Methods to enhance embryo quality and recovery rates in superovulated beef cows

by

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DEDICATION

I dedicate my thesis to my husband, Greg. His endless love, support, and encouragement has helped me get where I am today in this chapter of life. I am forever grateful for his patience with my hectic schedule and taking care of the day to day details of life that I have so often overlooked during this endeavor. I look forward to whatever journey lies ahead in life for us, knowing we will enjoy it together in love and laughter.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

AI	Artificial Insemination
AngII	Angiotensin II
AP	Anterior Pituitary
ART	Advanced Reproductive Technologies
CL	Corpus Luteum
DFA	Dominant Follicle Ablation
ET	Embryo Transfer
FSH	Follicle Stimulating Hormone
FSH-R	Follicle Stimulating Hormone Receptor
GnRH	Gonadotropin Releasing Hormone
HP	High Progesterone
HPGA	Hypothalamic Pituitary Gonadal Axis
IGF-1	Insulin-like Growth Factor – 1
IVF	*In vitro* Fertilization
LH	Luteinizing Hormone
LH-R	Luteinizing Hormone Receptor
MOET	Multiple Ovulation Embryo Transfer
P4	Progesterone
PGF	Prostaglandin $F_{2\alpha}$
TQE	Transferrable Quality Embryos

UTJ	Utero-Tubal Junction
ZP	Zona Pellucida

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ABSTRACT

Methods to enhance embryo quality and recovery rates in superovulated beef cows were investigated. In the first study, uteri from superovulated heifers on day of recovery were harvested immediately following exsanguination and sectioned into 3 segments per horn plus the oviduct (8 sections per total uterus). Each section was thoroughly flushed through individual filters, searched and evaluated. Sectioning of the uterine horns impacted location of embryos with the majority of embryos located in the tip and middle third of each horn. In comparing these two sections, a greater percentage of the embryos were in the tip than in the middle third. Interestingly, embryos were recovered in both the oviduct and the base of the uterine horns. Based on these data, at time of embryo recovery, an embryo transfer (ET) practitioner should focus their recovery efforts towards the tip and middle third of the horns and potentially incorporating the body of the uterus to be include all potential embryo locations.

In the second study, effects of endogenous progesterone (P4) concentrations on embryo production were evaluated. Beef cows were superovulated following a CIDR-based presynchronization coupled with ultrasound-guided dominant follicle ablation (DFA). Comparison of high progesterone (HP) concentrations to low progesterone (LP) concentrations was achieved by administration of prostaglandin F2α (PGF) at DFA to the LP group while HP maintained their CL during superstimulation. Superovulation protocol included decreasing dosages of follicle stimulating hormone twice daily over 4 days and timed artificial insemination performed twice (12 hours apart). A greater proportion of

quality grade 1 embryos were recovered from the LP cows compared to the HP cows, which had a greater number of quality grade 3 and 4 (degenerate) embryos. Additionally, a greater proportion of embryos recovered were developmental stage 6 and 7 in the LP cows. It should be noted, however, that more total embryos were recovered from HP than LP cows. There were no differences in total transferrable quality embryos between treatments.

A third study was designed to evaluate the effects of stage and grade of embryos on embryonic sex. *In vivo*-derived embryos from d 7 superovulated recoveries were biopsied using a micromanipulator with a microsurgical blade and the sex was determined using PCR techniques. Despite differences in developmental stage between embryos, they were not found to impact sex determination. However, differences in quality grade impacted embryonic sex determination. Specifically, a greater proportion of quality grade 2 embryos were female while a greater proportion of grade 1 embryos were males.

In application of these studies, a practitioner with a cattle producer who wants to maximize embryo output and has recipient cows available for transferring fresh embryos at time of recovery could superstimulate the donor under the influence of uninhibited P4 concentration. This would be followed with recovery on d 7 with placement of the catheter at the base of each horn for horn flushing while potentially incorporating the uterus during the recovery to perform a thorough flush of the entire uterus. Ideally, this scenario should yield a greater number of embryos, however quality may be compromised, yet could be transferred fresh into recipients. Additionally, with the potential for an increase in quality grade 2 embryos from the recovery, our data suggests the potential for

an increased number of heifer calves. On the other hand, if a seed stock producer wants to cryopreserve the best embryos from their donor, our data would suggest implementing a superovulation protocol under the influence of LP, followed by horn flushing method in which the catheter is placed at the base of each horn on d 6 -7. Our results would suggest this recovery would produce a greater proportion of freezable, grade 1 embryos which could increase the probability of producing bulls.

CHAPTER 1

INTRODUCTION

A United States farmer produces enough food to feed 155 people in today's world (2017) and yet they, along with the rest of the world, are delegated the daunting task of doubling their production to feed 9 billion people by 2050 (Schechinger, 2016; USDA, 2017). Cattle producers, in particular, face the challenges of environmental change, limited forage availability, and volatile feed costs that negatively impact their efforts to efficiently produce a high quality, reliable protein source. In lieu of this, research efforts and advancements in technology have focused on both feed and reproductive efficiency to overcome this production deficit. Recent research has enabled producers to utilize alternative feed sources and improve feed efficiency with limited acres as the urban sprawl continues to decrease land availability for livestock (Loy and Lundy, 2014). Additionally, significant progress has been made in advanced reproductive technologies (ART), allowing producers to incorporate better genetics in their herds in efforts to improve the future progeny in both maternal and carcass traits. This has resulted in cattle that are more efficient in feed conversion than ever before and yet as we progress to meet the challenges ahead, we will continue to apply ART in innovative ways to improve reproductive efficiency as well.

Advanced reproductive technologies have been incorporated in the livestock industry since the late 1800s, starting with artificial insemination (AI), and has expanded to

include embryo transfer (ET), *in vitro* fertilization and maturation (IVF), nuclear transfer (cloning), and more recently, transgenic offspring (Betteridge, 2003). Livestock producers must weigh the benefits of improving their herds' genetics with the increased cost of production associated with their use. However, the reward of genetic advancements, disease control, and increased number of embryos resulting from integrating ART into a herd often outweighs the cost (Mapletoft, 2013). As such, AI, ET and IVF have become the most commercialized and commonly used aspects of ART in today's livestock industry.

In the United States, ET was integrated in the animal agriculture sector in the 1970s. During this time, the supply of good quality continental breeds was low and the demand was high, and embryo transfer allowed producers to expedite their production (Bo and Mapletoft, 2014). Embryo transfer was initiated as an opportunity to increase the progeny of valuable cattle who were becoming a scarcity due to multiple factors at that time (Mapletoft and Hasler, 2005). In the last 40 years, advancements in technique of non-surgical recoveries and transfers, increased hormone bioavailability, and ease of superovulation protocols by manipulation of the estrous cycle have all contributed to the increasing application of ET in the cattle industry.

One aspect of ET that has had minimal investigation since the adoption of non-surgical recovery, is where the embryos are located at time of recovery. Research has been performed on embryo development in the oviduct up to the point at which the embryos enter the uterus (Barnes and Eyestone, 1990; Dorniak et al., 2013). However, after entrance into the uterus, little is known as to the location of the embryos until time of implantation around day 17. At the time of embryo recovery of superovulated donors, one

of two different nonsurgical techniques are often employed for catheter placement within the uterus to recover the embryos. A Foley catheter is either placed at the point of the internal cervical os for a body flush or manipulated up each uterine horn for individual uterine horn flushes (Goncalves et al., 1987; Hasler, 2014). Depending on the placement of the catheter in a horn flush relative to the location of the embryos, there is the potential for embryos to be passed over in the body during the recovery. Identifying embryo location within the uterus at the time of recovery to assist in determining ideal catheter placement is warranted as we strive to improve recovery rates of transferrable quality embryos (TQE).

Despite the advancements made within ET in the last several decades, there has been no significant increase in number of TQE recovered by each superovulated donor. Manipulation of the donor's estrous cycle to recruit a new follicular wave at time of protocol initiation and use of exogenous hormones resulted in improved ovarian response at time of recovery and an increased recovery rate (Nasser et al., 1993; Bo et al., 1996; Baruselli et al., 2012). Despite this, over the past 15 years, the average number of TQEs recovered remains at an average of 6.5 according to the American Embryo Transfer Association survey (AETA, 2017). Research in recent years has evaluated hormonal interactions during the superstimulation period and how these hormones may impact resulting embryo production. Progesterone (P4) not only plays a critical role following ovulation for maintaining a pregnancy and elongating the conceptus (Forde et al., 2012), it also suppresses the surge of luteinizing hormone necessary for final maturation and ovulation of dominant follicles (Hatler et al., 2008). There is conflicting research involving

the impact P4 concentrations has on the growth of the pre-ovulatory follicular wave and the quality of embryos produced during either sub-luteal or natural luteal concentrations (Hatler et al., 2008; Cerri et al., 2011; Wiltbank et al., 2014; Huang et al., 2016). Whether sub-luteal concentrations of P4 can improve the total quality of embryos produced during superovulation protocols remains undetermined.

An additional area of interest within the ARTs is embryo sexing. Since the increased application of AI and commercialization of ET in the 1970s, many cattle producers desire to know the sex of the calf prior to birth for various economic reasons. One theory suggests there is a correlation between timing of insemination relative to ovulation and the resulting sex of the progeny. However, mixed results have been demonstrated on timing of insemination relative to the onset of estrus in cattle and resultant impact of the sex of the offspring (Gutierrez-Adan et al., 1999; Rorie et al., 1999). This hypothesis has also been applied in IVF-based research in which they found if fertilization of oocytes was delayed for several hours after extrusion of first polar body, there was a greater male to female ratio (Dominko and First, 1997; Gutierrez-Adan et al., 1999). With the use of embryo micromanipulation and polymerase chain reaction (PCR), sex determination has become more feasible (Shea, 1999). While there appears to be a correlation of stage of embryo relative to sex in an IVF embryo, applying this thought process and technology to a large pool of *in vivo* derived embryos following timed insemination is another area worth further investigation.

As we strive to meet the demands of the future to provide a quality, sustainable protein source, improving the efficiency of cattle reproduction is of utmost importance.

Through various avenues of ART, we have been able to improve the genetics of each generation. Yet, as the multiple ovulation embryo transfer (MOET) technology continues to expand in its application of proliferating these superior genetics, improving TQE with each recovery will be a significant component to improving reproductive efficiency.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review encompasses bovine female reproductive physiology and advanced reproductive technologies as they relate to superovulation. More specifically, it will include ovarian cell types and the hormones that regulate the bovine estrous cycle, especially broken down into the follicular and luteal phases. Furthermore, folliculogenesis and oocyte development will be discussed, followed with fertilization and embryo development leading to embryo migration. Finally, assisted reproductive technologies of bovine, specifically regarding superovulation and embryo transfer, with a brief discussion of embryo manipulation in relations to gender determination will be presented. This review concludes with a general discussion and rationale behind the research studies presented in this thesis.

2.2 Ovarian Cell Structures

2.2.1 Overview

The primary function of the ovary, the female gonad, is to produce hormones that aid in the control of the estrous cycle and produce oocytes, the female gamete. While the ovary contains several cell types, two main cell types that contribute to specific and significant roles aiding in proper ovarian function are theca and granulosa cells. These cell

types work harmoniously to make up the physical structure of ovarian follicles and play a key role in the steroidogenic feedback loops of the hypothalamic-pituitary-gonadal axis (HPGA). These two cell types work in tandem through the 'two-cell theory', in which androgens produced by the theca cells diffuse into the granulosa cells where they are converted to estrogens (Berndtson et al., 1995; Forde et al., 2011). As follicles ovulate, the theca cells and granulosa cells transition into luteal cells, which produce progesterone (P4).

2.2.2 Theca cells

Theca cells surround the cortex of a follicle and are comprised of two distinct, separate layers termed the theca externa and theca interna layers. The theca externa layer is mainly composed of loose connective tissue and provides support for the follicle by elongated fibroblasts and collagen fibers as reviewed by Senger (2012). The theca interna lies adjacent to the basement membrane surrounding granulosa cells and proliferates as the follicle increases in size (Knight et al., 2012). This interna layer is more vascularized and contains luteinizing hormone receptors (LH-R) and produces androgens, which provide the main steroidogenic components utilized by the granulosa cells. Theca cells develop LH-Rs near the time of antrum formation of the follicle (Xu et al., 1995). The main roles of luteinizing hormone (LH) are to stimulate continual growth of dominant follicles in a low (P4) environment (Taft et al., 1996), cause ovulation of mature follicles, and stimulate P4 production following luteinization. Progesterone production by the theca cells occurs primarily from the corpus luteum (CL) following ovulation, as well as just prior to luteinization, in the pre-ovulatory stage (Richards, 1980; Skinner, 2005; Skinner et al.,

2008). Luteinizing hormone, a glycoprotein, when bound to its receptor, initiates a cascade of events that lead to androgen synthesis. Cholesterol is the precursor, utilized by the theca cells during androgen synthesis, to produce testosterone and androstenedione. Through the 'two cell' theory, these two hormones are aromatized to estradiol by the granulosa cells.

2.2.3 Granulosa cells

Granulosa cells comprise the inner most layers of the ovarian follicle and contribute to the estrous cycle by primarily producing the steroid hormone estradiol in response to follicle stimulating hormone (FSH). Follicle stimulating hormone is released from the anterior pituitary (AP) and binds to follicle stimulating hormone receptors (FSH-R) on the granulosa cells to stimulate granulosa cell hyperplasia resulting in follicular growth. The follicle continues to increase in size, forming an antrum filled with follicular fluid produced by the granulosa cells. The oocyte resides within the antrum cavity and is surrounded by special granulosa cells, cumulus cells. As the antrum expands with follicular fluid, the dimensions of the follicle correlate to the described sizes of small (<5mm), medium (5-10mm), and large (>10mm) follicles (Skinner et al., 2008).

The steroidogenic capabilities and aromatase activity of granulosa cells increases with an increase in follicular size and then decline in activity as a follicle becomes atretic (McNatty et al., 1984; Skinner and Osteen, 1988). Binding of FSH activates aromatase that enables testosterone and androstenedione, produced by theca cells and diffused to granulosa cells, to be aromatized to estradiol (Richards, 1980; Skinner et al., 2008). It

should be noted that both androgens, testosterone and androstenedione, are equipotent in their precursor abilities for estradiol production in granulosa cells (McNatty et al., 1984). Additionally, FSH stimulates production of P4 via granulosa and theca cell interaction. Fortune (1986) proved several important interactions between granulosa and theca cells in androgen synthesis during the end of the luteal phase of the estrous cycle, when estradiol has a positive feedback to the hypothalamus. First, granulosa cells improve thecal production of androstenedione by providing progestin precursors, namely pregnenolone. In return, androgens synthesized by thecal cells enhance progestin production in the granulosa cells. Granulosa cells convert androstenedione to testosterone which increases production of pregnenolone (Fortune, 1986).

In addition to estradiol production, hyperplastic granulosa cells produce a glycoprotein called inhibin. As antral follicles increase in size, inhibin production also increases, resulting in elevated concentrations within the antral fluid (Taya et al., 1996; Knight et al., 2012). One role of inhibin includes negative feedback to the AP and suppresses FSH release. Meanwhile, as inhibin rises in concert with estrogen, it amplifies LH-induced androgen production of theca cells. This results in increased follicular androgen production leading to increased estrogen following aromatization (Glister et al., 2010).

Another hormone impacting granulosa cells is insulin-like growth factor 1 (IGF-1), which has a complex role within the ovary. Regarding granulosa cells, it promotes growth during decreased FSH concentration by increasing FSH-R expression, ultimately increasing the sensitivity of these cells to FSH and LH (Spicer and Echternkamp, 1995; Mihm et al.,

1997). As FSH declines, IGF-1 increases intrafollicularly along with estradiol production, signaling selection of the dominant follicle (Mihm et al., 1997). Moreover, IGF-1 also stimulates production of P4 by both granulosa and theca cells during the luteal phase as reviewed by Spicer and Echternkamp (1995). While the exact mechanism that enhances IGF-1 production in dominant follicles remains unknown, it is a readily available biomarker produced by granulosa cells that plays an important role during dominant follicle development as well as androgen production in theca cells.

2.2.4 Luteal cells

Luteal cells develop from theca and granulosa cells that luteinize following an LH surge from the AP. As follicles become dominant and are of at least 8mm in diameter, LH-R develop on granulosa cells and increase LH-R expression with an increasing size of follicle (Xu et al., 1995; Bao and Garverick, 1998). After acquiring LH-R, granulosa cells respond to the LH surge and luteinize into large luteal cells while theca externa cells dynamically change and become small luteal cells of the CL (Priedkalns et al., 1968). This luteinization causes a decline in estrogen production, due to a reduction in hormones promoting follicle development and aromatase activity that coincidentally had previously inhibited P4 production. As the granulosa and theca cells become luteal cells, there is a transition to an increase in P4 production, with large luteal cells producing majority of the P4 concentration and the small luteal cells contribution being minimal (Skinner and Osteen, 1988; Wiltbank et al., 2014). As a CL develops, small luteal cells undergo hyperplasia, while large luteal

cells undergo hypertrophy (Priedkalns et al., 1968; Henderson and Moon, 1979; Senger, 2012).

2.3 Steroidogenesis

2.3.1 Overview

The HPGA is the complex that regulates the mechanisms associated with the estrous cycle. The hypothalamus synthesizes and secretes gonadotropin releasing hormone (GnRH) into the portal vessels that carry blood from the hypothalamus stalk to the AP, which stimulate release of FSH and LH at the AP level (Kinder et al., 1996). From the AP, these pituitary hormones travel through the blood stream to act on the ovary (gonad) and lead to a cascade of events resulting in production of the steroidal hormones, estradiol, produced by antral follicles, and P4, produced by a CL. Dependent upon the phase of the estrous cycle, these steroidal hormones can have positive or negative feedback mechanisms to the hypothalamus and/or the AP regulating secretions of GnRH, or LH and FSH, respectively to allow for proper follicular wave development and eventual ovulation. The estrous cycle can be broken down into two phases, follicular and luteal. The follicular phase, encompassing proestrus and estrus, beginning at luteolysis of the CL and lasts approximately 4-6 days. The follicular phase is primarily under the control of estradiol due to the subsequent elimination of progesterone via luteolysis of CL. The luteal phase, comprised of metestrus and diestrus stages, begins at ovulation and lasts approximately 14-18 days (Priedkalns et al., 1968; Forde et al., 2011). In contrast, the luteal phase is under the influence of P4.

