids in the diet, we can alter the tissue fatty acid composition in cattle, especially in blood plasma, follicular fluid, endometrial tissue, adipose tissue, and even the conceptus or fetal tissues (Childs et al., 2008a; Zachut et al., 2010a; Cooke et al., 2014; Marques et al., 2017). The changes in tissue composition explain why isocaloric and isolipidic supplements or diets can still induce changes in reproductive performance.

As previously stated, calcium salts of fatty acids or seed oils are way to supplement fat while avoiding biohydrogenation. These are generated through the saponification of calcium ions with fatty acids to generate a substance that is relatively stable above a pH of 6.5, although incorporation of unsaturated fatty acids will reduce some stability of the soap (Sukhija and Palmquist, 1990). The pH of grass-fed ruminants ranges between 6 and 7, thus calcium salts are fairly stable in the pasture based ruminant (Grünberg and Constable, 2009). Calcium salts of

soybean oils (CSSOs) are available as commercial supplements and contain high amounts of PUFA like LA and some ALA.

Polyunsaturated Fatty Acids on Reproduction

Supplementing CSSOs to beef cattle has shown reproductive benefits in both *Bos indicus* and *Bos taurus* breeds. Supplementing CSSO for 21 days beginning at the time of FTAI increases pregnancy rates to FTAI in both sub-species (Lopes et al., 2009a; Lopes et al., 2011a; Brandao et al., 2018). In *Bos indicus* cattle, supplementing PUFAs through CSSOs increases CL volume and circulating progesterone levels early in the CL's lifespan, roughly days 6 and 7, after exposure to FTAI (Lopes et al., 2011a; Cooke et al., 2014; Cipriano et al., 2016b). Increased progesterone levels during luteinization alter endometrial gene expression to help prepare the uterus for supporting conceptus development and elongation (Spencer et al., 2016), so PUFA supplementation may be enhancing the uterine environment in better preparation for pregnancy establishment. Interestingly, progesterone levels were not elevated on day 7 in cattle receiving embryo transfer (Lopes et al., 2011a). The increase in plasma progesterone seen in cows exposed to FTAI but not embryo transfer may suggest that maternal-embryo crosstalk even at this early stage plays an important role in improving CL function of *Bos indicus* cattle supplemented with PUFAs. In contrast, *Bos taurus* cattle do not exhibit changes in plasma progesterone or CL volume on day 7 when supplemented with CSSOs beginning at FTAI (Brandao et al., 2018). Delayed rises in progesterone are linked with subfertility and impaired conceptus growth, so examining progesterone levels during luteinization may provide insight as to why supplementation with CSSOs improves pregnancy rates in *Bos taurus* cattle (Shelton et al., 1990a; Mann and Lamming, 2001; Wathes et al., 2003b).

Maternal PUFA supplementation also benefits conceptus growth. Dairy cattle, who underwent a super-ovulation protocol for *in vivo* embryo production while consuming n-6 PUFA supplement, had more embryos with increased cell numbers recovered from flushing compared to cows receiving a saturated fat supplement (Thangavelu et al., 2007). In the same study, embryos recovered from cows supplemented with n-3 PUFA had increased cell number, but less embryos were recovered. Supplementation with a rumen inert fish oil rich in n-3 fatty acids was able to increase conceptus length in *Bos taurus* beef cattle (Giller et al., 2018). In contrast, maternal supplementation with n-6 rich CSSOs increase conceptus length by day 15 of gestation in *Bos indicus* beef cattle, but not *Bos taurus* (Cipriano et al., 2016b; Brandao et al., 2018). However, in both species receiving CSSOs, conceptuses had increased IFNT transcripts, which was then reflected in the maternal leukocytes where select ISGs were up regulated (Cipriano et al., 2016b; Brandao et al., 2018). It is not clear why supplementation with CSSOs would improve conceptus development in *Bos taurus* cattle without the increased circulating progesterone seen in *Bos indicus*. Neither species exhibited changes in the gene expression of prostaglandin synthases in endometrial tissue or oxytocin and steroidogenic enzymes in luteal tissue, suggesting there may be some beneficial effects of PUFAs acting directly on the embryo or conceptus itself (Cooke et al., 2014; Brandao et al., 2018).

Prostaglandins

The metabolites of the long-chain PUFAs serve as the precursors to eicosanoids and can act as signaling molecules in the body. These metabolites are arguably how PUFA supplementation impacts reproductive function. Arachidonic acid is stored in phospholipid membranes of tissues, and comprises a large portion of phospholipids in endometrial tissue and the conceptus (Childs et al., 2008a; Cooke et al., 2014). Freed AA is processed into

prostaglandins, leukotrienes, and epoxyeicosatrienoic acids by cyclooxygenase, lipoxygenase, or epoxygenase enzymes respectively. The cyclooxygenase enzymes (COX1 and COX2; also known as prostaglandin G/H synthase) converts AA to prostaglandin H_2, which is then further transformed into prostaglandin D_2, I_2, E_2, or $F_{2\alpha}$ through specific prostaglandin synthases. The same process is performed using EPA, but instead produces series 3 prostaglandins. The n-6 AA generates series 2 prostaglandins which are more biologically active than those generated by the n-3 EPA, and promote inflammation and angiogenesis (Rustan and Drevon, 2005). Prostaglandin $F_{2\alpha}$ and PGE_2 influence several aspects of ruminant reproduction, such as ovulation, luteinization, and parturition (Mattos et al., 2000). Prostaglandins are also essential for conceptus elongation and implantation, but IFNT stimulates a transition from $PGF_{2\alpha}$ to PGE_2 production (Brooks et al., 2014).

Some studies have tried to address if the dietary alteration of tissue fatty acid composition can then alter prostaglandin synthesis. Mattos's group has been able to demonstrate that increasing n-3 content of the diet in dairy cattle can reduce endometrial $PGF_{2\alpha}$ production *in vitro* and *in vivo*, providing a potential method for reducing luteolysis prior to maternal recognition of pregnancy (Mattos et al., 2000; Santos et al., 2008a). However, supplementing n-3 has not shown the same responses in beef cattle. Some studies have not demonstrated reduced levels of the $PGF_{2\alpha}$ metabolite after n-3 supplementation in animals with normal progesterone levels (Wamsley et al., 2005; Childs et al., 2008b). In contrast, supplementing n-6 has shown to increase $PGF_{2\alpha}$ in dairy cattle, but does not always change $PGF_{2\alpha}$ metabolite or prostaglandin synthase transcripts in beef cattle with adequate luteal progesterone levels (Robinson et al., 2002; Petit et al., 2004; Cooke et al., 2014; Cipriano et al., 2016b; Brandao et al., 2018). The dairy cow has a much greater energy demand than the beef cow, thus she may be much more sensitive to

alterations in dietary fat. Dairy cattle also deposit more fat in the body cavity than beef cattle and exhibit altered lipid utilization, thus it may be harder to change tissue fatty acid composition enough to elicit a physiologic response in beef cattle (Pfuhl et al., 2007).

Fatty Acid Receptors

Fatty acids further mediate reproduction by binding to receptors and eliciting gene responses. Peroxisome proliferator-activated receptors (PPARs) are ligand induced transcription factors that activate transcription of genes associated with lipid and cholesterol metabolism, cell differentiation, and insulin sensitivity (Vitti et al., 2016). These receptors are relatively non-specific; thus, they can bind to and be activated by a variety of ligands, especially long-chain PUFAs or eicosanoids. Upon binding to a ligand, the receptor forms a dimer with retinoid X receptors (RXR) to form a complex capable of binding to the PPAR-responsive element, typically found in the promoters of those genes regulating lipid metabolism, insulin sensitivity, and cell proliferation (Vitti et al., 2016). The three main isoforms (PPARA, PPARD, PPARG) are present in a variety of reproductive tissues in both bovine and non-ruminant species including follicles and CL on the ovary, uterus, placenta, and testes (Froment et al., 2006a; Bionaz et al., 2013; Socha et al., 2017). The isoform PPARG is perhaps the most well studied in relation to bovine reproductive function. It plays a key role in conceptus elongation, as it is highly upregulated as the conceptus grows from ovoid to filamentous, and inhibition of PPARG can inhibit elongation in sheep (Brooks et al., 2015b; Ribeiro et al., 2016a). It is also highly expressed in granulosa cells of antral follicles and the CL, with levels of *PPARG* expression decreasing after ovulation and luteolysis (Froment et al., 2006a).

Fatty acids may also bind to free fatty acid receptors (FFARs) to elicit cellular responses primarily associated with maintaining energy homeostasis. These receptors are G-protein

coupled receptors, and can specifically bind to short, medium, or long-chain fatty acids (Miyamoto et al., 2016). The two receptors GPR40/FFAR1 and GPR120/FFAR4 bind to long-chain fatty acids, especially EPA or DHA and ALA, respectively, and both stimulate intracellular calcium release while GPR40/FFAR1 also stimulates cAMP (Miyamoto et al., 2016). The GPR120 receptor is also likely how n-3 fatty acids mediate anti-inflammatory responses by stimulating the production of PGE_2 (Oh et al., 2010; Liu et al., 2014). The role of these FFARs on modulating reproduction is not well known, but these receptors are present in bovine endometrial cells and in human placenta. Culture of bovine endometrial cells with PUFAs can stimulate intracellular calcium release (Lager et al., 2014; Valenzuela et al., 2019b). Free fatty acid receptors may play influence the mechanisms by which PUFA supplementation can enhance reproduction, but more research is needed.

The largest confounding issue with studying the nutritional impacts of reproduction is the animals' diet, which makes it difficult to discern the true impacts of various feedstuffs. As mentioned, biohydrogenation of fats already poses an issue with supplementing, but rumen-inert sources can by-pass this. Animals maintained on pasture are likely consuming different basal diets depending on the forages present in the available pasture. Forages are typically high in ALA, but chicory and white clover, for example, have greater levels of LA and lower levels of ALA than tall fescue, and all fatty acid content in forages decreases with increasing number of harvests (Clapham et al., 2005). To further complicate things, the type of feedstuff intended to supplement PUFAs can vary greatly in PUFA content (Rahbar et al., 2014). Flaxseed, for example, contains high levels of ALA, while fishmeal contains little ALA and more of the elongated n-3 fatty acids EPA and DHA. Soybean oil contains less LA and more ALA than sunflower or safflower oils. To truly understand the mechanisms by which PUFAs impact bovine

reproduction, utilizing similar feedstuffs to previous literature is important. Research has also placed a large emphasis on examining how PUFAs impact reproduction through prostaglandin synthesis, but as described here, there are other mechanism by which supplemental fat may be altering bovine reproduction.

SUMMARY AND IMPLICATIONS

Reproduction in cattle is a complex and intricate process of individual, yet also connected events. The cyclicity of a cow involves an intricate play of hormones signaling for events of follicular growth, ovulation, and luteal function. We now understand the basics of this process well enough to manipulate the estrous cycle to improve the ease of AI, which helps improve the efficiency of beef cattle systems. However, there is little adoption of this technology in current cow-calf systems. Reproductive management becomes more complex when we analyze external factors impacting reproductive efficiency such as the role that nutrition plays on an individual animal. General energy status is only the tip of the nutritional iceberg, and underneath lies complex interactions of different feed supplements on reproductive efficiency. While we have made some developments in understanding this, much is still unknown.

The development from oocyte to embryo alone is another complex journey, involving phases of maturation, fertilization, and early embryonic development and we have successfully manipulated these processes to be performed *in vitro* without any sort of influence from maternal cells. However, there has been little improvement in the developmental potential of IVP embryos which has pushed for better methods of selecting the best quality embryo. Unfortunately, the discoveries and technologies developed for this have not had successful application to the industry due to technological variation or difficulty in implementation. Improving reproductive

efficiency is not a problem that can be solved with a silver bullet; thus, we must take a more comprehensive approach.

This dissertation will examine how reproductive efficiency can be improved through the entire system: herd management, an individual cow basis, and in the *in vitro* production system. Fixed time artificial insemination is a unique tool that can improve the reproductive efficiency of beef cows, but its adoption into production systems is limited. The first study examines the question if decreasing the bull:cow ratio in systems using FTAI and natural service influences pregnancy rates. We hypothesize this is not the case, thus reducing bull related costs by decreasing bull:cow ratio can make adoption of FTAI more economically feasible. Once the herd's reproductive efficiency is improved, we can focus on the individual cow. As discussed previously, addition of PUFAs to cattle diets can benefit reproduction. The second experiment aims to address how adding rumen-inert PUFAs improves reproduction using the non-pregnant, cyclic cow as a model. Finally, once herd and individual animal reproductive efficiency is improved, *in vitro* embryo production becomes most feasible. The last study examines a non-invasive, objective method for selection of the most viable embryos using physical size parameters of zygotes. The technique examined here may provide embryologists with an easier to implement, objective selection method than current techniques available or may be used to further develop microfluidic systems that can sort quality embryos by size.

CHAPTER 3

A retrospective analysis of bull:cow ratio effects on pregnancy rates of beef cows previously enrolled in fixed-time artificial insemination protocols

INTRODUCTION

The use of estrous synchronization and FTAI can provide cattlemen with several benefits, including diversified genetics and economic benefits through shortened calving seasons, increased calf uniformity, and production of more pounds of calf per cow exposed to FTAI due to earlier calving (Odde, 1990; Holm et al., 2008; Rodgers et al., 2012). Unfortunately, adoption of FTAI protocols in the United States beef industry is poor. As of 2009, only about 7% of producers surveyed reported using estrous synchronization or artificial insemination in their operations (USDA, 2009). Though this number has likely increased over the past 10 years, the prevalence of FTAI in the beef industry is still extremely low, especially when compared to the dairy industry which reports 89.3% of all operations using some method of estrous synchronization and artificial insemination (USDA, 2018).

What prevents the adoption of these reproductive technologies? For most producers, labor and cost considerations are largest hinderances to adoption of management changes (USDA, 2009). Traditionally, costs related to labor implementation of estrous synchronization protocols come at a tradeoff: protocols utilizing few synchronization drugs require added labor with estrus detection, while more intensive protocols that do not require estrus detection have a

greater cost input for synchronization drugs. Improving economic feasibility is likely the easiest method to improve adoption of reproductive technologies in beef cattle production. Utilization of FTAI generally results in a gain of $49 per cow exposed (Rodgers et al., 2012). Altering bull-related costs, primarily through altering the bull:cow ratio, can increase the economic returns in a FTAI program (Rodgers et al., 2012). However, it is not known to what extend this ratio can be altered when animals are estrous synchronized and artificially inseminated. The current recommended bull:cow ratio is 20 to 30 cows in pasture for every one bull (Chenoweth, 2015; King, 2015). Another standard of recommendation is based upon the Auburn formula, recommending the number of cows serviced be proportional to bull age for those less than 36 months, or scrotal circumference of bulls greater than 36 months in a single sire setting (Wenzel et al., 2012). However, cattlemen tend to air on the lower end of these recommendations, with the average number of beef cows exposed to yearling bulls at 16.3 and for mature bulls 23.7, regardless of the use of synchronization and FTAI (USDA, 2009). The costs associated with working cattle and synchronization drugs are out of cattlemen's control. The most feasible way to improve the economic burden of implementing FTAI protocols will be to reduce costs associated with owning and using bulls.

