INVESTIGATION OF MECHANISMS ASSOCIATED WITH ESTABLISHMENT AND MAINTENANCE OF PREGNANCY IN BEEF CATTLE

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by

JACLYN N. KETCHUM

Dr. Michael F. Smith, Thesis Supervisor

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The undersigned appointed by the Dean of The Graduate School, have examined the thesis entitled:

INVESTIGATION OF MECHANISMS ASSOCIATED WITH ESTABLISHMENT AND MAINTENANCE OF PREGNANCY IN BEEF CATTLE

Presented by Jaclyn N. Ketchum

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance;

_____

Dr. Michael F. Smith

_____

Dr. M. Sofia Ortega

_____

Dr. Jordan M. Thomas

_____

Dr. Thomas W. Geary

_____

Dr. Scott E. Poock

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ATP	Adenosine Triphosphate
Ab	Antibody
AI	Artificial Insemination
BFV	Blood Flow Volume
BCS	Body Condition Score
BW	Body Weight
BMP15	Bone Morphogenetic Protein 15
CAR	Caruncular
CV	Coefficients of Variation
cDNA	Complementary Deoxyribonucleic Acid
CIDR®	Controlled Internal Drug Release
CL	Corpus Luteum
CDOs	Cumulus-denuded oocytes
CEOs	Cumulus-enclosed oocytes
CXCL10	C-X-C Motif Chemokine Ligand 10
CT	Cycle Threshold
cAMP	Cyclic Adenosine Monophosphate

COX-2	Cyclooxygenase-2
d	Day
DPP	Days Postpartum
DNA	Deoxyribonucleic Acid
ET	Embryo Transfer
ELISA	Enzyme-Linked Immunosorbent Assay
E2	Estradiol 17β
EB	Estradiol Benzoate
ECP	Estradiol Cypionate
ESR1	Estradiol Receptor Alpha
ESR2	Estradiol Receptor Beta
EtOH	Ethanol
EDTA	Ethylenediaminetetraacetic Acid
EEF1A1	Eukaryotic Translation Elongation Factor 1 Alpha 1
FTAI	Fixed Time Artificial Insemination
FTET	Fixed Time Embryo Transfer
FSH	Follicle Stimulating Hormone
g	Gram

GVB	Germinal Vesicle Breakdown
GnRH	Gonadotropin Releasing Hormone
h	Hour
IFNT	Interferon-tau
IGLL1	Immunoglobulin Lambda-Like Polypeptide 1
IVF	In Vitro Fertilization
INCAR	Intercaruncular
ISG	Interferon Stimulated Gene
IFNT	Interferon Tau
ISG15	Interferon-Stimulated Gene 15
IM	Intramuscularly
KDa	Kilodaltons
L	Liter
LPE	Long Proestrus
LH	Luteinizing Hormone
MMP19	Matrix Metalloproteinase-19
MGA	Melengestrol acetate
mRNA	Messenger Ribonucleic Acid

MI	Metaphase I
MII	Metaphase II
µl	Microliter
µm	Micrometer
mg	Milligram
mL	Milliliter
mm	Millimeter
MX1	MX Dynamin Like GTPase 1
MX2	MX Dynamin Like GTPase 2
MYL12A	Myosin Light Chain 12A
ng	Nanogram
nm	Nanometer
NPPC	Natriuretic Peptide C
NPRC2	Natriuretic Peptide Receptor 2
OAS1	2'-5'-Oligoadenylate Synthetase 1
OXTR	Oxytocin Receptor
pg	Picogram
PLAU	Plasminogen Activator, Urokinase

PCR	Polymerase Chain Reaction
PAG	Pregnancy Associated Glycoprotein
P4	Progesterone
PR	Progesterone Receptor
PG	Prostaglandin $F_{2\alpha}$
RIA	Radioimmunoassay
ROC	Receiver Operating Characteristic
RI	Resistance Index
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
Se	Sensitivity
SLPI	Secretory Leukocyte Protease Inhibitor
Sp	Specificity
SPE	Short Proestrus
SLC2A1	Solute Carrier Family 2, Facilitated Glucose Transporter Member 1
SLC5A1	Solute Carrier Family 5 Member 1
STAI	Split Time Artificial Insemination
SEM	Standard Error of the Mean

TAMV	Time Averaged Maximum Velocity
ULF	Uterine Luminal Fluid
Wt	Weight
yr	Year

ABSTRACT

Preovulatory estradiol concentrations and expression of estrus have been shown to be associated with greater pregnancy rates in beef cattle. Experiment 1 was designed to determine if supplemental estradiol (E2) at GnRH-induced ovulation would decrease pregnancy loss in postpartum beef cows. The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation in 326 cows. The protocol included administration of GnRH (GnRH1) on day -9, treatment with exogenous progesterone via a CIDR® (controlled internal drug release) from day -9 to day -2, administration of prostaglandin $F_{2\alpha}$ (PG) on day -2, and an additional administration of GnRH (GnRH2) 48 h after PG (day 0) in cows that did not express estrus by that time. At 48 h post PG, cows were either estrual (Adequate endogenous E2 for estrus expression; n = 105) and assigned to the Positive Control group or nonestrual and randomly assigned to either no treatment (Low endogenous E2; Negative Control; n = 112) or administration of a 2 mL IM dose of 0.1 mg estradiol 17-β (Low endogenous E2 + adequate E2 supplementation; Estradiol; n = 109). To examine the timing of embryonic loss in the preceding treatments genetically identical pools of embryos, of similar developmental stage and quality grade, were transferred on day 7. To determine if there was an effect of E2 supplementation on the timing of pregnancy failure, interferon-stimulated gene expression in peripheral white blood cells and circulating progesterone, circulating pregnancy associated glycoprotein concentrations, and transrectal ultrasonography were used to detect evidence of a conceptus/fetus on days 19, 30, and 55/58, respectively. In regards to presence of a conceptus/fetus on specific days, there was no treatment by day interaction ($P = 0.9400$); however, there was a main effect of day ($P < 0.0001$), and there was a tendency for an

effect of treatment ($P = 0.0800$). Regardless of treatment, the proportion of cows that had evidence of a conceptus/fetus on days 7, 19, 30, and 55/58 was 100 ± 0.02^{a}, 40 ± 0.02^{b}, $32 \pm 0.02^{c,d}$, and 26 ± 0.02^{D}, respectively ($^{a,b,c,d}P < 0.05$; $^{D,d}P = 0.0856$). When the tendency for treatment as a main effect was evaluated, the Positive Control and Estradiol groups had similar ($P = 0.7095$) overall proportions of cows considered pregnant. The Negative Control group had lower ($P = 0.0388$) and tended to have lower ($P = 0.0786$) overall proportions of cows considered pregnant than the Positive Control and Estradiol groups, respectively. There was no treatment by Period of Loss interaction ($P = 0.9690$), nor a main effect of treatment ($P = 1.000$) when analyzing loss. There was a main effect of Period of Loss ($P < 0.0001$) such that greater ($P < 0.0001$) loss occurred in Period A (day 7-19) than both Period B (day 19-30) and C (day 30-55/58). The amount of loss that occurred in Periods B and C were similar ($P = 0.1441$). In summary, estradiol supplementation, at 48 h post PG, to nonestrual cows may potentially improve pregnancy rates; however, a second replicate is needed to solidify this conclusion.

Recently, a timed AI protocol (i.e. Split-time AI) was developed in which heifers that express estrus are inseminated at a predetermined time (e.g. 66 h post PG) and heifers that are nonestrual at that time are inseminated 24 h later (e.g. 90 h). Experiment 2 was designed to test the hypothesis that delaying the first timepoint from 66 h to 72 h would increase estrous response and pregnancy rate when using sex-sorted semen. Estrus was synchronized in 794 heifers across 4 locations with the 14-d CIDR®-PG protocol. Heifers were administered a progesterone intravaginal insert (CIDR®) on day 0, which was removed on day 14. On day 30, PG was administered, intramuscularly, and estrus detection aids were applied. Split-Time AI was performed based on estrous status. Within

location, heifers were blocked based on breed composition, source, sire, RTS, and BW and assigned within block to one of two experimental approaches: Approach 66: heifers that were estrual prior to 66 h after PG administration were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h), with GnRH administered to heifers that were nonestrual by that time; or Approach 72: heifers that were estrual prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that were nonestrual by that time. Within approach, heifers were preassigned to receive either SexedULTRA 4M™ sex-sorted or conventional semen. Overall estrous response did not differ between approaches. However, the proportion of heifers that were estrual by the first timepoint (66 h or 72 h following PG administration) was greater ($P < 0.0001$) with Approach 72 (76%; 302/395) compared to Approach 66 (61%; 242/399). Pregnancy rates to STAI differed ($P = 0.0005$) between semen type but were not affected by an approach x semen type interaction or by approach. Conventional semen (59%; 240/404) pregnancy rates to STAI were greater than sex-sorted semen (48%; 187/390). Among heifers that were estrual by Timepoint 1, pregnancy rates tended ($P = 0.08$) to differ between semen type (Conventional: 62% [174/280]; Sex-sorted: 55% [146/264]). Pregnancy rates of nonestrual heifers were reduced ($P < 0.01$) with sex-sorted semen (25%; 15/61) compared to conventional semen (41%; 23/56). In summary, when using sex-sorted or conventional semen for STAI in heifers following the 14-d CIDR®-PG protocol, pregnancy rates did not differ when timing of STAI was delayed by 6 hours. However, the proportion of estrual heifers prior to the first timepoint for STAI was greater when using the later timepoints.

CHAPTER 1

INTRODUCTION

Estrous synchronization and artificial insemination are technologies that can increase profitability in a beef operation. These technologies allow for genetic advancement and increase the proportion of females that conceive early in the breeding season. Early conceiving females not only calve earlier in the calving season, but also have an extended interval before the breeding season to return to cyclicity. These females have improved longevity and wean more pounds of calf compared to herd mates that calve later in the calving season. Weaning more pounds is a result of calves being born earlier in the calving season; therefore, being older and heavier at weaning (Cushman et al., 2013).

While the preceding technologies benefit the beef industry, less than 10% of beef producers in the USA utilize artificial insemination and(or) estrous synchronization. The associated time and labor have posed obstacles for integrating these techniques into the beef industry at a high rate (National Animal Health Monitoring System, 2008). Therefore, efforts have been directed towards the development of protocols that synchronize ovulation such that a group of females can be bred at a predetermined time, also known as Fixed -Time Artificial Insemination (FTAI). FTAI protocols require controlling the length of the luteal phase and follicular waves, culminating in precise synchronization of ovulation and semen deposition. Most FTAI protocols require the administration of Gonadotropin Releasing Hormone (GnRH) at the beginning of a

protocol to synchronize follicular waves and at the end of a protocol to induce ovulation of an oocyte that will be fertilized.

Substantial progress has been made in the application of artificial insemination to beef heifers and cows. FTAI allows every female an opportunity to conceive on the first day of the breeding season, while artificial insemination alone provides producers the opportunity to inseminate females to a genetically superior bull. Furthermore, with the commercialization of sex-sorted semen, producers can plan matings such that only genetically superior females or males are born. However, a current limitation to the use of sex-sorted semen for FTAI, in a beef herd, is that the pregnancy rate is approximately 0.75 of that with conventional semen, assuming detection of estrus and that AI is performed at the optimal time for sex-sorted semen. However, with the development of the SexedULTRA-4M™ technology the preceding statement may underestimate the pregnancy rate of sex-sorted semen compared to conventional semen. Further advancements that would allow the pregnancy rate of sex-sorted semen to more closely approximate that of conventional semen in a FTAI protocol would provide producers with an opportunity to increase the productivity, efficiency, and profitability of their operation.

The progress made regarding pregnancy rate following synchronization of estrus and ovulation is promising; however, there remains opportunity for improvement. For example, at FTAI there are normally heifers and cows that have and have not expressed estrus. A meta-analysis evaluated how expression of estrus influenced pregnancy rates following various FTAI protocols in heifers and cows (Richardson et al., 2016). Pregnancy rates were 27% greater for females that expressed estrus compared to those

that did not express estrus. Furthermore, estrus expression, when compared to no estrus expression reduced pregnancy loss from day 32 to 60 in Holstein cattle following FTAI (Pereira et al., 2014).

Since estrous expression increased pregnancy rates to FTAI in beef cattle, investigators have focused on strategies to increase the proportion of beef females that express estrus by the time of FTAI. One approach has been to increase the length of proestrus, which is the interval from onset of luteolysis to the preovulatory gonadotropin surge. Several experiments have shown a positive association between the length of proestrus and pregnancy rate following FTAI or embryo transfer (Bridges et al., 2010; Cruppe, 2015; Mussard et al., 2007). The length of proestrus can be manipulated via the administration of exogenous hormones commonly utilized in synchronization protocols. For example, removing a Controlled Internal Drug Release (CIDR®) intravaginal implant containing progesterone on day 5 (5-day CO-Synch + CIDR® protocol) instead of day 7 (7-day CO-Synch + CIDR® protocol) provides the dominant follicle more time to develop in a low progesterone (e.g. proestrus) and increased circulating estradiol endocrine environment before estrous expression or GnRH-induced ovulation. When the 5-day CO-Synch + CIDR® protocol was administered to yearling heifers, pregnancy rates were similar to the 7-day CO-Synch (Wilson, 2007). When females were exposed to either a 66 hour (h) or 54 h proestrus, those experiencing the 66 h proestrus had greater pregnancy rates (Busch et al., 2008). Increased or similar pregnancy rates to FTAI were reported in young cows and mature cows, respectively, that experienced a 56 h proestrus compared to a 72 h proestrus following a 7-day CO-Synch + CIDR® protocol (Dobbins et al., 2009).