2.3.2 Gonadotropin releasing hormone

The main hypothalamic hormone involved in reproduction is GnRH. Gonadotropin releasing hormone stimulates FSH and LH release into the blood stream to allow attachment to corresponding receptors on granulosa cells and theca cells, respectfully. The release of GnRH is governed by two control centers within the hypothalamus, the tonic center and surge center. The tonic center, made up of two nuclei (ventromedial nucleus and arcuate nucleus), releases GnRH in a small pulsatile or episodic fashion and maintains basal concentrations (Gorski, 1970). Tonic GnRH release is associated with small pulses of FSH and LH secretions, over days to weeks, which allows for growth and development of follicles. The tonic center is considered spontaneously rhythmic with no known stimulus to induce GnRH secretions, primarily stimulating release of FSH. In comparison to the tonic center, the surge center is composed of 3 nuclei (preoptic nucleus, anterior hypothalamic area and suprachiasmic nucleus) which is regulated by hormonal concentrations of progesterone and estradiol (Gorski, 1970). Elevated P4, during the luteal phase, provides a negative feedback to the hypothalamus, suppressing the surge center. Meanwhile following luteolysis, estradiol rises providing a positive feedback to the surge center to stimulate LH pulsations at the AP level. Because of estradiol stimulation, GnRH increases in secretions and 'primes' the AP during proestrus to increase the quantity of LH released once the GnRH surge occurs. During estrus the surge of GnRH from the surge center stimulate the increased frequency of pulsatile LH known as the pre-ovulatory surge of LH from the AP (Hansel and Convey, 1983; Senger, 2012).

2.3.3 Follicle stimulating hormone

Follicle stimulating hormone, a heterodimeric glycoprotein with an α-subunit, common to all glycoprotein hormones, and a β-subunit unique to FSH, is synthesized and secreted from the AP in a pulsatile fashion following stimulation from GnRH (Ryan et al., 1988; Weck et al., 1998). FSH stimulates follicular recruitment prior to the development of a new follicular wave. Receptors for FSH are present on granulosa cells of small (<5mm) and medium sized (5-10mm) follicles. Once bound, FSH stimulates aromatase activity which converts androgens to estradiol (Ryan et al., 1988). Following the initiation of a new follicular wave with growth of recruited follicles and a dominant follicle is selected, FSH concentrations decline to nadir levels. As follicles grow, they produce estradiol, which causes a negative feedback to the hypothalamus and AP, along with inhibin that results in suppressed FSH release from the AP. Together these two hormones suppress FSH release (Mihm et al., 1997; Beg et al., 2003).

2.3.4 Luteinizing Hormone

Luteinizing hormone, another heterodimeric glycoprotein, like FSH, but with a unique β subunit, is released from the anterior pituitary to aid dominant follicle maturation and ovulation induction. Luteinizing hormone is released in a surge of increased pulsation following a surge of GnRH during estrus or secreted in low amplitudes from the tonic center near dominant follicle deviation in the luteal phase (Ryan et al., 1988; Ginther et al., 2001b, a). Receptors for LH are located on the theca cells and pre-ovulatory granulosa

cells. There is a transient rise in LH at the time of follicle deviation (> 8.5 mm in diameter) when LH-R are active on theca cells, which aids in post-deviation growth of the dominant follicle and intrafollicular production of estradiol, IGF-1, and androstenedione (Ginther et al., 2001a, b). To aid in follicle maturation, LH stimulates theca cell production of androgens that are aromatized in the granulosa cells to estrogens (Ginther et al., 2001b). Ovulation of a follicle results from a surge of LH converting theca and granulosa cells to luteal cells, which downregulates aromatase activity leading to the transition from estradiol production to P4 production. During this transformation, cumulus cells surrounding the oocyte undergo expansion while supporting oocyte maturation as it resumes meiosis (Aparicio et al., 2011; Aardema et al., 2013).

Luteinizing hormone is regulated by P4 and estradiol. Progesterone provides a negative feedback to the hypothalamus during the luteal phase resulting in the surge center to remain quiescent and LH to remain low in pulsations from tonic center. However, there is a dose dependent relationship between LH and P4, specifically during mid-luteal phase when P4 concentrations are highest (average concentration of 5-8 ng/mL) with a low LH pulse frequency (approximately 1 pulse every 4 hours;(Rahe et al., 1980). As P4 concentrations decrease towards end of the luteal phase with concentrations around 1-2 ng/mL, LH increases frequency of pulses (20-30 pulses in 24 hours;(Rahe et al., 1980). Meanwhile, estradiol has a positive feedback to the GnRH surge center of the hypothalamus in the presence of low concentrations of P4, specifically following luteolysis of the CL. As P4 declines, estradiol stimulates the surge center to release GnRH to the AP which in turn releases a surge of LH to the granulosa and theca cells.

2.3.5 Estradiol

One of the main steroidogenic hormones produced from the ovary is estradiol, synthesized in growing follicles by the granulosa cells. Following deviation, the dominant follicle has greater concentrations of estradiol than the cohort follicles. During the luteal phase of the estrous cycle, estradiol has a negative feedback to the AP on FSH secretion. The increased production of estradiol from granulosa cells, as they proliferate, suppresses FSH from the AP and ultimately leads to atresia of subordinate follicles (Fortune et al., 2001). Estrogens contribute to follicular development by stimulating granulosa cell proliferation, thus increasing granulosa cell FSH and LH receptors and responsiveness, and ultimately resulting in heightened intrafollicular steroidogenesis from both theca and granulosa cells (Richards, 1980). The dominant follicle thrives without FSH because of the granulosa cell heightened sensitivity to FSH from estrogen production (Ginther et al., 2001a). As luteolysis occurs and P4 declines, estradiol transitions from a negative feedback to a positive feedback to the surge center of the hypothalamus and stimulates the cascade of events leading to increased LH pulsatility (Kinder et al., 1996). Estradiol concentrations decline following ovulation as the luteal phase initiates and P4 begins to increase in circulation.

2.3.6 Progesterone

Progesterone, the primary steroid hormone produced by the CL during the luteal phase of the estrous cycle, is synthesized from pregnenolone, a derivative of cholesterol.

Pregnenolone, an androstenedione precursor, is produced in both theca and granulosa cells following cytochrome P450 side-chain cleavage enzyme break down of cholesterol (Fortune, 1986). Following synthesis, pregnenolone is utilized by theca cells to produce androstenedione (Fortune, 1986; Bao and Garverick, 1998). Luteinizing hormone binding to theca cells increases conversion of pregnenolone to androstenedione and testosterone, which enhances the ability of granulosa cells to produce more pregnenolone and ultimately the conversion of androstenedione to estradiol via cytochrome P450 aromatase enzyme (Fortune, 1986). Both small and large luteal cells, formerly theca and granulosa cells, have the steroidogenic ability to produce P4 once they luteinize by having the capability to convert pregnenolone to P4 via their smooth endoplasmic reticulum as reviewed by Senger (2012).

Progesterone is necessary for ovulation of a healthy oocyte, maintenance of uterine quiescence (Ulberg et al., 1951), survivability of an embryo, and, in a non-pregnant cow, regulate timing of luteal regression (Ulberg et al., 1951; Garrett et al., 1988; Inskeep, 2004). The secretion of P4 by luteal cells increases as the CL grows and peaks in maturation in the non-pregnant female by approximately day 8-11 of the estrous cycle (Wiltbank et al., 2014). Progesterone concentration in the body is impacted by the rate of metabolism through the liver with some speculation that increased feed intake and/or increased liver blood flow decreases circulating P4 (Inskeep, 2004; Wiltbank et al., 2014).

Progesterone concentration impacts follicle development though a negative feedback to the hypothalamus on GnRH, thus keeping GnRH in low frequencies of pulses, or at a ‘tonic’ state (Kinder et al., 1996). Based on synchronization and superstimulation

protocols, P4 has been found to have a concentration dependent impact. Beg et al., (2003) reported that exogenous P4, in conjunction with endogenous P4 concentrations, decreased the LH concentrations that normally occurred at the time of follicle deviation. This decrease in LH correlated with a delay in follicle deviation as FSH levels remain higher and estradiol levels were suppressed (Beg et al., 2003). Additionally, during the luteal phase, when P4 is the highest, the largest follicle present in each wave failed to ovulate due to lack of LH in circulation which leads to those follicles becoming atretic (Inskeep, 2004). At concentrations below those produced by a CL, subluteal, LH was noted to have an increased pulsatility along with increased concentrations of estradiol compared to concentrations during the luteal phase (Hatler et al., 2008).

The luteal phase of the estrous cycle ends when luteolysis of the CL occurs and P4 concentration decreases, typically around day 16-18 of the estrous cycle. Luteolysis is induced by secretion of prostaglandin $F_{2\alpha}$ (PGF) from the uterine endometrium. Through a vascular counter-current exchange mechanism, PGF is transferred from the uterine vein, that lies in close proximity to the ovarian artery, and crosses into the ovarian artery to reach the target CL and induce luteolysis (Forde et al., 2011). This is an important anatomical relationship as PGF is highly degraded in the pulmonary system (Davis et al., 1985). The greater the concentration of PGF diffusing through the counter-current exchange, the more likely luteolysis will ensue and the follicular phase will begin.

2.4 Ovarian Follicular Development

2.4.1 Overview

The bovine estrous cycle is 18-24 days in length with an average of 21 days, and consist of the follicular and luteal phases, as previously mentioned. The reproductive phases are highly controlled by the HPGA through proper signaling of key hormones, which regulate the activity of the ovaries and the estrous cycle to stimulate folliculogenesis, oocyte development, and ovulation.

Bovine are mono-ovulatory species, however, multiple small follicles (8-41 in a recruitment) are recruited in follicular waves (2-3) throughout the luteal phase to obtain one pre-ovulatory follicle (Bao and Garverick, 1998; Adams et al., 2008). During the luteal phase, increased P4 provides negative feedback on the GnRH surge center inhibiting the dominant follicle from the first and second waves (in a 3-wave cow) and become atretic as there is not enough LH in circulation to begin final maturation. As P4 declines at the end of the luteal phase, LH increases in both pulse amplitude and frequency to stimulate ovulation of the dominant follicle (Rahe et al., 1980).

2.4.2 Oocyte development

In most domestic species, including bovine, the female is born with a set number of oocytes that were formed during fetal development. Oogenesis, development of the ovum, is initiated in the fetal ovaries around day 60-70 with primordial germ cells undergo mitosis to form oogonia and reaches maximal numbers near day 150 of gestation when mitosis ceases. During this time, primary oocytes develop from some of the oogonia

entering meiosis and arrest at prophase I, while others will develop postnatally (Ginther, 1993). It is estimated that there are about 2,100,000 to 2,700,000 primordial follicles by day 170 in gestation of a bovine fetus (Erickson, 1966a, b). However, this number is reduced to 130,000 at time of birth due to increased rate of oogonia degeneration and apoptosis. Degenerative changes that occur after meiosis usually are associated with condensation of chromatin that occurred during the first meiosis of oocytes (Erickson, 1966b). Across other domestic species, there are large variations of oocyte pool sizes that develop during fetal development or immediately after birth as in rodents (Richards, 1980; Paulini et al., 2014).

Oocyte maturation can be divided into two steps. The first step occurs during fetal development and entails the oocyte developing to prophase I of the first meiotic division, where activity is arrested (Sirard, 2001). The arrested oocytes are called primary oocytes and are surrounded by a single layer of flattened granulosa cells, which are referred to as primordial follicles (Fortune, 1994; Paulini et al., 2014). During this time, the chromatin within the oocyte reaches the dictyate phase, which corresponds to time between chromatin condensation and interphase. The second step of oocyte maturation follows stimulation from the LH surge during the follicular phase (Aardema et al., 2013). The LH surge stimulates resumption of meiosis from prophase I to arrested metaphase II with one polar body extruded, where the oocyte remains until sperm activation (Sirard, 2001).

The growth of oocytes as they prepare for the second stage of maturation occurs in conjunction with maturation of the follicle surrounding it. From the primordial to primary follicle stage, the single layer of granulosa cells become cuboidal. Transitioning to

secondary follicles is the result of increased layers of granulosa cells, stimulating oocyte growth along with the initiation of zona pellucida (ZP) formation (Fair, 2003). In terms of actual growth of the oocyte, Hyttel et al., (1997) found an intrazonal diameter of 100 µm was necessary for resumption of meiotic activity and metaphase II arrested oocytes have a diameter of 110 µm, equivalent to a 3-mm primary follicle (Hyttel et al., 1997; Aerts and Bols, 2010). The growth of oocytes varies across species with smaller species, such as rodents, having a more rapid growth rate than larger mammals. The ZP develops via protein secretions between the oocyte and the surrounding granulosa cells (Paulini et al., 2014), along with other key components necessary for fertilization (Sirard, 2001). In the bovine, the ZP consists of several glycoproteins, which all play a role in sperm binding activity and fertilization (Suzuki et al., 2015). From the secondary stage of development, follicles develop into tertiary, with small and then large antrum cavities, and finally mature antral follicles or 'Graafian follicles' (Fair, 2003; Aerts and Bols, 2010) .

In summary, the oocyte development and maturation in bovine is an intricate process that spans from embryonic development to mature, adulthood and relies on the surrounding ovarian environment to activate further developmental progress.

2.4.3 Folliculogenesis

In the late 1980s, researchers observed both hormonal concentrations and ultrasonagraphic evaluations of the ovaries throughout interovulatory intervals. In general, cattle have either 2 or 3 follicular waves in an estrous cycle, but the reason for differences cannot be fully explained. *Bos indicus* breeds are known to predominantly have 3 waves

per cycle, with a range of 2-4; meanwhile *Bos taurus* breeds generally have 2 waves with a similar range (Sirois and Fortune, 1988; Sartori and Barros, 2011). In 2-wave cattle, the average estrous cycle is shorter than compared to 3-wave bovine, 20 and 23 days, respectively (Ginther et al., 1989b). With each follicular wave, a group of cohort follicles are recruited. The number of follicles recruited are often breed dependent, as evident by *Bos indicus* breeds averaging 41.5 follicles recruited compared to 24 follicles in *Bos taurus* breeds. Furthermore, the follicle diameter at time of ovulation and subsequent CL varies between breeds, with *Bos indicus* ovulating smaller follicles, 10-12 mm, resulting in smaller CLs than *Bos taurus* ovulating 16-20mm follicles (Sirois and Fortune, 1988; Sartori and Barros, 2011).

The first follicular wave, the anovulatory wave, begins at or within a day of ovulation. The second wave, in a 2-wave cycle, the ovulatory wave, begins around day 9, with ovulation occuring approximately on day 20 (Ginther et al., 1989a). Dominant follicle selection in each wave is not clearly understood; however, Ginther et al., (1989a) found that once a follicle reached 7 mm, the rate of growth was faster than the subordinant follicles, often to an average size of 15.8 mm. Most of the subordinant follicles, regardless of wave, very rarely exceed 6 mm before they become atretic (Ginther et al., 1989a). A 3-wave cycle is similar to a 2-wave, with the addition of another anovulatory wave prior to the ovulatory wave. The surges of FSH prior to the detection of a new follicular wave occur at day 0 (ovulation), and approximately on days 8 and 14, with ovulation occuring approximately on day 23 (Adams et al., 1992). Near the time of follicle deviation, there is a transient increase in circulating LH. Luteinizing hormone receptors are primarily on theca

interna cells of small antral follicles until the follicle reaches the size of 9 mm or greater in *Bos taurus* and 5-6 mm in *Bos indicus*, at which point granulosa cells develop LH receptors and are responsive to FSH and LH (Xu et al., 1995; Barros et al., 2010). Once the dominant follicle is selected and continues to grow, recruitment of other follicles is inhibited (Sirois and Fortune, 1990).

Following deviation and growth of the dominant follicle in the ovulatory wave of the cycle, regression of the CL and declining concentration of P4 should ensue. During this time, the AP increases both pulse frequency and amplitude of LH following P4 declining and estradiol concentration rising. Luteinizing hormone causes maturation of the dominant follicle and initiates transformation of theca and granulosa cells to luteal cells which occurs with ovulation.

2.4.4 Ovulation

Ovulation of the female gamete occurs at the end of the follicular phase of the estrous cycle, following a decline in P4 and a surge of LH. As previously mentioned, estradiol concentrations increase inversely of P4 concentrations and have a positive feedback to the surge center of the hypothalamus, stimulating a cascade of events leading to increased release of LH. Additionally, estradiol primes the granulosa cells of the dominant follicle by promoting gap junction formation, increased cell mitosis, and increased receptor expression for FSH and LH as reviewed by (Geary et al., 2013). Another important factor in the ovulation process is angiotensin II (AngII), which was found to have increased levels in the follicular fluid following the LH surge (Acosta et al., 2000). While the

exact role of AngII is not completely understood, without AngII involved, it has been observed that ovulation does not ensue or is significantly delayed (Ferreira et al., 2007).

In terms of the physical changes occurring during this time, blood vessels associated with the theca interna layer become congested as ovulation nears (Priedkalns et al., 1968). Concurrently, the basement membrane separating theca and granulosa cells disintegrates in select sections. During ovulation, as the follicular fluid dissipates, the follicle collapses and allows the theca interna and granulosa cells to blend as they undergo luteinization to form a CL, as reviewed by Senger (2012). Theca and granulosa cells luteinize to form the small and large luteal cells, respectively, meanwhile the steroidogenic production transitions to P4 production and the oocyte is released to the oviduct for potential fertilization.

2.4.5 Fertilization

Following ovulation, the oocyte passes along the fimbria of the infundibulum to the ampulla portion of the oviduct, where the cumulus-oocyte complex firmly attaches (Kolle et al., 2009). Cumulus cells surrounding the oocyte start to break away from the oocyte, thereby exposing the ZP to oviductal fluid and aiding in preparation for fertilization (Coy et al., 2008). As the cumulus cells degrade, the oocyte progresses through the ampulla with assistance from ciliated cells, while under the influence of P4 (Kolle et al., 2009).

Sperm develop ‘reservoirs’ in primarily two locations, the cervix and the utero-tubular junction (UTJ) following insemination. Sperm migrate from the cervix to the UTJ at varying speeds and attach to the epithelium of the isthmus. During migration through the

uterus, sperm undergo capacitation to become competent for fertilization. Capacitation involves stripping off seminal plasma proteins that were coated on the sperm heads during interaction with seminal plasma at time of ejaculation. Capacitation is completed in the oviduct and allows specific zona binding proteins to be exposed on the sperm membrane. Sperm attach or 'dock' to the epithelium of the isthmus upon arrival, which triggers a signal within the sperm to promote viability (Kolle et al., 2009; Senger, 2012).