The recommended 1:25 bull:cow ratio may not even be reaching the bulls full breeding potential, according to Rupp and colleagues (Rupp et al., 1977). They reported no changes in pregnancy or estrus rates when cows were on pasture with bulls in ratios of 1:25, 1:44, or 1:60 bulls per cow. However, this work was done in a purely natural service setting, and recommendations need to be adjusted when females are exposed to estrous synchronization and FTAI. Current recommendations for bull:cow ratios after synchronization are adjusted to 1:25 bulls per cow (Healy et al., 1993; Chenoweth, 2015). However, the data provided by Healy is

based only on estrous synchronization of heifers immediately exposed to natural service. To our knowledge, there is no literature examining how the bull:cow ratios influence pregnancy rates after cows have been synchronized and artificially inseminated using an FTAI protocol. In theory, the bull:cow ratio can be reduced by half in order to double the number of cows serviced by a single bull, as nearly half of the cows should become pregnant to FTAI. Cows that remain non-pregnant will be returning to estrus in a synchronized manner, with the majority returning 20 to 23 days post FTAI (Larson et al., 2009), and put added pressure on the bull to breed many females in a much shorter amount of time. This retrospective analysis aimed at assessing how the number of cows exposed per single bull influences pregnancy rates after cows have been enrolled in an estrous synchronization and FTAI protocols and questions the necessity of utilizing bulls in cow-calf production systems.

MATERIALS AND METHODS

Animals

Commercial beef cattle (primarily Angus × Simmental crosses) from the Virginia Department of Corrections (VDOC) across 17 locations were enrolled in this study. Multiparous cows and heifers (average age per group = 5.11 ± 0.14 years; n = 14,868) were exposed to an estrous synchronization and FTAI protocol, typically the 7-day CO-synch + CIDR. Since these herds are utilized for experimental purposes, only data from breeding seasons that utilized frozen semen and synchronization protocols with progestin releasing intravaginal devices were included in the analysis. Fall breeding to FTAI occurs in mid to late December, while FTAI in the spring season occurs in early May. After FTAI, groups of cattle (47 ± 1 head/group) were maintained on pasture with bulls to breed any females not pregnant from FTAI. Mature, commercial bulls were diagnosed as fertile from breeding soundness exams prior to initiation of the breeding

season. Bulls were maintained in pastures with cows for an approximate 70-day breeding season. Pregnancy diagnosis was performed by transrectal ultrasonography at least twice in each location, starting on 45 to 55 days post FTAI and again 35 to 55 days after bulls were removed.

Data

Data from individual cows in VDOC herds were available from fall and spring breeding seasons from 2010 to 2017, for a total of 13 breeding seasons. Data included cow ID, age, body condition score (BCS) at start of synchronization, semen sire, inseminator, natural service sire(s), days pregnant at pregnancy diagnosis (1 to 3 pregnancy diagnosis dates per location) and previous calving data. Each animal was classified as bred by FTAI; bred by natural service following first return to estrus; or bred in late season (following second return to estrus or later) based on fetal age at pregnancy diagnosis via transrectal ultrasonography. Animals with incomplete data on natural service sire, fetal age at pregnancy checks, or having been removed from a group during the breeding season, were excluded from analysis.

The unit of analysis was group of animals (n = 392), as classified by VDOC records or by natural service sire mating. Groups were composed of heifers or mature cows based on provided age and records on previous calving information. Mature cows were defined as being 2 years of age and older and/or had calving data from earlier in the year. Each group was also categorized by sire setting: either single sire for groups exposed to one bull, or multiple sires for groups exposed to 2 or more bulls at once. For each group, the total number of cows bred following FTAI, first estrus natural service, or late season natural service were calculated. The total number of cows exposed per bull was calculated by dividing the total number of cows in a group by the number of bulls to whom cows were exposed. The number of open cows per bull was calculated

by dividing the number of cows classified as pregnant following first return to estrus or later by the number of bulls to whom cows were exposed on pasture.

Statistical Analysis

Statistics were analyzed in R version 3.3.1. Linear regression was performed to analyze the relationship between breeding success and total cow to bull ratio or open cow to bull ratio (R Core Team, 2016). Models also included interactions between the total number of cows or number of open cows exposed per bull and either sire setting, season, BCS, or age (heifer or mature cow). Student's t-test (or Welch's t-test when appropriate for unequal variances) was performed to assess differences in pregnancy rates between seasons and BCS between seasons. All percentage data were given an arcsine transformation. Significance was set at $P < 0.05$, and tendencies at $0.05 < P < 0.1$.

RESULTS AND DISCUSSION

The bull:cow ratios are listed in Table 3-1. As was expected with the 50% pregnancy rate to FTAI, the number of open animals exposed to bulls during their first return to estrus was nearly half that of the total number of animals exposed to bulls. The ratio of total number of cows exposed per bull followed the typical producer recommendation of 20 to 30 cows per bull. However, with an average of half the herd becoming pregnant to FTAI, the actual number of animals needing to be serviced by a bull is reduced to about 15. Previous work done by Healy and colleagues identified the ideal bull:cow ratio for estrous synchronized heifers to be 1:25 (Healy et al., 1993), based off economic evaluation and pregnancy rates of varying bull:cow ratios. While the economic analysis is outdated, their recommendation for bull:cow ratios after synchronization still exceeds our observed number of open animals exposed. Based on our

results, it is possible to recommend that cattlemen, using FTAI in combination with natural service, decrease bull:cow ratios to at least 1 bull per every 50 cows exposed to FTAI, for a resulting ratio of 1 bull per 25 open cows.

Interactions of total ratio with BCS, use of multiple or single sire, and age were not significant ($P > 0.34$). We observed a significant interaction between total number of cows exposed per bull and season ($P = 0.03$). There was also tendency for an interaction between the number of open cows and season ($P = 0.08$). Thus, we decided to analyze the data from spring and fall breeding seasons separately. In spring breeding seasons, there were no effects of BCS, age, or sire setting nor interactions with the total number of cows or number of open cows exposed on first return to estrus pregnancy rates ($P \geq 0.27$). In fall seasons, there was no effect of BCS, age, or sire setting and the interactions with total number of cows on pregnancy rates for first return to estrus ($P \geq 0.15$). We observed a tendency for effect of sire setting ($P = 0.057$) and the interaction between sire setting and number of open cows exposed per bull ($P = 0.086$) in fall data (Figure 3-2). When the fall data was separated by single or multiple sire herds, there were no effects of BCS or age ($P \geq 0.51$).

There was a significant ($P < 0.01$) negative correlation between the number of total cows exposed per bull and pregnancy rates to first return to estrus for fall breeding seasons, but not in spring ($P = 0.90$); however, the regression for fall data yielded an r^2 of 0.04 representing a poor relationship (Figure 3-1). We observed a significant ($P < 0.001$) negative correlation between the number of open cows exposed per bull and pregnancy rates to first return to estrus in herds using a single sire in the fall seasons. Again, it was a poor relationship with an r^2 of 0.11 (Figure 3-4). However, the number of open cows exposed per bull was not correlated in the spring breeding season nor in fall breeding season groups using multiple sires ($P \geq 0.12$) Although decreasing

the bull:cow ratio negatively impacted pregnancy success in the fall breeding seasons and in fall groups using a single sire, the bull:cow ratio only represented between 5 and 11 % of the variation in the data. Given the use of healthy bulls of proven fertility, this indicates that the bull is not the limiting factor in the success of a FTAI and natural service program. Thus, increasing the number of females serviced by a single sire will likely not have an impact on pregnancy rates. As previously stated, decreasing the bull:cow ratio to 1:50 or 1:60 would achieve a bull to open cow ratio of 1:25 or 1:30, following recommendations (Healy et al., 1993; Chenoweth, 2015; King, 2015). Provided the bull is proven fertile, decreasing the bull:cow ratio will likely have more economic benefit by reducing the bull associated costs than would be detrimental from any potential decreases in pregnancy rates to first service.

Reduced bull:cow ratios, however, comes with added risk and may not be applicable to all situations. In order to ensure productivity, increased emphasis should be placed on the reproductive capability of individual bulls, as well as practicality of implementing this research. Bull servicing capacity and general health are crucial to account for if reducing the number of natural service bulls used on operations. While utilizing bulls with greater libido does not necessarily correspond with increased pregnancy rates, there is an increase in the number of animals serviced, which may be beneficial if bull:cow ratios are decreased (Chenoweth, 1997). Scrotal circumference is a moderately heritable trait and positively correlated with semen output and semen quality (Brito, 2015). Continual selection of bulls with sufficient scrotal circumference can ensure breeding success after decreasing bull:cow ratios. Yearly breeding soundness exams for producers using only one bull are highly recommended for maintaining good pregnancy rates, especially if choosing increase the number of females exposed. While all bulls used underwent a breeding soundness exam to be diagnosed as fertile, there may have been

a larger variation in bull libido and fertility in the fall, explaining our observed significant negative correlation in fall single sire groups, but no influence of single or multiple sire settings in the spring. Unfortunately, we did not have records on individual bull performance to test this hypothesis.

Pregnancy rates to FTAI averaged 54.9 ± 0.7 % overall, but ranged from 14.3% to 83.3% by group. There was a significant effect of season on pregnancy rates, with fall breeding seasons having increased pregnancy rates (92.4 ± 0.5% vs. 89.8 ± 0.6 %; $P < 0.001$). Pregnancy rates to FTAI and pregnancy rates following first return to estrus did not differ by season ($P \geq 0.61$). We did observe greater pregnancy rates following second return to estrus or later in fall breeding seasons compared to spring (64.9 ± 1.6% vs. 57.8 ± 1.4%; $P < 0.01$). Although we observed a significant negative correlation of the number of open cows exposed per bull, there was a tendency for overall pregnancy rates in the fall season to be slightly greater in single sire groups, further demonstrating that the bull:cow ratio is not the limiting factor to pregnancy success (single sire: 92.9 ± 0.77% vs multiple sire: 91.7 ± 0.69%; $P = 0.054$).

Profitability of fall or spring calving systems varies greatly and has a large geographic influence. A study observing calving season impacts on a national scale noted that fall calving seasons are more beneficial to Southern regions of the U.S., spring calving is best for far North regions, and in the upper southern region, which includes Virginia, there is no benefit to either (Bradford et al., 2016). In contrast, a study focusing on much more local and extensive economic impacts in Tennessee noted fall calving seasons are more profitable (Henry et al., 2016). This second study considered other economic factors like feed cost, while Bradford et al. examined only weaning weight and yearling weights. However, neither study examined if there are differences in fertility of cows calving in these seasons. When focus is shifted to the maternal

side of a cow-calf operation, fall-calving systems produce more fertile cows as demonstrated by increased calving rates, decreased calving intervals, and increased pregnancy rates (King and Macleod, 1984; Caldwell et al., 2013; Campbell et al., 2013). In agreement with our study, we also observed increased pregnancy rates to fall-calving systems.

Seasonal heat stress can affect female and male fertility, and it may be playing a role in the observed seasonal differences in pregnancy rates, especially since the decreased pregnancy rates are observed later in the breeding season (Collier et al., 2017; Wolfenson and Roth, 2018). Removal of bulls from pastures with cows for fall breeding typically occurs between March and April, while bulls are removed from cows during late July and August for spring breeding. Condition of animals entering the breeding season is more likely explains our observed increase in fall pregnancy rates. Body condition score is an important factor influencing conception rates, and cows with a BCS greater than 5, assessed preceding synchronization, achieve greater pregnancy rates to FTAI than cows with a score less than or equal to 5 (Stevenson et al., 2015). In the current study, fall cows had a significantly greater BCS prior to synchronization compared to cows in the spring breeding groups (5.85 ± 0.04 vs. 5.19 ± 0.05; $P < 0.01$). Caldwell et al. also noted, in agreement with our results, increased body condition scores of fall calving animals (Caldwell et al., 2013). Body condition score at parturition also influences pregnancy rates in the succeeding season (Spitzer et al., 1995). Fall breeding cows in this study typical calve around October and likely have improved BCS at parturition compared to spring breeding cows which calve in February.

Decreasing the bull:cow ratio may not be feasible for every producer. With roughly 80% of beef operations managing less than 50 head (USDA, 2019), producers likely only use one bull to begin with. In this scenario, beef operations may benefit from using only FTAI eliminating

natural service entirely. Others may not be as willing or able to entirely eliminate the bull. Nonetheless, implementing FTAI and decreasing the bull:cow ratio reduces breeding costs per cow (Rodgers et al., 2012), and may provide an opportunity for cattlemen to purchase a bull of proven genetics and increased price. Interestingly, we observed that pregnancy rates to FTAI were greater than pregnancy rates to natural service on first return to estrus (54.9 ± 0.6 % vs. 51.3 ± 0.9%). Our results indicate the sole use of a FTAI system with multiple inseminations may be more economically feasible than maintaining a bull for smaller beef cow-calf operations. In the U.S., 54% of beef cattle operations own less than 20 head (USDA, 2019). Assuming FTAI costs at $33.19 (Rodgers et al., 2012), 50% pregnancy rates, and a herd of 20 head undergoing 4 rounds of FTAI, cost of breeding alone would reach $1,226.92; nearly half of a bull's purchase value (Rodgers et al., 2012).

Pessoa and colleagues examined reproductive efficiency of beef cattle having undergone resynchronization protocols followed by a second round of FTAI and noted increased pregnancy rates by day 60 of the breeding season compared to animals exposed to one round of FTAI and then natural service (Pessoa et al., 2018). Crepaldi and colleagues took this one step further and compared 3 consecutive rounds of FTAI, 2 rounds of FTAI plus natural service, and 1 round of FTAI plus natural service. They achieved overall pregnancy rates of 87% in a 64 day breeding season, and observed similar pregnancy rates between the 3 rounds of FTAI and 2 rounds plus natural service, both of which yielded greater pregnancy rates than 1 round of FTAI plus natural service (Crepaldi et al., 2017). These studies demonstrate that it may be possible to entirely eliminate natural service sires from beef cattle production systems. Furthermore, even with only 2 rounds of FTAI, there is a significant increase in the number pregnancies occurring earlier in the breeding season and the breeding season can be shortened dramatically, helping to contribute

to the economic benefits of FTAI (Holm et al., 2008; Rodgers et al., 2012). More studies need to be conducted to evaluate the feasibility of these in U.S. production systems, especially since estradiol, used in both studies, is not approved for use in estrous synchronization of cattle. The downside of resynchronization followed by FTAI is that it requires pregnancy diagnosis which will incur more costs, in addition to more synchronization drugs. A more in-depth economic analysis is needed to determine if the economic gains can outweigh the costs; however, for small herds, eliminating the bull entirely may be profitable.

This retrospective study aimed at determining if the number of cows exposed per bull affects pregnancy success after estrous synchronization and FTAI in beef cattle. While decreasing the bull:cow ratio had a negative correlation with pregnancy rates, only about 1-5% of the variation can be attributed to the bull:cow ratio. As seen in our data, bull:cow ratios remained similar to the 1:30 recommendation, yet after FTAI, the number of open cows that need servicing is reduced by half. Therefore, we recommend that a bull:cow ratio of at least 1:50 be used when implementing estrous synchronization and FTAI in combination with natural service. Decreasing the bull:cow ratio may help alleviate the economic burden with estrous synchronization and FTAI and encourage adoption of FTAI protocols by cattlemen.