In an attempt to understand how preovulatory estradiol may influence the establishment and maintenance of pregnancy, estradiol supplementation has been studied in both dairy and beef cows with mixed results. Administration of 1 mg of estradiol cypionate (ECP) 24 hours following the final prostaglandin $F_{2\alpha}$ (PG) injection in the CO-Synch protocol resulted in no improvements in fertility despite an increase in the proportion of females that expressed estrus (Hillegass et al., 2008). Estradiol cypionate (0.5 mg, IM) was administered 24 hours prior to AI to beef cows exposed to the CO-Synch + CIDR® protocol. Females that were induced to ovulate follicles < 12.2 mm in diameter and received ECP treatment had increased pregnancy rates compared to control cows. However; females induced to ovulate larger follicles did not have a higher pregnancy rate following ECP treatment (Jinks et al., 2013). ECP was incorporated into the CO-Synch protocol by administering 0.25 mg of ECP at the second GnRH injection in an attempt to determine if fertility could be increased in crossbred beef cows that were presynchronized with a single injection of PG. Pregnancy rate at 35 and 70 days did not differ between treatments (Howard et al., 2007). However, when ECP was administered to females at the second GnRH injection in the Ovsynch protocol and then artificially inseminated 10 hours later, those receiving ECP tended to have higher conception rates (68%) than females only receiving GnRH (57.5%) (Ahmadzadeh et al., 2003). Timing of insemination following ECP treatment and the physiological maturity of the ovulatory follicle may explain the conflicting results of the two previously mentioned studies.

Split-time AI (STAI) is another approach that has been used to increase the proportion of beef females that show estrus prior to insemination. With STAI only females that express estrus, as determined by an activated estrus detection device (e.g.

Estrotect® patch), are inseminated at the predetermined time point, normally 66 hours after PG administration for the 14-day CIDR®-PG protocol for heifers and for the 7-day CO-Synch + CIDR® protocol for cows. Females that have not expressed estrus by this time are afforded an additional 20- 24 hours (i.e. 90 h after PG-induced luteolysis) to express estrus, at which time all females are inseminated regardless of estrous expression. Females that have not expressed estrus by 90 h are administered GnRH at insemination. STAI resulted in more heifers or cows expressing estrus by the time of insemination. This approach improved pregnancy rates in beef heifers following a 14-d CIDR®-PG protocol (Thomas et al., 2014a) and a MGA-PG protocol (Knickmeyer et al., 2018) by increasing the proportion of heifers allowed to express estrus before AI.

It is not clear whether increased pregnancy rates with STAI, when using sex-sorted semen, are primarily due to increased estrus expression, deposition of sex-sorted semen closer to the time of ovulation, or a combination thereof. However, implementing a STAI program allows producers to inseminate a larger proportion of females at a more optimal time and affords a greater proportion of females time to express estrus. Delaying insemination with sex-sorted semen may improve pregnancy rates. Flow cytometric sorting and subsequent freeze-thaw results in precapacitation of sex-sorted sperm cells and potentially reduced sperm cell lifespan within the reproductive tract (Mocé et al., 2006). Sales et al. (2011) reported higher pregnancy rates to sex-sorted semen when time of FTAI was delayed from 54 to 60 h following progestin implant removal. These authors also reported that pregnancy rates to sex-sorted semen were increased when FTAI was performed closer to ovulation.

Previous work from our laboratories has shown that increased preovulatory concentrations of estradiol resulted in a higher pregnancy rate following embryo transfer in postpartum beef cows (Atkins et al., 2013; Jinks et al., 2013; Ciernia, 2019). The hypothesis that underlies the study in Chapter 3 is that administration of exogenous estradiol coincident with induced ovulation will not affect maternal recognition of pregnancy (i.e. interferon-stimulated gene expression), but reduce pregnancy loss thereafter as determined by pregnancy associated glycoproteins (PAGs) in maternal blood and transrectal ultrasonography. Therefore, the objective of chapter 3 was to determine the effect of exogenous estradiol administration on pregnancy establishment and maintenance in cows induced to ovulate with GnRH. An alternative strategy to estradiol supplementation for increasing pregnancy rate following FTAI with sex-sorted semen is to delay the time of insemination in a STAI protocol. This would allow further exposure of the maternal environment (e.g. uterus) to circulating estradiol in a proportion of the females and also allow deposition of sex-sorted semen closer to the time of ovulation. Therefore, we hypothesized that by delaying the initial insemination of heifers in a STAI protocol from 66 to 72 hours, the proportion of heifers that were estrual at insemination would be increased and that pregnancy rates to sex-sorted compared to conventional semen may be improved if using a later set of timepoints for STAI. Consequently, the objective of chapter 4 was to determine if delaying the time of insemination of sex-sorted semen by 6 h would increase estrous response and pregnancy rates in beef heifers with the goal of developing an approach to more effectively use sex-sorted semen in heifers.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

Estrous synchronization and artificial insemination are technologies that can increase profitability in a beef operation. These technologies allow for rapid genetic advancement and an increased proportion of females that conceive earlier in the breeding season. Early conceiving heifers not only calve earlier in the calving season, but also have more time to return to estrus before the start of the subsequent breeding season. Heifers that gave birth to their first calf in the first 21 days of the calving season, weaned more pounds, and had improved longevity compared to herd mates that calved later in the calving season (Cushman et al., 2013). Weaning more pounds is a result of calves being born earlier in the calving season; therefore, being older and heavier at weaning (Cushman et al., 2013).

While the preceding technologies have been beneficial for increasing the proportion of females that conceive early, less than 10% of beef producers in the USA utilize artificial insemination or estrous synchronization (National Animal Health Monitoring System, 2008). The associated time and labor have posed obstacles for integrating these techniques in cow calf operations at a high rate (National Animal Health Monitoring System, 2008). Therefore, efforts have been made to develop protocols that precisely synchronize ovulation, thereby eliminating estrous detection. Consequently, a group of females can be bred at a predetermined time, also known as Fixed-Time Artificial Insemination (FTAI). In order for FTAI to yield acceptable pregnancy rates, luteal phase length and follicular waves must be managed with exogenous hormones. The

luteal phase can be shortened by administering prostaglandin $F_{2\alpha}$ (PG) to advance estrous expression. Alternatively, estrus and ovulation can be delayed by the administration of exogenous progestins such as a CIDR® (Controlled Internal Drug Release; an intravaginal insert containing progesterone) or melengestrol acetate (MGA; an orally active progestin). To synchronize a follicular wave, FTAI protocols in North America frequently include administration of gonadotropin releasing hormone (GnRH) at the beginning of the protocol. When GnRH is administered at the beginning of a FTAI protocol, its role is to induce ovulation of a dominant follicle and thereby synchronize a follicular wave to allow the time of ovulation to be controlled more precisely. However, the purpose of a GnRH injection at the end of a FTAI protocol is to induce ovulation of a competent oocyte in heifers or cows that have not expressed estrus by the time of insemination. The purpose of this chapter is to review the scientific literature pertaining to follicular determinants of fertility, with particular emphasis on the role of estrous expression and preovulatory estradiol.

2.2 Follicular determinants of fertility

2.2.1 Effect of ovulatory follicle size on the establishment of pregnancy

Acquisition of ovulatory capacity in bovine follicles occurs between 7 and 10 mm in diameter in *Bos taurus* and *Bos indicus* cattle (Vasconcelos et al., 2001; Gimenes et al., 2008) and ovulatory follicle size at spontaneous or GnRH-induced ovulation is variable (Perry et al., 2005). In a FTAI protocol, administration of GnRH can induce ovulation in a wide range of dominant follicle sizes (Perry et al., 2005; Pohler et al., 2012), provided the granulosa cells have acquired LH receptors. In postpartum beef cows, Lamb et al.

(2001) reported that pregnancy rate to FTAI was decreased following GnRH-induced ovulation of follicles ≤ 12 mm (Lamb et al., 2001). When dominant follicles < 11.3 mm were induced to ovulate in postpartum beef cows, pregnancy was less likely to occur and embryo survival, after the initial pregnancy diagnosis, was reduced (Perry et al., 2005). However, when estrus and ovulation occurred spontaneously, dominant follicle size had no impact on pregnancy establishment or maintenance. After implementation of the Ovsynch protocol (GnRH was administered on day -9, followed 7 days later by prostaglandin $F_{2\alpha}$ and a second dose of GnRH 48 h after PG with all animals inseminated artificially 4–8 h after the second dose of GnRH) and FTAI in Holstein cows, more females became pregnant when estradiol concentration and pre-ovulatory follicle size were greater on day of FTAI (Lopes et al., 2007). Vasconselos et al. (2001) utilized follicle aspiration or no aspiration on day -5 or -6 of the OvSynch protocol (GnRH1 on day -9, PG on day -2, and GnRH2 on day 0) to create differences in dominant follicle diameter at GnRH2. The purpose was to understand how dominant follicle size impacts progesterone production and size of the subsequent CL formed following GnRH-induced ovulation. Aspiration decreased ovulatory follicle size and serum estradiol at GnRH2 and also decreased subsequent luteal volume, serum progesterone and pregnancy per AI compared to non-aspirated cows. Reduction of pregnancy rates have been reported in beef and dairy cattle by a number of investigators when physiologically immature follicles were induced to ovulate (Dias et al., 2009; Meneghetti et al., 2009; Perry et al., 2007; Mussard et al., 2007; Bridges et al., 2010; Sá Filho et al., 2010a; Sá Filho et al., 2009; Waldmann et al., 2006). In summary, the physiological maturity of the ovulatory follicle has an effect on pregnancy rate following FTAI, and not dominant follicle size.

The mechanisms underlying the decreased pregnancy rate and late embryonic survival following GnRH-induced ovulation (Perry et al., 2005) have not been elucidated, but may be due to an effect of the preovulatory follicle on acquisition of oocyte competence and(or) an effect on the preparation of the maternal environment for the establishment and maintenance of pregnancy. Atkins et al. (2013) conducted a reciprocal embryo transfer study to distinguish between follicular effects on oocyte competence and the uterine environment on pregnancy rate following GnRH-induced ovulation in postpartum beef cows. The investigators employed path analysis (Wright, 1934) to describe the relationships among the complex array of factors affecting pregnancy success. Following artificial insemination, embryos recovered from donors that had not expressed estrus and ovulated either a large or small dominant follicle were transferred fresh on day 7 into recipient females that had ovulated either a large or small dominant follicle. In donor cows, ovulatory follicle size had a positive effect on fertilization rate, circulating estradiol at GnRH-induced ovulation (day 0), and embryo survival on day 7; whereas, in recipient cows pregnancy rate was positively affected by circulating estradiol at GnRH-induced ovulation and circulating progesterone on day 7, and largely independent of donor cow effects. These results indicate that fertilization rate and embryonic survival before day 7 were influenced by dominant follicle growth rate and the follicle size of the donor cow; however maintenance of pregnancy after day 7 in recipient cows was dependent on ovulatory follicle size, production of preovulatory estradiol and the subsequent production of progesterone (Atkins et al., 2013).

2.2.2 Effect of the follicular microenvironment on oocyte competence

During folliculogenesis there is bi-directional communication between the oocyte and surrounding follicular cells in numerous species, which has a role in acquisition of oocyte competence (Fair, 2003; Gosden, 2002). The follicular microenvironment can affect the ability of the oocyte to develop into a viable embryo following fertilization (reviewed by Pohler et al., 2012). Increased oocyte competence was associated with bovine oocytes having a larger diameter (Otoi et al., 1997). Furthermore, increased oocyte competence was reported in oocytes that were obtained from larger follicles (Arlotto et al., 1996; Atkins et al., 2013; Hendriksen et al., 2000). Atkins et al. (2013) reported postpartum cows that were induced to ovulate a small dominant follicle had reduced fertilization rates and lower embryo quality.

Acquisition of oocyte competence: The development and survival of an embryo requires ovulation of a competent oocyte (Krisher, 2004). A competent oocyte, as defined by Sirard (2006), resumes meiosis after the preovulatory gonadotropin surge, proceeds through several rounds of cleavage divisions following fertilization, becomes a blastocyst, undergoes placentation, and results in the birth of a live offspring (Sirard et al., 2006). Oocyte competence has been defined as the ability of the oocyte to become meiotically, cytoplasmically, and molecularly mature (Fair et al., 1997). Spontaneous resumption of meiosis (i.e. nuclear maturation) can occur when an oocyte enclosed in a dominant follicle is exposed to a preovulatory gonadotropin surge or when the cumulus oocyte complex is separated from the mural granulosa cells. Interaction with spermatozoa and formation of a zygote occurs after resumption of meiosis and progression to metaphase II (Sirard, 2001). While bovine oocytes that have progressed to metaphase II

are more likely to cleave following fertilization (Bhak et al., 2006), the cytoplasmic maturity of the oocyte and maturity of the antral follicle that houses said oocyte are more indicative of its ability to reach the blastocyst stage than nuclear maturation alone.