Once the oocyte enters the ampulla, the sperm, which have developed a reservoir at the caudal aspect of the isthmus, become hyper-activated and are released to continue transport to the ampulla (Miller, 2015). The sperm migrate against the beating cilia with the assistance of smooth muscle contractions of the oviduct. Contractility of the oviduct is positively influenced by estrogen and PGF, meanwhile P4 leads to relaxation of the oviductal smooth muscle resulting in decreased speed of transportation (Kolle et al., 2009). Capacitated sperm enter the remaining cumulus cell layers and bind to the ZP. Binding of sperm to ZP is enhanced when glycoproteins ZP3 complexes with ZP4 (Suzuki et al., 2015). Upon fusion of the sperm to the ZP, an acrosomal reaction occurs that releases various enzymes which enhances binding and allows the sperm to penetrate the ZP (Hyttel et al., 1990; Senger, 2012). Following penetration of the ZP, the sperm binds and fuses with the ovum plasma membrane. Cortical granules within the peripheral cytoplasm of the oocyte are released at this time and lead to a cortical reaction which includes ZP hardening along with destroying sperm receptors in the ZP that blocks polyspermy from occurring (Florman and Ducibella, 2006).

2.5 Embryology

2.5.1 Embryo development and migration

Following fertilization of the ovum by penetration of the sperm, embryonic development begins. Fusion of the gametes' pronuclei activates the second meiotic division, generally within 4 hours after fertilization. This activation coincides with the extrusion of the second polar body (Hyttel et al., 1988). Within 24 hours after fertilization, a 2-cell zygote forms (Hyttel et al., 1990). Cleavage to 4-cell and 8-cell stages generally occur approximately 48 hours and 60 hours post-fertilization, respectively, and the 16-cell stage occurs 21-30 hours thereafter. The zygote develops to a 16-cell structure within the oviduct over about four days followed with migration into the uterus around day 5 in cattle (Sirard and Lambert, 1985; Barnes and Eyestone, 1990; Bauersachs and Wolf, 2015). Following a 16-cell structure, the nomenclature changes to reflect the visual changes in size and conformation that occur. The following development results in individual cells becoming indistinguishable as cellular compaction occurs. The following stage is termed a morula, at which time the embryo usually migrates into the uterus. While this is the process that occurs in cattle, migration and eventual implantation is specie specific (Rizos et al., 2002; Dorniak et al., 2013).

With migration into the uterus, the compact morula develops into a blastocyst, which contains a blastocoel, fluid filled cavity, separating an inner cell mass and a monolayer of trophectoderm, which will become the embryo proper and the placenta, respectively. During this growth phase, the blastocyst hatches from the ZP around days 8-10. Elongation that ensues involves growing into an ovoid structure followed by a

filamentous shape by day 15 and occupies most of the uterine horn ipsilateral to the CL (Dorniak et al., 2013; Spencer et al., 2017). With growth and elongation of the embryo and associated extraembryonic membranes, the embryo is termed a conceptus (Dorniak et al., 2013; Bauersachs and Wolf, 2015).

Near time of implantation (day 19), after approximately 2 weeks of 'free-life' within the uterus, the embryo, more specifically the trophectoderm layer, begins to form apposition with the endometrium and secretes a signal, interferon tau, which binds to the endometrium and inhibits endometrial PGF secretions, thus allowing for P4 production and maintenance of the CL (Sandra et al., 2017; Spencer et al., 2017). Interferon tau is the maternal recognition of pregnancy for bovine and ovine. In other species, there are similar signals known to be released by the embryo near implantation for recognition and maintenance of pregnancy, however in many species this phenomenon is unknown. Aside from knowledge that the conceptus undergoes growth and reside on the original side of ovulation, there is little known about location within the uterus during the growth phase, which is often when recovery and transfer of embryos occurs.

2.6 Assisted Reproductive Technologies

2.6.1 Overview

Assisted reproductive technologies (ART) are continuing to be integrated into more livestock operations as producers take their best animals, often based on genomics or personal preferences, to increase the number of elite offspring from particular matings. Two of the main ART utilized currently include multiple ovulation embryo transfer (MOET)

and artificial insemination (AI) with sex-sorted or conventional, unaltered semen. Additionally, the technology is available to trans-vaginally aspirate dominant follicles (DFA) to reset the follicular waves for MOET or aspirate oocytes for *in vitro* fertilization (IVF). The technologies to be discussed in further detail are MOET and DFA.

2.6.2 History

The first documentation of successful embryo transfer (ET) occurred in 1890 when Walter Heape performed a transfer of two Angora rabbit embryos into a Belgian doe and resulted in live Angora and Belgian babies (Betteridge, 2003). There were no more recorded reports of successful transfers until the 1920s which again involved rabbits. In the 1930s and 1940s, ET expanded to sheep and goats. The first report of successful embryo transfer in cattle occurred in the late 1940s. A veterinarian surgeon by the name of L.E.A. Rowson became heavily involved in the first artificial insemination center for cattle in Cambridge, England, and would later become one of the founding fathers of ET in livestock and a founding president of the International Embryo Transfer Society, an organization developed to allow for open discussion across the world in regards to ET (Mapletoft, 2013). While Europe was working through difficulties and challenges with ET, the United States was also developing the technology in the late 1940s (Betteridge, 2003), and in 1951, the first ET calf was born in Wisconsin following surgical transfer (Betteridge, 1981).

In the 1970s, the ET industry became commercialized in North America with private veterinary practitioners and small companies working together to develop the technology

and techniques that are currently utilized today. Through the use of ET, producers have been able to make genetic improvements, control disease spread, and partake in exporting and/or importing embryos (Mapletoft, 2013). While techniques for recovering and transferring embryos have also improved since commercialization, recovery rates of transferrable quality embryos (TQE) have not seen as much improvements in the last 20 years.

2.6.3 Embryo recovery and transfer

Embryo transfer begins with the selection of the donor, that has more desirable genetics or physical traits compared to herdmates. With bovine, once the donor is selecetd, she is typically subjected to a superstimulation protocol. In other species, such as equine, the donor mare is not superovulated as it is difficult to achieve desirable results (Betteridge, 2003). In bovine, single embryo collections can be conducted following natural estrus with AI or natural mating, however it is not commonly practiced due to the availability of exogenous hormones, customized protocols, and success of superovulation in cattle (Mapletoft and Hasler, 2006). Following a superstimulation protocol, to be discussed in the following section, the donor is inseminated either by AI or natural service between 1 and 3 times on 12-hour intervals. Embryo recovery is then performed 6-7 days after the initial breeding by instilling a balanced media with a surfactant-type component into the donor's uterus which is then filtered to capture the embryos. Following recovery of the embryos, they are evaluated and graded based on stage of development and quality according to International Embryo Transfer Society guidelines.

Knowing the normal progression of embryo development and migration is important when conducting an embryo recovery to be able to collect embryos of ideal developmental stages for either transferring or cryopreserving. The time to recovery should be at least 5 days post fertilization for the embryos to be in the uterus and capable of being recovered. Additionally, it has been shown the more developed the embryo is in terms of a blastocoel cavity, the more challenging it is for cryopreserving (Ochota et al., 2017). Therefore, embryo recoveries are usually conducted between day 6-8 post insemination, when the embryos should be at the most ideal stage for transferring or cryopreserving (Lawson et al., 1975; Kocyigit and Cevik, 2016).

Recovery technique has improved since the commercialization of ET. In the early 1970s, embryo recoveries were performed under general anesthesia with a mid-ventral surgical approach, which limited the use to those with adequate facilities and capabilities to accommodate recumbant bovine (Mapletoft, 2013). By the late 1970s, non-surgical approach to recovery of embryos became a viable, successful procedure. Transferring of embryos followed the non-surgical approach shortly after successful recoveries were performed (Rowe et al., 1980). Research attempted to localize embryos within the uterus at recovery to assist in the pioneering of catheter placement of non-surgical recoveries (Newcomb et al., 1976). Newcomb et al., (1976) ligated the uterus 10 cm from the utero-tubal junction and then flushed the base of the horns followed by each of the ligated tips. Through segmenting each horn into two parts, a greater number of the embryos were located in each of the tips of the horns compared to the base of the horns. They also found embryos had migrated to the uterine body section at time of recovery. Additionally,

embryos were still located in the oviduct on d 6-8, albeit significantly more on d 6 than d 7 or 8 recoveries (Newcomb et al., 1976). Based on these findings, from a non-surgical approach, the focus of the recovery should be towards the tip of the horns to improve recovery rates. An alternative approach compared recovery rates of non-surgical technique to *in vitro* technique from excised uteri of slaughtered cows. Recovery rates from the excised uteri were higher than the non-surgical approach, however this technique is not generally practiced due to sacrificing the donor's uterus (Deleeuw, 1992). Despite the morbidness of an excised uterus recovery, if embryos of no signficant genetic value were required for demonstrations or teaching, this would be an option with the potential for increased recovery rates.

Progress continued in the area of recovery techniques in the early 1980s, with the adoption of the more efficient, less invasive, nonsurgical technique (Shelton et al., 1979; Goncalves et al., 1987). With the advent of non-surgical techniques, ET practitioners could take their services to the farm, which lead to a drastic increase in demand (Hasler, 1992). There are two techniques utilized for catheter placement in the uterus to recover embryos. One technique executed is a 'body flush', which entails placement of the catheter at the level of the internal cervical os and flushing media through both horns at the same time (Hasler, 2014). With this approach, both horns can be flushed simultaneously as well as the body. The other technique employed is a 'horn flush', in which the catheter is placed in each uterine horn of the uterus, about the level of the external bifurcation or as far cranial as it will go (Rowe et al., 1980). With this technique, the recovery media is localized to the cranial aspect of the horn where the embryos initially enter the uterus and are potentially

located, if the embryos have not migrated towards the uterine body (Greve et al., 1977). As mentioned previously, migration of embryos from time of entrance into the uterus until implantation time remains unknown. With superovulation, the assumption is made that migration of the embryos into the uterus occurs over an extended period, consequently some embryos may enter in the uterus even a day earlier pending ovulation timing and migrate farther down the uterus. Therefore, there is some debate about these two placements. Potentially, "horn flushing" may bypass embryos pending location of migration into or near the uterine body and "body flushing" may not adequately distend each horn to recover all embryos that potentially may be localized towards the uterine tips. Embryo migration has been evaluated minimally but has not been applied to practical approaches based on nonsurgical recoveries routinely done today. While limited research on this topic was done in the 1970s (Newcomb et al., 1976; Greve et al., 1977), there is still much to be learned.

2.6.4 Superovulation

Follicle stimulating hormone is essential to stimulate follicular recruitment (Adams et al., 1992), therefore, it is the foundation for superstimulation of the estrous cycle by adminstering exogenously. Important factors of exogenous gonadotropins that allow for diversification of protocols include: type and concentration of gonadotropin administered, dosage, administration interval, and timing of initial treatment (Bo and Mapletoft, 2014). Follicle stimulating hormone preparations used in cattle are most commonly derived from porcine pituitary extracts, due to a risk of transmitting prion disease with bovine pituitary

extract. With the extracted FSH preparations available, there are differences in purity of FSH, since it is sourced from the AP where both FSH and LH are secreted. Depending on the preparation, some pharmaceutical FSH products are more contaminanted than others with LH (Bo and Mapletoft, 2014).

While there is no 'one size fits all' superovulation protocol, as evident by the lack of progress made in increasing TQE recovered in recent years, there are several key components commonly applied (Mapletoft and Bo, 2013). First, initiating exogenous FSH in conjunction with endogenous FSH release has been shown to have a positive effect on the superstimulation response and led to an increased recovery rate of embryos (Nasser et al., 1993). Second, administering FSH over the course of several days resulted in delayed dominant follicle divergence, enabling more of recruited follicles to continue to grow and acquire LH receptors, without normal follicle selection occurring (Adams et al., 1993). Third, the length of treatment, days of FSH administration, has been researched and shown that longer days of exposure to FSH, 7 vs. 4, resulted in an increase number of embryos recovered without compromise to quality. The prolonged exposure to FSH may have allowed for an increase in number of follicles able to mature (Dias et al., 2013). Lastly, with the variations in purity of FSH productions, those that have a higher ratio of contamination with LH are sometimes incorporated in the second half of stimulation protocols to increase LH's availability to mature follicles. Applying these described components of FSH with the other key hormones of the estrous cycle in correct synchrony are capable of achieving increased superovulation results.

2.6.4.1 Follicular wave emergence

It has been thoroughly studied and repetitively shown that initiating superovulation protocols concurrent with follicular wave emergence improves embryo recovery results (Nasser et al., 1993; Bo et al., 1995; Bo et al., 2006; Bo et al., 2012). As previously mentioned, a new follicular wave is initiated following a dominant follicle ovulating or becoming atretic. The original criteria for initiating superovulation treatment coincided with the donors' anticipated second follicular wave of the estrous cycle, 8-12 days after estrus (Ginther et al., 1989b; Bo and Mapletoft, 2014). These criteria were mainly due to lack of exogenous hormones or technology capable of manipulating the estrous cycle. Through several different techniques, manipulation of the estrous cycle can initiate a new follicular wave emergence. Bo et al., (1995) investigated several ways to remove the dominant follicle and allow for a new wave emergence. These included ultrasound-guided follicle ablation (DFA) of follicles greater than 5 mm; hormonal therapy to luteinize or cause atresia of present follicles with human chorion gonadotropin or GnRH analogs; or administering progesterone and estradiol valerate to reduce follicular size. All of these methods were investigated with ultrasound monitoring and found to have increased follicular recruitment at various times following the treatments. (Bo et al., 1995)

Research has been performed on DFA to synchronize a new follicular wave for either artificial insemination or superovulation protocols (Bergfelt et al., 1994; Bergfelt et al., 1997; Baracaldo et al., 2000). Bergfelt et al., (1994) found an increase in FSH by the first day following ablation and an increase in follicles recruited on the second day following

DFA. Similar results have been obtained in other studies with a new follicular wave emergence within 2 days following the procedure of removing follicles greater than 5 mm in diameter. By removing the follicles, the negative feedback of estradiol and inhibin produced by the follicles to the AP is removed, thus enabling a release of FSH for a new follicular recruitment (Bergfelt et al., 1997; Baracaldo et al., 2000). This has proven to be a reliable method with repeatable results of initiating a new follicular wave and recovering adequate number of TQE.

Exogenous GnRH administration has also been used to manipulate the estrous cycle to induce a new follicular wave. After exogenous GnRH administration, a dominant follicle present should ovulate and/or luteinize and induce a new follicular wave to begin approximately 2 days later (Mapletoft and Bo, 2013). If a dominant follicle is not present with LH receptors, the GnRH will not eliminate the negative feedback of estradiol and inhibin which may influence protocol response. It has been reported, GnRH administration will induce ovulation 66 percent of the time in suckled beef cows (Geary et al., 2000). Therefore, accuracy and consistency of this treatment is less than the other forms of manipulation discussed due to success occurring only if ovulation or luteinization transpires (Martinez et al., 1999). This treatment is often coupled with a P4 device inserted 2 or 3 days prior to GnRH to better ensure a dominant follicle presence and receptivity to the GnRH (Mapletoft and Bo, 2013).

An additional option that is illegal in some countries including the United States, but has shown success for initiating a new follicular wave is the combination of P4 and estradiol -17β. Estradiol-17β overrides the HPGA and suppresses all growth on the ovaries,

inducing complete follicular atresia. With the administration of estradiol along with P4, it takes approximately 4 days to develop another FSH surge and subsequent follicular wave emergence. Initiating exogenous FSH at day 4 following this treatment resulted in superovulatory responses similar to protocols that began at the start of the second follicular wave (Bo et al., 1995, 1996). These various manipulations to the estrous cycle allowing a new follicular wave to emerge in synchrony with superstimulation have all shown successful results with similar numbers of total quality embryos (TQE) at recovery (Mapletoft and Bo, 2013).

2.6.4.2 Progesterone influence

While emergence of a new follicular wave at initiation of superstimulation is a key factor to improving recovery results, the influence of hormones in circulation also play an important role in the resulting embryos. Progesterone, as previously described, has a significant impact on the fate of follicles developing during the luteal phase of the estrous cycle. In looking simply at differing number of waves in an estrous cycle, follicles at all stages are exposed to P4 for different lengths of time. Length of follicle exposure to P4 relative to the number of follicular waves has been investigated to evaluate impact on oocyte competence. In two different studies, higher pregnancy rates were noted in cows with three follicular waves compared to two waves (Ahmad et al., 1997; Townson et al., 2002). Based on these studies, the increased length of exposure to progesterone can negatively impact oocyte competency. With P4 present, estradiol concentrations will remain low and LH will be suppressed, thus delaying maturation and ovulation of dominant follicles. Three wave cows develop and regress follicles approximately 1-3 days quicker

than two wave cows. The shorter exposure time to P4, due to luteolysis, during the ovulatory wave of 3 wave cows, results in an increased length of exposure time to increasing concentration of estradiol, which will stimulate surge of LH to initiate maturation of the follicle. Meanwhile, an increased time of maturation leads to increased diameter of the dominant follicle, which has been positively correlated with improved oocyte quality and ovulation (Baruselli et al., 2012).

Aside from length of exposure time to P4, follicle development and maturation is also impacted by concentration of P4. Research has shown the impact of P4 is concentration dependent with increased concentrations resulting in significant suppression of even the tonic pulses of LH, while decreased concentrations, subluteal levels, results in increased estradiol and LH pulsatility in the face of P4 (Bergfeld et al., 1995; Hatler et al., 2008). Subluteal concentrations of P4 are achieved by removing the endogenous source and relying on only an exogenous source, such as a controlled intravaginal release device (CIDR). Long et al., (2008) found in ovariectomized cows that CIDRs initially provided 3.6 – 4.2 ng/ml P4 which declined over 7 days to 1.2-1.7 ng/ml. In many synchronization protocols, CIDRs are commonly used during the synchronization period to provide 'subluteal' P4 concentration and control estrus timing. In application of subluteal concentrations to AI protocols, several studies found increased dominant follicle size and increased ovulation rates when the only P4 source was a CIDR, compared to counterparts that had a CL and CIDR present during the synchronization period (Carvalho et al., 2008; Cerri et al., 2011). Pegorer et al., (2010) found similar results with increased dominant follicle diameters in the subluteal P4 cows but failed to find any improvements in ovulation

rates or subsequent pregnancies in this group. The increased follicle size and ovulations in subluteal P4 is the result of increased estradiol secretions and LH pulsations over a longer timespan with less of a negative feedback from P4. The improved ovulation rate found in several of these studies can be attributed to the lengthened maturation of the follicle and increased follicle size which are both associated with increasing ovulation rates.