Table 3-1. Bull to cow ratios for combined data, fall and spring data, and single and multiple sires setting data for combined and seasonal data.

	Mean	Min	Max
Fall Breeding Seasons			
Total cows exposed	**1:33**	**1:10**	**1:73**
Single sire	1:35	1:10	1:61
Multiple sire	1:31	1:14	1:73
Open cows exposed	**1:16**	**1:2**	**1:40**
Single sire	1:16	1:2	1:40
Multiple sire	1:15	1:6	1:39
Spring Breeding Seasons			
Total cows exposed	**1:30**	**1:10**	**1:62**
Single sire	1:36	1:14	1:61
Multiple sire	1:25	1:10	1:62
Open cows exposed	**1:14**	**1:4**	**1:44**
Single sire	1:16	1:5	1:44
Multiple sire	1:12	1:4	1:36
Combined Seasons			
Total cows exposed	**1:31**	**1:9**	**1:73**
Single sire	1:34	1:9	1:61
Multiple sire	1:28	1:9	1:73
Open cows exposed	**1:14**	**1:2**	**1:44**
Single sire	1:15	1:2	1:44
Multiple sire	1:13	1:3	1:39

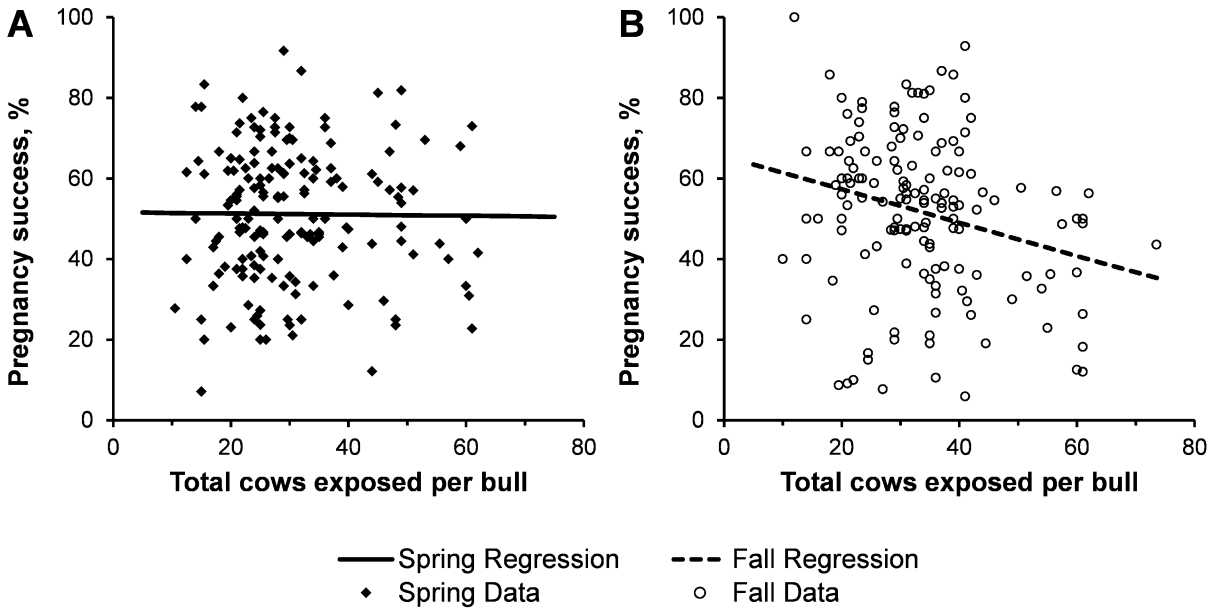

Figure 3-1. Pregnancy success on first return to estrus following fixed-time artificial insemination compared to the total number of cows exposed per bull for spring (A) and fall (B) seasons. There was no effect of ratio in spring ($P = 0.90$; $r2 = -0.01$; $\arcsin(\sqrt{y}) = -0.0001x + 0.80$), but there was a negative correlation in fall ($P < 0.01$; $r2 = 0.04$; $\arcsin(\sqrt{y}) = -0.0042x + 0.94$).

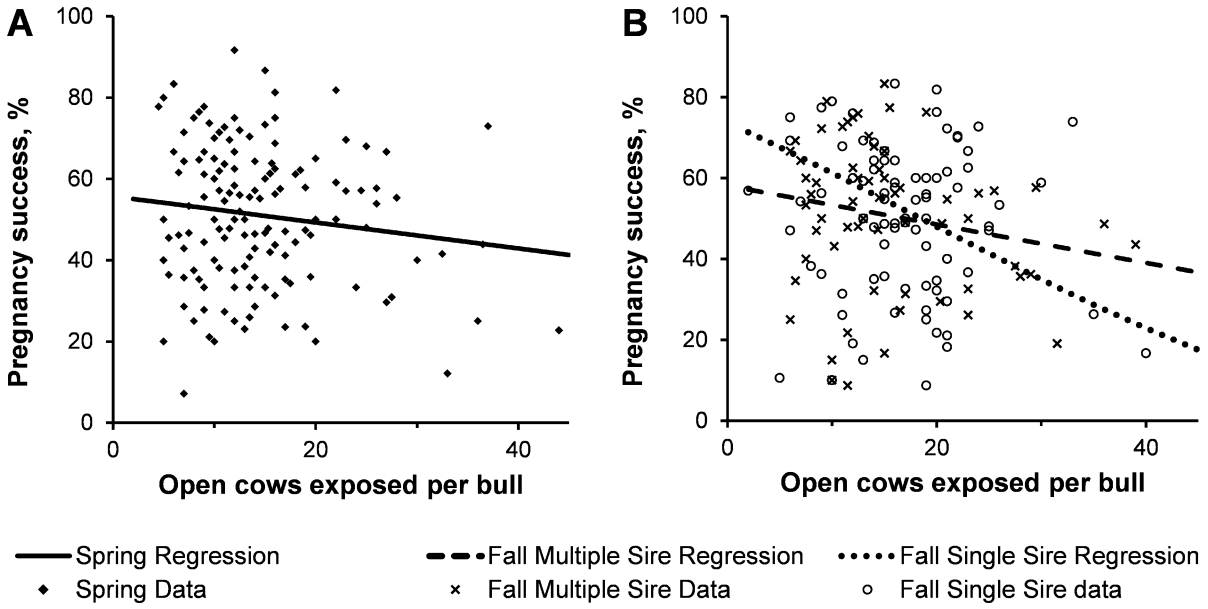

Figure 3-2. Pregnancy success on first return to estrus following fixed-time artificial insemination compared to the number of open cows exposed per bull for spring (A) and fall (B) seasons. There was no effect of ratio in spring (P = 0.10; r2 = 0.01; arcsine(√(y)) = -0.0001x + 0.80). There was a tendency for effect of sire (P = 0.058) and interaction of sire and ratio in the fall (P = 0.086). When separated by sire setting, there was a negative correlation in herds with a single sire (P < 0.001; r2 = 0.12; arcsine(√(y)) = -0.0133x + 1.03) and no effect of ratio in herds with multiple sires (P = 0.12; r2 = 0.02; arcsine(√(y)) = -0.0048x + 0.87).

CHAPTER 4

Influences of Calcium Salts of Soybean Oil on Reproductive Parameters in Cyclic Beef Cows

INTRODUCTION

Supplementing fat into ruminant diets is an efficient way to boost energy content, but the effects of different types of fat is proving to be more complex than simply increasing energy content (Staples et al., 1998a). Polyunsaturated fatty acids of both n-3 and n-6 series are able to alter reproductive performance of dairy cattle, but less research has focused on supplemental PUFA to beef cattle (Santos et al., 2008b; Moallem, 2018). It has been previously demonstrated that daily supplementation with n-6 rich CSSO beginning at the time of artificial insemination for 21 days can improve pregnancy rates in both *Bos indicus* and *Bos taurus* beef cows (Lopes et al., 2009b; Lopes et al., 2011b; Brandao et al., 2018). In *Bos indicus* cows, the increased pregnancy rates can be attributed to increased progesterone, incorporation of n-6 fatty acids into maternal and conceptus tissues as well as improved interferon-tau production by a larger conceptus 15 days post-insemination (Cooke et al., 2014; Cipriano et al., 2016a). In *Bos taurus* cows, by day 15 post-insemination there are no differences in plasma progesterone, conceptus length or endometrial gene expression, but there is increased interferon-tau transcripts in the conceptus (Brandao et al., 2018).

Polyunsaturated fatty acids and their metabolites may also mediate reproduction by functioning as ligands to PPARs. The three major PPAR isoforms (PPARG, PPARA, PPARD)

primarily act as transcription factors to regulate genes associated with lipid metabolism, cell differentiation, and insulin sensitivity (Vitti et al., 2016). All three isoforms are present in various reproductive tissues in cattle, such as the ovary, placenta, and endometrium (Froment et al., 2006b; Bionaz et al., 2013; Socha et al., 2017). The PPARG isoform is also upregulated during conceptus elongation in cows (Ribeiro et al., 2016b). However, there is little research determining the role PPARs play in the bovine endometrium, especially in response to fatty acid supplementation.

It is unclear how supplementation with rumen-inert CSSO during early pregnancy enhances reproductive function in *Bos taurus* cows. What is more uncertain is if there are changes in the maternal environment supporting improved conceptus development, or if these supplements are acting directly on the conceptus itself. This study aimed to evaluate how CSSO supplementation changes the maternal environment to improve reproductive function, with the hypothesis that CSSO supplementation will change reproductive parameters in favor of supporting pregnancy establishment. We evaluated how ovarian dynamics, plasma progesterone concentration, uterine gene expression, and tissue fatty acid concentrations change between non-pregnant, cyclic cows receiving supplemental saturated fat or CSSO.

MATERIALS AND METHODS

All experimental protocols involving cattle were reviewed and approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University, protocol #17-120.

Animals and Treatments

Twenty-five multiparous commercial beef cows were stratified by body weight (658.05 ± 16.64 kg) and body condition score (7.52 ± 0.18) and randomly assigned to one of two treatment supplements: 1) 0.7 kg of corn gluten feed + 0.1 kg of prilled saturated fats (Energy Booster, Milk Specialties, Eden Prairie, MN ; CON, n = 12), or 2) 0.7 kg of corn gluten feed + 0.1 kg of CSSO (Essentiom, Church and Dwight Co., Inc., Princeton, NJ; TRT, n = 13). Cows were maintained as one group on the same pasture and given ad libitum access to forage and water. Treatments supplements were provided daily in feed pans to individual cows separated into holding pens from d-21 to d28, either in the afternoon or immediately after tissue sample collection. One sample of each supplement (combined corn gluten feed and commercial supplement) was conserved frozen at -20°C until thawed and dried in a forced-air oven (55°C, 72 h) to determine DM content. Once dried, samples were ground in a hammer-miller (Willey Mill) to pass a 1mm sieve and sent to Dairy One Forage Laboratory (Ithaca, NY) for chemical analysis. Nutritional composition/characterization of both supplements is listed in Table 4-1. Supplements were formulated to be isonitrogenous, isocaloric, and isolipidic.

Estrous synchronization, superovulation and follicle aspiration

Animals were allowed two weeks of acclimation to supplements, after which cows underwent a super ovulation protocol (d-5 to d0) for collection of follicular fluid as described below. Immediately after follicular aspirations (d0), cows were enrolled in a 7-day CO-synch + CIDR estrus synchronization protocol. Briefly, an injection of GnRH (100 µg, Factrel, Zoetis, Florham Park, NJ) was administered on d0 after aspirations and an intravaginal progestin releasing device (CIDR, Zoetis) was inserted. On d7, CIDR was removed and an injection of prostaglandin (25 mg, Lutalyse, Zoetis) was administered. Three days later (d10) an injection of GnRH was administered and cows were left to cycle without insemination. Diameter of the

dominant follicle was assessed on d7 and CL volume was assessed on d14 via transrectal ultrasonography. Uterine biopsies were collected on days 18 and 28.

Animals underwent a super-stimulation protocol and follicle aspirations to collect follicular fluid for fatty acid analysis. Cows received epidural anesthesia prior to each follicle ablation or aspiration with lidocaine 2% HCl. On d-7, the two largest follicles were ablated using a 17" double lumen follicle aspiration needle guided with a transvaginal ultrasound probe in a commercial holder with needle guide. Immediately after ablations, a CIDR was inserted. The super-stimulation protocol began on d-5. Cows received a total of 400 mg (20 mL) of FSH (Folltropin-V, Vetoquinol, Lavaltrie, Québec, Canada), dispersed into two daily intramuscular injections, at approximately 8 am and 5 pm, in decreasing dosage over four days (4mL, 3 mL, 2 mL, 1mL; 20 mg/mL). Two injections of prostaglandin (25 mg) were given with the last two injections of FSH. On d0, follicle aspirations were performed and afterwards an injection of GnRH (100 μg, Factrel, Zoetis) was administered to ovulate remaining follicles. Follicular fluid from all visible follicles was collected with the use of an ultrasound guided needle using negative pressure generated from a 12 mL syringe connected directly to the needle. Fluid collected from all follicles were pooled, generating one sample per animal. Needles were thoroughly flushed with disinfectant and saline between each animal. Recovered follicular fluid was stored in 15 mL conical tubes on ice until returning to the lab, where it was centrifuged at 1500 × *g* for 15 minutes to remove cellular debris. The supernatant was isolated and stored at -20°C until fatty acid analysis. Samples containing excess blood were excluded from analysis.

Plasma Collection

Blood samples were obtained via jugular or coccygeal venipuncture on days 0, 7,10,11, 12, 13, 14, 15, 16, 17, 18, 25, and 28. Blood was collected into a 10 mL Vacutainer tube with

sodium heparin (Becton Dickinson, Franklin Lakes, NJ), centrifuged at 1,500 × *g* for 15 minutes, and plasma was siphoned off the top and stored at -20°C until analysis.

Uterine Biopsy

Uterine biopsies were collected on days 18 and 28 of the experiment, corresponding to days 8 and 18 of the estrous cycle, for RNA extraction and gene quantification. Epidural anesthesia (Lidocaine 2% HCl) was administered to each cow prior to biopsy. Biopsy samples were taken from the anterior-most portion of both ipsilateral and contralateral uterine horns to the CL using uterine biopsy punch forceps. Biopsy samples were immediately snap frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Plasma Progesterone Analysis

Plasma progesterone was analyzed using a chemiluminescent enzyme immunoassay (Immulite 2000 XPi Platform, Siemens Medical Solutions, Inc USA). Samples were analyzed as singletons in a single assay with an intra-assay CV of 5.98%.