For an oocyte to become competent both nuclear and cytoplasmic maturation must occur. The ability to condense chromosomes, complete meiosis I, and progress to the metaphase II stage is referred to as nuclear or meiotic maturation. However, the ultrastructural changes that allow an oocyte to be fertilized and progress to the blastocyst stage is defined as cytoplasmic maturation. A brief description of both aspects of oocyte maturation is provided below.

Nuclear (Meiotic) Maturation: A growing oocyte is generally considered to be meiotically incompetent in most species. By day 80 of gestation, bovine oocytes, within the fetal ovary, enter meiotic prophase I, which has several stages (e.g. leptotene, zygotene, and pachytene) that must be completed before being arrested at the diplotene stage. Transcriptional activity is minimal by the early pachytene stage but increases by the diplotene stage. An oocyte will remain at this stage of meiosis until the follicle in which it is housed responds to the preovulatory gonadotropin surge or becomes atretic. If an antral follicle responds to a luteinizing hormone (LH) surge, germinal vesicle breakdown (GVB, i.e. nuclear envelope breakdown) occurs and meiosis will resume, culminating in polar body expulsion and progression to metaphase II. (Fair, 2003; van den Hurk & Zhao, 2005).

An oocyte housed in a primordial follicle lacks a zona pellucida but will be surrounded by a single layer of pregranulosa cells that have a squamous shape. These pregranulosa cells have intimate contact with the oocyte and form transzonal processes

that span the zona pellucida following its formation (Baena & Terasaki, 2019). Primordial follicles are present in a fetal ovary by day 90 of gestation in cattle; however, primordial follicle activation cannot occur for another 40 to 50 days (Fortune et al., 2011). The transformation of granulosa cells from squamous to cuboidal shape is triggered by primordial follicle activation around 140 days of gestation. This also causes proliferation of granulosa cells (Fair, 2003). During progression from the primordial follicle stage to the secondary follicle stage there is formation of the zona pellucida, acquisition of cortical granules within the cytoplasm, a second layer of granulosa cells and the first detection of RNA synthesis (Fair et al., 1997). Differentiation of somatic cells into theca externa and interna, proliferation of granulosa cells, formation of cumulus cells, along with the first appearance of a fluid filled antrum are all characteristics of a tertiary follicle (Erickson & Shimasaki, 2000; Gilchrist et al., 2004). Activation of transcription in an oocyte occurs at the secondary follicle stage and continues until the tertiary follicle stage. A tertiary follicle, having a diameter of approximately 3 mm, will house an oocyte that is 110 µm in diameter (Hyttel et al., 1997). Parallel growth occurs between the early oocyte and follicle, however oocyte growth will plateau (120-130 µm) once the bovine follicle reaches a diameter of 3 mm, while the follicle has the potential to grow up to 15-20 mm (Fair, 2003).

Nuclear maturation occurs when the oocyte gains the ability to condense its chromatin followed by formation of the metaphase I (MI) plate, and subsequently the metaphase II (MII) plate and expulsion of a polar body. Arrest of the oocyte's chromatin at the MII stage signals the final event of meiotic maturation (Sirard, 2001). While 100 µm is the minimal intra-zonal diameter at which a bovine oocyte will resume meiosis, as

mentioned above, a bovine oocyte continues to acquire meiotic competence up to a diameter of 110 µm (Hyttel et al., 1997). This size of oocyte can be aspirated from a bovine follicle that is approximately 3 mm. Bovine follicles, with a diameter ranging from 2-3 mm to 15 mm, contain oocytes that are still acquiring mRNA and proteins, which likely explains why oocytes from follicles < 2 to 3 mm contain oocytes that are not meiotically competent (Arlotto et al., 1996; Fair et al., 1995). Dominant follicles at a more advanced stage produced oocytes that were more meiotically competent. This was also the case for follicles that were exposed to a surge of LH compared to those in which the LH surge had not occurred (Sirard et al., 2006). Otoi et al. (1997) reported an increase in the percentage of oocytes that underwent GVB when oocyte intra-zonal diameter was 110 µm compared to 100 µm. These oocytes were housed in follicles that had diameters of 1-7 mm. They also reported that oocytes with a diameter of 120-125 µm had the greatest potential to reach MII and develop into a blastocysts followed by those with a diameter of 115-120 µm. Oocytes with an intra-zonal diameter of 100-115 µm had the same potential to reach MII and were less likely to develop to the blastocyst stage than those with a diameter of 115-120 µm (Otoi et al., 1997). In summary, acquisition of meiotic competence is a gradual process that is dependent upon an increase in oocyte and follicular diameter.

Cytoplasmic Maturation: Both nuclear and cytoplasmic maturation occur in large antral follicles; therefore, these processes are heavily influenced by the follicular milieu with bidirectional communication between the oocyte and follicular cells playing a major role (Yamada & Isaji, 2011). Nuclear maturation is characterized by chromosomal segregation; whereas, cytoplasmic maturation is characterized by storage of mRNA and

proteins, transcription factors involved in the maturation process, and organelle reorganization as well as the ability to undergo fertilization and embryogenesis. During the progression from the germinal vesicle stage to metaphase II, there is movement of mitochondria, ribosomes, endoplasmic reticulum, cortical granules and the Golgi complex within an oocyte. The movement of these organelles is facilitated by cytoskeletal microfilaments and microtubules located in the cytoplasm (Ferreira et al., 2009).

Among oocytes with equivalent meiotic competence, those with an intrazonal diameter greater than 115 µm were more likely to reach the blastocyst stage following in vitro fertilization than oocytes with an intrazonal diameter of 105-114 µm. Therefore, while an oocyte housed in a smaller follicle (> 3 mm) may have obtained nuclear competency, it may be cytoplasmically immature and will need to complete cytoplasmic maturation to obtain complete developmental competence (Arlotto et al., 1996).

Effect of follicle diameter on developmental competence of embryos: Developmental competence, the ability of an embryo to become a blastocyst, was greater when oocytes were obtained from a follicle that had a diameter of 6-8 mm compared to follicles that were less than 6 mm. Oocytes derived from follicles greater than 13 mm had even greater oocyte competence (Hendriksen et al., 2000). Lonergan et al. (1994) also reported that oocytes collected from follicles greater than 6 mm were more likely to reach the blastocyst stage and were surrounded by more layers of cumulus cells than oocytes from follicles that were 2-6 mm (Lonergan et al., 1994). Differences were reported in the number of oocytes that developed to the blastocyst stage, with oocytes recovered from follicles greater than 6 mm having the highest blastocyst rate and those collected from

follicles less than 2 mm having the lowest blastocyst rate. However, there were no differences between follicle class size and the number of hatched blastocysts (Tan & Lu, 1990). When oocytes were aspirated from follicles ≤ 2.7 mm, there was no development; however, oocytes from 2.7-5 mm and ≥ 5 mm follicles had a similar number of oocytes develop to the morula stage following fertilization (Blondin & Sirard, 1994). Pavlok et al. (1992) reported that oocytes aspirated from 1-2 mm diameter follicles had impaired competence and were unable to cleave past the 8-cell stage. However follicles in the > 2-8 mm diameter range had similar developmental competence (Pavlok et al., 1992). Therefore, a more competent oocyte is generally obtained from a follicle ≥ 6 mm.

Metabolism in the bovine cumulus-oocyte complex: One form of communication between cumulus cells and an oocyte is through gap-junctions where nutrients, such as ATP and pyruvate, and small molecular weight molecules (< 1000 KDa) are transferred from cumulus cells to the immature oocyte (Anderson & Albertini, 1976; Buccione et al., 1990). Premature breakdown of gap junctional communication hindered cytoplasmic maturation. The opposite (improved competence as well as increased blastocyst rates) was observed when gap junctional communication was prolonged. Maintenance of gap junctions is important not only for transfer of nutrients, but also for transcription and remodeling chromatin. A study by Luciano et al. (2011) reported that when communication between follicular cells and oocytes was prematurely interrupted, oocytes not only failed to acquire full developmental competence, as they were still acquiring molecules and transcripts, but they also experienced abnormal chromatin condensation, which can hinder the functional differentiation of large-scale chromatin structure. This

could represent an epigenetic mechanism for aberrant global gene expression during the differentiation of the developing oocyte (Luciano et al., 2011).

Glycolysis, the pentose phosphate pathway, and the hexosamine biosynthesis pathway are three pathways that the bovine oocyte utilizes to metabolize glucose with glycolysis being the main pathway (Thompson et al., 2019). Once the product of glycolysis, pyruvate, is transported to the oocyte it enters the tricarboxylic acid cycle and undergoes oxidative phosphorylation to produce ATP. From hours 0-24 of maturation, glucose metabolism increased in both prepubertal heifers and adult cows, however at hour 12 of maturation, glucose metabolism in the oocyte was much lower in prepubertal heifers than adult cows. Similarly peak oxidative metabolism of both pyruvate and glutamine was lower in prepubertal heifers than adult cows (Steeves et al., 1999). Oocytes from prepubertal heifers have decreased cytoplasmic maturation compared to oocytes from mature cows. The preceding data suggests glucose metabolism within the cumulus cells may play a role in the ability of the oocyte to acquire developmental competence.

Importance of ATP to oocyte competence: Mitochondrial ATP, not only provides the oocyte with energy but also is used by adenylate cyclase to produce cAMP, which aids in maintaining meiotic arrest at the germinal vesicle stage. Stojkovi et al. (2001) reported higher morula and blastocyst rates in oocytes that morphologically were superior when compared to oocytes that were morphologically poor. The morphologically good oocytes also had increased ATP content (Stojkovic et al., 2001). In heat stressed animals, mitochondria-associated genes were impaired. These genes play a role in the mitochondrial DNA transcription, replication, and encoding of oxidative phosphorylation

complexes that are involved in ATP production. When ATP concentrations decrease below their required threshold, both oocyte maturation and embryonic development are impaired. This is one mechanism contributing to decreased fertilization and blastocyst rates in oocytes that are harvested during the summer months (Roth, 2018).

Oocyte is dependent upon the cumulus cells for pyruvate: Glucose is required in order for the bovine cumulus-oocyte complex to reach nuclear and cytoplasmic maturation. However, the oocyte itself is inefficient at metabolizing glucose and therefore relies on oxidative phosphorylation for the production of ATP. Proliferating cumulus cells and post-compaction embryos utilize high quantities of glucose that are metabolized through aerobic glycolysis, resulting in high quantities of lactate. However, the preferred carboxylic acid that enters the tricarboxylic acid cycle in the oocyte is pyruvate (Thompson et al., 2019). Geshi (2000) performed a study where cumulus-enclosed oocytes (CEOs) and cumulus-denuded oocytes (CDOs) were cultured for 24 hours in culture media either with or without sodium pyruvate. Cumulus-denuded oocytes cultured with sodium pyruvate had higher maturation and normal fertilization rates when compared to CDOs not cultured in sodium pyruvate. Blastocysts produced from CDOs cultured in sodium pyruvate developed into normal calves (Geshi et al., 2000). Cytoplasmic maturation and resumption of meiosis demands mitochondrial organization and continued metabolic activity (Calarco, 1995; Cummins, 1998; Hyttel et al., 1989; Loos et al., 1989; Steeves & Gardner, 1999; Van Blerkom et al., 1995; Van Blerkom & Runner, 1984). Stojkovic et al. (2001) classified slaughterhouse derived oocytes into four categories based on how homogenous the oocyte cytoplasm appeared and number of surrounding cumulus cells. Oocytes with the most homogenous cytoplasm and most

cumulus cell layers made up category 1, while oocytes that had a heterogeneously pigmented cytoplasm and almost no cumulus cells composed category 4. Category 1 nonmatured oocytes had greater ATP content than all other categories other than category 2. Matured oocytes belonging to categories 1, 2, and 3 all had similar ATP content, while category 4 matured oocytes had less ATP content then all other categories. Following IVF, category 1 and 2 oocytes resulted in the highest cleavage and blastocyst rates. Expanded blastocysts from category 1 oocytes had greater ATP content than the other categories and were more likely to hatch, a mechanical process dependent on energy. The ATP content was positively correlated to total cell number at the blastocyst stage, indicating that embryos with lower ATP levels may have lagging development and therefore fewer cells (Stojkovic et al., 2001). Meiotic and cytoplasmic maturation are energy intensive processes, therefore cumulus-oocyte complexes must be efficient at converting glucose and pyruvate into ATP.

2.3 Effect of preovulatory estradiol on the maternal environment

2.3.1 Physiological role of estradiol during the preovulatory period

Estradiol is synthesized in bovine ovarian follicles under gonadotropin regulation, described by the two cell-two gonadotropin model (Fortune and Quirk, 1988). Briefly, LH stimulates the production of androgen (i.e. androstenedione) in bovine theca interna cells, via the delta 5 pathway, and androstenedione is rapidly converted to testosterone in granulosa cells. Bovine granulosa cells respond to FSH and convert testosterone to estradiol by aromatase, and there is a progressive increase in estradiol synthesis during the 48 hours following the onset of luteolysis in cattle. Preovulatory estradiol has several

physiological roles in the establishment of pregnancy, including the expression of estrus (Coe & Allrich, 1989), induction of the preovulatory gonadotropin surge (Chenault et al., 1975), facilitating the transport of sperm (Hawk & Conley, 1975), and inducing endometrial progesterone receptors (Zelinski et al., 1982). In cattle, after initiation of the preovulatory gonadotropin surge, aromatase gene expression in granulosa cells decreased within 3.5 to 6 hours leading to a decrease in estradiol concentration in follicular fluid by 6 to 24 hours (Komar et al., 2001). In addition, the preovulatory gonadotropin surge induced a decrease in expression of 17 alpha-hydroxylase (CYP17A1) and aromatase (CYP19A1) in the theca and granulosa layer of the preovulatory follicle, respectively (Smith et al., 1994). Furthermore there was an increase in side-chain cleavage enzyme (P450scc/CYP11A1) and 3 β-hydroxysteroid dehydrogenase enzyme (3 β-HSD) expression in the luteinizing theca and granulosa cells (Smith et al., 1994). The preceding steroidogenic enzyme changes account for the rapid decrease in circulating concentration of estradiol following the preovulatory gonadotropin surge and the subsequent increase in progesterone during the early luteal phase.