While the application of subluteal P4 to MOET, is relatively new, the studies conducted have resulted in conflicting data. EL-Sherry et al., (2010) compared the number and size of follicles developed in cows that had a CL growing verses cows with lysed CLs and DFA performed prior to FSH stimulation (subluteal group). Results showed a significant increase in follicles developed at the end of the FSH treatment as well as increased estradiol concentrations with the subluteal cows. Other research investigated superstimulation during the first follicular wave of the estrous cycle, when the CL is developing and increasing P4 production, thus creating its own subluteal P4 concentrations. Both Nasser et al., (2011) and Rivera et al., (2011), found similar results with exogenous P4 having beneficial effects on embryo quality during FSH treatments compared to the cows stimulated in the first follicular wave without any exogenous P4. Interestingly, previous superovulation research recommends initiating FSH treatment at the start of the second follicular wave versus the first due to a developed CL providing consistent, steady P4 concentrations during the superstimulation protocol that resulted in more consistent and improved recovery results. With that in mind, the results from these latter studies, suggesting exogenous P4 improved embryo quality during superstimulation of the first follicular wave, supersede the developing CL and naturally low P4

concentrations that occur during the early luteal phase. These variabilities across studies imply that further research is warranted to determine the impact subluteal P4 has on TQE in MOET programs.

2.6.5 Embryo manipulation

Technology advancements in reproduction have enabled genetic testing at the embryonic development stages prior to transferring or cryopreserving embryos. The capability of micromanipulation to biopsy embryos has allowed for testing of sex, genomics, and genetic defects (Mapletoft, 2013). While only a minimal amount of embryo is required for analysis, micromanipulation involves a high level of skill as the procedure disrupts the ZP integrity, decreasing viability of the embryo (Mapletoft and Hasler, 2005). Biopsy involves removing 10-20% (approximately 15 cells) of the inner cell mass using a microblade or aspirating individual blastomeres with an aspiration pipette from a young embryo with degenerative or loose cells within the perivitelline space (Shea, 1999; Machaty et al., 2012). Many producers desire to know the sex of the embryos prior to being transferred for economic purpose of managing future resources or preparing for potential retention of the calf once born (Shea, 1999). Sex determination tests have substantially improved over the years. Initially karyotyping was performed, which took one week to achieve results (Avery et al., 1991). In today's world, DNA amplification utilizing polymerase chain reaction (PCR) which can be performed in a couple of hours and what is now commonly used (Thibier and Nibart, 1995). Polymerase chain reaction technology allows practitioners to perform this procedure in designated labs with the proper

equipment. Even though PCR is very accurate (>90%), there are errors that can occur due to contamination of biopsy, insufficient DNA, and failure of reagent activity (Shea, 1999). Some of the other diagnostic testing, including genomic testing, requires the biopsies to be sent to designated labs.

2.6.5.1 Sex determination

Fetal sex is determined at time of fertilization with the female gamete providing an X-chromosome and the male gamete providing either an X- or Y-chromosome. At the present time there is no known predilection for either X or Y bearing spermatozoa to be superior to the other in terms of fertilizing ability (Dominko and First, 1997). Knowing that bulls are more likely to be associated with dystocia, cattle producers can utilize the sex of the offspring to make management decisions at time of parturition (Morris et al., 1986; Dematawewa and Berger, 1997). Additionally, this information can be applied economically in respects to marketing the future offspring (Kaimio et al., 2013; Mikkola et al., 2015).

There is a wide-based theory that breeding early in estrus leads to increase in female offspring while breeding later in estrus results in more male offspring (Moor et al., 1981; Dominko and First, 1997). Support for this theory has been in several *in vitro* fertilization studies. Gutierrez –Adan et al., (1999) and Dominko and First, (1996) performed studies with fertilization relative to maturation of *in vitro* derived oocytes. The results showed an increase of female embryos from the early fertilization and male embryos representing a greater proportion of the delayed fertilization (Gutierrez-Adan et al., 1999). In application to AI, staggering AI of synchronized cattle by several hours in one

study failed to find a significant difference in the sex ratio of the calves born (Rorie et al., 1999).

There appears to be several factors contributing to this theory. First, it has been shown that the sex of the embryo plays a role in developmental speed, regardless of time of insemination. Male embryos are fast-cleaving in their development, while female embryos are considered slow-cleaving relative to their counterparts (Xu et al., 1992). Based on this notion, at the time of embryo recovery male embryos are more likely to be larger and further in developmental stage than the female embryos in the same recovery (Dominko and First, 1997; Gutierrez-Adan et al., 1999; Jimenez et al., 2003). In a study looking at 235 frozen/thawed day 7 IVF embryos, there was no significant difference in sex ratios with an overall 1:1 sex ratio (Carvalho et al., 1996). However, when evaluating the data by stage of embryo in relation to sex, there were significant differences noted. In the expanded blastocysts and hatched blastocysts stages, males were 68% and 100%, respectfully. Meanwhile, in the morula population, only 24% were males (Carvalho et al., 1996). However, in a 6-year retrospective study of IVF embryos, Shea (1999) failed to find evidence of embryo stage affecting sex. While quality was found to influence sex, the results were not significant enough to base embryo selection off of in future recoveries (Shea, 1999).

In reviewing all these studies, there is inconsistent evidence of different developmental rates of male and female zygotes by the different stages of embryonic development recorded. Additionally, the impact of timing of breeding relative to ovulation has conflicting evidence about its impact on gender of the offspring. Further investigation

into both timing of breeding and developmental stage and their relationship to embryo sex are warranted as it relates to MOET programs. Finding a correlation between the stage or grade of embryo at recovery and its gender could potentially change the way practitioners and producers evaluate their embryos.

2.6.6 Conclusion

A more indepth knowledge of the female bovine reproductive physiology has led to integration of ART in the livestock industry and an increased number of offspring from genetically superior animals. There are many important factors involved for a normal estrous cycle, under the control of the HPGA, to occur including ovarian function of recruitment and development of follicles, along with maturation and ovulation of dominant follicles. Assisted reproductive technologies enable manipulation of any of those factors mentioned to improve genetic progress. Embryo transfer, in particular, has been a growing branch of ART since the 1970s. And while the application of MOET continues to grow in the cattle industry, the resulting TQE production per superovulation event has remained relatively stagnant. Several components of MOET have the potential to impact the results which have not been fully investigated including: hormones administered during the protocol, individual donor differences, and potentially techniques of recovering embryos. While manipulation of the estrous cycle to initiate follicular wave emergence has been well documented with repeatable results, the influence of subluteal P4 concentrations during MOET have not. In regards to recovery technique, catheter placement is generally based the practitioner's preference, at least until further research provides incite into location of

embryos at that time of recovery. Lastly, additional technology enables embryos produced both *in vivo* and *in vitro* methods to be manipulated for sex determination and can provide beneficial information to producers.

2.7 Statement of Problem

Since the 1970s, MOET research has been conducted to simplify protocols while achieving acceptable production results (Mapletoft et al., 2015). With the advancements of technology, the bovine estrous cycle can be manipulated or altered to be more applicable for on-farm ART to be performed. Non-surgical embryo recoveries are performed everyday across the world, however there has been limited research regarding embryo location at the time of recovery. There are two general techniques to catheter placement for embryo recoveries, either a uterine body flush or individual horn flush. A better understanding of where exactly the embryos are located at the time of recovery would help determine which technique is more advantageous in recovering embryos. In regard to increasing TQE recovered, more recent superstimulation studies have focused on P4 and the impact various P4 concentrations have on the quality of embryos recovered. Subluteal P4 concentrations have been reported to allow estradiol to increase in circulating concentrations and increase LH pulsatility prior to luteolysis, resulting in improved dominant follicle growth and in some reports improved ovulation rates. However, subluteal P4 concentrations impact on embryos produced during superovulation has produced conflicting results. Further research into this area is warranted as veterinarians

strive to improve recovery results. Finally, another area of ART that cattle producers often want to know is sex determination of embryos. Despite theories that time of insemination relative to oocyte maturation impacts the sex of the offspring, research in both *in vitro* and *in vivo* studies have provided inconsistent results. Most of the previous studies utilized *in vitro* models and have shown some relationships between development stage and sex; however, determining if there is any association of development stage and/or quality at time of *in vivo* embryo recovery to sex remains undetermined. In conclusion, efforts to improve embryo recovery technique and results, along with any potential relationship between *in vivo* recovered embryos and their sex have resulted in minimal improvements since the 1970s and are still areas for further research.

The goal of the studies conducted in this thesis was to determine methods to enhance the quality and recovery rates in superovulated beef cows. Given the timeless debate on recovery technique, we attempted to determine migration of embryos at the time of recovery within segments of the uterine horns of superovulated heifers. Another area that was focused on included the impact P4 has during superstimulation and the effects of recovery rate and quality of embryos developed under the influence of increased P4 or sub-luteal concentrations. With the embryos recovered during the previously mentioned study, we performed PCR on biopsies from all transferrable embryos to determine if there was any correlation with stage or quality and gender.

CHAPTER 3

LOCALIZATION OF BOVINE EMBRYOS IN THE UTERUS AT TIME OF RECOVERY FOLLOWING SUPERSTIMULATION IN BEEF HEIFERS

3.1 Abstract

Since the advent of non-surgical embryo recoveries, there has always been a question as to the exact location of bovine embryos at the time of recovery. Despite thousands of embryo recoveries performed each year, there is little research describing the location of embryos on d 6-7 post ovulation following a superstimulation protocol. The objective was to study the location of embryos within the uterus at the time of a recovery following superovulation. Non-pregnant, yearling beef heifers (n=12; 577 ± 31 kg) were divided into three replicates and superovulated using a timed, 17-d, CIDR-based protocol consisting of GnRH and PGF and decreasing total dosage of 240mg FSH administered twice daily for 4 days. Heifers were artificially inseminated (AI) twice (12 hours apart) based on a timed-AI protocol using frozen-thawed semen from a single bull collection. At 6.5 d after first AI, heifers were harvested at a commercial abattoir. Immediately following exsanguination, the reproductive tract was removed from the carcass and uterine tracts were measured and sectioned into 4 segments per horn consisting of the oviduct and 3 equidistant segments of the horn with the body split in half between the last sections of each horn (8 total segments per tract). Each section was isolated in a sealed bag and preserved in commercial flush media, flushed through individual filters, and evaluated for

total embryos in each section. Ovaries were evaluated for quantity of corpora lutea (CL). Data were analyzed using the MIXED procedures of SAS, with results of each horn individually evaluated and then combined to evaluate the sections of both uterine horns together. Not surprisingly, results indicate that the total recoveries in each section of the uterus differed in the number of embryos recovered ($P < 0.0001$; Table 3.1). However, embryos could be recovered in all sections, including the oviduct and the uterine body. Overall, the tip of horn contained a greater number and proportion of the embryos, followed by the middle third of the horn, then body and oviduct. Of the total embryos recovered, 33.9 percent were found in the middle third. With this relatively large proportion of the embryo population being found in this area, there would seem to be merit in altering MOET collection procedures. In summary, these data indicate that embryos can be located throughout the uterine tract with the majority located in the tip of the horn and the middle third section. Given that many practitioners focus on the cranial third of the horn, these data may have implications on catheter placement to maximize recovery rates in multiple ovulation embryo transfer (MOET) programs.

3.2 Introduction

Since the 1970s, the embryo transfer (ET) industry has been working to improve embryo recovery rates in superovulated donors. Research-based improvements have been made in superovulation protocols and recovery techniques. With superovulation protocols, manipulating the estrous cycle to initiate stimulation protocols at the time of a new follicular wave emergence resulted in drastic improvement in embryos recovered

(Nasser et al., 1993; Bo et al., 1996; Baracaldo et al., 2000). In regard to recovery technique, growing interest to transition recovery and transfer techniques from surgical to non-surgical occurred in the late 1970s. As non-surgical approaches to ET were becoming more feasible, Newcomb et al., (1976) conducted several experiments on egg migration in the uterus on d 3-7 via a surgical approach. When the uterus was ligated 10 cm from the utero-tubal junction and each section was flushed separately, greater number of embryos were found in the tip of uterus compared to the base of the uterus (Newcomb et al., 1976). With these findings, it was suggested that in non-surgical recoveries emphasis should be on flushing the tip of the horns with media. Additionally, the adoption of non-surgical techniques enabled ET to be performed on-farm, which led to a significant growth in the industry (Hasler, 1992).

Currently with conventional ET, veterinary practitioners conduct non-surgical embryo recoveries approximately 6-7 days following fertilization. Post fertilization in the oviduct, embryonic development from zygote to a 16-cell embryo occurs over approximately 4 days in the oviduct (Betteridge and Flechon, 1988; Barnes and Eyestone, 1990). Migration into the uterus occurs around day 5, when the embryo has further developed and undergone compaction to form the morula (Dorniak et al., 2013). This time interval to recovery allows embryos to ideally be at a compact and mature development stage (Lawson et al., 1975; Trounson et al., 1976). There are two general approaches for catheter placement within the uterus when recovering embryos via non-surgical method. One approach involves placing the catheter at the level of the internal cervical os and flushing both horns and body simultaneously, known as a “body flush” (Hasler, 2014).

Application of body flushes are more advantageous for donors with smaller uterine tracts (heifers). However, there is an increase amount of flushing media required to fully distend both uterine horns and the uterine body simultaneously for adequate recovery. The other approach involves placing the catheter in each horn (separately) to the level of at least the external bifurcation or as far cranially as possible, otherwise known as "horn flushing" (Rowe et al., 1980). While the catheter must be manipulated through the cervix and up a horn two different times during a recovery, less flushing media can be used as area of focus is limited to distal part of each horn. Without a better understanding of where the embryos are located at this stage of development, there is the potential for embryos to be left in the uterus if the catheter and recovery occur cranial to their location. The objective of this study was to determine the location of embryos within the uterus from superovulated heifers at the time of recovery by sectioning the uterus and excising the reproductive tract. We hypothesized that majority of the embryos would be located towards the tip of the uterus.

3.3 Materials and Methods

3.3.1 General

All protocols and procedures used were approved by the Iowa State University Institutional Animal Care and Use Committee. The project was conducted at the Iowa State University Zumwalt Station Research Center in Ames, Iowa during January – February 2016 with the harvesting of the reproductive tracts occurring at Amend Packing Company, Des

Moines, Iowa. The project utilized single-sourced heifers from the Iowa State University McNay Beef Research Farm.

3.3.2 Animals and protocol

Non-pregnant, purebred Angus yearling beef heifers (n=12; 577 ± 31 kg) were used in this study. Prior to treatment, all heifers were subjected to a trans-rectal reproductive ultrasound to confirm normal anatomy and ovarian activity. The heifers were assigned to 1 of 3 replicates based on weight and were penned in these replicate groups for the duration of the study. Initiation of superovulation protocols for each replicate were staggered one week apart. All heifers were subjected to superstimulation by utilizing a timed, 17-d, CIDR (EAZI-BREED™, Zoetis Inc., Kalamazoo, MI)-based protocol with GnRH (Cystorelin®, Merial LLC, Duluth, GA) and PGF (Lutalyse®, Kalamazoo, MI) with decreasing total dosage of 240mg FSH (Folltropin-V®, Vetoquinol, Quebec) administered twice daily for 4 days (Experimental Design, Figure 3.1). Heifers were artificially inseminated (AI) following a timed-AI schedule in conjunction with observed signs of standing estrus, with a second AI 12 hours later by the same technician with one unit of frozen semen from a single bull collection at each insemination. A dose of GnRH (150 μg) was administered at the first breeding. At 6 d post-initial AI, trans-rectal ultrasound was performed to assess the number of CLs on each ovary. Approximately 12 hours later, the heifers were taken to a commercial abattoir where they were slaughtered via captive bolt and exsanguination and reproductive tracts were harvested within 15 minutes. Within the next 30 minutes, the length of each tract was measured in its natural state and each horn was sectioned into

even thirds with the uterine body split between the caudal third of each horn (Uterine Sections, Figure 3.2). Zip ties were placed at the edges of these sections and the uterus was cut distal to the zip tie, for a total of 4 sections per side of the uterus with oviduct as a separate section, with a total of 8 sections per heifer. Once in individual bags and labeled accordingly, commercial flush media was added to preserve embryos and tissue for transportation to the lab. Ovaries were removed and placed in corresponding bags for CL counts. Samples were transported in a cooler at room temperature to the lab.

3.3.2 Embryo recovery

Each section of uterus was filleted open in its bag and with a catheter tip syringe, approximately 60 mL flushing media was used to lavage the tissue and bag it was contained it. The oviducts were flushed, starting at the infundibulum, with 5 mL flushing media followed by air into a corresponding bag to be filtered. All media from each section was then filtered through EmCon filters. Each filter from each section was thoroughly rinsed into labeled square gridded search dishes and searched for embryos. All recovered embryos were evaluated according to International Embryo Transfer Society standards by American Embryo Transfer Association certified personnel. Ovaries were evaluated for number of CLs and anovulatory follicles to determine recovery rate. Structures were marked with pins and confirmed via scalpel blade dissection.

3.3.3 Statistical analysis

Data was analyzed using SAS 9.4 (SAS institute Inc., Cary, NC) using the MIXED procedure with heifer serving as the experimental unit and uterine section as the fixed main effect. Animal within replicate (group) served as the random effect. Both section length and total number of CLs were initially included as covariates, but section length was subsequently removed due to lack of significance ($P = 0.70$). Statistical significance was determined at $P \leq 0.05$.

3.4 Results

3.4.1 Embryo location

In total, embryos were found in all sections of the uterine tracts, including the oviducts. As combined total recovery of both horns, there was a significant difference in location of embryos among sections ($P < 0.001$; Table 3.1). In terms of total proportion of embryos recovered, there was a significant difference between the tip of the uterine horn (Section 2; Figure 3.2 for reference) and the middle third (Section 3; $P = 0.05$). However, both sections were greater than the oviduct and body ($P < 0.05$). There was no difference between the percentage of embryos found in the oviduct (Section 1) and the caudal third (uterine body; section 4; $P = 0.90$). In comparison of total number of embryos recovered, there was not a significant difference in the sections 2 and 3 of the uterus ($P = 0.37$), nor between sections 1 and 4 ($P = 0.65$). However, there was a significant difference between the total findings in sections 2 and 3 relative to sections 1 and 4 ($P < 0.05$). There was a

significant difference between section 1 and 2 along with 1 and 3 ($P = 0.0005$, $P = 0.006$; respectively). Furthermore, there were significant differences between sections 2 and 4 and between sections 3 and 4 ($P = 0.002$, $P = 0.017$; respectively).