Fatty Acid Extraction and Quantification

Lipids were extracted from blood plasma and follicular fluid using the method of Hara and Radin with modifications described by Corl et al. (Hara and Radin, 1978; Corl et al., 2001). Nonadecanoic acid (19:0) free fatty acid was used as internal standard (Nu-Chek Prep, Inc., Elysian, MN), and 10 µL was added to 1 mL plasma or follicular fluid. Lipids were extracted from 1 mL of sample with 3 mL of 3:2 (v/v) hexane-isopropanol solution, followed by 2 mL sodium sulfate (66.7 g/L). The upper organic phase was transferred to a test tube containing 0.5 g anhydrous sodium sulfate, while the lower phase was re-extracted with 3 mL of 7:2 (v/v) hexane-isopropanol solution. Upper phase was added to the tube with anhydrous sodium sulfate and left

to dry for 30 min. The solution was evaporated under N_2 gas, and transmethylation was performed using 1% methanolic-sulfuric acid as previously described (Perfield et al., 2006). Fatty acid methyl esters were then analyzed via gas chromatography using a DB-225 column (100 m × 0.25 mm ID and 0.2 μm film; Aglient, Santa Clara, CA).

RNA Extraction and RT-qPCR

The RNA from uterine biopsies was extracted using chloroform-phenol extraction and purified using the PureLink RNA Mini Kit (Thermo Fisher Scientific Inc., Waltham, MA). RNA quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.) measuring absorbance at 260 and 280 nm. All samples contained a 260:280 ratio greater than 1.8. Four ng of RNA was incubated in RNAase-free DNAse I (Applied Biosystems, Inc., Foster City, CA) and then reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.). Complimentary DNA was amplified with primers for the genes *PPARA*, *PPARD*, *PPARG*, *PTGES*, and *AKR1B1*, described in Table 4-2, and SYBR Green PCR Master Mix (Applied Biosystems, Inc.). Reactions were performed in 20 μL volume and in duplicate. Amplification consisted of 40 cycles of a 2-step amplification protocol (95°C for 15 s followed by 60°C for 1 min). Primer efficiencies ranged from 75 to 101%. Amplification reactions were followed by melting curve analysis to ensure amplification of a single product. Negative controls were run in the same manner but with the absence of reverse transcriptase. Relative gene expression was assessed with the $2^{[-ddCt]}$ method using *GAPDH* as the housekeeping gene.

Statistical Analysis

Follicle diameter, CL volume, and follicular fluid fatty acid data were analyzed using the GLM procedure of SAS v. 9.4 (SAS Institute, Inc., Cary, NC) with treatment as the fixed effect. Plasma progesterone and plasma fatty acids were analyzed using the Mixed Procedure and models included terms of treatment, day, and treatment by day interaction. Data on gene transcript fold change was given a cubed root transformation. A Mixed Procedure with repeated measures was used to analyze fold change in uterine transcript expression for each transcript analyzed, with fixed effects of treatment, day, side of uterine horn relative to CL, the interaction among the three, and all appropriate lower level interactions. The covariance structure used for plasma progesterone and gene expression was first order autoregressive, while covariance structure for plasma fatty acids was compound symmetry heterogenous based of the variance and covariances provided by an unstructured model and the smallest Akaike information criterion. The specified subject in all repeated measures models was animal (treatment). Tukey's adjustment for post-hoc comparisons was used when applicable. Significance was set at $P < 0.05$ and tendencies determined at $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

Analysis revealed that CON supplements had numerically greater total fatty acid content than TRT, which was unexpected as the commercial supplements used in this study have previously shown to be isolipidic (Brandao et al., 2018). Brandao and colleagues utilized a mixture of corn and soybean meal, while this study utilized only corn gluten feed, but each treatment received equal amounts and this is unlikely to offset lipid content between groups. Multiple supplement samples from several days likely would have yielded more similar lipid content, unfortunately only one composite sample of each fat supplement and corn gluten feed

was available for analysis from this experiment. Nonetheless, we observed no differences in total plasma fatty acids suggesting our diets were isolipidic ($P = 0.59$, Table 4-4).

In this study, we aimed to use non-pregnant animals as a model for studying components of the maternal environment in response to CSSO supplementation. In comparison to the study by Brandao and colleagues, we see the same changes in plasma fatty acid composition in response to CSSO supplementation, particularly increasing n-6, decreasing n-3, and altered n-6:n-3 ratios (Brandao et al., 2018). We did however observe greater concentrations of individual fatty acids, likely due to increased corn supplementation (for enhanced palatability to ensure treatment consumption) and a longer total period of supplementation. Furthermore, our results agree with their findings on follicle diameter, CL volume, and plasma progesterone. Thus, our data indicates that any changes observed in the non-pregnant cow receiving CSSO will reflect changes occurring in the pregnant cow.

Ovarian Dynamics and Progesterone

We observed no differences in diameter of the dominant follicle (17.02 ± 1.37 mm, $P = 0.91$) or CL volume (1.79 ± 0.33 cm^3, $P = 0.87$) between treatments. Plasma progesterone concentrations, presented in Figure 4-1, did not differ between treatments ($P = 0.28$), nor was there a treatment by day interaction ($P = 0.60$). There was an effect of day for plasma progesterone concentrations ($P < 0.01$), which is to be expected as progesterone concentrations are increasing daily during the course of luteinization. Calcium salts of soybean oil have been shown to increase circulating progesterone in *Bos indicus* cattle (Cipriano et al., 2016a); however, *Bos indicus* breeds have naturally decreased circulating concentrations of steroid hormones and are more susceptible to changes in hormone concentrations with intervention,

likely due to decreased hormone metabolism (Sartori et al., 2010). Our lack of findings is in agreement with studies performed in *Bos taurus* beef breeds fed commercial CSSO (Moriel et al., 2014; Brandao et al., 2018). In addition, we observed no differences in dominant follicle diameter prior to ovulation or CL volume during luteinization, which supports the observed lack of difference in circulating progesterone concentration, as the size of the ovulatory follicle is related to the size of CL and its progesterone production (Perry et al., 2005; Mann, 2009).

Changes to the post-ovulatory rise in progesterone concentration may alter endometrial secretions and ultimately affect conceptus growth, and delayed post-ovulatory rise of progesterone compromises conceptus development and cow fertility (Shelton et al., 1990b; Mann and Lamming, 2001; Wathes et al., 2003a). Thus, this study examined if changes in the post-ovulatory rise in progesterone are contributing to the observed increased pregnancy rates and conceptus development in previous studies (Cipriano et al., 2016a; Brandao et al., 2018). However, no differences in treatment or treatment by day interaction were observed during the duration of this study ($P \geq 0.28$). Animals in our study did receive CSSO supplement at an earlier time point relative to those in previous studies. It may be that initial incorporation of n-6 fatty acids into ovarian tissue needs to occur during luteinization to potentially alter plasma progesterone concentration during luteinization, as opposed to mobilizing stores that may already be present after receiving supplement for a few weeks.

Fatty Acid Concentrations

Identified follicular fluid fatty acids are presented in Table 4-3. Our identified fatty acid composition is in agreement with previous studies in dairy and beef cattle, where the predominant follicular fluid fatty acids are palmitic, oleic, steric, linoleic, and alpha-linoleic

acids (Childs et al., 2008a; Bender et al., 2010; Zachut et al., 2010b). In our study, TRT cows had nearly doubled the LA to ALA ratios compared to CON animals ($P < 0.01$). Likely due to the small sample size and large variation since samples were excluded due to blood contamination, there were no differences in LA or total n-6 PUFAs between CON and TRT cows ($P \geq 0.31$). However, the total n-6:n-3 ratio was elevated in TRT animals by nearly double ($P < 0.01$). This increased n-6:n-3 ratio was likely driven by the significant reductions in n-3 fatty acids in TRT animals, especially ALA ($P = 0.041$), EPA ($P = 0.01$), and total concentration of n-3 fatty acids ($P = 0.029$).

Identified plasma fatty acids are presented in Table 4-4. Total fatty acids concentrations did not differ between treatments ($P = 0.59$). This was expected as supplements were formulated to be isolipidic based on previous studies by our group (Brandao et al., 2018). Similarly, as observed in follicular fluid, the predominant fatty acids in plasma were palmitic, oleic, stearic, linoleic, and alpha-linoleic acids. Total fatty acid content increased over time ($P = 0.01$). In addition, total PUFA ($P < 0.01$) and total n-6 ($P < 0.01$) concentrations increased over time in both groups. Corn is relatively high in LA to begin with, thus likely increasing total n-6 tissue concentration in the controls over time (Moallem, 2018). However, the TRT group had a tendency for greater increases in LA ($P = 0.06$) and total n-6 fatty acids ($P = 0.08$) over time compared to CON animals. As expected, total n-6 fatty acids ($P < 0.01$) and the n-6:n-3 ratio ($P < 0.01$) were increased in TRT cows, driven by elevated concentrations of LA ($P < 0.01$) and decreased total n-3 fatty acids ($P < 0.01$).

Interestingly, LA concentrations increased in plasma, while the concentrations of LA's metabolite AA did not differ statistically between treatments. Other studies utilizing similar CSSO supplements also demonstrated elevated concentrations of LA without changes in AA in

plasma and maternal tissues (Cooke et al., 2014; Brandao et al., 2018). The beneficial effects of n-6 supplementation on reproduction in *Bos taurus* cattle are not derived from changes in AA or the eicosanoids produced from it, but rather increased abundance of LA itself. Incorporation of PUFA into cellular membranes alters membrane fluidity which can then impact membrane function through an altered ability for membrane reorganization and interaction of lipids with functional membrane proteins (Shaikh and Edidin, 2008). Long-chain fatty acids also act as signaling molecules, and could be altering PPAR expression, as examined in our uterine biopsies. It is also known that fatty acids can bind to G-protein coupled receptors, deemed FFAR, to mediate energy homeostasis (Briscoe et al., 2003). Recently, scientists have discovered that these FFAR are present in the bovine endometrium and are activated by long chain fatty acids (Valenzuela et al., 2019a). This may provide a mechanism of action for LA that is independent of AA and its eicosanoids, but further research is needed to divulge the role of these FFAR in bovine reproduction.

The observed reductions in plasma AA may indicate reduced activity of the fatty acid desaturase enzymes by the liver, which are the first rate-limiting steps of n-6 and n-3 fatty acid metabolism. Holman (1964) noted that LA and ALA are competitive inhibitors of each other for access to the desaturase enzymes. Inclusion of just 1% ALA into the diet of rodents inhibited the desaturation of LA into AA (Holman, 1964). While the CSSO supplement used provides mostly LA, it also increases the ALA content relative to the control diet on an as-fed basis (Table 4-1). This slight inclusion of ALA may be inhibiting desaturase activity of LA. Studies examining the kinetics of desaturase enzymes used the rodent as a model, when in fact the activity of elongation and desaturation enzymes in the bovine is reduced in comparison to the rat (St John et al., 1991).

Thus, addition of these fat supplements may be altering the kinetic activity of desaturase enzymes in beef cattle to generate stores of LA in the tissues.

This hypothesis is further supported when we examine the ratio of products to precursors (AA:LA or EPA:ALA) in blood plasma between treatments and over time, presented in Table 4-4. This method has been used to provide non-invasive insight to enzyme activity (Vessby et al., 2002). We found a decreased ratio of AA to LA in the TRT group ($P < 0.01$) and a treatment by day interaction ($P = 0.01$) with CON groups increasing product to precursor ratio while TRT cows remained constant, confirming that the TRT group is having reduced conversion of n-6 fatty acids. Unfortunately, we cannot directly assess delta-6 desaturase activity on LA without having quantified plasma concentrations of dihomo-γ-linoleic acid, but as delta-6 desaturase is the limiting step in n-6 fatty acid metabolism, it is likely to be the altered enzymatic step. Conversely, the ratio of EPA to ALA did not differ between groups ($P = 0.78$), but decreased over time in both groups ($P = 0.02$). Total n-6 fatty acids increased in both groups, explaining the decrease in ALA to EPA conversion overtime, as an increasing abundance of n-6 fatty acids will compete more with n-3 fatty acids for desaturation. It appears in beef cows supplemented with CSSO, there are alterations in delta-6 desaturase activity, favoring the production of EPA from ALA over AA from LA, either from the slightly elevated ALA concentration compared to CON diets inhibiting LA desaturation, or potentially as an effort to compensate for the abundance of LA replacing ALA in tissues. Whether this shift in lipid metabolism aids reproduction directly or is only acting to alter tissue fatty acid compositions remains uncertain.

Uterine Gene Expression

Expression of all PPAR isoforms (*PPARA, PPARD, PPARG*) increased over time ($P < 0.01$), but did not differ between treatments ($P \geq 0.73$), uterine horn relative to the CL ($P \geq 0.11$), nor was there an interaction between day, treatment, and uterine horn relative to CL or any lower order interactions ($P \geq 0.11$, Figure 4-2). There is little information on the expression of PPARs in the bovine endometrium, however recent work performed by Socha and colleagues (2017) demonstrated that only *PPARG* changes throughout the estrous cycle, with the greatest transcript expression occurring during estrus, while *PPARA* and *PPARD* did not change throughout the estrous cycle (Socha et al., 2017). In our study, we observed increases in all PPAR isoform transcripts over time; however, we believe this is more attributed to increasing fatty acid content of tissues over time rather than changes associated with the estrous cycle. In mice, *PPARG* is also upregulated in those fed a high fat diet (Sikder et al., 2018). In cattle, supplementation with n-3 PUFA generated a moderate increased expression of *PPARA* and *PPARD*, but not *PPARG* in uterine biopsies and cultured endometrial cells (MacLaren et al., 2006; Coyne et al., 2008). However, to our knowledge there has been no work examining the influence on n-6 PUFAs on PPAR expression in the bovine uterus. As we did not see differential expression between treatments, we believe the increase in PPAR expression is most likely an effect of increasing total plasma fatty acids over time. However, it should be noted that PPAR can be activated in response to inflammation, and since the same animals were used for biopsies, we cannot rule out the possibility that elevated PPAR expression may be in response to inflammation caused by the initial biopsy (Korbecki et al., 2019).

There was no difference in expression of prostaglandin E_2 (*PTGES*) or $F_{2\alpha}$ synthases (*AKR1B1*) between treatments ($P \geq 0.30$), nor was there an interaction between treatment, day, and uterine horn relative to the CL ($P \geq 0.15$, Figure 4-2). There was no change in *PTGES*

transcripts from d18 to d28 (d8 and 18 of estrous cycle; $P = 0.24$). This is in agreement with previous studies that did not observe changes in prostaglandin E synthase in response to supplementation of CSSO to beef cattle (Cipriano et al., 2016a; Brandao et al., 2018). We observed an increase in *AKR1B1* transcripts over time ($P < 0.001$), which is expected, as prostaglandin $F_{2\alpha}$ drives regression of the corpus luteum by day 18 of the estrous cycle when maternal recognition of pregnancy does not occur. As stated, we had not observed changes in plasma AA, the primary precursor for series 2 prostaglandins, between treatments ($P = 0.19$). Supplementation with CSSO does not impact transcript expression of prostaglandin synthases in the bovine endometrium. However, further analysis of protein expression would be necessary to confirm that uterine prostaglandin secretion is not altered by CSSO supplementation in beef cows.