Estradiol has an essential intrafollicular role in the establishment of pregnancy. The intrafollicular role of estradiol includes the following: 1) increased granulosa cell proliferation in many species (Drummond & Findlay, 1999; Goldenberg et al., 1972), 2) facilitation of cell cycle progression from G1 to S in granulosa cells (Quirk et al., 2006), 3) inhibition of granulosa cell apoptosis (Quirk et al., 2006), 4) formation of gap junctions among granulosa cells (Merk et al., 1972), 5) regulation of the expression of steroidogenic enzymes (Gore-Langton and Armstrong, 1994), 6) increased stimulatory action of FSH on aromatase activity (Zhuang et al., 1982), 7) control of oocyte meiotic

resumption by regulating natriuretic peptide C (NPPC) and natriuretic peptide receptor 2 (NPR2) (Liu et al., 2017; Zhang et al., 2011; Zhuang et al., 1982), and 8) enhanced progestin synthesis following gonadotropin stimulation (Welsh et al., 1983).

A reciprocal embryo transfer study was performed to determine the effect of ovulatory follicle size on the establishment of pregnancy in postpartum beef cows (Atkins et al., 2013). In addition to grouping the cows by ovulatory follicle size, the cows were also grouped according to low or high circulating estradiol on day 0 (GnRH-induced ovulation) resulting in the following treatment groups: Low-Low, High-Low, Low-High, and High-High where the first classification (Low or High) refers to circulating estradiol in donor cows and the second classification refers to recipient cows (Jinks et al., 2013). When embryos were transferred into recipients (day 7) that had high preovulatory estradiol at GnRH-induced ovulation, pregnancy establishment was increased compared to embryos transferred into recipients that had low preovulatory estradiol, regardless of the estradiol concentration at GnRH-induced ovulation in the donor cows. Increased preovulatory estradiol also increased the probability that a viable embryo, and not an unfertilized oocyte, was recovered from donor females (Jinks et al., 2013).

Estradiol and estrus expression: At FTAI there are normally heifers and cows that have and have not expressed estrus. A meta-analysis, including 10,116 beef females from 22 different studies, evaluated how expression of estrus influenced pregnancy rates following various FTAI protocols in heifers and cows (Richardson et al., 2016). Pregnancy rates were 27% greater for females that expressed estrus compared to those that did not express estrus. While expression of estrus contributed to the establishment of pregnancy, little to no repeatability in expression of estrus from one year to another was

found. However, factors such as body condition score (BCS; 1 = emaciated and 9 = obese) and estrus-cycling status (cycling or non-cycling) did influence estrus expression. A larger proportion of estrus-cycling cows and those females with a BCS of ≤ 4 did not express estrus compared to anestrous cows and those with a BCS > 4 respectively (Richardson et al., 2016). Females that exhibited estrus had greater concentrations of preovulatory estradiol compared to cows that did not exhibit estrus (Perry & Perry, 2008a). Furthermore, estrus expression, when compared to no estrus expression reduced pregnancy loss from day 32-60 in Holstein cattle following FTAI (Pereira et al., 2014).

Since estrus expression increased pregnancy rates to FTAI in beef cattle, investigators have focused on strategies to increase the proportion of beef heifers and cows that express estrus by the time of FTAI. One approach has been to increase the length of proestrus, which is the interval from onset of luteolysis to the preovulatory gonadotropin surge. Several experiments have shown a positive association between the length of proestrus and pregnancy rate following FTAI or embryo transfer (Bridges et al., 2010; Cruppe, 2015; Mussard et al., 2007). The length of proestrus can be manipulated via the administration of exogenous hormones commonly utilized in synchronization protocols. For example, removing a CIDR® on day 5 (5-day CO-Synch + CIDR® protocol) instead of day 7 (7-day CO-Synch + CIDR® protocol) coupled with PG administration and delaying FTAI until 72 h later provides the dominant follicle more time to develop in a low progesterone (e.g. proestrus) and increased circulating estradiol endocrine environment before estrus expression or GnRH-induced ovulation. However, the 5-day CO-Synch + CIDR® protocol requires two injections of PG, approximately 8 h apart, at CIDR® removal to accomplish complete luteolysis, since heifers or cows that

ovulate in response to GnRH administration at CIDR® insertion will have corpora lutea that are less responsive to PG-induced luteolysis.

Final development of a preovulatory follicle is initiated by the increased pulse frequency of LH (Ireland & Roche, 1983) that occurs following decreased circulating progesterone concentrations (Cruppe, 2015; Ireland & Roche, 1983; Kinder et al., 1996). The increased pulse frequency of LH secretion causes circulating estradiol to reach preovulatory concentrations (Kaneko et al., 1991). Increasing the length of proestrus lengthens the period of high frequency/low amplitude LH stimulation of a preovulatory follicle and a concomitant increase in preovulatory concentrations of estradiol. A lengthened proestrus provides a hormonal environment conducive to the growth of a mature dominant follicle; therefore, not only increasing preovulatory estradiol concentrations but also fertility (Day et al., 2019). For example, when the 5-day CO-Synch + CIDR® protocol was administered to yearling heifers, pregnancy rates were similar when compared to the 7-day CO-Synch (Wilson, 2007). When females were exposed to either a 66 h or 54 h proestrus, those experiencing the 66 h proestrus had greater pregnancy rates (Busch et al., 2008). Increased or similar pregnancy rates to FTAI were reported in young cows and mature cows, respectively, that experienced a 56 h proestrus when compared to a 72 h proestrus following a 7-day CO-Synch + CIDR® protocol (Dobbins et al., 2009). When proestrus length was manipulated in both a FTAI and FTET setting, females that experienced proestrus for 3 days (LPE) had greater pregnancy rates for both AI and ET compared to females that experienced a 1.5 day proestrus (SPE) (LPE-AI, 69.9%; LPE-ET, 55.7%; SPE-AI, 51.2%; SPE-ET, 43.7%) (Cruppe, 2015). However, Wilson et al. (2010) reported the lack of a significant

difference in estrous response, interval to estrus, synchronized conception rates, and pregnancy rates to AI between cows administered the 7-day Select Synch +CIDR® or the 5-day Select Synch + CIDR® protocols.

Split-time AI (STAI) is a second approach that has been used to increase the proportion of beef females that show estrus prior to insemination. With STAI only females that express estrus, as determined by an activated estrus detection device (e.g. Estrotect® patch), are inseminated at the predetermined time point, normally 66 hours after PG administration for the 14-day CIDR®-PG protocol for heifers or 66 hours for the 7-day CO-Synch + CIDR® protocol for cows. Females that have not expressed estrus by this time are allowed 24 h (i.e. 90 h after PG-induced luteolysis) to express estrus, at which time all females are inseminated regardless of estrus expression. Females that have not expressed estrus by 90 h are administered GnRH at insemination. STAI results in more heifers or cows expressing estrus by the time of insemination. Thomas et al. (2014a) reported higher pregnancy rates in heifers exposed to a 14-d CIDR®-PG and STAI (54%) compared to heifers that were all inseminated at a fixed time (46%).

Estrus Expression and Sex-Sorted Semen: The STAI approach may be especially practical when using sex-sorted semen, in which pregnancy rates are generally lower compared to conventional semen, particularly for heifers that have not expressed estrus. The development and enhancement of flow cytometry allowed for separation of X and Y bearing spermatozoa resulting in the commercialization of sex-sorted semen (Garner & Seidel, 2008). The decreased pregnancy rates observed with sex-sorted semen have been attributed to fewer sperm/dose and(or) the detrimental effect of flow cytometric sorting on sperm lifespan. Frijters et al. (2009) conducted a study to determine the impact of the

preceding factors on the decline in pregnancy rates to sex-sorted semen in cattle. Females were inseminated with sex-sorted semen from seven different Holstein bulls in which pregnancy rate was decreased by 13.6% compared to conventional semen. A third of that decrease (5%) was attributed to the sorting process while two thirds (8.6%) of the decrease in pregnancy rate could be attributed to the decreased number of sperm/dose. The effects of reduced sperm numbers per straw and the sorting process differed among bulls (Frijters et al., 2009).

Thomas et al. (2014b) compared pregnancy rates to FTAI with conventional semen, FTAI with sex-sorted semen, and STAI with sex-sorted semen in beef cows following the 7-d CO-Synch-CIDR® protocol. Among females that expressed estrus, FTAI with conventional semen resulted in the highest pregnancy rate (77%) compared to cows receiving sex-sorted semen at either the standard time (66 h post PG-induced luteolysis and CIDR® removal: 51%) or following STAI (42%). Cows that failed to express estrus and received conventional semen at FTAI (66 h) had a 37% pregnancy rate while those females that failed to express estrus and received sex-sorted semen at FTAI (66 h) had a pregnancy rate of 3%. Those females that failed to express estrus at 66 h and received sex-sorted semen at 90 h achieved a pregnancy rate of 36% (Thomas et al., 2014b). When STAI was performed in beef heifers that were synchronized with the 14-d CIDR®-PG protocol, the pregnancy rate with conventional semen (25.0×10^6 live cells per 0.5 mL straw) was 60% compared to 52% for sex-sorted semen (4.0×10^6 live cells per 0.25 mL straw). In the preceding study, the proportion of heifers that expressed estrus did not differ between groups. (Thomas et al., 2017). The results of the above studies show the importance of expression of estrus in females that will be inseminated with sex-

sorted semen and that by affording females that have not expressed estrus by 66 h an additional 24 h to express estrus, pregnancy rates to sex-sorted semen can be improved.

It is not clear whether increased pregnancy rates with STAI are due to increased estrus expression and(or) deposition of sex-sorted semen closer to the time of ovulation. Delaying insemination with sex-sorted semen may improve pregnancy rates. A reason pregnancy rates might be increased when sex-sorted semen is deposited closer to ovulation is that the flow cytometry process is generally believed to partially capacitate sperm. Sales et al. (2011) reported higher pregnancy rates to sex-sorted semen when time of FTAI was delayed from 54 to 60 h following progestin implant removal. They also reported that pregnancy rates to sex-sorted semen increased when FTAI was performed closer to ovulation. Pregnancy rates were greatest for females inseminated 0-12 h before ovulation (37.9%) while pregnancy rates in females that were inseminated either 12.1-24 h (19.4%) or > 24 h (5.8%) before ovulation were significantly lower (Sales et al., 2011). Dairy heifers inseminated with sex-sorted semen 16.1 to 24 h after onset of estrus had higher pregnancy rates (51.8%) than heifers inseminated 12 to 16 h after the onset of estrus (37.7%). However, the pregnancy rates for dairy heifers in which insemination was delayed to 24.1 to 30 h after onset of estrus did not differ from either of the previous insemination time points (45.5%; Sá Filho et al., 2010b). When sex-sorted semen was used to inseminate superstimulated Nelore cows, fewer transferable embryos were recovered when insemination occurred 12 and 24 h after administration of porcine LH (2.4 +/- 1.8) than when insemination was delayed to 18 and 30 h (4.5 +/- 3.0) (Soares et al., 2011). Conversely, Hall et al. (2017) reported that despite more cows exhibiting estrus before FTAI, there was no difference in pregnancy rates to sex-sorted semen when

insemination was delayed from 72 to 80 h following PG-induced luteolysis and CIDR® removal with a 5-day CO-Synch + CIDR® protocol in postpartum cows (Hall et al., 2017). Further research to determine optimum timing of insemination with sex-sorted semen and whether that timing differs between semen sorting processes could be beneficial to both the beef and dairy industries.

In vitro fertilization, with sex-sorted semen, followed by timed embryo transfer is an alternative strategy for achieving higher pregnancy rates compared to FTAI with sex-sorted semen. A study was conducted to compare the number of pregnancies created by in vitro fertilization and timed embryo transfer (IVF/FTET) or FTAI, in which both methods utilized sex-sorted semen from the same bull. Pregnancy rates were greater with IVF/FTET (35.4%; 345/974) compared to FTAI (30%; 293/974; Pellegrino et al., 2016). Sex-sorted semen has potential to have a large impact on both the beef and dairy industries. Development of management tools such as STAI or delayed insemination have potential to increase the proportion of females that conceive to sex-sorted semen. However, more work is needed to determine if pregnancy rates with sex-sorted semen and conventional semen.