As total number of CLs increased, so did the number of structures recovered ($P < 0.01$). Based on CL counts and total embryos recovered per uterus, the average recovery rate was 49% with the highest recovery at 92% and the lowest at 0.

3.5 Discussion

3.5.1 General

The objective of this study was to determine the location of embryos at the time of recovery in the uterus of superovulated heifers. While research has focused on the environment of the oviduct and the uterus during the formation and development of embryos (Hyttel et al., 1990; Sirard, 2001; Rizos et al., 2002), little is known about the location of embryos after entering the uterus relative to time of embryo recovery during MOET. To achieve this, heifers were slaughtered at the time when the non-surgical recovery would have occurred (d 6.5). Based on previous work using slaughtered cows, the uterus was removed from the carcass as soon as possible following exsanguination in efforts to eliminate the negative effects of pH levels declining following death (Deleeuw, 1992). After harvesting the uterus, measurements and sectioning was immediately performed to decrease any potential post-mortem movement of embryos. While our recovery rate was not 100 percent even with *ex vivo* recovery, we assume that not all ova

are captured in the infundibulum with donors who overstimulate. The results of this study are applicable to veterinary practitioners as they palpate the reproductive tract during an embryo recovery and correlate these similar sections *in vivo* to determine their approach to catheter placement for adequate flushing techniques.

3.5.2 Embryo quality

The stages and grades of embryos found in this current study were not included due to a substantial amount of poor quality embryos. Despite the pre-breeding semen evaluation, observation of standing estrus, and an experienced AI technician, the resulting embryos were poor. A study by Deleeuw (1992) evaluating embryo recoveries from slaughtered cows found poor viability in the embryos recovered. They attributed it to the drastic decrease in pH of the uterine environment immediately following slaughter as the tissue switch from aerobic to anaerobic metabolism and pH declines (Deleeuw, 1992). However, in another study involving embryo recovery from slaughtered cows on d 4 post-estrus and subsequently placed in culture media for further growth *in vitro*, there was no reference made to pH levels impacting embryo quality (Dias et al., 2013). In the current study, while pH levels were not documented, efforts were made to quickly excise the uterus, section it, and add flush media to the tissues to prevent any potential post-mortem effects.

3.5.3 Embryo location

As the ET industry transitioned from surgical to non-surgical approaches of both embryo recoveries and transfers, there was questions raised about how successful the recoveries would be via the less invasive, non-surgical approach (Newcomb et al., 1976; Rowe et al., 1980; Hasler, 2014). Newcomb et al., (1976) investigated this topic through several experiments. In one experiment, embryos were recovered at d 3-7 post estrus via a surgical approach with oviductal flushing conducted separate from the uterus. They found that as days post-estrus increased, there were greater number of stages 4-7 embryos, along with a greater number of degenerate embryos found in the uterus. Interestingly, Newcomb et al., (1976) found a significant number of embryos in the oviduct on d 6, compared to d 7-8. Similarly, in this present study, there was a small percentage of total embryos found in the oviduct on d 6.5 (5.8%; Table 3.1). Based on these findings, it appears there is the potential for embryos to remain in the oviduct at the time of recovery. If time from ovulation to recovery was delayed, there is the potential for an increase in embryos being recovered in the uterus, however the embryos recovered could be more mature and limit some of the versatility of embryos being tolerant of cryopreservation. Further investigation into the percentage of quality embryos still in the oviduct at time of recovery may be of merit.

In Newcomb et al., (1976), ligation of the uterus into two sections revealed a greater proportion of embryos in the tip of the uterine horn compared to the oviducts and the base of the uterus. While most embryos were in the uterine tip section, their experiment did find embryos in the caudal half of the uterus (uterine base section). It

should be noted, this previous experiment was all performed via surgical approach *in vivo* (Newcomb et al., 1976). The current study went a step further and sectioned the uterus into thirds, which allowed for a more precise localization of embryos. Accounting for individual animal variation, the total length of each uterus was taken into consideration and each uterus was evenly sectioned into thirds from the utero-tubal junction to the internal cervical os. These variations in section lengths between heifers were evaluated but not found to impact the migration of the embryos at the time of recovery. The results of our study also found embryos in all four sections of the uterus, with the largest percentages in the tip and middle third (53.5 and 33.9%, respectively). Furthermore, embryos were found in the base of the horns. Based on these results, placement of the catheter close to the tip of the horn will potentially fail to recover embryos that have migrated closer to the uterine body. It is suggestive that if a horn flush is to be conducted, placing the catheter at the level of the uterine bifurcation should allow recovery of majority of the embryos, but there is the potential for some to be in the uterine body. While this study utilized nulliparous, yearling heifers, re-creation of it using mature, multiparous cows would be of value to compare embryo migration between the two reproductive statuses.

It is apparent there is a relationship between the number of CLs on the ovaries and embryos recovered. However, this was not correlated with the number of embryos per section. It would seem logical that as number of CLs increase, the number of ovulations increase, therefore the number of embryos to be recovered would subsequently increase. With an opportunity to recover more embryos, we would suspect there to be an increase

in number of embryos in the tip and middle third of the horn, based on our findings. Repeating this study with a larger sample size, would potentially allow for more conclusive results. Additionally, even though CLs were accounted for, progesterone concentrations were not evaluated in this study. Progesterone is known to cause relaxation of the smooth muscle of the oviduct, which leads to a decreased rate of transport through the oviduct (Bennett et al., 1988). With superovulated cows, whether progesterone concentrations impact migration of embryos through the oviduct or uterus remains unknown.

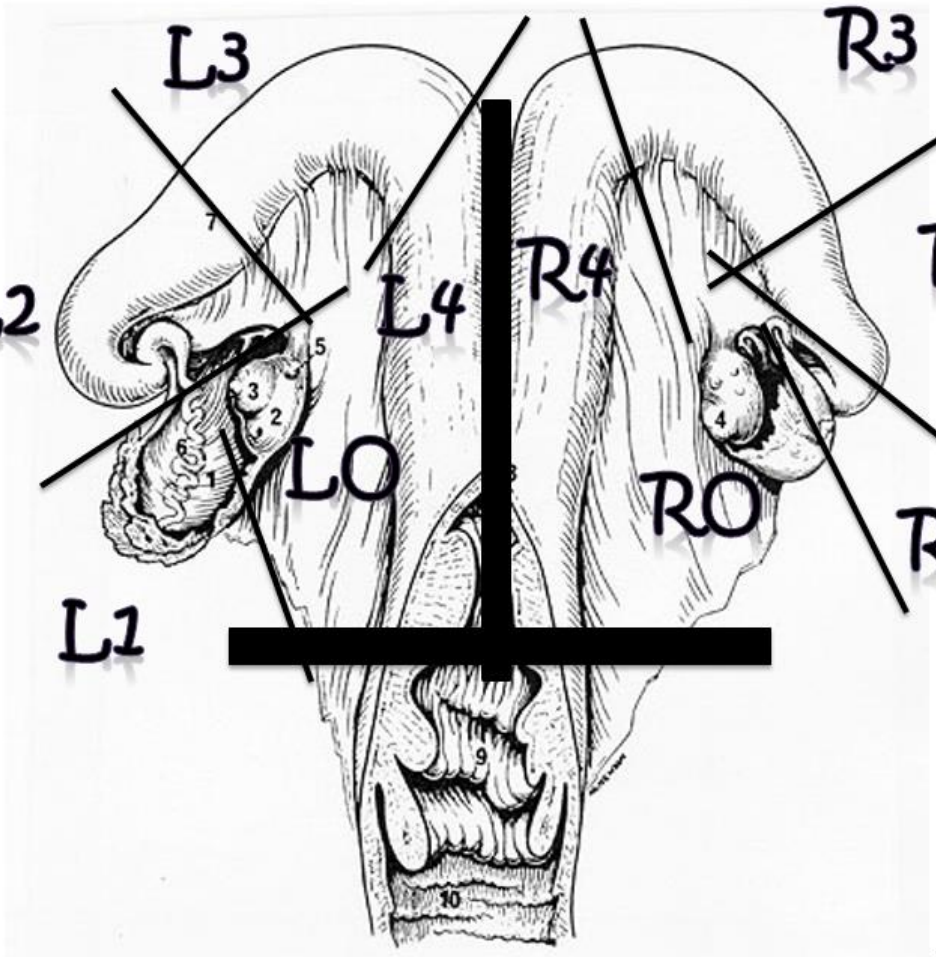
3.6 Conclusion

Based on these data, at the time of embryo recovery from superovulated heifers, embryos can be located throughout the uterus with the majority localized towards the tip and middle third of the uterine horn. In regard to catheter placement for recovering embryos, the closer the catheter is towards the tip, the increased risk of missing embryos. However, knowing that most embryos are in the tip and middle third of the horns emphasizes the need to perform a body flush and achieve full distention of the horns to maximize recovery the tip of each uterine horn. Regardless of which approach to take, our results suggest the focus of the recovery should be towards the tip and middle third of the horn to improve recovery rates while potentially incorporating the body of the uterus to maximize recovery efforts. Further research is warranted in regard to whether the developmental stage and/or quality of the embryo impacts its migration, the age of donor,

or to what extent progesterone concentrations may influence the location of embryos at time of recovery.

Day	Time	Administration
0	AM	CIDR-insertion + 25mg PGF2α
4	AM	150 µg GnRH
5	PM	40 mg FSH
6	AM/PM	40 mg FSH / 30 mg FSH
7	AM/PM	30 mg FSH / 30 mg FSH
8	AM/PM	30 mg FSH / 20 mg FSH + 25 mg PGF2α
9	AM	20 mg FSH + 25 mg PGF2α + CIDR-removal
10	PM	AI + 150 µg GnRH
11	AM	AI
16	PM	Ultrasound Ovaries, document CLs
17	AM	Slaughter, harvest and process uterus, locate embryos

Figure 3.1. Experimental design of superovulated heifers.



Key[1]:

LO: Left Ovary	RO: Right Ovary
L1: Left Oviduct	R1: Right Oviduct
L2-L4: Sections of left uterine horn	R2-R4: Sections of right uterine horn

Figure 3.2. Depiction of experimental design for sectioning and identification of each horn of the uterus for localization of embryos at time of recovery.

[1] Each horn was divided into 4 sections, the oviduct (L1/R1), and 3 equal lengths of uterine horn (L2-4/R2-4) with the uterine body bisected in half. Each section was thoroughly flushed with flushing media and searched for recovered embryos. Ovaries (L0/R0) were evaluated for CLs and anovulatory follicles.

Table 3.1. Recovery of embryos within each section of the uterus.

	Section[1]					
	1	2	3	4	SEM[2]	P-value
Total Recovery % [3]	5.8 [c]	53.5[a]	33.9 [b]	6.8 [c]	9.6	<0.001
Left Recovery %	9.5 [bc]	59.1[a]	25.8 [b]	5.6 [c]	7.0	<0.001
Right Recovery %	0 [b]	52.1[a]	41.7 [a]	6.3 [b]	11.9	0.01
Total Recovered [3]	0.33 [b]	3.17[a]	2.50 [a]	0.67 [b]	0.52	<0.001
Left Recovered	0.33 [b]	2.08[a]	1.08 [b]	0.25 [b]	0.30	<0.001
Right Recovered	0 [b]	1.08[ab]	1.42 [a]	0.42 [ab]	0.41	0.08

[a,b,c] Within each row LSMEANS that do not share a common superscript differ at $P \leq 0.05$.

[1] Section of Uterine tract: 1=oviduct; 2=tip of horn; 3=middle third of horn; 4=base of horn

[2] SEM: n=12

[3] Combined totals of left and right sides for each section.

CHAPTER 4

EFFECTS OF ENDOGENOUS PROGESTERONE DURING OVARIAN FOLLICLE SUPERSTIMULATION ON EMBRYO QUALITY AND QUANTITY IN BEEF COWS

4.1 Abstract

Despite modifications in techniques and protocols used for multiple ovulation embryo transfer (MOET) in the last couple of decades, total quality embryos (TQE) recovered has remained relatively unchanged. The objective of this study was to evaluate the effects of endogenous progesterone (P4) during beef cow superstimulation on embryo quality and quantity. Thirty non-pregnant beef cows were sorted by breed, body condition, and age into 1 of 5 replicates. Presynchronization was staggered so each rep began treatment on subsequent days using a 5 d CO-Synch + CIDR protocol, followed by ultrasound-guided dominant follicle ablation (DFA) and CIDR insertion (d 0) nine days after estrus with confirmation of a corpus luteum. Within each replicate, one-half of the cows were assigned a low progesterone (LP) treatment and administered PGF at time of DFA, while the other half were in the high progesterone (HP) control treatment. On d 1, cows began a timed, 13-d, superovulation CIDR-based protocol with a total 320mg FSH, administered twice daily in decreasing amounts over 4 days. Cows were artificially inseminated (AI) twice (12 hours apart) on d 6 (2 days after CIDR removal and PGF) using frozen-thawed semen from a single bull collection. Non-surgical embryo recovery was performed on each replicate 7 days after first AI and embryos were evaluated by

International Embryo Transfer Society standards. Blood samples were collected to evaluate P4 and estradiol concentrations each morning that the cows were handled, prior to administration of any hormones. Results revealed a greater number of total embryos recovered from the HP than the LP cows (19.26 vs. 10.74, $P = 0.01$). Additionally, the HP cows had greater number Stage 4 embryos along with increased amount of quality Grade 3 and 4 embryos than the LP group (5.76 vs 2.20, $P = 0.002$; 1.87 vs 0.61, $P = 0.01$; 8.22 vs 2.89, $P= 0.01$, respectively). However, LP cows had a higher percentage of Grade 1 embryos (58.22 vs 37.32, $P = 0.03$) as well as a greater percentage of Stage 7 and 6 TQEs (18.47 vs 1.22, $P = 0.01$; 10.37 vs 3.19, $P = 0.03$). Serum P4 concentrations had a significant treatment x day interaction ($P = 0.002$), as evident on d 2 and d 3, the concentration of P4 was more reduced in the LP than the HP group (P = 0.02), whereas all other days were relatively similar. Estradiol concentrations had a significant treatment by day interaction ($P < 0.0001$). More specifically, on d 2-4, estradiol was greater in concentration in the LP cows ($P \leq 0.04$) compared to d 6 in which the HP cows had greater concentrations of estradiol ($P < 0.0001$). In addition, treatment and day relative to treatment protocol had significant effects on the results ($P = 0.018$, $P <0.0001$; respectively). These data indicate that with removal of endogenous progesterone during superovulation, the percentage of embryos recovered are of higher quality grade (grade 1s) and are potentially more advanced in their development (stages 6 and7) on a single recovery. However, while poorer in quality (grades 3 and 4), these data also indicate endogenous progesterone presence during follicle maturation results in a greater number of total embryos recovered.

4.2 Introduction

In the past 40 years, the embryo transfer (ET) industry has grown in popularity across the United States. In the late 1980s, multiple ovulation embryo transfer (MOET) in bovine became a feasible process and producers were able to reduce generation intervals and increase genetic selection intensity by using their best females with highly proven bulls (Mapletoft and Hasler, 2005). According to the American Embryo Transfer Association (AETA) statistics, in 2003 there were just under 35,000 embryo recoveries performed on US cattle, while in 2015, AETA recorded nearly 42,000 embryo recoveries performed with an average of 6.5 TQE per recovery during that time span (AETA, 2017a, b). Despite the gradual growth in embryo recoveries performed, TQE recovered have stayed relatively stagnant. These data highlight the inherent need to increase both quality and quantity of embryos through MOET to increase return on investment for producers as well as to feed a growing global population. Research has focused on manipulation of estrous cycles to initiate emergence of follicular waves in conjunction with initiating a superstimulation protocol, which resulted in improvements in total embryos recovered, but minimal increase in TQE (Nasser et al., 1993; Baracaldo et al., 2000; Mapletoft and Bo, 2013).

Since the early 1990s, research has been conducted involving the interactions of the steroid hormones during the superstimulation protocol and impact on embryos produced. Progesterone (P4) has been shown to negatively impact GnRH secretions and specifically control the release of luteinizing hormone (LH) and estradiol production via concentration

dependency of P4, regardless if the source of P4 is endogenous or exogenous (Bergfeld et al., 1995; Kinder et al., 1996). This knowledge was applied to artificial insemination (AI) protocols, by removal of endogenous P4 at the beginning of protocol in conjunction with CIDR insertion to maintain subluteal concentrations. Subluteal P4 concentrations, even at 0.1-1.0 ng/ml in one study (Hatler et al., 2008), have been reported to effectively block LH and ovulation while others reported increased diameter of dominant follicles (Carvalho et al., 2008; Hatler et al., 2008; Pegorer et al., 2011) for females subjected to subluteal concentrations of P4 during the ovulatory follicle wave. Progesterone impacts in MOET research suggests subluteal P4 concentrations allows for irregular LH pulses, causing disturbance in nuclear maturation of oocytes (Nasser et al., 2011; Baruselli et al., 2012). Changes observed by subluteal P4 included decreased TQE compared to those supplemented with exogenous P4 (Hatler et al., 2008; Nasser et al., 2011; Rivera et al., 2011). These previous studies mainly focused on the first follicular wave of the estrous cycle, when P4 is still relatively low. This warrants further investigation into the impact of various concentrations of P4 on subsequent follicular waves in a cycle.

The objective of this study was to determine the effects of removal of endogenous progesterone on the overall embryo quality and quantity recovered follow a superovulation protocol. It was hypothesized that cows in the low progesterone (LP) treatment would produce more mature embryos with improved quality due to exposure to increased estradiol concentrations earlier in development resulting in increased follicle maturation compared to the high progesterone (HP) treatment group.

4.3 Materials and Methods

4.3.1 General

All protocols and procedures used were approved by the Iowa State University Institutional Animal Care and Use Committee. The project was conducted at the Iowa State University Zumwalt Station Research Center in Ames, Iowa in June and July 2016. This project sourced cows from the Iowa State University McNay and Beef Teaching herds.

4.3.2 Animals and treatments

Non-pregnant, non-lactating Black Angus and Simmental cows (n=30; 5.43 ± 2.87 years of age; 595 ± 79 kg BW; 5.6 ± 0.6 BCS) were used in this study. Prior to enrollment, all cattle were subjected to trans-rectal reproductive ultrasound to confirm normal ovarian activity and cyclicity, along with body weight (BW) and body conditioning score (BCS) assessment. At initiation of study, cows were stratified by breed, age, and BCS and assigned to 1 of 5 replicates. For the duration of this study, all cattle were housed together in an open pasture with ad lib hay, grass pasture, and a mineral and salt supplement.