CONCLUSION

Supplementation of CSSO to beef cattle at the time of artificial insemination can improve pregnancy rates and conceptus development, but it is unclear why this occurs. This study aimed to identify changes in the maternal environment that may benefit pregnancy establishment and conceptus development using the non-pregnant cyclic cow as a model. We did not observe changes in dominant follicle diameter, CL volume, or plasma progesterone between cows receiving CSSO and those receiving prilled saturated fat. Nor did we observe changes in gene expression of prostaglandin synthases or PPAR isoforms in endometrial tissue. There was an increase in all PPAR transcripts over time which we attributed to the accumulation of plasma fatty acids over time. The most pronounced changes were in plasma and follicular fluid fatty acid composition, where supplementation with n-6 rich CSSO increases n-6 fatty acids while reducing n-3 fatty acids. We also observed changes in delta-6 desaturase activity through

analysis of plasma fatty acid ratios. Supplementation with CSSO did not affect any of the reproductive parameters examined here, thus we believe that CSSO supplementation may directly benefit conceptus development through increased LA abundance in maternal tissues. Increased abundance of LA in the endometrium phospholipids, as seen in *Bos indicus*, would increase the LA content of extracellular vesicles secreted from the uterus (Cooke et al., 2014; Bridi et al., 2020). These would be directly taken up by the conceptus, allowing it to increase production of AA from increased stores of LA to drive its own development, potentially through increasing the necessary *PPARG* expression (Brooks et al., 2015a; Ribeiro et al., 2016b). There is an abundance of desaturase enzyme transcripts in the elongating ruminant conceptus, and extracellular vesicles are known to facilitate the maternal-embryo crosstalk (Brooks et al., 2016; Bridi et al., 2020). Future work should examine the conceptus's response to altered maternal tissue lipid concentrations, as well as how altered fatty acid composition and lipid enzyme activity can impact *Bos taurus* beef cow reproduction independent of eicosanoids production.

Table 4-1. Nutrient composition (on dry-matter basis) and fatty acid content (% of total fatty acids) of supplements[1,2]

Item	CON	TRT
Nutrient composition, DM basis		
Metabolizable energy, Mcal/kg	3.52	3.59
Net Energy for Maintenance, Mcal/kg	2.21	2.25
Total Digestible Nutrients, %	83	84
Crude Protein, %	29.6	32.8
Neutral detergent Fiber, %	12.2	10.9
Total Fatty Acids, %	13.91	10.9
Ca, %	0.36	1.35
P, %	0.55	0.57
Fatty Acid content, %		
Lauric (12:0)	0.65	0.14
Myristic (14:0)	2.84	0.56
Palmitic (16:0)	27.9	26.86
Palmitoleic (16:1)	0.39	0.14
Steric (18:0)	37.16	4.32
Oleic (18:1, *n*-9)	11.10	26.07
Linoleic (18:2, *n*-6)	11.71	36.10
α-Linolenic (18:3, *n*-3)	0.85	3.31
Arachidic (20:0)	0.58	0.29
Gadoleic (20:1, *n*-11)	0.11	0.18
Eicosapentaenoic (EPA; 20:5, *n*-3)	0.03	0.10
Behenic (22:0)	0.19	0.23
Docosahexaenoic (DHA; 22:6, *n*-3)	0.00	0.00
Lignoceric (24:0)	0.11	0.15
Other	6.37	1.56
Saturated	69.43	32.55
PUFA	12.59	39.51

[1]Treatments were provided daily and animals were supplemented individually

[2]CON = 0.7 kg/hd/d of corn gluten feed + 0.1 kg/hd/d prilled saturated fat (Energy Booster, Milk Specialties, Eden Prairie, MN), n = 12; TRT = 0.7 kg/hd/d of corn gluten feed + 0.1 kg/hd/d calcium salts of soybean oil (Essentiom, Church and Dwight Co., Inc., Princeton, NJ), n = 13

Table 4-2. Nucleotide sequence of bovine-specific primers and accession number of transcripts analyzed using reverse-transcription PCR.

Target Gene		Sequence	Accession number
PPARA	F	GCCTCTGGCTACCACTACGG	NM_001034036.1
	R	ACGAATCGCGTTATGGGACA	
PPARD	F	TGGTACTCACGCAGTGGCTT	NM_001083636.1
	R	GCAGTTCCCGTCAGCCTCTT	
PPARG	F	CATAATGCCATCAGGTTTGGGCG	NM_181024.2
	R	TCAAGATCGCCCTCGCCTTT	
AKR1B1	F	CAGCAGAGCTCAAGCAGTCA	NM_001012519.1
	R	CACTTTGCCTGGAGGGGACTTC	
PTGES	F	CGCTGCTGGTCATCAAAAT	NM_174443.2
	R	GGAAGGGGTAGATGGTCTCC	
GAPDH	F	TCGGAGTGAACGGATTCGGC	NM_001034034.2
	R	ACTTGCCGTGGGTGGAATCA	

Table 4-3. Mean concentration of identified fatty acids (μg/mL) in follicular fluid of super stimulated cows supplemented with either prilled saturated fat (CON, n = 6) or calcium salts of soybean oil (TRT, n = 5)[1,2,3]

Identified Fatty Acids	CON	TRT	SEM	*P* - value
Myristic (14:0)	6.75	5.43	0.89	0.30
Palmitic (16:0)	66.34	57.25	4.51	0.17
Palmitoleic (16:1, *n*-7)	7.54	5.34	0.52	0.01
Steric (18:0)	80.25	65.73	7.14	0.17
Oleic (18:1, *n*-9)	87.93	60.87	6.53	0.01
Vaccenic (18:1, *n*-7)	4.87	4.03	0.39	0.15
Linoleic (18:2, *n*-6)	96.37	120.09	16.31	0.31
α-Linolenic (18:3, *n*-3)	45.35	30.52	4.60	0.04
Arachidic (20:0)	0.543	0.46	0.04	0.15
Gondoic (20:1, *n*-9)	1.77	1.71	0.03	0.13
Eicosadienoic (20:2, *n*-6)	0.58	0.49	0.02	0.01
Arachidonic (20:4, *n*-6)	14.61	11.70	1.12	0.09
Eicosatrienoic (20:3, *n*-3)	3.63	2.25	0.75	0.19
Eicosapentaenoic (EPA; 20:5, *n*-3)	9.93	7.35	0.59	0.01
Behenic (22:0)	0.64	0.60	0.06	0.63
Erucic (22:1, *n*-9)	0.69	0.67	0.03	0.56
Docosahexaenoic (DHA; 22:6, *n*-3)	2.70	2.35	0.14	0.10
Lignoceric (24:0)	0.80	0.66	0.07	0.21
Nervonic (24:1, *n*-9)	0.47	0.34	0.04	0.05
Total identified fatty acids	431.04	377.83	39.12	0.34
Total n-3	60.88	42.47	5.23	0.03
Total n-6	111.56	132.29	17.23	0.40
Total PUFA	172.44	174.76	21.56	0.94
Ratio *n*-6:*n*-3	1.84	3.09	0.23	> 0.01

[1]Treatments were provided daily and animals were supplemented individually

[2]CON: 0.7 kg/hd/d of corn gluten feed + 0.1 kg/hd/d prilled saturated fat (Energy Booster, Milk Specialties, Eden Prairie, MN); TRT: 0.7 kg/hd/d of corn gluten feed + 0.1 kg/hd/d calcium salts of soybean oil (Essentiom, Church and Dwight Co., Inc., Princeton, NJ)

[3]On d-7, the two largest follicles were ablated to synchronize the follicular wave and a CIDR was inserted. Beginning d-5 until d-2, cows received two daily injections of follicle stimulating hormone (400 mg total; Folltropin-V, Vetoquinol, Lavaltrie, Québec, Canada) in decreasing dosage (4mL, 3 mL, 2 mL, 1mL; 20 mg/mL). Two injections of prostaglandin (25 mg, Lutalyse, Zoetis, Florham Park, NJ) were given with the last two injections of FSH. On day 0, follicle aspirations were performed and an injection of GnRH (100 μg, Factrel, Zoetis) was administered.

Table 4-4. Mean concentration of identified fatty acids (μg/mL) in blood plasma of cows supplemented with either prilled saturated fat (CON, n = 12) or calcium salts of soybean oil (TRT, n = 13) on days 0 or 25 of the study[1,2]. Values are presented as mean ± SEM with *P*-values for effects of treatment (Trt), day, and treatment by day interaction (Trt × Day).

Fatty Acid	**Supplement**				***P* - value**		
	CON		**TRT**				
	D0	**D25**	**D0**	**D25**	**Trt**	**Day**	**Trt × Day**
Myristic (14:0)	12.81 ± 0.62	17.69 ± 1.58	11.34 ± 0.62	15.48 ± 1.27	0.13	< 0.01	0.72
Myristoleic (14:1, *n*-5)	1.02 ± 0.09	1.19 ± 0.16	1.10 ± 0.09	1.23 ± 0.14	0.67	0.15	0.86
Palmitic (16:0)	277.07 ± 12.88	327.42 ± 14.39	265.75 ± 12.88	330.41 ± 13.70	0.76	< 0.01	0.60
Palmitoleic (16:1, *n*-7)	33.70 ± 1.99	40.96 ± 2.72	23.89 ± 1.97	27.88 ± 2.53	< 0.01	< 0.01	0.41
Steric (18:0)	331.77 ± 12.09	399.17 ± 21.96	297.72 ± 11.98	367.58 ± 19.45	0.04	< 0.01	0.95
Oleic (18:1, *n*-9)	358.88 ± 16.12	345.42 ± 19.28	284.73 ± 16.09	277.17 ± 18.22	< 0.01	0.53	0.86
Vaccenic (18:1, *n*-7)	19.76 ± 1.01	22.04 ± 1.45	18.13 ± 1.01	20.77 ± 1.34	0.27	0.04	0.87
Linoleic (18:2, *n*-6)	407.73 ± 30.36	518.76 ± 42.48	614.43 ± 30.17	846.91 ± 39.39	< 0.01	< 0.01	0.06
α-Linolenic (18:3, *n*-3)	188.96 ± 8.60	217.06 ± 14.17	128.47 ± 8.56	138.19 ± 12.80	< 0.01	0.08	0.38
Arachidic (20:0)	1.43 ± 0.07	2.77 ± 0.20	1.22 ± 0.07	2.46 ± 0.15	0.05	< 0.01	0.72
Gondoic (20:1, *n*-9)	2.18 ± 0.05	2.22 ± 0.10	2.12 ± 0.05	2.31 ± 0.09	0.89	0.12	0.31
Eicosadienoic (20:2, *n*-6)	1.27 ± 0.08	1.66 ± 0.10	1.18 ± 0.07	1.46 ± 0.09	0.13	< 0.01	0.48
Arachidonic (20:4, *n*-6)	59.86 ± 2.98	79.97 ± 4.53	54.57 ± 2.94	73.32 ± 4.15	0.19	< 0.01	0.82
Eicosatrienoic (20:3, *n*-3)	0.51 ± 0.04	0.75 ± 0.07	0.44 ± 0.04	0.55 ± 0.06	0.04	< 0.01	0.12
Eicosapentaenoic (20:5, *n*-3)	42.87 ± 2.16	46.19 ± 2.78	30.38 ± 2.16	29.23 ± 2.61	< 0.01	0.65	0.36
Behenic (22:0)	2.73 ± 0.14	4.00 ± 0.19	2.76 ± 0.14	3.99 ± 0.18	0.95	< 0.01	0.91
Erucic (22:1, *n*-9)	0.62 ± 0.01	0.64 ± 0.01	0.62 ± 0.01	0.64 ± 0.01	0.99	0.19	0.88
Docosahexaenoic (22:6, *n*-3)	8.30 ± 0.46	8.89 ± 0.50	7.51 ± 0.45	7.57 ± 0.48	0.09	0.28	0.37

Lignoceric (24:0)	3.98 ± 0.18	6.05 ± 0.30	3.94 ± 0.18	5.81 ± 0.27	0.54	< 0.01	0.71
Nervonic (24:1, *n*-9)	2.43 ± 0.15	3.21 ± 0.24	2.34 ± 0.14	3.24 ± 0.22	0.91	< 0.01	0.72
Total identified fatty acids	1757.88 ± 74.33	2046.05 ± 107.53	1753.11 ± 74.33	2151.78 ± 99.23	0.59	0.01	0.54
Total *n*-3	240.64 ± 9.72	272.90 ± 16.11	166.44 ± 9.72	176.30 ± 14.54	< 0.01	0.11	0.38
Total *n*-6	468.85 ± 32.29	600.39 ± 45.53	669.97 ± 32.09	921.37 ± 42.17	< 0.01	< 0.01	0.08
Total PUFA	709.49 ± 39.99	873.29 ± 59.92	837.00 ± 39.82	1096.63 ± 54.99	< 0.01	< 0.01	0.29
Ratio *n*-6:*n*-3	1.95 ± 0.16	2.23 ± 0.08	4.07 ± 0.16	5.43 ± 0.08	< 0.01	< 0.01	< 0.01
Ratio AA:LA	0.15 ± 0.01	0.16 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	< 0.01	0.28	0.01
Ratio EPA:ALA	0.23 ± 0.02	0.22 ± 0.02	0.25 ± 0.01	0.22 ± 0.02	0.78	0.02	0.45

[1]Treatments were provided daily and animals were supplemented individually

[2]CON: 0.7 kg/hd/d of corn gluten feed + 0.1 kg/hd/d prilled saturated fat (Energy Booster, Milk Specialties, Eden Prairie, MN); TRT: 0.7 kg/hd/d of corn gluten feed + 0.1 kg/hd/d calcium salts of soybean oil (Essentiom, Church and Dwight Co., Inc., Princeton, NJ)

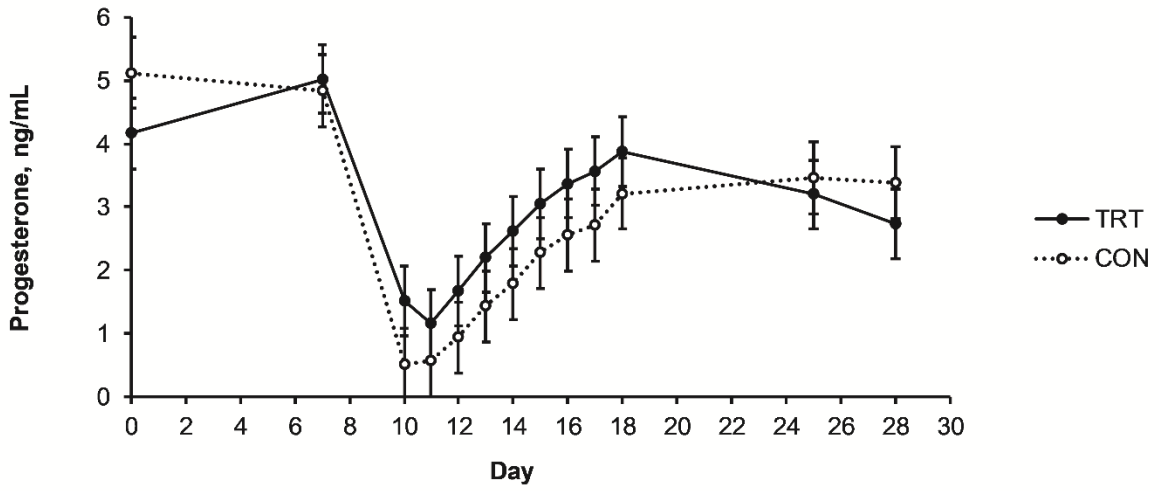

Figure 4-1. Plasma progesterone concentrations (ng/mL) of cows over the course of the study. Animals were individually supplemented with 0.7 kg/hd/d of corn gluten feed and either: 0.1 kg/hd/d prilled saturated fat (CON, Energy Booster, Milk Specialties, Eden Prairie, MN; n = 12), or 0.1 kg/hd/d calcium salts of soybean oil (TRT, Essentiom, Church and Dwight Co., Inc., Princeton, NJ; n = 13) daily over the course of the study. There was an effect of day ($P < 0.01$), but no effects of treatment ($P = 0.28$) or treatment by day interaction ($P = 0.60$).