2.3.2 Effect of estradiol on the uterus

The requirement of preovulatory estradiol for the establishment of pregnancy following embryo transfer has been shown in ovariectomized sheep (Miller et al., 1977) and cattle (Madsen et al., 2015). Miller and colleagues (1977) administered different sequences of estradiol and progesterone to ovariectomized ewes to identify a profile of circulating estradiol and progesterone administration that resulted in a normal pregnancy rate following embryo transfer (Figure 2.1). Following ovariectomy, ewes were

	Priming Progesterone	**Preovulatory Estradiol**	**Maintenance Progesterone**	**% Ewes with Normal Embryos**
	Days 3-14 (5 mg, 2x/day)	Days 15-16 (35 mg over 5 injections)	Days 18-33 (5 mg, 2x/day)	Days 27 and 34
Group 1				77.8%
Group 2				0%
Group 3				16%

Figure 2.1. Effect of administration of ovarian steroids on the establishment of pregnancy in ovariectomized ewes (Miller et al., 1977 modified by McLean, 2018)

administered the following steroid treatments: Group 1) Priming progesterone administration (5 mg; twice daily) on days 3 to 14 (day 0 = start of experiment coinciding with a single, 25 µg injection of estradiol) to simulate circulating progesterone prior to luteolysis, estradiol (35 µg over 5 injections) on days 15 to 16 to simulate changes in preovulatory estradiol, and maintenance progesterone (5 mg; twice daily) on days 18 to 33 to simulate circulating concentrations of progesterone during a cycle when a ewe received an embryo, Group 2) Priming progesterone administration on days 3 to 14, no estradiol on days 15 to 16, and maintenance progesterone on days 18 to 33, and Group 3) No priming progesterone administration on days 3 to 14, estradiol on days 15 to 16, and maintenance progesterone on days 18 to 33. When ewes received priming progesterone, estradiol, and maintenance progesterone (Group 1) the pregnancy rate was 77.8%.

However, when ewes were administered priming progesterone followed by maintenance progesterone and no estradiol (Group 2) none of the ewes maintained pregnancy (pregnancy rate = 0%) following embryo transfer. The pregnancy rate in ewes that received estradiol injections followed by maintenance progesterone and no priming progesterone (Group 3) had a pregnancy rate of 16%. In summary, exposure of the uterus to estradiol was required for the establishment of pregnancy following embryo transfer. Furthermore, progesterone priming before estradiol treatment further increased pregnancy rate in ovariectomized ewes (Miller et al., 1977).

In another study with ovariectomized ewes, by the same research group, when maintenance progesterone was administered, but estrual estradiol was removed from the regimen, endometrial protein synthesis rate, RNA/DNA ratio, and uterine weight were decreased compared to ewes that received estrual estradiol. The same decrease of protein

synthesis rate and the RNA:DNA ratio was observed in the oviduct when estradiol was omitted. The authors suggested that changes in RNA:DNA ratio may influence glandular secretions. Their results also suggested that the presence and(or) absence of priming progesterone and estrual estradiol influence the uterine environment in ways that are either supportive or destructive to the development and survival of embryos (Miller & Moore, 1976).

In a related study, ovariectomized sheep were exposed to the previous steroid treatment regimen with the addition of maintenance estradiol where maintenance estradiol along with maintenance progesterone mimicked early pregnancy (Miller et al., 1977). Embryo transfer was performed 4 days following mating and ewes were sacrificed either 6 or 13 days after embryo transfer. The removal of maintenance estradiol did not influence the number of embryos that developed normally. However, when estrual estradiol or priming progesterone was removed, embryos stopped developing normally within one to two days. The amount of protein in the uterine lumen, rate of protein synthesis, and RNA:DNA ratio were decreased at the time of embryo transfer in ewes that did not receive estrual estradiol. In ewes that received estrual estradiol, protein synthesis and RNA:DNA ratios were increased. However, ewes that received maintenance estradiol had increased protein synthesis, but without estrual estradiol did not experience improved embryo survival rates. Therefore, these changes may not be the mechanism associated with embryo survival. The concentration of progesterone receptors was decreased in ewes that did not receive priming progesterone and those ewes that did not receive estrual estradiol. The authors suggested that endometrial sensitivity to

progesterone may be the mechanism by which estrual estradiol influences embryo development (Miller et al., 1977).

In cattle, the importance of preovulatory estradiol in the establishment and maintenance of pregnancy was demonstrated by administering no estrogen, estradiol benzoate (EB), or estradiol cypionate (ECP) during a simulated preovulatory period in ovariectomized multiparous beef cows. Ovariectomy occurred on day 0 of the estrous cycle and the subsequent luteal phase was mimicked by administering a CIDR® immediately after surgery and then replaced every seven days for 3 weeks. Nine days prior to the mimicked onset of estrus, a CIDR® was administered and removed seven days later. At this time PG was administered and the preovulatory gonadotropin surge, that normally occurs near the onset of estrus, was mimicked on day 0 via the administration of GnRH. Females received one of the three following treatments: Group 1. ECP (2.5 mg) administered in sesame oil 36 h prior to GnRH, Group 2. EB (1.2 mg) administered in sesame oil 12 h prior to GnRH, and Group 3. Control- no ECP or EB. In each of the preceding groups, injectable progesterone (10 mg/mL in sesame oil) was administered intramuscularly in increasing twice daily doses (20, 40, 80, 120 mg) on days 3, 4, 5, and 6 respectively to simulate the early luteal phase. From days 7 to 29 exogenous progesterone, in the form of one or more CIDRs® was administered. On the seventh day following GnRH administration each cow received an embryo. Interferon stimulated gene (ISG; *ISG15*, *MX2*, and *OAS1*) expression in peripheral white blood cells was measured on day 19 and ISG expression was greater in females treated with ECP or EB than the control group. Madsen et al. (2015) also measured PSPB from days 22 to 28 and reported that on day 24, pregnancy rates (based on PSPB concentration) were greater in cows that

received estradiol treatment, however pregnancy rates, as determined by PSPB, did not differ from days 25-28. Transrectal ultrasonography was performed on d 29 and females exposed to ECP or EB had a greater pregnancy rate than the control group. The embryo loss that occurred between d 19, as indicated by ISG expression, and d 29 was greatest in the control cows (62%), followed by the ECP group (50%). Females exposed to EB treatment had the least amount of loss (39.7%; Madsen et al., 2015). The embryonic loss in this study appears to be concentrated around days 22-24 of gestation which coincides with early stages of placentation. Therefore, while the mechanism is not known, preovulatory estradiol may play a role in both placental attachment and embryonic growth.

Estrus, estradiol and uterine pH: When the uterine pH of cows that either did or did not express estrus was measured, following a CO-Synch protocol, females not expressing estrus had lower uterine pH (pH = 6.72) than females that did not express estrus (pH = 7.0). In females that expressed estrus there was a transient decline in uterine pH from 36 hours prior to the onset of estrus until estrus. From the time of onset of estrus to approximately 6 hours after the onset of estrus uterine pH increased rapidly. Neither the decrease in uterine pH prior to estrus nor the rise after estrus was observed in females that did not express estrus (Perry & Perry, 2008b). The effect of preovulatory estradiol on the uterine environment, more specifically uterine pH, was studied in females that were either treated with 1 mg of estradiol cypionate 36 hours prior to the final injection of GnRH in a CO-Synch protocol or not. Females that expressed estrus and received ECP treatment had a higher uterine pH (7.0 ± 0.07) then estrual females that were not administered ECP (pH = 6.72 ± 0.10; $P = 0.02$) and nonestrual females that were

administered ECP (6.81 ± 0.09; $P = 0.06$; Perry & Perry, 2008a). Bolzenius and others (2016) found that as uterine pH became more acidic at the time of FTAI, pregnancy rates increased. They also noted that NA^{+}/H^{+} exchanger isoforms 1, 2, and 3 play a role in altering uterine pH during the onset of estrus (Bolzenius et al., 2016). A transient alteration in uterine pH around the time of estrus may provide a mechanism for increasing longevity of sperm in the reproductive tract. Jones and Bavister (2000) reported that as pH decreased, motility of bull sperm decreased and longevity increase.

Hawk (1983) demonstrated that in order for sperm to be efficiently transported in the female reproductive tract, females needed exposure to estradiol (Hawk, 1983). This point was further demonstrated in ovariectomized females, when exogenous estradiol was required for the transport of sperm (Allison & Robinson, 1972). Estrus initiation (or the second administration of GnRH) occurs approximately 30 h prior to ovulation (Pursley et al., 1995; Vasconcelos et al., 1999). At initiation of estrus, uterine pH was lower which may have led to a greater longevity of sperm through a transient decrease in motility. The peak in uterine pH observed 6 hours after estrus, may play a role in aiding in sperm transport as Goltz (1988) reported that as pH increased so did sperm motility (Goltz et al., 1988). In a FTAI scenario, where ovulation can be induced in the absence of elevated estradiol concentrations, the ability of a sperm to survive until ovulation may be compromised as the interval of insemination to ovulation is lengthened.

Estradiol and uterine blood flow: Endocrine, paracrine, and autocrine factors are all involved in the development and growth of not only the embryo but also the placenta. The bovine fetus receives nutrients through both hematotroph and histotroph. Hematotroph allows exchange of nutrients between fetal and maternal circulation (Bazer

et al., 1990). Since most reproductive hormones elicit their effects through an endocrine manner, the use of doppler ultrasonography has become a popular way to noninvasively investigate the reproductive tract and the vasculature that supports it. Bollwein et al. (2000) used transrectal doppler ultrasonography to characterize uterine blood flow over two consecutive estrous cycles in four Deutsch Fleckvieh cows, three of which were multiparous while one was nulliparous. Uterine blood flow was greatest on day -3 (Day 0 = ovulation) and circulating concentrations of estradiol were greatest on day -2 (Bollwein et al., 2000). Similarly, Rawy et al. (2018) measured uterine blood flow in 6 lactating Holstein-Friesian cows via transrectal doppler ultrasonography following the intramuscular administration of 10 mg of estradiol benzoate. They reported a positive correlation between circulating concentrations of estradiol and both uterine blood flow and uterine artery diameter (Rawy et al., 2018).

A positive correlation between uterine blood flow and estradiol concentration was found when uterine blood flow was monitored in nonpregnant cyclic cows. When estrus is defined as day 0, blood flow increased two days prior to estrus and remained high until the day after estrus. This corresponded with elevated estradiol concentrations. Uterine blood flow decreased until day 6 which was then followed by a brief increase from day 7-15. Estradiol concentrations increased transiently on days 9-12 and 13-18. A decrease in uterine blood flow on d 16 preceded the increase in blood flow two days prior to estrus. Progesterone concentrations increased on day 5 and remained elevated until day -2. Uterine blood flow in pregnant cows was similar to that of nonpregnant cows up to 13 days post mating at which time blood flow to the gravid horn increased. On the nineteenth day of pregnancy, the flow of blood to the gravid horn resembled that of day

13. From days 25 to 30 blood flow again increased to the gravid horn and decreased in the nongravid horn. Progesterone concentrations and uterine blood flow to the gravid horn was correlated positively. The increase in blood flow to the gravid horn, caused by the preimplantation conceptus, was similar to blood flow observed when estradiol was elevated. This increase in blood flow may also increase the blood flow to the CL found on the ipsilateral horn and therefore may increase progesterone secretion which would aid in maintaining pregnancy (Ford & Chenault, 1981). When blood flow to the ovary that contains the CL decreases due to natural (Niswender et al., 1976) or PG- induced luteal regression (Nett et al., 1976), a decrease in progesterone synthesis was also observed. Therefore, the preimplantation conceptus may secrete a factor that acts similar to estradiol to cause dilation of the utero-ovarian vasculature that is ipsilateral to the pregnancy allowing the maintenance of pregnancy (Ford & Chenault, 1981).

Estradiol and uterine gene expression: Bridges and others (2012) researched how the concentration of preovulatory estradiol impacted conceptus development and uterine gene expression in beef heifers. They created different preovulatory concentrations of estradiol by altering proestrus length. Cows in the high estradiol (HiE2) treatment were administered PG 2.5 days prior to GnRH administration (d0); whereas, the low estradiol (LoE2) treatment group was administered PG 1.5 days prior to GnRH administration. Heifers received embryos via embryo transfer on day 7 and were slaughtered on day 15.5. Treatment groups were confirmed by the circulating preovulatory estradiol concentration being higher in the HiE2 treatment than the LoE2 treatment. Nonpregnant animals had higher estradiol concentrations at d-2 and a greater abundance of nuclear progesterone receptors than pregnant animals; however, these animals had lower estradiol

concentrations at d0. While progesterone concentration, conceptus development, and IFNT levels did not differ between treatments, pregnant heifers had higher progesterone concentrations at day 4, 7, and 15.5. LoE2 treatment heifers expressed fewer nuclear progesterone receptors in the glandular epithelium and had decreased mRNA estradiol receptor alpha (ESR1) concentrations in the uterine endometrium than the HiE2 treatment. Nonpregnant heifers had higher ESR1 concentrations than pregnant heifers at day 15.5. The lack of a difference in progesterone concentration, conceptus development, and IFNT expression, but the presence of a difference in PGR and ESR1 between treatment groups indicate that preovulatory estradiol may have prolonged effects on the uterus that would impact conceptus development past day 15.5 but before day 30 (Bridges et al., 2012). The aforementioned proestrus lengths and protocol were used in a previous study; however, instead of performing embryo transfer, females were artificially inseminated 12 hours after GnRH administration to create Long Proestrus (LPE) and Short Proestrus (SPE) treatment groups. Females in the LPE treatment had greater pregnancy rates at day 30 (50.0%, $P < 0.01$) than those in the SPE treatment (2.6%). The SPE treatment had an increased incidence of short luteal phases and lower progesterone concentrations during the mid- luteal phase of the subsequent estrous cycle. In both experiments follicular diameter was similar between treatments, suggesting that other follicular characteristics may be determinant of follicular maturity (Bridges et al., 2010).