All cattle were pre-synchronized using a 5-day CO Sync + CIDR protocol, with initiation of presynchronization occurring on subsequent days for each replicate (Superovulation Protocol, Figure 4.1). This was achieved using gonadotropin releasing hormone (GnRH), (Factrel®, Zoetis, Kalamazoo, MI); progesterone intravaginal insert (CIDR; Zoetis, Kalamazoo, MI), and prostaglandin F2α (PGF) (Lutalyse®, Zoetis, Kalamazoo, MI).

Estrus detection was achieved through a combination of Estrotect™ patches and twice-daily visual monitoring for estrus activity. Nine days after GnRH-induced ovulation, all cows were subjected to ultrasound-guided dominant follicle ablation (DFA) immediately followed by implantation of a CIDR (d 0) and confirmation of a corpus luteum (CL). Follicular ablation was utilized to enable all cows in the study to synchronize follicular wave development within their replicate and decrease variance in stimulation treatment relative to stage of estrous cycle. Dominant follicle ablation was performed using an Aloka SSD-900V ultrasound with 7.5 MHz micro-convex probe and a 20 gauge, 3 inch OPU-IVF needle contained in an aspiration case.

Within each replicate, cows were assigned to either 1) a high progesterone (HP; n=15 total) or 2) a low progesterone (LP; n=15 total) treatment. Specifically, LP cows were administered PGF to remove endogenous P4 immediately following DFA completion, while HP cows received no additional hormonal administration at that time. The following day (d 1) a timed, 13-d superstimulation, CIDR-based protocol was initiated (Superovulation Protocol, Figure 4.1). This protocol utilized a total of 320mg of purified porcine pituitary extract follicle stimulating hormone (FSH) (Folltropin-V®, Vetoquinol, Quebec, Cananda) administered twice daily in decreasing amounts over 4 days; GnRH (Cystorelin®, Merial LLC, Duluth, GA) with PGF administered concurrently with the last FSH injections. Cows were artificially inseminated (AI) twice (12 hours apart) according to timed-AI protocol in conjunction with signs of estrus on d 6. Inseminations were completed by 2 certified technicians (one inseminating in AM and other inseminating in PM) using one unit frozen-

thawed semen at each insemination from a single bull collection previously observed to have high success rates in other superovulation research in our lab.

4.3.2. Embryo recovery and evaluation

On d 13, seven days after initial timed-AI, trans-rectal ultrasound was performed to assess number of CLs and anovulatory follicles. Directly after ultrasound, a non-surgical embryo recovery was performed using a closed, gravity-flow uterine horn flush system with 2 liters of commercial grade flush media. All embryos recovered were evaluated by American Embryo Transfer Association certified personnel according to International Embryo Transfer Society standards. Transferrable quality embryos are regarded as quality Grades 1 – 3 and are considered acceptable to transfer to recipients following recovery. Freezable quality embryos includes only quality Grades 1 and 2, which have a high degree of viability through cryopreservation (Kennedy et al., 1983; Shea et al., 1983).

4.3.2 Plasma collection

Blood samples were collected via coccygeal venipuncture prior to hormone treatments on d 0 – 4, 6, and 13 to evaluate both estradiol and progesterone concentrations. Blood was collected into 6 mL EDTA vacutainers (10.8mg EDTA; BD Vacutainer™, Becton, Dickinson and Co., Franklin Lakes, NJ) and immediately placed on ice. Samples were centrifuged, within 1 hour of collection, at 3500 x *g* for 20 minutes. Plasma was aliquoted into 5-mL polystyrene tubes and frozen at -20^{o}C until hormone analyses were performed. Plasma samples were analyzed for P4 concentrations to confirm a

reduction in concentrations in LP cows due to luteal regression resulting from PGF administration. Progesterone serum concentrations were analyzed via radioimmunoassay (RIA) using methods described by (Engel et al., 2008). The high and low pool inter-assay CVs were 6.43% and 8.9%, respectively. The average intra-assay CV was 4.5%. Estradiol-17β concentrations for each day of FSH treatment and at time of AI were analyzed via RIA methodology described by (Perry and Perry, 2008). Across two assays, the average intra-assay CV was 5.52% and the inter-assay CV for a pooled sample was 5.07%.

4.3.3. Statistical analysis

Data was analyzed in SAS 9.4 (SAS institute Inc., Cary, NC) using the MIXED procedure with cow as the experimental unit and the treatment and replicate as the main fixed effects. Covariates for ovarian and embryo variables of interest included treatment, breed, and age. Other covariates analyzed were BCS and BW, however they were not found to be significant and not included in the model ($P \geq 0.16$). Flush order nested in replicate was included as a random effect. Hormone concentrations were evaluated using MIXED procedure of SAS for REPEATED measures. For repeated model variables, the covariance structures of compound symmetry, heterogeneous compound symmetry, first order autoregressive, unstructured, and ante-dependence were compared. The covariance structure with the smallest Bayesian Information Criterion was used for the final analysis, which was ante-dependence. This model included fixed effects of treatment, day, and treatment x day interactions with breed and age as covariates.

Statistical significance was determined to be at $P \leq 0.05$, with tendencies established at *P*-value > 0.05 and ≤ 0.10. It should be noted four cows were removed from the dataset for various, non-treatment related reasons.

4.4 Results

4.4.1 Hormone Analysis

As expected, there was a treatment x day interaction for P4 concentrations over the duration of the sampling period ($P < 0.05$). Specifically, concentrations of P4 were less in LP than HP on d 2 and 3 of the superstimulation protocol ($P = 0.02$; Figure 4.2). Also, as expected, there was a significant effect of day on P4 concentrations ($P < 0.0001$)

In regards to estradiol concentrations, there was a treatment x day interaction during the sampling period ($P < 0.0001$). In particular, LP had greater concentrations of estradiol than HP on d 2 through d 4 and HP had greater concentrations of estradiol on d 6 than LP (Figure 4.3). Moreover, there was a significant treatment effect ($P = 0.018$) and significant day effect ($P < 0.0001$) during the sampling period.

4.4.2 Embryo recovery

Overall, there was a difference in total number of embryos recovered between treatments with HP recovering a greater number than LP ($P = 0.01$; Table 4.1). However, there was no difference in the overall number of TQE or number of freezable embryos ($P > 0.1$; Table 4.1). Conversely, when analyzed as a proportion of each flush there was a greater proportion of freezable embryos in LP cows ($P = 0.03$). There was a higher

proportion of LP embryos being grade 1 ($P = 0.03$). In addition, LP group had a greater number ($P = 0.04$) and proportion ($P = 0.02$) of stage 7 embryos (Table 4.2). Additionally, LP had a greater proportion of stage 6 transferrable and freezable embryos ($P = 0.03$ and $P = 0.05$, respectively) compared to HP cows. However, there was a greater number of stage 4 embryos ($P < 0.01$) and an overall greater percentage of stage 4 transferrable embryos per flush ($P = 0.03$) in the HP cows. Furthermore, HP had significantly greater number of grade 3 ($P = 0.01$; Table 4.3) and grade 4 embryos (degenerate and unfertilized; $P = 0.01$).

4.5 Discussion

4.5.1 General

Despite advancements in superovulation protocols since the 1970s, improvement in TQE per flush has been nearly nonexistent. Recent studies reviewing the progress made in the ET industry highlight the user-friendly protocols, availability of commercial hormone therapies, new techniques to manipulate the estrous cycle, and advancements in embryo manipulation (Mapletoft and Hasler, 2005; Bo and Mapletoft, 2014; Mapletoft et al., 2015). Hormone interactions and the impact they have on follicular development and maturation, and consequently embryo production, is an area that continues to lack repeatable results and a clear understanding. Breed, age, and body condition can all impact results of an embryo recovery (Betteridge, 2003). In this study, we accounted for these variables when forming replicates.

4.5.2 Superstimulation protocols

In previous studies, initiating superovulatory protocols concurrent with follicular wave emergence improves embryo recovery results (Nasser et al., 1993; Bo et al., 1995; Bo et al., 2006; Bo et al., 2012). The original protocol for initiating superovulatory treatment coincides with the donors' anticipated second follicular wave of the estrous cycle, 8-12 days after estrus (Ginther et al., 1989b; Bo and Mapletoft, 2014). However, researchers have found other approaches to adequately stimulate donors with techniques to appropriately time endogenous release of FSH followed by a new follicular wave with proven effectiveness and similar numbers of TQE at recovery (Mapletoft and Bo, 2013). These approaches include exogenous GnRH (Martinez et al., 1999; Bo et al., 2006; Bo and Mapletoft, 2014); administration of estradiol-17β with P4 (Bo et al., 1995, 1996), and ultrasound guided follicle ablation (DFA) of all follicles > 5mm (Bergfelt et al., 1997; Baracaldo et al., 2000) to induce a new follicular wave. The present study utilized DFA at d 0, which produced results comparable to studies referenced as evident by our average of 7.8 and 11 TQE per recovery from the LP and HP cows, respectively.

4.5.3 Hormone analysis

Previous research has identified that P4, even at subluteal concentrations, provides a dose-dependent influence on the suppression of LH and estradiol (Bergfeld et al., 1995; Hatler et al., 2008). To induce subluteal P4 concentrations, endogenous P4 is removed and exogenous P4 is administered, such as a CIDR, which is a routine component in synchronization and superstimulation protocols (Bergfelt et al., 1991; Goulding et al.,

1994; Bergfeld et al., 1995; Bo et al., 1995; Carvalho et al., 2008). In ovariectomized cows exposed to CIDRs, Long et al. (2009) found plasma P4 concentrations of 4 ng/ml after 24 hour of insertion, which proceeded to decline gradually to 1.4 ng/mL by day 7 of insertion. In the current study, LP cows had significantly lower P4 concentrations on d 2 and 3 of the protocol, following administration of PGF and CIDR insertion on d 0 compared to the HP cows that did not receive PGF. Based on these results, we can conclude the LP cows achieved subluteal P4 concentrations during the time of stimulation.

In several studies, superovulation protocols were initiated during the first follicular wave of the estrous cycle, when endogenous P4 concentrations are reduced as the CL is developing (Nasser et al., 2011; Rivera et al., 2011). Conclusions from those studies found decreased TQEs and decreased number of embryos in cows with reduced P4, which is potentially attributed to insufficient regulation of LH pulsatility during the treatment period. In the present study, superstimulation was initiated during the second follicular wave following DFA when P4 ideally would be near peak concentration in the estrous cycle. In comparison to the previous studies, the different concentrations of P4 achieved in our study, did not result in any substantial differences in recovery of TQE in either treatment. While the P4 concentrations in the LP group never reached as low of concentrations as observed in previous studies, differences in concentrations were found between the two treatment on d 2 and 3. In our study, we used new CIDRS and removed endogenous P4 by the treatments described. To further alter concentrations of P4, treatment with once used CIDRs as described by Long et al. (2009) or identifying CIDRS impregnated with reduced P4 concentrations may afford a more reduced P4

concentration. Future research into more reduced or even higher concentrations of P4 is warranted to find that ideal concentration that enables the synchrony among hormones resulting in increased TQE.

In the current study, we noted increased estradiol in the LP cows during the time of superstimulation (d 1-4), followed with a drastic increase in the HP cows at time of AI (d 6). From a non-stimulated embryo recovery perspective, greater concentrations of estradiol were also documented with subluteal P4 concentrations while not affecting embryo quality (Cerri et al., 2011). Bergfeld et al., (1995) revealed similar conclusions with increased estradiol resulting from reduced P4. In contrast to Cerri et al., (2011), the current study showed a significant increase in estradiol concentration in the HP group at time of AI. The observed differences in estradiol concentrations between treatments were as anticipated. Reduced P4 in LP cows during superstimulation theoretically suppresses endogenous FSH early in the treatment due to the reduced P4 enabling estradiol secretions during the superstimulation period and stimulating LH to increase in pulsatility and induce earlier follicle maturation (Carvalho et al., 2008; Hatler et al., 2008). This cascade results in a diminished overall response to exogenous FSH in the reduced P4 cows and ultimately fewer recruited follicles with potentially increased estradiol production per follicle. Conversely, the increased number of total follicles of HP, as evident by the increased number of total embryos recovered compared to LP, hypothetically secreting less estradiol per follicle, had greater combined total estradiol effect at time of AI. With suppressed estradiol during the 4 days of FSH treatment, the greater number of follicles recruited were building up estradiol that when all P4 was

removed the estradiol secreted drastically increased in a narrower window of time. Hypothetically, the estradiol per follicle may have been less in the HP while the combined total was greater. We did not measure follicle diameter during superstimulation due to risk of altering infundibulum placement relative to the anticipated enlarged ovary. Had measurements of dominant follicles near ovulation been documented, we may be able to find a correlation between estradiol production per follicle under subluteal P4 control compared to estradiol production from follicles under uninhibited P4 control.

While LH was not measured in the current study, based on previous studies and greater estradiol concentration during stimulation, it is likely LH had increased pulsatility in the LP cows. Based on the later staged embryos produced from LP cows, reduced P4, with increased estradiol and potentially increased LH, enabled earlier development and ovulation of more mature follicles compared to the HP cows. Further investigation of LH response to subluteal P4 concentrations in MOET programs is warranted.

4.5.4 Embryo evaluation

Nasser et al., (2011) compared total quality embryos (TQE) recovered in relation to P4 concentration in *Bos indicus* cattle when superstimulation protocol was initiated during the first follicular wave with or without exogenous P4. Exogenous P4 improved embryo quality in terms of fewer unfertilized oocytes with significantly more TQE (Nasser et al., 2011). Nasser et al., (2011) attributed the improved TQEs to more consistent P4 concentrations, with the exogenous source, delaying the LH surge until later, when P4 has been removed. Results from Rivera et al., (2011) further supports the findings that donors

under the influence of increased P4 concentrations produce greater percentages of TQE and freezable embryos. However, in that study, there were no differences in the number of embryos recovered due to treatment.

In the current study, there were differences in the proportion of quality of embryos produced, however there was no difference in percent of TQEs recovered between the treatments in our study. The LP treatment recovered a greater percent of freezable embryos per recovery, along with a greater percent of those embryos being quality grade 1. Additionally, the LP treatment recovered embryos with a greater percent of more mature embryos, Stages 6 and 7, compared to the HP group that recovered significantly more stage 4 embryos. Interestingly, HP had significantly more total number of embryos, albeit of poorer quality (3 and 4). The proportional differences in quality to quantity could be associated with follicular development and estradiol impact.

4.6 Conclusion

In conclusion, these data indicate subluteal P4 concentrations during stimulation of MOET programs results in increased quality grade 1 embryos produced meanwhile greater quantity of embryos were produced under uninhibited P4. With subluteal plasma P4 concentrations, theoretically, follicle maturation occurs over a larger timespan due to a significantly increased estradiol concentration, resulting in earlier onset of estrus and more developed embryos at time of recovery. In contrast, a higher P4 plasma concentration enabled a larger recruitment of follicles that were potentially not as mature at time of AI, due to a smaller exposure time to estradiol and LH prior to ovulation and AI,

which suggests that ovulation occurs in a smaller timeframe resulting in a more uniform group of embryos with decreased quality. In application of these results, it may be advantageous for a practitioner with a donor that repeatedly produces poorer quality embryos to utilize a subluteal P4 protocol. Another avenue with these results would be to utilize a HP protocol if recipients are available to allow for transferring of fresh grade 3 embryos that would otherwise be of no value if cryopreservation is the only option.

Day	Time	Administration	Ultrasound/Blood Collection
-17	AM	CIDR[1] insertion + 100mcg GnRH[2]	…..
-12	PM	CIDR-removal + 25 mg PGF[3]	…..
-11	AM	25 mg PGF	…..
-9	AM	100mcg GnRH	…..
0	AM	DFA + CIDR insertion +/- 25 mg PGF	U/S[4] + Initial P4[5]
1	AM/PM	50 mg FSH[6] / 50 mg FSH	P4 + Estradiol
2	AM/PM	40 mg FSH / 40 mg FSH	P4 + Estradiol
3	AM/PM	40 mg FSH / 40 mg FSH	P4 + Estradiol
4	AM/PM	30 mg FSH + 25 mg PGF / 30 mg FSH + 25 mg PGF + CIDR-removal	P4 + Estradiol
6	AM/PM	AI[7] + 100 mcg GnRH, AI	P4 + Estradiol
13	AM	Embryo Recovery + PGF (post -flush)	U/S + P4

Figure 4.1. Experimental design of superovulated cows with or without PGF administered on d 0 concurrently with dominant follicle ablation (DFA).
[1]CIDR: Intravaginal progesterone device
[2]GnRH: Gonadotropin releasing hormone
[3]PGF: Prostaglandin $F_{2\alpha}$
[4]U/S: Ultrasound
[5]P4: Progesterone
[6]FSH: Follicle stimulating hormone
[7]AI: Artificial Insemination

Table 4.1. Embryo recovery results for beef cows after treatment with Prostaglandin $F_{2\alpha}$

	Treatment[1]			
Total Embryos	LP	HP	SEM[2]	P-value[3]
Total Embryos, no.	10.74	19.26	1.978	0.01
Total Quality Embryos (TQE[4]), no.	7.86	11.00	1.433	0.13
% of recovered[5]	77.25	59.16	7.690	0.11
Freezable Embryos[6], no.	7.24	9.13	1.30	0.31
% of Flush	72.35	48.57	7.401	0.03
% of TQE[7]	93.33	83.70	3.049	0.04

[1] LP = PGF2α administered at start of superstimulation; HP = No induced luteolysis and maintained endogenous CL throughout superstimulation.

[2] Greater SEM presented: Lo P4 (n = 14); Hi P4 (n= 12).

[3] *P*-values of the main effects of treatment, day, treatment x day interaction, breed and age ($P \leq 0.05$; considered statistically significant).

[4] Transferrable Quality Embryos: Total number of embryos graded by International Embryo Transfer Society guidelines: 1= Excellent or good, 2= Fair, 3= Poor; able to be transferred fresh.

[5] Percent of total embryos in each flush.

[6] Freezable Embryos: Total number of embryos graded by International Embryo Transfer Society guidelines: 1= Excellent or good, 2= Fair.

[7] Proportion of TQE that were graded as either quality 1 or 2.