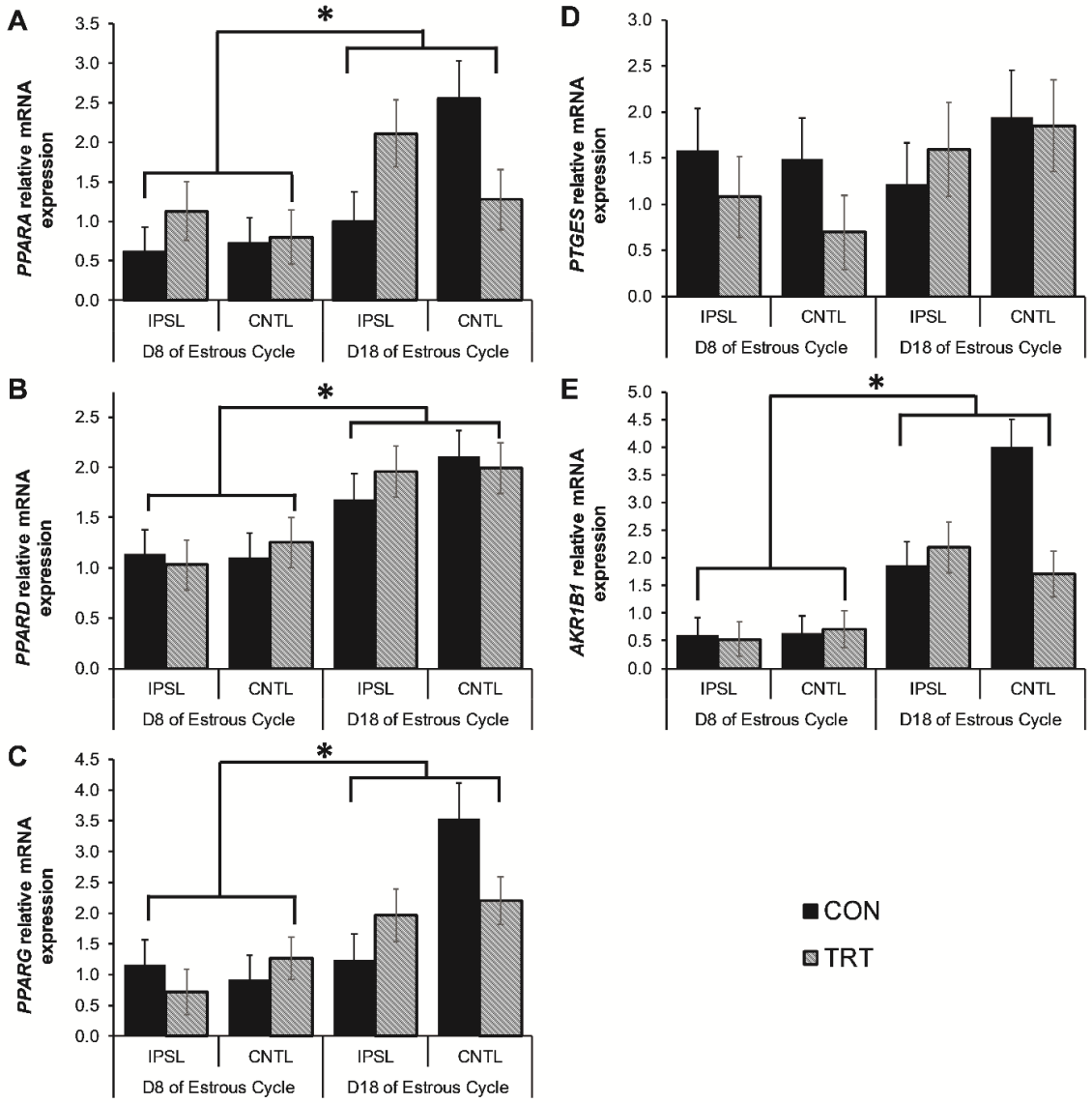

Figure 4-2. Uterine expression of A) *PPARA*, B) *PPARD*, C) *PPARG*, D) *PTGES*, or E) *AKR1B1* transcripts relative to housekeeping gene, *GAPDH*. Cows individually received 0.7 kg/hd/d of corn gluten feed and either: 0.1 kg/hd/d prilled saturated fat (CON, Energy Booster, Milk Specialties, Eden Prairie, MN; n = 12), or 0.1 kg/hd/d calcium salts of soybean oil (TRT, Essentiom, Church and Dwight Co., Inc., Princeton, NJ; n = 13) daily. Uterine biopsies were collected on days 8 and 18 of the estrous cycle (days 18 and 28 of the study) in uterine horns ipsilateral (IPSL) or contralateral. * $P < 0.05$.

CHAPTER 5

Physical parameters of bovine activated oocytes and zygotes as predictors of development success

INTRODUCTION

Embryo transfer in livestock production systems is a growing business, allowing for drastic genetic improvement in a short span of time and increasing the number of offspring from desired genetic lines. The number of IVP bovine embryos has been increasing steadily over the past 20 years, and the number of bovine IVP embryos generated and transferred surpasses that of in-vivo derived embryos by nearly double (Viana, 2018). Unfortunately, IVP embryos tend to be of inferior quality and produce lower pregnancy rates than their in-vivo counterparts (Hasler, 2000; Ealy et al., 2019). Within the past 20 years, little change in the methodology for producing and selecting suitable embryos for transfer has occurred. Quality is traditionally assessed by morphology; however, a given score is highly subjective and can vary based on an embryologist's experience (Farin et al., 1995; Baxter Bendus et al., 2006).

Recently there has been a push for more objective methods for embryo evaluation. Common endpoints used in research, like blastomere number and proportion of apoptotic cells (Knijn et al., 2003; Gomez et al., 2009), chromosomal abnormalities (Viuff et al., 2000; Booth et al., 2003; Liang et al., 2013), or genetic/epigenetic analyses (Balasubramanian et al., 2007; O'Doherty et al., 2012), are inapplicable to industry practices because of their terminal nature. Thus, there is a need for non-invasive techniques to obtain objective evaluation. Non-invasive

research has focused on markers of embryo metabolism (Tejera et al., 2012; Gardner and Wale, 2013) and morphokinetics of developing embryos (Lundin et al., 2001; Cruz et al., 2012; Coticchio et al., 2017). The transition of these technologies to application in livestock production and human clinics lags due to technological challenges and variability, as well as lack of agreement with post-transfer success (Gutierrez-Adan et al., 2015; Sanchez et al., 2017). The objectives of this study were to investigate if physical parameters of artificially activated oocytes and presumptive zygotes can be indicative of development potential for bovine embryos. Activated oocytes were first used as a model, and then a second study was performed with IVF zygotes to assess the validity of outcomes.

MATERIALS AND METHODS

Oocyte Collection and Maturation

Bovine ovaries were obtained from Brown Packing Co. (Gaffney, SC) and COCs were incubated in 500 µL of TCM-199 medium with Earle's salts maturation medium supplemented with 10% [v/v] fetal bovine serum, 25 µg/mL bovine FSH (Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada), 2 µg/mL estradiol (Sigma-Aldrich, St. Louis, MO), 22 µg/mL sodium pyruvate, 1mM glutamine, and 25 µg/mL gentamicin sulfate at 38.5°C in 5% CO_2 in humidified air, as previously described (Xie et al., 2017; Wooldridge and Ealy, 2019). Maturation of COCs intended for activation lasted 24 hours, while COCs for IVF matured 21-23 hours.

Artificial Oocyte Activation

Artificial activation was performed as previously described (Susko-Parrish et al., 1994) with some modifications to generate gynogenetic parthenotes (n = 723; 8 replicates). Matured

COCs were denuded by vortexing in HEPES buffered synthetic oviductal fluid (HEPES-SOF; (Denicol et al., 2014) with hyaluronidase (1,000 U/mL) for 4 minutes. Oocytes with visible polar bodies and evenly distributed cytoplasm underwent activation. Oocytes were incubated in 5 μM ionomycin (Sigma-Aldrich, St. Louis, MO) in HEPES-SOF for 5 minutes, washed in HEPES-SOF, and immediately placed in 2mM 6-dimethylaminopurine (6-DMAP; Chemodex, St. Gallen, Switzerland) in SOF-Be1 culture medium (Fields et al., 2011) for 3 hours at 38.5°C under 5% CO_2, 5% O_2, and 90% N_2 in humidified air. After activation, oocytes were placed in 5 μL droplets of SOF-Be1 under oil and photographed individually using a Nikon camera on an inverted microscope. Activated oocytes were then group cultured in 50 μL droplets of SOF-Be1 under oil at 38.5°C under 5% CO_2, 5% O_2, and 90% N_2 in a polyester micromesh with 300 μm openings (Spectra Mesh woven filters, Spectrum Labs, Repligen), enabling identification of individual oocytes during culture (Figure 5-1). The mesh was prepared by cutting into 4 × 6 mm squares and melting edges of mesh to the culture plate with a hot awl. Plates were rinsed with water and ethanol to remove debris, then sterilized in ethanol 1 hour. Plates dried at least 3 hours in a sterile hood before culture droplets were added over mesh squares. Development to the blastocyst stage was assessed on days 7 and 8 of culture.

In Vitro Fertilization

In-vitro fertilization to produce bovine embryos (n = 875; 11 replicates) was performed as previously described (Fields et al., 2011; Xie et al., 2017; Wooldridge and Ealy, 2019), with some modifications. Matured oocytes incubated in SOF-FERT fertilization media (Sakatani et al., 2012) with sperm purified from frozen-thawed semen washed by a BoviPure™ density-gradient (Nidacon; Spectrum Technologies Healdsburg, CA, USA) for 16-19 hours. Semen contained a mixture of 4 Holstein bulls (donated by Select Sires, Inc, Plain City, OH).

Presumptive IVF zygotes were denuded, individually photographed, and group cultured in a micromesh as described above. Development to the blastocyst stage was assessed on days 7 and 8 of culture.

Blastomere Staining

Nuclear staining was performed on day 8 of culture on IVF blastocysts (n = 87) to assess total blastomere number. Blastocysts were fixed in 4% paraformaldehyde in PBS-PVP for 15 minutes, permeabilized with 0.25% Triton X-100 [v/v] in PBS-PVP for 20 minutes, then incubated with 1 µg/mL of Hoechst 33342 (Life Technologies) for 15 minutes at room temperature. Stained embryos were mounted on a glass slide with coverslip. Total blastomere number was assessed using the cell count function of Fiji (Schindelin et al., 2012).

Image Analysis

Each image was analyzed using Fiji (Schindelin et al., 2012) with scale based off the image of a hemocytometer grid taken at similar magnification. On each activated oocyte/presumptive zygote, the ZP thickness, ooplasm area, and diameter (including ZP) were measured (Figure 5-2). An average of 2 measurements were collected for ZP thickness and diameter.

Statistical Analysis

Statistics were performed using R v. 3.6.1 (R Core Team, 2016) with the lme4 package (Bates et al., 2015). Residual estimates of area and ZP thickness in relation to diameter were used to address collinearity. Predictors were also scaled by Z-statistic. Development rates on days 7 and 8 were assessed with a generalized linear mixed effects model and blastomere numbers from IVF embryos were assessed with a linear mixed effects model. Fixed effects were

diameter, residual estimate of area, and residual estimate of ZP thickness, and random effects were culture drop and replicate. Significant interactions remained in the model. *P*-values were obtained using Wald tests, with significance set at $P < 0.05$ and tendencies set as $0.05 < P \leq 0.1$.

RESULTS

Size Parameters

Size parameter measurements are displayed in Table 5-1. There was a positive correlation between diameter and ooplasm area in activated oocytes (r = 0.69, $P < 0.01$) and IVF zygotes (r = 0.67, $P < 0.001$). A positive correlation existed between ZP thickness and diameter in activated oocytes (r = 0.35, $P < 0.01$) and IVF zygotes (r = 0.32, $P < 0.001$). There was little correlation between area and ZP thickness in activated oocytes (r = 0.08, $P = 0.03$) and IVF zygotes (r = -0.03, $P = 0.34$).

Blastocyst Development

Overall day 7 blastocyst rates were 18.1 ± 1.4 % for activated oocytes and 9.8 ± 1 % for IVF zygotes. Day 8 blastocysts rates were 33.5 ± 1.8 % for activated oocytes and 23.1 ± 1.4 % for IVF zygotes. Diameter had a significant effect on the probability of development in activated oocytes on d 7 ($P < 0.01$) and d 8 ($P < 0.001$; Figure 5-3), and tended to effect development potential on d 8 ($P = 0.08$) in IVF zygotes but not d 7 ($P = 0.11$). For both oocytes and zygotes, a larger diameter was more beneficial. Zona pellucida thickness also affected probability of development in activated oocytes on d 7 ($P < 0.001$) and d 8 ($P < 0.001$; Figure 5-3), and in IVF zygotes on d 8 ($P = 0.044$) but not d 7 ($P = 0.65$; Figure 5-4). For both oocytes and zygotes, a thinner ZP was beneficial. Area had no effect on either day, neither for activated oocytes nor IVF zygotes ($P \geq 0.17$). There was a significant interaction between diameter and ZP thickness on

days 7 ($P = 0.02$) and 8 ($P < 0.01$; Figure 5-4) observed only in IVF zygotes. Zygotes with larger diameter and thicker ZP as well as zygotes with smaller diameter and thinner ZP were more likely to develop to the blastocyst stage.

Blastomere Number

Blastomere number averaged 119.61 ± 4.40 cells per blastocyst. There was an effect of area on the total number of blastomeres on d 8 of culture, with a positive but weak correlation ($P = 0.01$; conditional $R^2 = 0.09$; Figure 5-5). There was no effect of diameter or ZP thickness ($P \geq 0.21$).

DISCUSSION

Several studies have aimed at correlating size parameters of bovine oocytes and blastocysts with development. These studies have only obtained observations prior to fertilization or at the blastocyst stage, while our data was collected immediately after activation or fertilization. Our findings indicate that zygotes and oocytes with larger diameters are more likely to develop, and is in agreement with previous research (Fair et al., 1995; Otoi et al., 1997). These studies, however, examined bovine ooplasm diameter prior to fertilization. The ooplasm shrinks in response to maturation, removal of cumulus cells, and fertilization and has undergone drastic changes by the time our measurements were taken (Suzuki et al., 1994; Tartia et al., 2009; Walls et al., 2016). This may explain why we did not observe an effect of ooplasm area on development potential while others have. We did observe a positive correlation between diameter and area, which likely represents a larger ooplasm area prior to fertilization. The significant effect of outer diameter seen in our study may be an artifact of larger oocyte area prior to fertilization.