Steroid hormones of ovarian origin, such as estradiol, are transported in blood until they bind to their specific cytoplasmic receptor in a target cell. The steroid receptor complex can then translocate to the nucleus and initiate changes in gene expression (Gorski et al., 1968). The nuclear receptor becomes activated once the protein chaperone

molecules disassociate and dimerization occurs. This is followed by the steroid-receptor complex being bound to the steroid response element which tends to be located in the promoter region of the gene (Tsai & O'Malley, 1994). The nuclear estradiol receptor has two forms, ESR1 and ESR2, which are different genes. Estradiol binds to these nuclear receptors with high affinity, however, the DNA-binding affinity differs (Hall & McDonnell, 1999). The uterus, mammary gland, testes, pituitary, liver, kidney, heart, and skeletal muscle all express ESR1 mRNA while transcripts for ESR2 are mainly found in the ovary and prostate (Beker-van Woudenberg et al., 2004). The cytoplasmic model of estradiol receptors can be found in both the endometrial and myometrial layers of the uterus however the different layers respond differently to estradiol (Jackson & Chalkley, 1974). There are four pathways by which estradiol can elicit its biological effects 1) the classical ligand-dependent pathway, 2) the ligand-independent pathway, 3) the ERE-independent pathway, and 4) the cell-surface (nongenomic) signaling (Hall et al., 2001). The progesterone receptor is found in 2 isoforms, PR-A and PR-B, which is the result of a single gene having differing promoter starts sites resulting in different PR mRNA (Edwards, 2005). The functionality of the PR isoforms differs; however, they are generally coexpressed in target tissues (Stormshak & Bishop, 2008).

In an attempt to better understand the role of preovulatory estradiol and postovulatory progesterone at the cellular level, Zelinksi and others characterized cytoplasmic progesterone receptors in the bovine endometrium during proestrus and diestrus. During proestrus and early metestrus both estradiol and progesterone endometrial cytoplasmic receptor concentrations were increased compared to females during diestrus. However, later in the luteal phase, the concentration of endometrial

cytoplasmic estradiol receptors decreased. They concluded that estradiol can induce the synthesis of cytoplasmic estrogen and progesterone receptors in the endometrium (Zelinski et al., 1982). Their results further support work from Koligian and Stormshak that concluded progesterone has an inhibitory effect on the replenishment of cytoplasmic estradiol receptors during the luteal phase in the ovine endometrium (Koligian & Stormshak, 1977).

A study to determine how estrus expression influenced endometrial, conceptus, and luteal gene expression was conducted in Nelore cattle exposed to an E2 and P4 based FTAI protocol (Davoodi et al., 2016). Females were artificially inseminated on day 0 and sacrificed 19 days later at which time only gravid uteri were studied. In the CL of females that expressed estrus, genes that were related to apoptosis, progesterone synthesis, and the prostaglandin receptor were downregulated compared to females not expressing estrus. Females not expressing estrus had smaller conceptuses compared to females that expressed estrus. A larger conceptus, as observed in females that expressed estrus, may be beneficial as IFNT- stimulated gene expression may be enhanced since a larger conceptus would occupy a greater amount of luminal space. In the day 19 conceptuses of females expressing estrus there were four genes that were differentially expressed (*ISG15*, *PLAU*, *BMP15*, and *EEF1A1*). Endometrial transcripts related to prostaglandin synthesis (*OTR* and *COX-2*) as well as the immune system and cell adhesion (*CXCL10*, *IGLL1*, *MX1*, *MX2*, *MMP19*, *MYL12A*, and *SLPI*) were influenced by the expression of estrus. *CXCL10* in the ruminant promotes adhesion and pulls trophoblast cells to the endometrium while *MMP19* aids in conceptus/endometrial interactions. *MYL12A* regulates the signaling generated by protrusion and adhesion as well as stabilizing cell-

cell junctions. *SLPI* stops the activation of the transcription factor NF-kB which could lead to the down regulation of *COX2*. *COX2* is an enzyme that is needed for PG synthesis. *SLPI* and the downregulation of *OTR* may both play a role in preventing *COX2* from synthesizing PG which would favor the maintenance of the CL. This study showed that changes in reproductive gene expression around the preimplantation period was altered in a way that was favorable towards the elongating conceptus when estrus was expressed near FTAI (Davoodi et al., 2016).

Conceptuses, resulting from the artificial insemination of beef females that were induced to ovulate, were collected on day 16 of gestation from females that were grouped according to preovulatory estradiol concentrations (high or low; Northrop et al., 2018). Intercaruncular (INCAR) and caruncular (CAR) tissues were collected so that total cellular RNA could be extracted and protein, glucose, and IFNT was measured in uterine luminal fluid (ULF). Preovulatory estradiol concentration did not have an effect on the recovery rate of conceptus, protein concentration in ULF, IFNT, nor apoptosis in the trophectoderm. However, both the CAR and INCAR tissues of females with high preovulatory estradiol concentrations had greater abundance of *SLC2A1* and *SLC5A1*. Both *SLC2A1* and *SLC5A1* are glucose transporters found in the uterus. Glucose is transported into the endometrial epithelial cells through both the basal and lateral membrane via *SLC2A1*, which is a facilitative glucose transporter that is thought to be regulated by not only progesterone but also IFNT. Between days 10 and 16 of gestation, the sodium dependent glucose transporter *SLC5A1* moves glucose from the endometrial epithelial cells through the apical membrane and into ULF. Glucose transport into the ULF is critical for conceptus growth and development. It is thought that these

transporters work cooperatively to provide the conceptus with optimal levels of glucose. However the effect of the greater abundance of these glucose transporters on conceptus recovery rate was not apparent on day 16 of gestation, therefore it is possible that these transporters may elicit their effect later in gestation (Northrop et al., 2018).

Estradiol and uterine endometrial thickness: Cows that naturally expressed estrus experienced increases in and maximum endometrial thickness at 114 h and 96 h prior to ovulation, respectively (Sugiura et al., 2018). Endometrial thickness began to decrease 6 h following ovulation. Progesterone concentrations began to decrease 138 h prior to ovulation while estradiol concentration began to increase 132 h prior to ovulation. Cows induced to express estrus via exposure to a CIDR® for 5-7 days followed by administration of PG at CIDR® removal, experienced decreased progesterone concentration and increased endometrial thickness 144 h prior to ovulation while estradiol concentrations began to increase 102 h prior to ovulation. These results indicate that as progesterone concentrations decrease endometrial thickness will increase, however estradiol may also play a role in sustaining and/or enhancing these changes as endometrial thickness was most strongly correlated with the Estradiol: Progesterone ratio in both natural and induced estrus groups (Sugiura et al., 2018). Endometrial thickness was measured by transrectal ultrasonography, in lactating Holstein cows administered the Ovsynch protocol. Endometrial measurements were taken once per day starting at PG-induced luteolysis and ending 2 days following GnRH and AI. The thickness of the endometrium increased from 7 to 9.5 mm following PG, remained thick (> 9 mm) for two days and then became thinner on both day 1 (8 mm) and 2 (7.4 mm) following the second GnRH injection. The supplementation of 1 mg of estradiol- 17β eight hours prior to the

second GnRH injection in the Ovsynch protocol increased P/AI in females with thinner endometrium. However; in females that had an endometrial thickness measurement of > 8 mm 48 hours following PG administration, estradiol supplementation did not improve P/AI (Souza et al., 2011).

2.3.3 Estradiol supplementation

Estradiol supplementation has been studied in both the dairy and beef industry in attempts to better understand how preovulatory estradiol may influence the establishment and maintenance of pregnancy. Results from both dairy and beef vary.

Estradiol supplementation in dairy cattle: Detection of estrus in dairy cattle is challenging due to the type of housing, feet, and leg problems, and decreased circulating concentrations of preovulatory estradiol due to increased metabolism by the liver (Binelli et al., 2019). Estradiol supplementation around the time of AI has been studied in dairy cattle not only to increase the incidence of estrus expression, but to examine the effect on pregnancy rate to FTAI. In an experiment conducted by Souza et al. (2007), 1 mg of estradiol -17β was administered 8 h prior to the GnRH-induced ovulation that preceded AI in the Ovsynch protocol. The purpose of the estradiol injection was to mimic the physiological increase in preovulatory estradiol, while not disrupting the natural decline in estradiol following the preovulatory gonadotropin surge. While no overall differences were detected in pregnancy rates between the control and estradiol supplemented cows, the pregnancy rates of the females that were in lower body condition, that ovulated a follicle 15-19 mm in diameter, or that were primiparous were increased in the estradiol -17β supplemented cows. However no difference in pregnancy loss was detected when comparing females supplemented with estradiol -17β to the control cows (Souza et al.,

2007). In another experiment, Hillegass administered 1 mg of estradiol cypionate 24 hours following the final PG injection in the CO-Synch protocol. Similar to the previous study no improvements in fertility were observed when estradiol cypionate was administered even though there was an increase in the proportion of females that expressed estrus (Hillegass et al., 2008). Conversely, Brusveen et al. (2009) reported, the administration of 0.5 mg of estradiol-17β at the time of final administration of GnRH in the Ovsynch protocol did not influence pregnancy rates when different ovulatory follicle class sizes were analyzed even though the proportion of females that expressed estrus was increased (Brusveen et al., 2009). In the same study, when estrual females that did not ovulate, or had incomplete luteal regression where removed, estradiol administration tended to have a negative effect on pregnancy rates (Brusveen et al., 2009). In summary, although the supplementation of estradiol around the time of FTAI increased the proportion of females that expressed estrus, pregnancy establishment and maintenance were not increased.

Estradiol supplementation in beef cattle: Estradiol cypionate (0.5mg, IM) was administered 24 hours prior to AI to beef cows exposed to the CO-Synch + CIDR® protocol (Jinks et al., 2013). Females that were induced to ovulate follicles < 12.2 mm benefited from ECP treatment as pregnancy rates were increased compared to control cows. However; females induced to ovulate larger follicles did not benefit from ECP treatment (Jinks et al., 2013). During the follicular phase it is thought that GnRH-induced ovulation before the endogenous preovulatory gonadotropin surge may cause estradiol secretion to decrease earlier than normal and therefore fertility may be adversely affected. Howard et al. (2007) incorporated ECP into the CO-Synch protocol by

administering 0.25 mg of ECP at the second GnRH injection in an attempt to determine if fertility could be increased in crossbred beef cows that were presynchronized with a single injection of PG. Body weight, BCS, and age were used to pair cows that were then randomly assigned to either receive ECP at the second GnRH or GnRH alone. Females were immediately artificially inseminated following treatment. Bulls were turned out with cows 14 days following AI. Pregnancy was detected on day 35 and 70 via ultrasonography. Pregnancy rate at 35 and 70 days did not differ between treatments (Howard et al., 2007). However, when ECP was administered to females at the second GnRH injection in the Ovsynch protocol and then artificially inseminated 10 hours later, those receiving ECP tended to have higher conception rates (68%) than females only receiving GnRH (57.5%) (Ahmadzadeh et al., 2003). Timing of insemination following ECP treatment may explain the conflicting results of the two previously mentioned studies.

Use of estradiol in South American synchronization protocols: Estrus synchronization protocols in South America often involve the use of both progesterone and estradiol. Unlike in the United States of America, estradiol use is permitted in South America and thus allows for alternative synchronization protocols in both *Bos indicus* and *Bos taurus* cattle. A typical estradiol/progesterone based FTAI synchronization protocol involves administration of estradiol benzoate, on day 0 to induce follicular atresia and synchronize follicular waves coincident with insertion of an exogenous progesterone releasing device. The use of estradiol at the beginning of a protocol may be slightly more efficient at synchronizing follicular waves than GnRH since GnRH can only eliciting its effect on follicles that have acquired LH receptors in the granulosa layer

(50-65% ovulation rate when administered to dairy cows at random stages of the estrous cycle; Vasconcelos et al., 1999; Thatcher et al., 2002; Giordano et al., 2013; Wiltbank and Pusley, 2014). Alternatively, estradiol will have a negative feedback on FSH to cause antral follicles to become atretic, regardless of size; however; 25-30% of cows may ovulate a persistent follicle due to incomplete regression of the previous follicular wave (Monterio et al., 2015; Melo et al., 2016).

Exogenous progesterone inserts will typically be removed after 7, 8, or 9 days and PG will be administered to ensure luteolysis. An estrogen (ECP or EB) is frequently administered at or following progesterone insert removal to simulate the preovulatory rise in estradiol resulting in induction of a preovulatory gonadotropin surge. To synchronize ovulation the following approaches have been used: 1) Administration of ECP at exogenous progesterone insert removal, 2) Administration of EB 24 h following exogenous progesterone insert removal, 3) Administration of LH 54 h after exogenous progesterone insert removal, and 4) GnRH administration at the time of FTAI. Pregnancy per AI in estradiol/progesterone treatments range between 40 and 60% (reviewed by Bó et al., 2018).