Table 4.2. Embryo recovery characteristics based on development stage for beef cows after treatment with Prostaglandin $F_{2\alpha}$

	Treatment[1]			
Embryo Stage	LP	HP	SEM[2]	P-value[3]
Stage 7, no.	1.79	0.00	0.616	0.04
% of Recovery [4]	12.88	0.00	3.638	0.02
% of TQE [5]	18.47	1.22	5.056	0.01
% of Freezable [6]	19.09	1.75	5.286	0.01
Stage 6, no.	0.89	0.48	0.367	0.27
% of Recovery	7.93	3.08	3.102	0.13
% of TQE	10.37	3.19	3.790	0.03
% of Freezable	10.55	4.02	3.793	0.05
Stage 5, no.	3.01	4.92	1.024	0.19
% of Recovery	27.09	22.78	9.069	0.68
% of TQE	38.76	35.72	10.99	0.82
% of Freezable	44.11	44.12	10.600	1.00
Stage 4, no.	2.20	5.76	0.708	<0.01
% of Recovery	26.78	31.21	7.755	0.69
% of TQE	29.87	57.56	8.497	0.03
% of Freezable	26.19	50.53	9.355	0.08

[1] LP = PGF2α administered at start of superstimulation; HP= No induced luteolysis and maintained CL throughout superstimulation.

[2] Greater SEM presented: LP (n = 14); HP (n: = 12).

[3] P-values of the main effects of treatment, day, treatment x day interaction, breed and age ($P \leq 0.05$; considered statistically significant).

[4] Percent of total embryos in each flush.

[5] Transferrable Quality Embryos: Total number of embryos graded by International Embryo Transfer Society guidelines: 1= Excellent or good, 2= Fair, 3= Poor; able to be transferred fresh.

[6] Freezable: Total number of embryos graded by International Embryo Transfer Society guidelines: 1= Excellent or good, and 2= Fair.

Table 4.3. Embryo recovery characteristics based on quality grade for beef cows after treatment with Prostaglandin $F_{2\alpha}$

	Treatment[1]			
Embryo Grade	LP	HP	SEM[2]	P-value[3]
Grade 1, no.	6.00	7.08	1.142	0.52
% of Recovery[4]	58.22	37.32	6.682	0.03
% of TQE[5]	77.42	65.14	5.779	0.14
% of Freezable[6]	83.02	75.69	5.543	0.35
Grade 2, no.	1.23	2.04	0.479	0.22
% of Recovery	14.19	11.35	4.223	0.60
% of TQE	15.85	18.38	4.966	0.70
% of Freezable	16.98	24.31	5.543	0.35
Grade 3, no.	0.61	1.87	0.333	0.01
% of Recovery	4.90	10.59	2.150	0.07
% of TQE	6.67	16.30	3.049	0.04
Grade 4, no.	2.89	8.22	1.363	0.01
Degenerate, no.	1.22	2.58	0.605	0.12
Nonfertilized, no.	1.64	5.60	1.202	0.03
% of Flush	22.75	40.84	7.690	0.11

[1] LP= PGF2α administered at start of superstimulation; HP = No induced luteolysis and maintained CL throughout superstimulation.

[2] Greater SEM presented: LP (n = 14); HP (n= 12).

[3] *P*-values of the main effects of treatment, day, treatment x day interaction, breed and age ($P \leq 0.05$; considered statistically significant).

[4] Percent of total embryos in each flush.

[5] Transferrable Quality Embryos: Total number of embryos graded by International Embryo Transfer Society guidelines: 1= Excellent or good, 2= Fair, 3= Poor; able to be transferred fresh.

[6] Freezable: Total number of embryos graded by International Embryo Transfer Society guidelines: 1= Excellent or good, and 2= Fair.

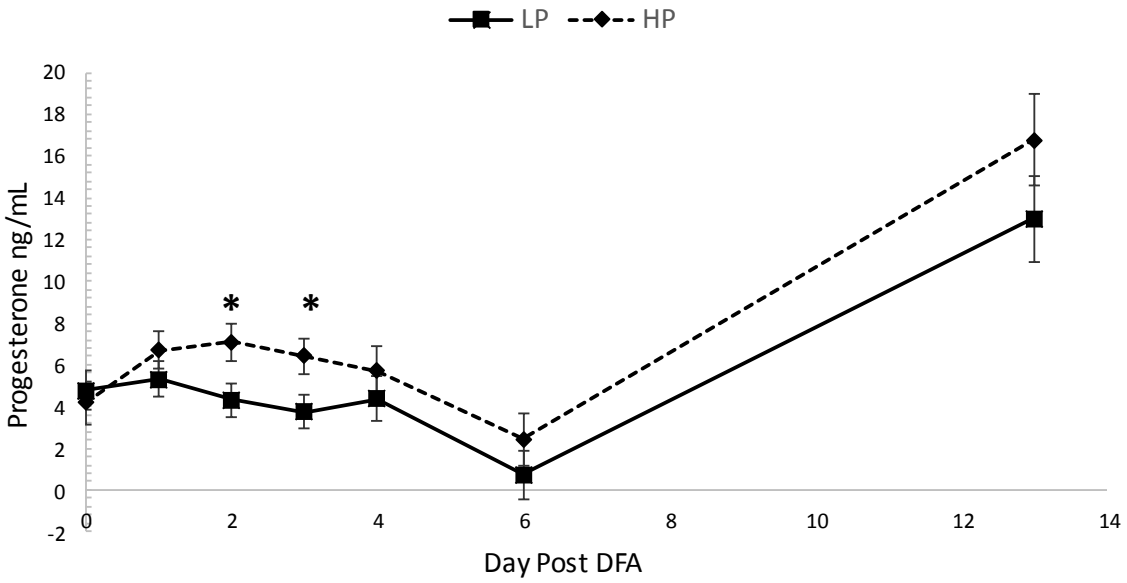


Figure 4.2. Effect of treatment (LP = PGF2α administered at start of superstimulation; HP = No induced luteolysis and maintained CL throughout) on progesterone concentrations 13 d post dominant follicle ablation and treatment. Effects of day ($P < 0.0001$), and treatment x day interaction ($P < 0.002$) were observed. *P*-value for treatment was ($P = 0.12$). Days on which P4 was significantly different between treatments ($P < 0.05$) are denoted by *.

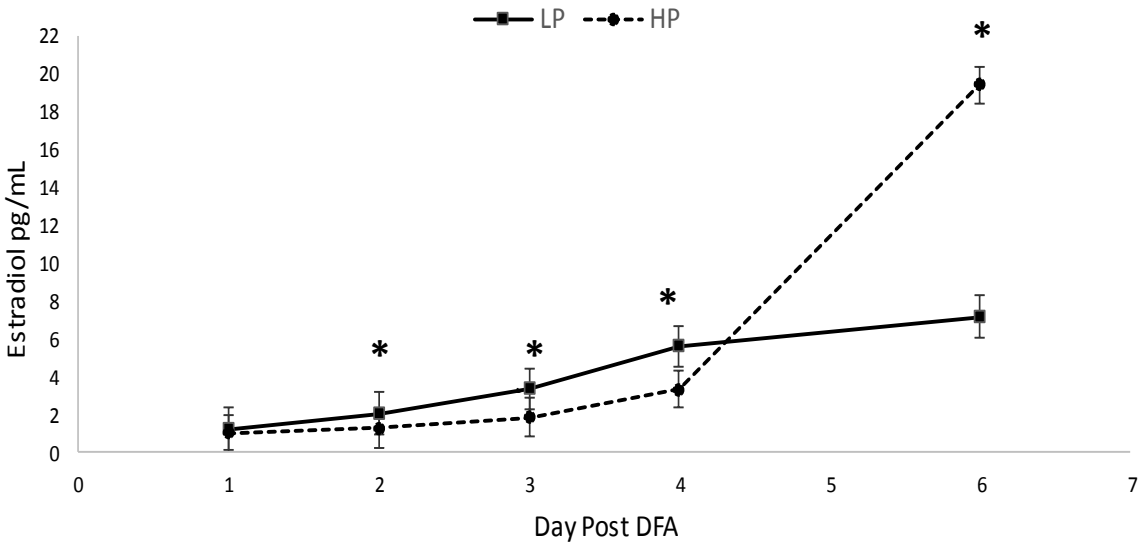


Figure 4.3. Effect of endogenous progesterone during superstimulation on estradiol concentrations in beef cows. Treatments were: LP = PGF2α administered at start of superstimulation; HP = No induced luteolysis and maintained CL throughout. Effects of treatment ($P = 0.018$), day ($P < 0.0001$), and treatment x day interaction ($P < 0.0001$) were observed. Days on which estradiol was significantly different between treatments ($P < 0.05$) are denoted by *.

CHAPTER 5

EFFECT OF EMBRYO STAGE AND GRADE ON EMBRYONIC SEX IN SUPEROVULATED BEEF COWS

5.1 Abstract

For cattle producers, knowing the sex of their offspring prior to parturition can have economic advantages from both a management and marketing aspect. The objective of this study was to determine if there is a relationship between the developmental stage and/or embryo quality at time of recovery to the embryonic sex. Non-pregnant, non-lactating Angus and Simmental mature beef cows (n = 29) were superovulated using a 13-d, CIDR-based protocol following ultrasound guided dominant follicle ablation. Cows were artificially inseminated (AI) twice (12 hours apart) on d 6, 36 hours after CIDR-removal and PGF2α, using frozen-thawed semen from a single bull collection observed to have high success rates in previous superovulation research in our lab. Embryo recovery occurred 7 days after first AI via non-surgical flush and embryos were evaluated by International Embryo Transfer Society standards, resulting in an average recovery rate of 9 transferrable quality embryos per donor. Embryos of quality grade 1-3, (n = 265) were biopsied utilizing a micromanipulator with a microsurgical blade to excise approximately 15-20% of the inner cell mass of each embryo. Subsequently, embryo sex was determined by conducting polymerase chain reaction (PCR) on the biopsies, and following electrophoresis, the gels were analyzed to detect male-specific Y-chromosome. Data were analyzed using the MIXED procedures of SAS. Stage of embryo did not have an impact on sex (P = 0.6).

However, grade impacted sex (P = 0.03) with grade 1 embryos being proportionately more male (51.2%), while grade 2 embryos were a higher proportion of females (75.2%). In summary, these data suggest that quality of embryos is a better predictor of sex than stage within a recovery.

5.2 Introduction

Knowing the sex of offspring prior to parturition can be economically beneficial to cattle producers. For instance, in the dairy industry there is a strong interest in heifer calves for production either as a replacement or marketed to another producer as feeders or replacements (Seidel, 2003; Kaimio et al., 2013; Mikkola et al., 2015). Conversely, in some sectors of the beef industry, bulls are preferred for supplying feedlots with steers, while heifers are desired for retention as replacements in operations to aid in increased future progeny (Dahlen et al., 2014).

In addition to marketing benefits, fetal sex determination may assist in improving management at time of parturition. Research has repeatedly shown greater dystocia rates in bull calves leading to a higher mortality rate compared to heifers (Morris et al., 1986; Dematawewa and Berger, 1997). Knowing what the sex of the offspring is prior to parturition allows for better preparation and early intervention if necessary.

In natural mating scenarios, there is a 51% probability the conception will result in a bull calf (Seidel, 2003). For producers to influence the sex of the offspring, sex-sorted semen is the main resource available, which provides a 90% accuracy in producing the desired sex (Seidel, 2003). Timing of insemination has also been a hypothetical method

employed by producers to influence fetal sex. However, there are conflicting results as to whether time of insemination can impact the sex ratio of offspring (Avery et al., 1991; Gutierrez-Adan et al., 1999; Rorie et al., 1999).

For those producers requesting sex determination prior to parturition, there are several options, all with some level of variability in the accuracy of the results. Trans-rectal ultrasound is one of the most common and less invasive methods utilized by trained veterinarians with ultrasound capabilities to determine the sex of fetuses within the 55-98 days post ovulation (Curran and Ginther, 1991; Ali, 2004). The remaining options for sex determination involves micromanipulation at the embryonic stage of development, with several different tests utilized over the past few decades. These various methods include cytoplasmic analysis (Wintenbergertorres and Popescu, 1980), male specific antigens (White et al., 1987), and X-linked enzyme activity (Williams, 1986), however they are not commonly used due to impracticality and inconsistent results. Currently, the most common and reliable method for embryonic sex determination is DNA analysis via polymerase chain reaction (PCR; (Thibier and Nibart, 1995; Shea, 1999).

Research has attempted to identify any relationship between developmental stage and/or embryo quality to the embryonic sex. Several studies have been conducted with *in vitro* (IVF) embryos to determine if the stage of embryo development at time of transfer has any correlation with fetal sex (Avery et al., 1991; Xu et al., 1992; Carvalho et al., 1996; Gutierrez-Adan et al., 1999). The main findings of these studies are that male embryos develop at a faster rate than female embryos, if insemination occurred at the same time (Avery et al., 1991; Xu et al., 1992). However, exact timing of ovulation and associated

fertilization is nearly impossible to analyze *in vivo*, particularly in superovulated bovine that may ovulate over a period of 24 h. The objective of this study was to determine if there is a relationship between embryonic stage and/or quality to embryonic sex from conventional *in vivo* derived recoveries since limited research has been evaluated with this relationship in conventional embryos. It was hypothesized there would be no significant difference in sex of embryos due to embryo stage or quality grade.

5.3 Materials and Methods

5.3.1 General

All protocols and procedures used were approved by the Iowa State University Institutional Animal Care and Use Committee. This study was conducted at Iowa State University Lab Animal Research Station, Ames, IA. The project utilized embryos recovered from a superovulation study conducted at Iowa State University Zumwalt Station, Ames, IA in August 2016 (Wiley et al., 2017).

Embryos were recovered from Angus and Simmental cows (n=29) following a timed, 13-d superstimulation CIDR (Eazi-Breed™CIDR®, Zoetis, Kalamazoo, MI)-based protocol initiated 1 d after ultrasound guided dominant follicle ablation (Wiley et al., 2017). Cows were AI twice (12 hours apart) according to timed-AI schedule, 1.5 d after CIDR-removal and second prostaglandin F2α (Lutalyse®, Zoetis, Kalamazoo, MI), concurrent with signs of estrus observed in all cows. Each insemination utilized one unit of frozen-thawed conventional semen sourced from a single bull collection known to have high success rates in previous superovulation research in our lab. Non-surgical embryo recovery was

performed on d 7 after initial timed-AI and embryos were evaluated according to International Embryo Transfer Society standards by American Embryo Transfer Association certified personnel.

5.3.2 Embryo biopsy

Following evaluation and washing, the embryos were placed in micro-drops of splitting media (ViGro™Splitting Plus Solution; Bioniche Animal Health) used to immobilize embryos for micromanipulation. Biopsying was achieved using a Bioniche Animal Health twinning system with a micromanipulator and an Olympus CKX41 microscope. A microsurgical blade excised through the zona pellucida to allow removal of approximately 4-8 cells from the inner cell mass of the intact embryo contained in a micro-drop of splitting media. Each biopsy was washed with ViGro™ Retrieval Supplement media (Bioniche Animal Health) and immediately transferred to labeled micro centrifuge tube with 8 µl sterile water. Each tube was submerged in liquid nitrogen to 'snap-freeze' the samples and then were placed in racks at -18^{o}C for approximately 1 month, until time allowed for PCR analysis.

Embryo sexing was performed with a commercial polymerase chain reaction (PCR) kit using primers specific to the Y-chromosome determinant (YCD) according to the manufacturer's instructions (Herr et al., 1995). Following electrophoresis, the gels were placed on a UV transilluminator to determine Y-chromosome presence or absence. If the sample failed to produce distinguishable bands, results were not included in the dataset. As previously stated, a total of 203 embryos were determined with definitive sex via PCR.

5.3.3 Statistical analysis

Data was analyzed in SAS 9.4 (SAS institute Inc., Cary, NC) using the MIXED procedure with embryo as the experimental unit and the embryo grade, stage, sex, and flush group (5 groups) as the fixed effects. Animal was nested in group as a random effect. Statistical significance was acknowledged at $P \leq 0.05$. It should be noted that a total of 62 embryo biopsies were excluded from the dataset due to inconclusive PCR results.

5.4 Results

There was no difference in sex due to stage of embryo ($P = 0.6$; Table 5.1). However, sex differed because of quality grade ($P = 0.03$; Table 5.2). Specifically, there was a greater percent of females within grade 2 embryos compared to grade 1s. Meanwhile, there was a greater percentage of males within grade 1 embryos compared to grade 2s (75.2 vs. 48.8, respectively; $P = 0.01$).

5.5 Discussion

5.5.1 General

Sex determination of offspring prior to parturition is a growing sector of advanced reproductive technologies. Utilizing sex-sorted semen is one option available that allows 90% accuracy in determining the sex of the offspring from that insemination. However, sex-sorted semen has its limitations with decreased AI conception rates and decreased embryo yield when incorporated in MOET programs due to the processing required to sort

semen and the traditionally low concentration of sorted sperm per insemination dose (Kaimio et al., 2013). The remaining options of determining sex of offspring are micromanipulation of embryos prior to embryo transfer and fetal sexing via trans-rectal ultrasound during a specific time of gestation. As MOET technology continues to be incorporated in more reproductive programs, sexing of embryos will continue to be a requested service and PCR has become the most relevant technology for early embryonic sex determination.

5.5.2 Embryo biopsy

With PCR, embryo sexing has become a simpler, more reliable technology that can be offered by private practitioners. Regardless of how the biopsy is obtained, excising a small portion of the embryo or aspirating a few cells with a glass pipette, 4 to 5 cells from the inner cell mass is all that is necessary for DNA amplification with 95% sensitivity (Thibier and Nibart, 1995). However, embryo biopsy can cause a decrease in conception rate due to alterations made to the structure of the embryo (Gustafsson et al., 1994). Several sexing studies were conducted using different biopsy techniques and found technique differences in obtaining the biopsy do not affect the resulting sex determination (Thibier and Nibart, 1995; Shea, 1999). While PCR is a reliable and accurate technology with a 95% efficiency in actual sex determined according to Thibier and Nibart (1995), there are samples that fail to have definitive results by lacking distinguishable bands correlating with male specific band and the autosomal band (female). In the current study, 203 of the 265 embryos evaluated had distinguishable results, while 62 biopsies were

removed from the dataset due to inconclusive results. Causes for failed results include insufficient DNA in the biopsy or absence of cells, lack of amplification from a reagent failure, or contamination of biopsies (Thibier and Nibart, 1995; Shea, 1999).