Other studies have examined blastocyst diameter and demonstrate that larger blastocyst diameters are correlated with increased cell number (Mori et al., 2002; Hoelker et al., 2006). Yet, it's important to note the stage at which measurements are taken. A larger diameter blastocyst is in a more developed stage, and logically should have more cells. Our study did not measure blastocyst diameter, but measured ooplasm area after fertilization and found that larger areas have increased total blastomere number at d 8 of culture. Our research is novel to previous work in that we can draw a correlation between an objective size parameter and cell numbers before the blastocyst stage has been reached. It is important to note however that the correlation between the area and cell number was weak and only total blastomere number was assessed in this study.

Zona pellucida thickness in relation to development competence has been well-studied in humans, while the majority of work in bovine focuses on ultrastructural aspects of the ZP. In humans, ZP thickness decreases with successful fertilization and is correlated with embryo quality (Bertrand et al., 1995; Balakier et al., 2012). Our observation of increased likelihood of development with thin ZP may be reflective of the increased probability of development associated with successful fertilization. While there is little evidence that these findings in humans are applicable to bovine, other species including pig (Hirao et al., 1994), buffalo (Raghu et al., 2002), and human (Durinzi et al., 1995) seem to exhibit similar trends relating large diameters of oocytes to developmental competence. It is plausible that trends in ZP thickness are conserved across species as well.

An interaction between ZP thickness and diameter was only observed in IVF embryos. while novel, this finding emphasizes that parthenogenetic embryos may not always be the best models for developmental studies. First, some biological processes, like oocyte activation differ

between activated oocytes and IVF zygotes. Fertilized oocytes experience oscillating calcium levels, while artificially activated oocytes undergo only one initial rise in intracellular calcium, causing differences in cortical granule exocytosis (Soloy et al., 1997; Nakada and Mizuno, 1998). Secondly, artificial activation does not reflect the male contribution to development success. The biggest known paternal influences on development rates in bovine IVF are individual sire and semen quality (Ortega et al., 2018; Siqueira et al., 2018), but less is known about how oocyte size can influence aspects of fertilization involving sperm, like sperm-oocyte-binding, effective polyspermy block, pronuclear formation, and subsequent epigenetic reprogramming. There is little to no relation between ooplasm diameter and sperm penetration (Otoi et al., 1997), and female imprinted genes are dependent on oocyte growth (Hirao et al., 1994) but more research is needed. While we do find agreement in maternal parameters such as oocyte size and ZP thickness among the two models, care should be taken when using only parthenogenetic embryos in place of IVF embryos.

One hypothesis for the observed interaction between diameter and ZP thickness is that there is an ideal, median area size which is important for development success. A small oocyte with a thin ZP likely has a more similar ooplasm area compared to a large oocyte with a thick ZP. While increasing ooplasm is beneficial for development, there may reach a point where too large is detrimental. Otoi and colleagues noted that ooplasm diameters above 130 μm did not develop to the blastocyst stage (Otoi et al., 1997); however their sample size for this size group was small and results should be extrapolated with care. A study by Kyogoku and colleagues reported that large cytoplasmic size is linked to more error-prone chromosome segregation in oocytes (Kyogoku and Kitajima, 2017). Our observations that a large diameter with a thick ZP is beneficial maybe be due to a slightly reduced area inside the ZP.

The lack of agreement between parameters affecting probability of development on d 7 and d 8 for IVF embryos is likely due to the lower development rates for IVF embryos weakening the power of the data set. These development rates are not too far below normal, however the process of individually photographing and culturing a large number of embryos (typically 100-150 at a time) does prolong exposure to atmospheric conditions. Hundreds more observations in the IVF embryos are likely necessary to see stronger agreement between d 7 and d 8 results, as well as between the activated and IVF data. Nonetheless, we do begin to see some congruency between activated and IVF parameters on d 8.

The methods used in this study are simple and easy to implement, but have great potential for improvement. The polyester micromesh allows for beneficial group culture with easy identification of individuals. However, it does lend some limitations. While the majority of oocytes and zygotes remain in place, some will occasionally drift into openings with other zygotes. It is also not always conducive to taking proper measurements, as sometimes edges of zygotes are not clearly visible. To combat this problem, we photographed zygotes in 5 µL SOF-Be1 droplets under oil. This approach does increase exposure time to atmospheric conditions. Culture dishes utilizing a well-of-the well system (Vajta et al., 2008) may be more optimal for photographing large quantities of zygotes and reducing time outside the incubator. This was not done in this study as, wells that are too deep or jagged disrupt optics and produce inaccurate measurements. The ever-increasing prevalence of artificial intelligence and machine learning in biological sciences is going to play a major factor in future non-invasive selection methods. Measurements, currently done manually, could be automated and software developed for ease of use. Advancements in predictive modeling and further exploration of other potential physical

parameters will better develop predictive abilities. While our methodology is rudimentary, there is unlimited potential for growth.

This study demonstrates that physical parameters of bovine zygotes have potential as markers for developmental competence. Zygotes with larger diameters and thinner ZP are more likely to develop to the blastocyst stage. Area after fertilization had no effect on development potential, but larger area was associated with increased blastomere numbers. Interestingly, there is an interaction between the zona thickness and diameter not observed in activated oocytes. The techniques used in this study can only be improved with the advancements of micro-fabrication technology and machine learning, and in the near future could be transformed into an effective and easy to use system to predict viability of embryos. Future work assessing the applicability of these results to post transfer outcomes is crucial for advancement of this methodology.

Table 5-1. Size parameter measurements for activated oocytes and in-vitro produced zygotes. Parameters included outer diameter including zona pellucida (ZP), ZP thickness, and ooplasm area.

Parameter	Mean	SEM	Range
Activated Oocytes			
Diameter (µm)	154.27	0.26	131.30 – 173.20
ZP (µm)	11.99	0.06	6.09 – 17.70
Area (μm^2)	10,453.56	34.24	7,262.97 – 14,358.84
IVP Zygotes			
Diameter (µm)	151.22	0.23	130.36 – 171.57
ZP (µm)	11.75	0.05	7.23 – 17.48
Area (μm^2)	9,856.69	32.28	6,421.91 – 13,814.83

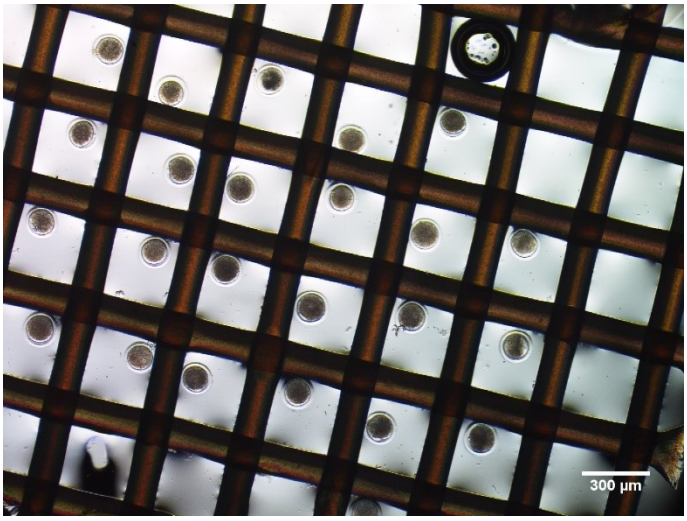

Figure 5-1. Presumptive zygotes in micromesh with 300µm openings. After mesh is adhered to plate and sterilized, culture medium droplets are place over mesh. One zygote per square of mesh opening.

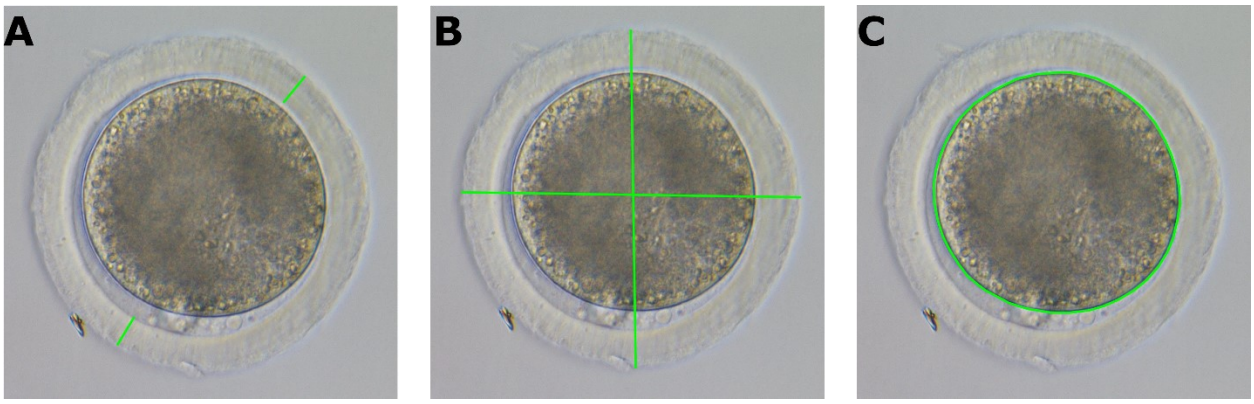

Figure 5-2. Size parameters of activated oocytes and zygotes: A) Zona pellucida thickness, average of 2 measurements; B) outer diameter, average of 2 perpendicular measurements; C) ooplasm area.

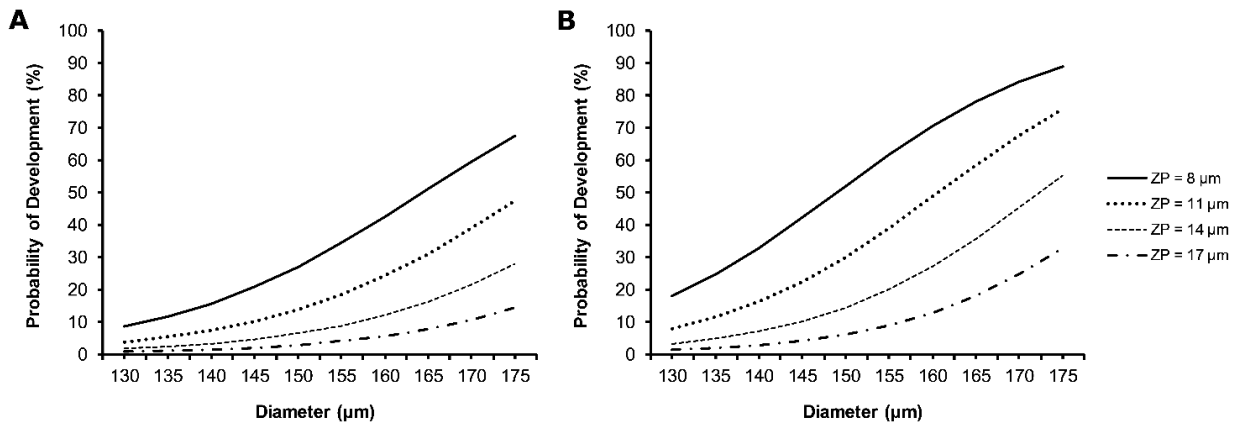

Figure 5-3. Probability of development to the blastocyst stage for artificially activated oocytes on day 7 (panel A) and day 8 (panel B) across varying diameters and zona pellucida (ZP) thickness, given average area (10,453.6 µm2). Diameter and ZP thickness effected probability of development for both days ($P < 0.01$). Area had no effect ($P \geq 0.21$).

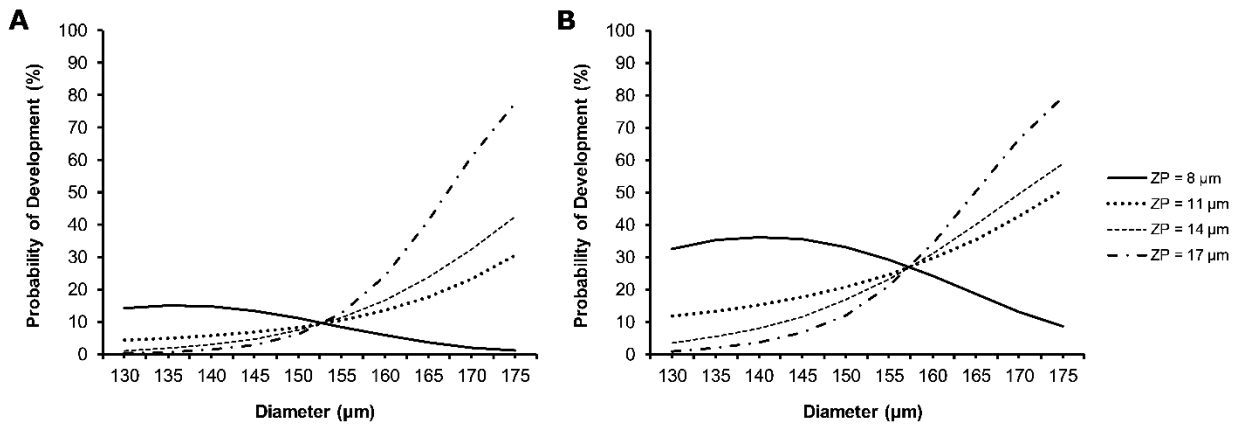

Figure 5-4. Probability of development to the blastocyst stage for in-vitro fertilized zygotes on day 7 (panel A) and day 8 (panel B) across varying diameters and zona pellucida (ZP) thickness, given average area (9,856.69 µm2). There was a tendency for an effect of diameter ($P = 0.08$) and an effect of ZP thickness ($P = 0.044$) on the probability of development for day 8. There was a significant interaction between ZP thickness and diameter on days 7 ($P = 0.02$) and 8 ($P < 0.01$). Area had no effect ($P \geq 0.17$).

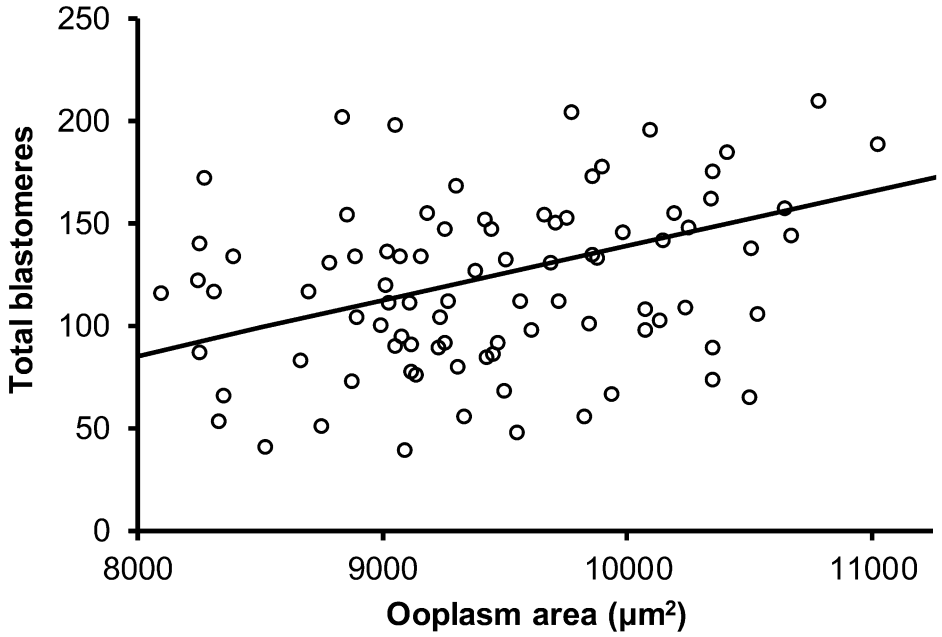

Figure 5-5. Total blastomere number correlated with ooplasm area. Trendline represents correlation between blastomere number and area accounting for average diameter (151.22 µm) and average zona pellucida (ZP) thickness (11.75 µm). There was an effect of area on blastomere number ($P = 0.01$), but not diameter nor ZP thickness ($P \geq 0.21$).