2.4 Conclusions

Estradiol is a steroid hormone, produced by ovarian follicles via the two cell-two gonadotropin model, that acts in concert with progesterone to aid in the establishment and maintenance of pregnancy. Estradiol has intra- and extrafollicular roles associated with follicle development, estrus expression, induction of the preovulatory gonadotropin surge, oviductal/uterine function, and several other areas related to reproduction. During

the preovulatory period estradiol has an intrafollicular role where it acts on granulosa cells to stimulate proliferation (Drummond & Findlay, 1999; Goldenberg et al., 1972), development (Quirk et al., 2006), and communication with the oocyte (Merk et al., 1972). Preovulatory estradiol also regulates expression of steroidogenic enzymes (Gore-Langton and Armstrong 1994), increases the ability of FSH to act on aromatase (Zhuang et al., 1982), regulates meiotic resumption of the oocyte via NPPC and NPR2 (Liu et al., 2017; Zhang et al., 2011), and enhances synthesis of progestins following gonadotropin stimulation (Welsh et al., 1983). Estradiol has also been reported to have an effect on the maternal environment (reviewed by Pohler et al. 2012). Estradiol has been shown to be higher in females that exhibit estrus, which itself improves pregnancy rates (Richardson et al., 2016) and therefore synchronization protocols that have longer proestrus periods provide a hormonal environment that is conducive to the development of a mature dominant follicle (Bridges et al., 2010; Cruppe, 2015; Mussard et al., 2007). Estradiol has several roles in the uterus and is required for a pregnancy to be established (Miller et al., 1977). Furthermore, it appears preovulatory estradiol may be playing a role in embryonic growth and placental attachment (Madsen et al., 2015). Estradiol influences uterine pH (Perry & Perry, 2008a), endometrial thickness (Souza et al., 2011), uterine blood flow (Bollwein et al., 2000; Ford & Chenault, 1981; Rawy et al., 2018), and uterine gene expression (Bridges et al., 2010; Davoodi et al., 2016; Koligian & Stormshak, 1977; Northrop et al., 2018; Zelinski et al., 1982). The supplementation of preovulatory estradiol in estrus synchronization protocols has been employed as a way to better understand the role of preovulatory estradiol on pregnancy rates and results seem to be dependent on the timing of supplementation in relation to insemination (Benelli et al.,

2019; Brusveen et al., 2009; Hillegass et al., 2008; Howard et al., 2007; Jinks et al., 2013; Souza et al., 2007). Estradiol plays several reproductively important roles and is crucial to the establishment of pregnancy.

CHAPTER 3

INFLUENCE OF PREOVULATORY ESTRADIOL ON THE ESTABLISHMENT AND MAINTENANCE OF PREGNANCY IN BEEF CATTLE

3.1 Abstract

An experiment was designed to determine if supplemental estradiol (E2) at GnRH-induced ovulation would decrease pregnancy loss in postpartum beef cows. The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation in 326 cows, which included administration of GnRH (GnRH1) on day -9, exposure of exogenous progesterone via a CIDR® (controlled internal drug release) from day -9 to day -2, administration of prostaglandin $F_{2\alpha}$ (PG) on day -2, and an additional administration of GnRH (GnRH2) 48 h after PG (day 0) in cows that did not express estrus by that time. At 48 h post PG, cows were either estrual (Adequate endogenous E2 for estrus expression; n = 105) and assigned to the Positive Control group or nonestrual and randomly assigned to either no treatment (Low endogenous E2; Negative Control; n = 112) or administration of a 2 mL IM dose of 0.1 mg estradiol 17-β (Low endogenous E2 + adequate E2 supplementation; Estradiol; n = 109). Administration of exogenous E2 increased circulating E2 into the physiological range for the preovulatory period and E2 remained elevated above baseline for approximately 8 h. To examine the timing of embryonic loss in the preceding treatments, genetically similar pools of embryos of similar developmental stage and quality grade were transferred on day 7. To determine if there was an effect of E2 supplementation on the timing of pregnancy failure, interferon-stimulated gene expression in peripheral white blood cells and circulating progesterone,

circulating pregnancy associated glycoprotein concentrations, and transrectal ultrasonography were used to detect evidence of a conceptus/fetus on days 19, 30, and 55/58, respectively. In regards to presence of a conceptus/fetus on specific days, there was no treatment by day interaction ($P = 0.9400$); however, there was a main effect of day ($P < 0.0001$) and there was a tendency for an effect of treatment ($P = 0.0800$). Regardless of treatment, the proportion of cows that had evidence of a conceptus/fetus on days 7, 19, 30, and 55/58 was 100 ± 0.02^{a}, 40 ± 0.02^{b}, $32 \pm 0.02^{c,d}$, and 26 ± 0.02^{D}, respectively ($^{a,b,c,d}P < 0.05$; $^{D,d}P = 0.0856$). When the tendency of treatment as a main effect was evaluated, the Positive Control and Estradiol groups did not have differing ($P = 0.7095$) overall proportions of cows considered pregnant. The Negative Control group had lower ($P = 0.0388$) and tended to have lower ($P = 0.0786$) overall proportions of cows considered pregnant than the Positive Control and Estradiol groups, respectively. There was no treatment by Period of Loss interaction ($P = 0.9690$), nor a main effect of treatment ($P = 1.000$) in terms of Period of Loss. There was a main effect of Period of Loss ($P < 0.0001$) such that a greater ($P < 0.0001$) amount of loss occurred in Period A (day 7-19) than both Period B (day 19-30) and C (day 30-55/58). The amount of loss that occurred in Periods B and C were similar ($P = 0.1441$). In summary, estradiol supplementation, at 48 h post PG, to nonestrual cows may potentially improve pregnancy rates, however a second replicate is needed to further examine this conclusion.

3.2 Introduction

Beef heifers and cows that express estrus by the time of FTAI have a higher pregnancy rate than females that do not express estrus (Richardson et al., 2016). Expression of estrus is positively associated with preovulatory secretion of estradiol (Coe

& Allrich, 1989) and is a marker of the physiological maturity of a dominant follicle (Perry et al., 2005). Several differences between cows that express estrus or have higher preovulatory circulating concentrations of estradiol compared to those induced to ovulate and have lower estradiol include: increased uterine contractions believed to affect sperm transport (Hawk 1983), a difference in uterine pH that may affect sperm motility and longevity (Perry and Perry, 2008b) increased oocyte competency as a result of a more efficient or mature glycolytic pathway in surrounding cumulus cells (Dickinson et al., 2018), increased fertilization success and embryo viability on day 7 (day 0 = GnRH-induced ovulation; Atkins et al., 2013), more appropriate timing of ovulation relative to insemination (Saacke et al., 2000), increased endometrial progesterone receptors (Zelinski et al., 1982) and increased postovulatory circulating progesterone concentrations (Atkins et al., 2013). However, the mechanisms and timing of the preceding effects of estrus/estradiol related to an improvement in pregnancy rate are unclear.

The overall hypothesis underlying the previous work from our laboratories is that ovulation of sub-optimal follicles (i.e. reduced estradiol secretion) will decrease both pregnancy rate and late embryonic/fetal survival (days 28 to 80 post breeding). These physiological responses are likely due to both a decrease in oocyte competence and inadequate estradiol stimulation in preparation of the maternal environment for the establishment and maintenance of pregnancy. From previous studies, we have determined that the size of the dominant follicle at induced ovulation, but not at spontaneous ovulation, affects the incidence of late embryonic/fetal mortality (Perry et al., 2005). Furthermore, induced ovulation of a physiologically immature follicle provides

insufficient preovulatory estradiol for optimal preparation of a maternal environment for pregnancy (Atkins et al., 2013; Jinks et al., 2013; Ciernia et al., 2018) and also results in release of an oocyte that has not yet acquired competence for fertilization; thus embryonic development is compromised (Atkins et al., 2013; Dickinson et al., 2018). However, a difference in conceptus development is not realized until after day 16 of gestation (Bridges et al., 2012; McLean et al., 2018; Northrop et al., 2018;). In order to elucidate the mechanism by which preovulatory estradiol affects uterine receptivity, in postpartum beef cows, we need to establish an experimental model to determine when embryonic mortality occurs following embryo transfer on day 7. The specific hypothesis was that administration of exogenous estradiol coincident with induced ovulation in cows not detected in estrus will not affect maternal recognition of pregnancy (i.e. interferon-stimulated gene expression) but reduce pregnancy loss thereafter as determined by pregnancy associated glycoproteins (PAGs) in maternal blood and transrectal ultrasonography. Therefore, the objective of this study was to determine the effect of exogenous estradiol administration on pregnancy establishment and timing of embryonic loss associated with insufficient preovulatory estradiol concentrations in beef cows induced to ovulate with GnRH.

3.3 Materials and Methods

3.3.1 Treatment groups

The following experimental design was used to determine if supplemental estradiol (E2) at GnRH-induced ovulation would decrease pregnancy loss in postpartum beef cows: 1) Estrual cows that ovulated spontaneously (i.e. Adequate endogenous E2 for

estrus expression; Positive Control; n = 105); 2) Nonestrual cows that were induced to ovulate with GnRH (i.e. Low endogenous E2; Negative Control; n = 112); and 3) Nonestrual cows that were induced to ovulate with GnRH but received supplemental exogenous E2 (i.e. Low endogenous E2 + adequate E2 supplementation; Estradiol; n = 109). To examine the timing of embryonic loss in the preceding treatments genetically identical pools of embryos, of similar developmental stage and quality grade, were transferred on day 7 (day 0 = estrous expression or GnRH-induced ovulation) into cows within each treatment. To determine if there was an effect of E2 supplementation on the timing of pregnancy failure, evidence of a conceptus or fetus was determined at the following time points: 1) Day 19 by using interferon-stimulated gene (ISG) expression and circulating progesterone, 2) Days 24 and 30 by using circulating concentrations of pregnancy associated glycoproteins (PAGs), and 3) Day 55/58 by using transrectal ultrasonography following estrus expression or GnRH-induced ovulation. In addition, circulating concentrations of progesterone were determined on days -2, 0, 7, 19, 24, 30, and 55/58 to further assist in determining the timing of embryonic loss.

3.3.2 Synchronization of ovulation

The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation. The protocol included administration of GnRH (Factrel®; IM; 100 µg; Zoetis; Kalamazoo, MI; GnRH1) on day -9 to induce ovulation of the dominant follicle and initiation of a new follicular wave in the majority of the cows. Exogenous progesterone, via an intravaginal implant in the form of a CIDR® (CIDR®; controlled internal drug release containing 1.38 g progesterone; Zoetis; Kalamazoo, MI) was administered from day -9 to day -2 to prevent spontaneous ovulation and induce cyclicity. Administration of

prostaglandin $F_{2\alpha}$ (Lutalyse® HighCon, IM; 25 mg; Zoetis; Kalamazoo, MI) (PG) occurred on day -2 to induce luteolysis, and an additional administration of GnRH (Factrel®; IM.; 100 µg; Zoetis; Kalamazoo, MI; GnRH2) was administered on day 0 (48 h after PG and CIDR removal) to induce ovulation. Ovulation was induced via administration of GnRH2 in only those cows assigned to the Negative Control and E2 treatment groups.

3.3.3 Animals

Cows were housed at Fort Keogh Livestock and Range Research Laboratory in Miles City; Montana and all procedures were approved by the Fort Keogh Animal Care and Use Committee (ACUC approval No. 120418-1). There was a total of 326 crossbred postpartum beef cows assigned to the experiment and for ease of handling they were managed as two herds with 168 cows in herd 1 and 158 cows in herd 2. The experimental treatment schedule (Figure 3.1) was identical for both herds. On day -9, cow body condition scores, based on a 1 to 9 scale (1 = emaciated; 9 = obese), and body weights were obtained. Estrus or ovulation of cows (n = 326) were synchronized using the 7-day CO-Synch + CIDR® protocol described above. All cows were observed for estrus expression on day 0, 7, 19, 24, and 30 with the aid of Estrotect® patches (Estrotect®; Estrotect Inc., Spring Valley, WI). Patch scores (0 = missing, 1 = 0-25% activated, 2 = 25-50% activated, 3 = 50-75% activated, 4 = 75-100% activated; (Pohler et al., 2016) were recorded and those females with a patch score ≥ 3 or 0 were classified as estrual. Cows that were nonestrual by 48 h post PG received GnRH2 and were randomly assigned to receive either no treatment or administration of a 2 mL IM dose of 0.1 mg estradiol 17-