5.5.3 Sex determination

Sexing of IVF derived embryos has advantages of monitoring maturation of oocytes, specific timing of fertilization, and development rates of embryos, all which areas have been hypothesized to impact the genetic make-up of embryos (Avery et al., 1991; Carvalho et al., 1996; Dominko and First, 1997; Gutierrez-Adan et al., 1999). Several IVF studies have noted correlations between embryonic development stage and sex. Carvalho et al., (1996) concluded male embryos develop at a faster rate than females. The early blastocysts (Stage 5) and blastocysts (Stage 6) had a greater proportion of males, while morulas (Stage 4) had a greater proportion of females. Xu et al., (1992) reported similar proportions of males developing to more advanced stages by d 8 after insemination. The reasoning for increased rate of development of males is unknown, however it is hypothesized that the increased growth rate in males is associated with their gonadal sex differentiation (Mittwoch, 1989; Avery et al., 1991). Furthermore, when the maturation state of oocytes was considered, early fertilization resulted in a higher ratio of females while delayed fertilization was found to produce a greater proportion of males (Dominko and First, 1997; Gutierrez-Adan et al., 1999). Potential reasoning for these findings are based off scientific evidence that oocytes develop a mechanism allowing for improved processing of Y-bearing spermatozoa during late maturation of metaphase II arrested oocytes. X and Y-bearing

spermatozoa produce different signaling messages and surface proteins, which allows oocytes early in maturation to process X-bearing spermatozoa easier; meanwhile, more mature oocytes process Y-bearing spermatozoa more effectively later in estrus (Moor et al., 1981; Dominko and First, 1997). The current study failed to find a relationship between stage and embryonic sex. With superovulation protocols, it is expected that a donor will ovulate multiple ova during a 24-36h window of time as opposed to non-stimulated donors. As such, the current study implemented two separate timed inseminations (12 hours apart) to have viable sperm at any point within this window of ovulation. Therefore, conceptually, timing of fertilization relative to ovulation was evenly spaced and would result in no significant difference in sex. Our results would support this hypothesis. Furthermore, since these embryos were *in vivo* derived, exact timing of fertilization and rate of development cannot be accounted for.

Embryo quality grade is an important component of embryo evaluation as it determines viability through cryopreservation or fresh transfer options. Quality grades take into account percentage of extruded cells, compactness of inner cell mass, shape, and color of cytoplasm to determine overall quality (Wright and Ellington, 1996). As the developmental stage of embryos increases with *in vivo* derived embryos, quality grades below a 1 are rarely appreciated, especially once the embryos develop to Stage 6 (blastocyst) and above. One reason for this is the blastocele cavity expands and fills the perivitelline space of the embryo making it difficult to appreciate any extruded cells or debris, which eliminates the potential for downgrading embryo quality. The results from the current study, with *in vivo* derived embryos, reflect these observations with all Stage 6

and 7 embryos obtaining a quality grade 1. Meanwhile, quality grade 2 embryos in this study were of Stages 4 and 5. Bernardi and Delouis (1996), evaluated the embryonic sex of ovine IVF embryos based on their rate of development at 207 hours post-insemination. Within the advanced development stages (hatched and hatching blastocysts) of the IVF embryos evaluated, there were embryos of quality grades 1-4 observed. They found the quality grades 1-3 of the expanded blastocysts embryos, had a significantly higher proportion of males (Bernardi and Delouis, 1996). In this instance, despite advanced developmental stages, variations in quality were observed and found association with embryonic sex. However, the quality grading of IVF embryos is not as well defined as conventional embryo grading, and IVF embryos are often graded harder due to increased cellular debris and extruded cells especially in the more developed stages, increasing the variation of quality grades even with further developed embryos (Barfield, 2015). We acknowledge in our study the relatively limited numbers of grade 2 and 3 embryos (n= 29 and 19, respectively) and feel further evaluation incorporating a larger dataset of *in vivo* derived embryos is warranted. Furthermore, due to the lack of variation in quality grade with more developed *in vivo* derived embryos as observed in this current study, it may be of value to evaluate the embryonic sex of embryo recoveries performed earlier, at d 6. These embryos would be less developed with potentially more variation in quality grade. This in turn would enhance our ability to determine if quality grade is still associated with sex determination.

5.6 Conclusion

In conclusion, these data indicate a relationship between quality grade of *in vivo* derived embryos and their genetic make-up, with a greater proportion of the quality grade 2 embryos being heifers and a greater proportion of quality grade 1 embryos being bulls. Meanwhile, there was no significant relationship between developmental stage at time of recovery and embryonic sex. Based on our findings, a cattle producer looking to increase their heifer offspring may select the quality grade 2 morula and early blastocyst embryos from a conventional embryo recovery to transfer.

Table 5.1. Sex determination by developmental stage of embryo[1]

	Development Stage					
	4	**5**	**6**	**7**	**SEM[2]**	**P-value[3]**
% Female	56.0	57.2	77.7	60.8	0.16	0.6
% Male	44.0	42.8	22.3	39.2	0.16	0.6

[1]Stage is based on the International Embryo Transfer Society Classification (1= Excellent or good, 2= Fair, 3= Poor).
[2]Greatest SEM presented: 4 (n = 78); 5 (n = 99); 6 (n = 11); 7 (n = 15).
[3]*P*-values of the main effect of developmental stage on sex ($P \leq 0.05$; considered statistically significant).

Table 5.2. Sex determination by embryo quality grade[1]

	Quality Grade				
	1	**2**	**3**	**SEM[2]**	**P-value[3]**
% Females	48.8 [a]	75.2 [b]	64.7 [ab]	0.13	0.03
% Males	51.2[a]	24.8[b]	35.3 [ab]	0.13	0.03

[a, b, c] Within each row: LSMEANS that do not share a common superscript differ at $P \leq 0.05$.

[1] Quality grade is based on the International Embryo Transfer Society Classification (1= Excellent or good, 2= Fair, 3= Poor).

[2] Greatest SEM presented: 1 (n=155); 2 (n=29); 3 (n=19).

[3] *P*-values of the main effect of grade ($P \leq 0.05$; considered statistically significant).

CHAPTER 6

GENERAL DISCUSSION

As MOET programs have continued to increase in application on livestock operations, veterinary practitioners strive to produce more TQE with each recovery. Improvements in the technology since the 1970s, including timing of initiation of protocols, implementation of exogenous hormones, recovery techniques and cryopreservation, have helped lay the foundation for where the industry is at today. Yet, the overall TQE recovery rate in cattle has remained fairly stagnant for at least the last 15 years with an average recovery of 6.5 TQE per conventional ET recovery, despite the improvements mentioned.

The studies conducted in this thesis were initiated to be applicable to a MOET practitioner with the goal of improving embryo quality and recovery rates. The research focused on comparing the effects of two different ranges of P4 concentrations during superstimulation protocols along with investigating the location of embryos at time of recovery in relations to catheter placement during a non-surgical recovery. Additionally, with growing interest by producers in embryo sexing, a study was conducted to investigate whether there is an association between developmental stage and/or quality grade of embryos and sex of *in vivo* derived embryos.

Embryo transfer practitioners generally have one of two approaches to recovery of embryos in relation to the placement of the Foley catheter. First, practitioners can manipulate the catheter up each horn close to the ovary to focus on the tip of the horn. The other option is to place the catheter in the uterine body, enabling them to flush both horns and body simultaneously. From a clinical perspective, a horn flush enables localizing

the area to be flushed towards the tip of each horn, where most of the embryos are suspected to be located and more efficient use of the flushing media with a disadvantage of manipulating the catheter up each horn. A body flush enables media to be flushed through the whole uterine tract including the body where there could potentially be embryos; however, a disadvantage is an increase in media used to thoroughly distend both horns. Additionally, the inability to fully distend a specific horn or section of the uterus for proper distention can hinder the recovery, regardless of which method is used. Despite valid argument behind each method, there has been no investigation into the location of the embryos at the time of a recovery to substantiate anecdotal preferences in technique.

As reported in Chapter 3, there were differences found in the number and proportion of embryos localized in different sections of the horn, with the tip and middle third containing the majority of the embryos. Interestingly, there were embryos recovered in the base of the horn. Additionally, CLs were found to have an influence on recovery of embryos, in that as the total number of CLs increased, the number of embryos recovered also increased. While we did not find a correlation between total number of CLs and location within specific sections of the horn, it would seem logical that an increase in CL numbers would be associated with an increase in embryos recovered in the tip and middle third where the majority of embryos were located. Further evaluation of CLs and any impact P4 may have on the migration of embryos during this time with a larger sample size is warranted. Moreover, we acknowledge that information of embryo stage and quality were not included, this is an area that would be worth investigating in the future to determine if the developmental stage of the embryo influences migration. Practitioners

can apply the results from this study in terms of the approach to catheter placement in a recovery, knowing where majority of the embryos should be located at time of recovery.

To improve number of TQEs, a second study was conducted to evaluate hormone interaction during superstimulation of a protocol. Research has repeatedly noted the negative feedback effects of P4 on LH pulsatility. Recent research on AI of Nellore cattle have noted that removing endogenous P4 during the maturation phase of the ovulatory follicle has resulted in increased size of the dominant follicle and improved ovulation rates, with at least one study reporting increased subsequent pregnancy rates. In theory, the same concept when applied to MOET could improve embryo quality through improved oocyte maturation. However, there are limited number of studies that have evaluated this concept, with superovulatory results conflicting those of the monovulatory studies.

Our results from this study demonstrated that subluteal P4 (LP) resulted in a greater number of quality grade 1 embryos along with increase in the developmental stage of the embryos compared to the high progesterone (HP) cows that recovered a greater total number of embryos, of which were poorer in quality. These data suggest subluteal P4, during stimulation, provides less of a negative feedback on estradiol production, enabling follicle maturation earlier in development. Theoretically, this results in earlier ovulation of primed oocytes that develop into a greater number of stage 7 embryos along with an increased percent of quality grade 1 embryos per recovery. Comparatively, uninhibited P4 concentrations maintained suppression of estradiol during follicular growth, theoretically allowing FSH to recruit and develop a greater number of follicles, that when P4 was removed at end of stimulation, follicles had a shorter exposure to estradiol.

Therefore, while more follicles were present, they were not as mature at ovulation resulting in less developed, poorer quality embryos. Applying these results, an ET practitioner may utilize a HP protocol when there are recipients available for fresh transfer for embryos not acceptable for cryopreservation. Conversely, a producer with no recipients available and only wants the highest quality embryos cryopreserved may lean towards a LP protocol. Future research into further manipulation of P4 levels to find protocols which increases *both* embryo quality and quantity is worth investigating.

The third study was designed as a follow-up to Chapter 4 by utilizing the *in vivo* derived embryos to determine if a relationship between embryo stage and/or quality grade with embryonic sex existed. Previous research, primarily using IVF embryos, concluded timing of insemination/fertilization relative to oocyte maturation impacts sex of embryo with a greater percentage of females produced when fertilization occurred early in the maturation of oocytes. Meanwhile, research also concluded male embryos develop at a faster rate than females when fertilized at the same time. With IVF, each step of the process from oocyte maturation to fertilization and development can be monitored.

In Chapter 5, our results did not find an association between development stage and sex. However, quality grade had a significant effect in relation to sex, with a greater proportion of females being quality grade 2 compared to a greater proportion of quality grade 1 embryos being males. We acknowledge that most of the grade 2 embryos were morula and early blastocyst stage (Stage 4 and 5) and the blastocysts and expanding blastocysts (Stage 6 and 7) were all grade 1 on d 7 recoveries. When evaluating further developed (stage 6 and above) *in vivo* derived embryos, very rarely are the quality grades

less than 1 due to the increased size of the blastocoel cavity filling the entire perivitelline space to where extruded cells and defects are not as easily distinguishable. Further investigation in sexing embryos from earlier recoveries (d 6) in which the embryos may not be advanced and potentially allow for increased variation of quality grades is warranted. However, from these data, a producer raising replacement heifers may be more apt for transferring the quality grade 2 embryos into recipients. Alternatively, a producer capitalizing on male offspring would potentially be more interested in quality grade 1 embryos.

Multiple ovulation embryo transfer is a multifaceted technology with many factors affecting results. These studies provide ET practitioners with a better understanding of the impact P4 concentrations have on superovulated donors regarding the quality and quantity of embryos produced. Furthermore, they provide insight into location of embryos at time of recovery, to potentially influence catheter placement to improve the recovery rates of TQE. Continuing to research the impact P4 concentrations have on estradiol secretions and LH pulsatility to find the ideal ratio where embryo recoveries result in both increased quality and quantity would be invaluable. Additionally, investigation into how the stage and/or grade of embryos potentially effects their migration at the time of recovery or whether time of recovery impacts the quality would also be influential in improving recovery rates of TQE. While embryo sexing will not impact TQE, it is valuable information that will continue to be in demand, especially if scientific evidence can validate an association between embryo stage or quality grade of embryos to the sex. The more

knowledge gained in all aspects of MOET programs, the better equipped ET practitioners can be to assist producers in achieving their production goals.

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CURRICULUM VITA

CAITLIN E. WILEY, DVM

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EDUCATION

Iowa State University, Ames, IA
Clinical Resident of Theriogenology — **July 2015 – Present**
Eligible and Registered for Board Examination: Aug. 2017

Iowa State University, Ames, IA
Masters of Science, Reproduction Physiology — **Aug. 2015 – July 2017**
Thesis: "Methods to enhance embryo quality and recovery rates in superovulated beef cows"

The Ohio State University, Columbus, OH
Doctor of Veterinary Medicine — **Sept. 2009 – May 2013**

The Ohio State University, Columbus, OH
Bachelor of Science in Agriculture — **Sept. 2005 – June 2009**
Graduated Cum Laude
Minors: Life Science, Agribusiness

PROFESSIONAL POSITIONS

Iowa State University, College of Veterinary Medicine
Adjunct Instructor, Theriogenology Resident — **July 2015 – Present**
Provide clinical services and after hour care for Lloyd Veterinary Medical Center's Theriogenology, Food Animal Medicine and Surgery, and ISU Veterinary Field Services.

Maria Stein Animal Clinic
Associate Veterinarian — **July 2013 – July 2015**
Performed herd work for cattle clients including pregnancy diagnosis, sick cow evaluation and treatments, and developed vaccination protocols. Provided care to small animal clients including routine monitoring, surgeries, and hospitalization monitoring.

LICENSES/CERTIFICATIONS

Ohio	**2013 – Present**
USDA Accreditation – Category II	**2013 – Present**
Iowa	**2015 – Present**
American Embryo Transfer Association Certified	**2017 – Present**
Indiana	**2013 – 2015**

TEACHING EXPERIENCE

IOWA STATE UNIVERSITY

VDPAM 450: Disturbances of Reproduction (3rd Year Veterinary Clinical Course) **2015 – Present**
Lectured on Post-Partum issues in Bovine and on Male Anatomy and Hormone Interactions.

VDPAM 416: Bovine Reproduction Evaluation Lab (3rd Year Veterinary Elective) **2015 – Present**
Providing hands-on palpation experience, using an ultrasound to diagnose pregnancies, and teaching students how to artificially inseminate cows.

VDPAM 351: Bovine ET and Related Technologies (3rd Year Veterinary Elective) **2015 – Present**
Lectured on Superovulation and Trans-rectal Ultrasounding. Assist with labs.

VDPAM 471 C, FA, EQ: Comparative, Food Animal, and Equine Reproduction Rotation (4th Year Veterinary Clinical Courses) **2015 – Present**
Provide hands-on clinical learning experience for students and after-hour emergency services.

VDPAM 476: Food Animal Field Service Rotation (4th Year Veterinary Clinical Courses) **2015 – Present**
Provide hands-on clinical learning experience for students and after-hour emergency services.

VPDAM 477: Food Animal Medicine/Surgery Rotation (4th Year Veterinary Clinical Courses) **2015 – Present**
Provide hands-on clinical learning experience for students and after-hour emergency services.

VDPAM 451: Clinical Embryo Transfer (4th Year Veterinary Elective) **2015 - Present**
Provide embryo recovery and transfer services for clients in conjunction with hands-on experience.

VDPAM 495: Adv. Small Ruminant Technologies (4th Year Veterinary Elective) **2015 - Present**
Assist fourth year students with laparoscopic artificial insemination and embryo recovery techniques on goats.

AN S 426: Beef Cattle Systems Management **2015 - Present**
Discuss and demonstrate breeding soundness exams on bulls

VDPAM 340: Clinical Foundations (1st – 2nd Year Veterinary Elective) **2016**
Demonstrated and assisted with basic bovine physical exam

PUBLICATIONS (IN PROGRESS)

ABSTRACTS

Wiley, Caitlin, Tyler Dohlman, Marianna Jahnke, Colby Redifer, Patrick J. Gunn. Effects of endogenous progesterone during ovarian follicle superstimulation on embryo quality and quantity in beef cows. Society for Theriogenology Meeting. 2017. Abstract submission.

Dickson, M.J., S.K. Kvidera, E.A. Horst, J.A. Ydstie, K.L. Bidne, **C.E. Wiley**, P.J. Gunn, A.F. Keating, L.H. Baumgard. Chronic lipopolysaccharide infusion has no impact on dominant follicular size but affects

17β-estradiol in lactating dairy cows. American Dairy Science Association Conference, Pittsburgh, PA. 2017. Abstract submission.

THESIS

Methods to enhance embryo quality and recovery rates in superovulated beef cows

-Effects of endogenous progesterone during ovarian follicle superstimulation on embryo quality and quantity in beef cows. (In manuscript preparation)

-Localization of embryos within the uterus at time of recovery following superstimulation on beef heifers. (In manuscript preparation)

-Relationship between embryo sex and stage and quality grade of embryos in superovulated beef cows. (In manuscript preparation)

EXTENTION RELATED OPPORTUNITIES

Iowa 4-H Beef Blast – Bull Breeding Soundness Exam Lecture and Lab
Iowa 4-H Beef Round-Up – Embryo Transfer Lecture
Veterinary Student Clubs – Theriogenology and Surgery Lunch Lectures

PROFESSIONAL DEVELOPMENT

American Embryo Transfer Association – Certified, 2017
Canine Theriogenology Seminar – 2016, Asheville, NC
SFT Preconference seminar involving reproductive procedures in canine.
The Replacement Heifer, from Birth to Pre-Calving – 2014, Phoenix, AZ
AABP Preconference seminar on heifer rearing

MEMBERSHIPS

American Association of Bovine Practitioners (AABP)
Society for Theriogenology (SFT)
American Embryo Transfer Association (AETA)
International Embryo Transfer Society (IETS)
American Veterinary Medical Association (AVMA)
Ohio Veterinary Medical Association (OVMA)