CHAPTER 6

Conclusions and Implications

There are several technologies available at the cattleman's disposal to help increase the reproductive efficiency of cow-calf systems and ultimately help improve the efficiency of beef production as a whole. With these technologies we can also mitigate issues associated with sub-fertility and early embryonic loss that prevent us from obtaining one healthy calf from each cow every year. We are beginning to better understand what hinders successful pregnancy establishment and encourage the use of reproductive technologies in production systems, but as demonstrated here, successful reproduction is a complex process and improving reproductive efficiency requires a more comprehensive approach. This comprehensive approach can be thought of a tiered cake. Any adoption of technology requires strong herd management at the base. From there one can focus on individual cow needs, and then begin utilizing *in vitro* embryo technologies. The goal of this work was to examine how we can improve reproductive efficiency by better utilizing assisted reproductive technologies that impact beef cows through these various tiers of broader herd management, individual cow physiology, and in the *in vitro* embryo production system.

The first study aimed to address the economic challenge of using FTAI in cow-calf systems to improve reproductive efficiency. Adoption of FTAI is poor in beef cattle systems compared to dairy, but it serves as a powerful tool to increase productivity and genetic merit of herds. The costs of implementing FTAI are the primary reason cattlemen are hesitant to adopt the technology; however, there may be potential for reducing bull related costs which can then make FTAI more economically feasible. To reduce bull related costs, one must increase the number of

cows serviced by a single bull, which can be at least doubled when FTAI is used in conjunction with natural service. Our study found that indeed the number of open animals exposed per bull was decreased by half after FTAI. We also found that there was little to no correlations between both the total number cows and number of open cows exposed per bull and pregnancy rates on first return to estrus. While there were some significant negative correlations, the bull:cow ratio explained only between 4 and 11% of the variation in that data, indicating the bull:cow ratio is not the limiting factor.

With this information, we can recommend to cattlemen that they can decrease the bull:cow ratio by half when using FTAI followed by natural service, and that bull:cow ratio is unlikely to affect pregnancy rates. By increasing the number of cows one bull can service, it may be possible for some herds to save money by reducing the number of bulls owned and either purchase greater genetic merit bulls or invest in other reproductive technologies like embryo transfer. Providing an effective means of cost savings by reducing bull costs may also encourage cattlemen who do not already implement FTAI to begin doing so. Further research should evaluate if the bull:cow ratio used after FTAI will affect pregnancy rates in a more controlled setting. Eliminating sources of variation such as individual bull or location seen in the retrospective study will help give a better understanding if bull:cow ratio really does influence pregnancy rates on first return to estrus. It may also identify if an individual sire can service more cows at once than another sire and if there are genetic traits associated with this that should be selected for. Other smaller herds may even be able remove the bull completely and use only a few rounds of FTAI. Future work should examine in more depth the economic impact of removing the bull entirely from small herds. This can also improve reproductive efficiency by encouraging cattlemen to increase selection pressure and remove sub-fertile animals from the

herd. Lastly this data can be used to better communicate with cattlemen and practitioners so that they may begin implementing our findings in their systems.

The second study narrows focus onto the individual cow's physiology and how CSSO supplementation alters it in favor pregnancy establishment. It's been established that supplementing the omega-6 rich CSSO for 21 days beginning at the time of FTAI enhances fertility of both *Bos indicus* and *Bos taurus* breeds of beef cows. In *Bos indicus* cows, increased progesterone and altered tissue fatty acid composition likely drives increased conceptus development. However, it is less clear how CSSO impacts *Bos taurus* animals, especially since there is increased pregnancy rates and conceptus IFNT production without increases in progesterone. This study did not find any changes in ovarian structures, plasma progesterone, or uterine gene expression between animals receiving saturated fat and CSSO. There were changes in plasma and follicular fluid fatty acid composition, as well as desaturase enzyme activity. This study was not able to pinpoint exactly what, if any, maternal changes are occurring to enhance fertility. It further indicates that CSSO supplementation may be more beneficial for the conceptus itself in *Bos taurus* cows. However, it should be noted cows in this study were consuming CSSO for much longer than cows in previous studies. Future work may need to verify results when CSSO is supplemented in a similar time frame as those studies.

Since this study only evaluated non-pregnant cows, the next step should be to more closely examine the conceptus's response to maternal CSSO supplementation. As demonstrated in our study and previous literature, supplementing CSSO increases tissue content of LA but not AA, and this is also true for endometrial tissue of *Bos indicus* beef cows (Cooke et al., 2014). I hypothesize that the increased abundance of LA in the endometrium phospholipids may be secreted directly to the conceptus via uterine extracellular vesicles (EVs), from which the

conceptus is utilize increased stores of LA to produce AA, which may stimulate PPARG to drive elongation and promote its own development. The ruminant conceptus does indeed produce very high levels of the desaturase enzymes necessary for converting LA to AA during the time of elongation and EVs play a vital role in uterine-conceptus crosstalk (Brooks et al., 2014; Bridi et al., 2020). First, fatty acid composition of *Bos taurus* endometrial and conceptus phospholipids should be evaluated to confirm that the same increases of LA with no changes in AA are occurring as they did in *Bos indicus*. Second, more in-depth analysis of differential gene expression of the *Bos taurus* conceptus in response to CSSO supplementation is needed, focusing on those playing a role in lipid metabolism and elongation. Evaluation of the *Bos taurus* conceptus at more time points may also help determine if conceptus growth is altered in response to maternal supplementation with CSSO. Early evaluation may reflect hastened development, but one should also take into consideration how these alterations will impact development later in gestation and through the animal's life. The early embryonic period involves cell lineage specification restructuring of genome methylation patterns; thus, it would be interesting to evaluate any potential fetal programming affects from supplemental CSSO.

The final study examined the potential for a non-invasive, objective method for embryo selection. Current selection methods are purely morphological, and attempts to generate objective markers of development potential fail to be successfully utilized in clinics and the livestock industry. The ability to accurately and non-biasedly select for embryos that will establish pregnancy would greatly improve the efficiency and justification for using *in vitro* produced embryos. This study examined if physical size parameters that can be digitally measured were indicative of development to the blastocyst stage. Indeed, we found that artificially activated oocytes and IVF zygotes were more likely to develop if they had larger

diameters or thinner zona pellucidae. We also found that cell area correlated with increased blastomere numbers on day 8. Most interestingly, there was an interaction between diameter and zona pellucida thickness in IVF zygotes that was not seen in activated oocytes. This is important to note because it emphasizes that the parthenogenetic embryo is not always a suitable model in place of an IVF embryo for *in vitro* studies of early development. This research generates the potential to develop automated software to measure zygotes and better machine learning algorithms that may be able to accurately predict developmental success.

This study only examined development to day 8 in culture. However, to fully understand if size parameters take influence developmental success, a study needs to evaluate size parameters in relation to pregnancy establishment. If there are no correlations between size and pregnancy establishment, this may not be a useful method for selection of embryos for transfer. However, it would be interesting to see if adding zygotes that are more likely to develop to a droplet of zygotes less likely to develop can improve the environment of the microdroplet and improve development of the lesser zygotes. Future work should also look at if these trends hold true for oocytes retrieved from cows that underwent ovarian stimulation and OPU. The oocytes in the current studies were collected from abattoir derived ovaries via slashing follicles open. This likely generates a more heterogenous population of gametes than would be collected from donors via OPU, and it would be interesting to see if the larger oocytes are still more likely to develop if they come from more uniform follicles. Finally, it would be interesting to develop a more accurate prediction model so that transfers can be performed earlier. Keeping the embryo in the *in vitro* environment negatively impacts its development, so transferring back to the maternal environment sooner may be beneficial.

Together these studies emphasize that the solution for improving reproductive efficiency of beef cows is not a silver bullet. First improving the adoption of assisted reproductive technologies is needed, and this requires altering some management practices to increase the economic feasibility. Second, improving the individual's cow performance will benefit the herd productivity and this can be done through nutritional supplements. Lastly, once herd management and individual animals are performing at their best, using *in vitro* embryo production becomes most beneficial, but the methods of embryo selection need to be improved to maximize the technology's success. Continual improvement of reproductive technologies and management techniques will help cattlemen address today's challenges with producing more beef while using less resources.

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APPENDIX A

Artificial Activation of Bovine Oocytes

Based off the protocols developed by Susko-Parrish et al., 1994

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Reagents

Ionomycin calcium salt from Streptomyces conglobatus (Sigma-Aldrich, I0634)

6-Dimethylaminopurine (6-DMAP; Chemodex, D0318)

Hyaluronidase (Sigma, H3884-50mg)

SOF-BE1 culture media and HEPES-SOF (refer to the Ealy Lab IVF protocol for recipes)

Reagent Preparation

1. Ionomycin
    a. Prepare a 5mM stock solution by dissolving 1 mg of ionomycin in 267.713 µL of DMSO
        i. Stock solution should be aliquoted in 3 – 4 µL aliquots in microcentrifuge tubes and stored at -20C
        ii. Stock solution can be kept at -20°C for several months or up to 6 weeks at 2 – 8C
    b. Prepare working solution immediately prior to use by diluting 3 µL of stock into 3 mL of HEPES-SOF in a 35mm petri dish.
2. 6-DMAP
    a. Prepare a 2 mM working solution by dissolving 1 mg in 3.0641 µL of SOF-Be1
        i. Solution should be made as needed and at least 2 days prior to activation, as 6-DMAP does not dissolve quickly
        ii. Store up to 2 weeks at 4°C. Protect from light
3. 2% Sucrose HEPES
    a. Dissolve 2 g of sucrose per 10 mL of HEPES-SOF
    b. Prepare fresh at least 1 day prior to activation
4. Hyaluronidase
    a. Dilute to 10,000 units/mL with saline
    b. Sterilize with a 0.2 µm filter

c. Prepare 100 µL aliquots into microcentrifuge tubes
d. Store at -20°C up to 2 years

General Preparation

1. Aliquot the necessary amount of HEPES-SOF into a 50 mL conical tube and place into a 38.5°C water bath at least 2 hours prior to activation. Be sure to aliquot enough HEPES-SOF to prepare:
    a. 10, 35 mm petri dishes filled half-way with HEPES-SOF
    b. 1, 35 mm petri dish with 3 mL of HEPES-SOF
2. 6-DMAP Drops
    a. Pipette 50 µL drops of SOF-BE1 with 2 mM 6-DMAP onto a 35 mm petri-plate
        i. The number of drops will depend on the number of oocytes being activated. One drop will contain between 20 and 30 oocytes.
    b. Flood plate with mineral or paraffin oil so that drops are completely covered.
    c. Place in incubator at 38.5°C with 5% CO_2 at least 2 hours prior to activation
3. Culture Drops
    a. Pipette 50 µL drops of regular SOF-BE1 onto a 35 mm petri-plate
        i. The number of drops will be the same as the number of 6-DMAP drops and contain 20 to 30 oocytes
    b. Flood plate with mineral or paraffin oil so that drops are completely covered.
    c. Place in incubator at 38.5°C with 5% CO_2 at least 2 hours prior to activation
4. SOF-BE1 washes
    a. Pipette SOF-BE1 into 2, 35 mm dish
    b. Cover each with a thin layer of mineral or paraffin oil
    c. Place in incubator at 38.5°C with 5% CO_2 at least 2 hours prior to activation

Protocol:

1. Mature oocytes in maturation media for 24 hours.
2. Denude oocytes using hyaluronidase
    a. Let hyaluronidase microcentrifuge tube thaw at room temperature. Pipette contents into a 35 mm plate containing HEPES-SOF.
    b. Using a 200 µL pipettor, pipette oocytes from maturation medium into the 35 mm plate containing HEPES with hyaluronidase.
    c. Pipette oocytes from the hyaluronidase into a 1.5 mL Eppendorf tube. Limit volume to 400 µL maximum.
    d. Vortex for 4 minutes.
    e. Pipette solution from the microcentrifuge tube into a dish of HEPES-SOF without hyaluronidase.
    f. Rinse the tube with hyaluronidase-free HEPES-SOF twice to collect any leftover oocytes.
3. Pipette from first HEPES-SOF wash into the second wash.
4. Pipette zygotes into the 35 mm plate of HEPES + 2% sucrose.
5. Select oocytes with visible polar body and dark, uniform cytoplasm.

    a. Placing selected oocytes in the third HEPES-SOF wash.
6. Move oocytes from HEPES wash into the 35 mm plate with 5 μM ionomycin.
7. Let sit for 5 min.
8. Immediately pipette oocytes into another HEPES wash
9. Send oocytes through 2 more HEPES washes
10. Pipette oocytes into the first SOF-BE1 wash.
11. Using a wiretrol or captrol, place 20 - 30 oocytes into one 50 μL droplet of 6-DMAP under oil. Repeat until all oocytes are in 6-DMAP.
12. Incubate 3 hours at 38.5°C with 5% CO_2.
13. Remove from 6-DMAP and place into a HEPES-SOF plate.
14. Send oocytes through 2 more HEPES-SOF washes and into the last SOF-BE1 wash.
15. Place 20 to 30 oocytes into each SOF-BE1 culture droplet
16. Culture at 38.5°C in 5% CO_2, 5% O_2 for development to blastocysts.

APPENDIX B

Culture Plate with Polyester Micromesh

For identification of individual zygotes during group culture

Materials

Spectra/Mesh® Woven Filters – Polyester, 300 µm opening (Repligen)

35 mm Corning™ Falcon™ Easy-Grip Tissue Culture Dishes

Needle or Awl

Tweezers

Bunsen burner

DI water in squeeze bottle

70% Ethanol in squeeze bottle

Protocol

1. Cut mesh into small squares, roughly 0.5 cm^2 or small enough to fit under a 50 µL droplet with at least 20 intact square openings
2. Place in desired location on 35 mm dish and hold in place by pressing flat with a pair of tweezers
3. Heat tip of awl in flame of the Bunsen burner until hot (about 1 minute)
4. Quickly and carefully melt one side of the square mesh to the dish while applying pressure with the tweezers
5. Reheat tip of the awl
6. Repeat melting the opposite side of the square mesh to the dish
7. Repeat until the desired number of meshes are secure.
    a. One mesh per culture droplet with enough openings to hold 20 to 30 zygotes
8. Rinse the dish thoroughly with DI water using a squeeze bottle, being sure to spray each mesh
9. Rinse the dish and lid with 70% ethanol using a squeeze bottle, being sure to rinse each mesh
10. Fill the dish completely with 70% ethanol and let sit for 30 min to 1 hour to sterilize
11. Dispose of ethanol and let dish dry in a sterile hood for several hours

12. Optional: when dry, let sit under UV light in the sterile hood for 20 to 30 minutes
13. Prepare plate for embryo culture or cover with lid and secure with parafilm for use later