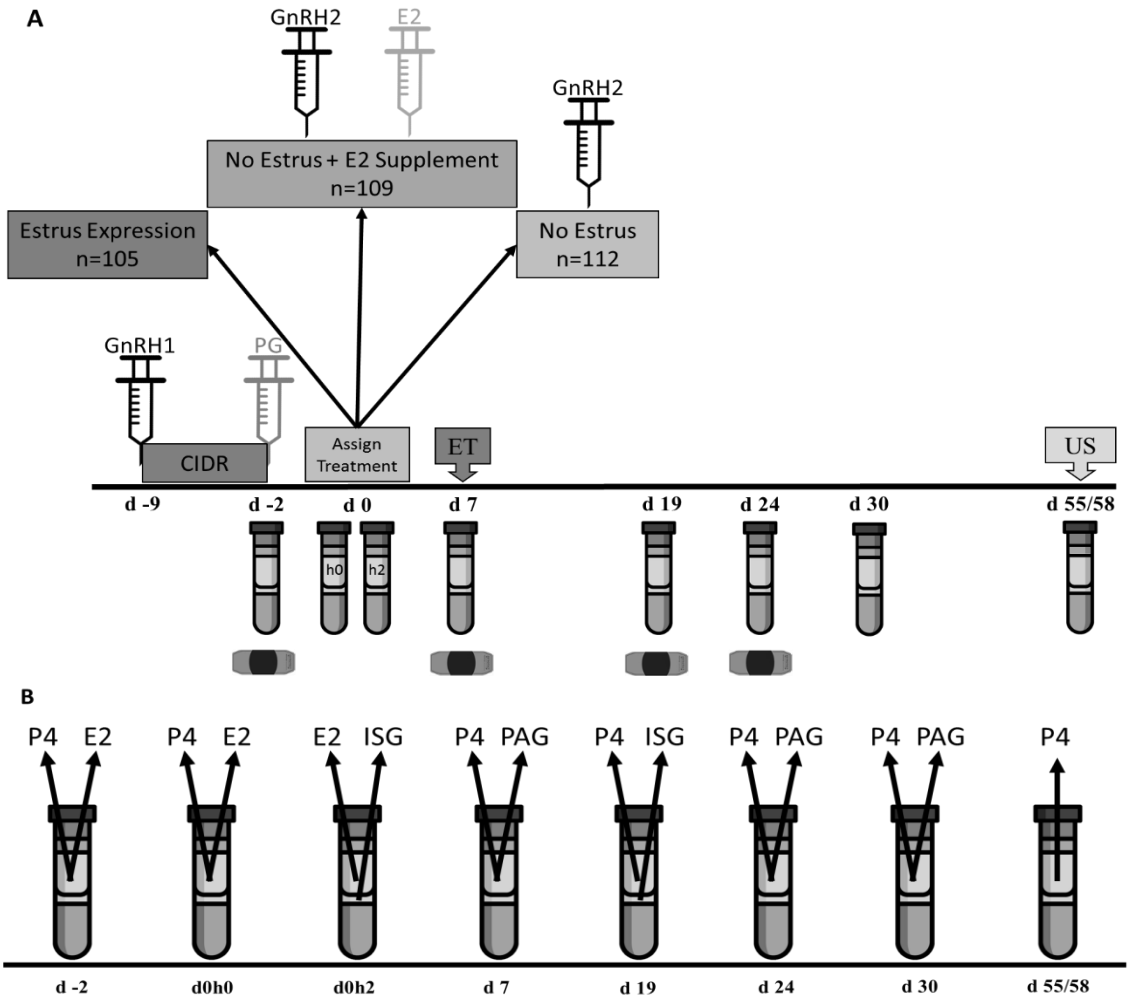


Figure 3.1. *Panel A)* The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation. The protocol required administration of GnRH1 on day -9, exposure of exogenous progesterone via an intravaginal implant in the form of a CIDR® from day -9 to day -2, and administration of prostaglandin $F_{2\alpha}$ (PG) on day -2. On day 0 cows were assigned to 1 of 3 treatment groups: 1) Estrual cows that ovulated spontaneously (i.e. Estrus expression group [n = 105]; Adequate endogenous estradiol for estrus expression; Positive Control); 2) Nonestrual cows that were induced to ovulate with GnRH2 (i.e. No estrus group [n = 112]; Low endogenous estradiol; Negative Control) and 3) Nonestrual cows that were induced to ovulate with GnRH2 but received supplemental exogenous estradiol (i.e. No estrus expression + estradiol supplement group [n = 109]; Low endogenous estradiol + estradiol supplement; Estradiol). Embryo transfer (ET) occurred on day 7. Pregnancy was detected via ultrasonography (US) on days 55/58 and 88/90. Estrus detection aids (Estrotect®) were applied on d -2, 7, 19, and 24. *Panel B)* Blood samples were collected on d-2, 0h0, 0h2, 7, 19, 24, 30 and 55/58. Progesterone (P4) was measured on d -2, d0h0, 7, 19, 24, 30, and 55/58. Estradiol (E2) was measured on d-2, 0h0, and 0h2 while Pregnancy Associated Glycoproteins (PAG) were measured on d 7, 24, and 30. Buffy coats were collected from d0h2 and 19 samples for measurement of Interferon-stimulated gene (ISG) expression.

β. This dose was determined following a preliminary study that was conducted at Fort Keogh Livestock as described below.

3.3.4 Preliminary experiments

The following preliminary experiments were conducted to determine the appropriate concentration of estradiol 17-β to administer on day 0 to mimic physiological preovulatory concentrations of estradiol at estrus. The preliminary experiments were conducted with identical experimental designs other than the doses of estradiol 17-β administered. As described above (see Synchronization of Ovulation), the 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation in 29 or 31 cows in preliminary experiment 1 and 2, respectively. Briefly, GnRH and CIDR® insertion occurred on day -9, administration of PG and CIDR® removal on day -2, and a second injection of GnRH occurred 48 h after PG injection (day 0). On day 0 ovarian scans were performed via ultrasound (Aloka 500V) to record dominant follicle diameter. Follicle diameter was measured by averaging follicular diameter at the widest point and at a right angle to the first measurement using the internal calipers. From the 29 or 31 cows that were exposed to the 7-day CO-Synch + CIDR® protocol, 9 or 10 cows, respectively were eliminated from the study due to expression of estrus prior to administration of GnRH2, having more than one dominant follicle, or not having a clear dominant follicle in preliminary experiment 1 or 2. Cows were blocked on dominant follicle size and assigned within block to receive 1 of 4 (0 mg: n = 5; 0.25 mg: n = 5; 0.5 mg: n = 5; 1.0 mg: n = 5) doses of estradiol 17-β in preliminary experiment 1 (see Appendix Figure 1) or 1 of 3 (0 mg: n = 7; 0.1 mg: n = 7; 0.2 mg: n = 7 mg) doses of estradiol 17-β in preliminary experiment 2. Estradiol 17-β was administered by intramuscular injection at GnRH2

injection and Estrotect® patches were applied on day -2. Blood samples were collected by venipuncture 0, 1, 2, 4, 6, 8, 12, and 24 hours after estradiol 17-β administration on day 0 to characterize the estradiol profile and patch scores were recorded at each bleed time. Samples were centrifuged (1200 x g for 20 minutes at 4˚C) and plasma was aspirated and stored at -20˚C until RIA as described below.

Estradiol 17-β (250 mg; Sigma E2758) was resuspended in 100 mL Absolute Ethanol (Sigma Ethyl Alcohol, Pure; E7023) by stirring for 30 minutes. Sesame oil was added to bring the total volume to 1 L resulting in a final concentration of 0.25 mg/mL in 10% EtOH sesame oil. Further dilutions were made by adding sesame oil. Mean circulating concentrations of estradiol 17-β for preliminary experiment 1 and 2 are shown in Appendix Figure 1 and Figure 3.2 respectively. The peak in estradiol 17-β concentration occurred between 1 and 2 hours for both 0.1 mg and 0.2 mg doses. Estradiol 17-β concentrations returned to baseline within 8 and 24 hours for 0.1 mg and 0.2 mg, respectively. The proportion of cows in replicate 2 that had an activated patch score by 24 h after injection was 0, 71, and 71% for the 0, 0.1 mg, and 0.2 mg doses, respectively. Based on these results it was determined that administration of 0.1 mg of estradiol 17-β would most closely approximate preovulatory concentrations of estradiol 17-β at estrus.

3.3.5 Embryo transfer

All embryos were *in vivo* produced from 65 donor cows at the USDA ARS, Fort Keogh Livestock and Range Research Laboratory. Donor cows were superovulated via a standard protocol and were artificially inseminated to one of 6 sires. Seven days

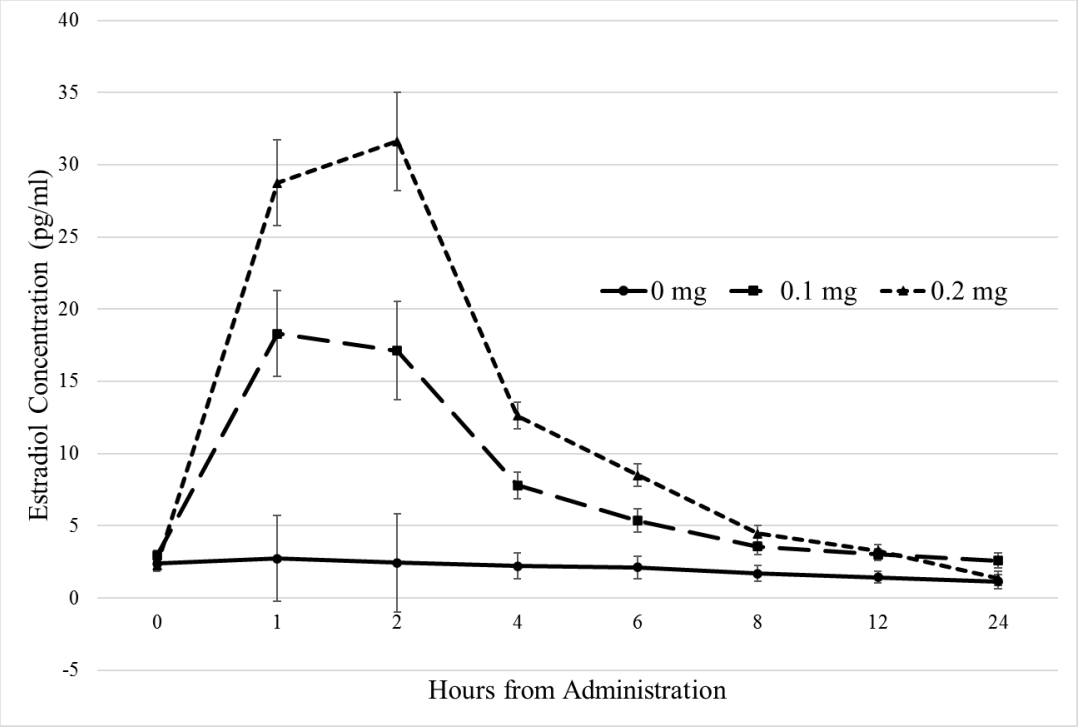


Figure 3.2. LSmean ± (SEM) circulating concentrations of estradiol 17-β following administration (IM) of 0 mg, 0.1 mg, or 0.2 mg of estradiol 17-β.

following insemination, embryos were collected nonsurgically and individually frozen in ethylene glycol for direct transfer on day 7. Embryos of the same developmental stage and quality grade from the same donor flush were frozen in pools of 3 straws per cane. On the day of transfer, embryos were thawed and transferred by one technician, into the uterine horn ipsilateral to the CL on day 7. All recipient cows (Positive Control, Negative Control, and Estradiol) received an *in vivo* produced embryo of similar stage and quality from the same donor flush on day 7. At ET (day 7) all cows received a new Estrotect® patch that was monitored and replaced on days 19 and 24 for comparison of the proportion of activated patches at day 7, 19, 24, and 30 among treatments. Determination of whether or not a cow had expressed estrus by the preceding days was determined by patch score as previously described.

3.3.6 Blood Sample Collection

Blood samples were collected on days -2, 0h0, 0h2, 7, 19, 24, 30 and 55/58 (Figure 3.1 *Panel B*). Blood samples from day -2, 0h0, and 0h2 were used for determination of E2 concentrations during the preovulatory period. Measurement of progesterone was determined in blood samples collected on days -2, 0h0, 7, 19, 24, 30, and 55/58 by RIA and PAGs were measured in plasma collected on days 7, 24 and 30 via an in-house sandwich ELISA and in day 30 samples via a commercial IDDEX ELISA. All blood samples were collected via venipuncture into 10-mL vacutainer tubes containing EDTA and were immediately placed on ice. Samples were centrifuged (1200 x g for 20 minutes at 4^{o}C) within 2 h of collection and plasma was aspirated and stored at -20˚C until RIA (progesterone and estradiol 17-β) or ELISA (PAG) analysis.

3.3.7 Assays

Progesterone and Estradiol 17-β: Concentration of progesterone was analyzed in plasma samples collected on day -2 (PG), d0h0, 7 (ET), 19, 24, 30, and 55/58 by RIA in duplicates (Pohler et al., 2016). Intraassay and interassay coefficients of variation (CV) were 1.67% and 4.02%, respectively, over 17 assays. Assay sensitivity was 0.05 ng/mL. Concentration of estradiol 17-β was analyzed in plasma samples collected on day -2, 0h0, and 0h2 by RIA in duplicates (Kirby et al., 1997). Intraassay and interassay coefficients of variation (CV) were 3.89% and 16.38%, respectively, over 13 assays. Assay sensitivity was 0.5 pg/mL.

PAGs: Circulating concentrations of PAGs have been used to diagnose pregnancy in ruminants (reviewed by Wallace et al., 2015) and to predict late embryonic mortality in cattle (Pohler et al., 2016; Reese et al., 2018). The first increase in circulating PAGs was detected by day 24 following insemination (Pohler et al., 2013). Concentrations of PAGs were determined on day 7, 24, and 30 in duplicates by using a previously validated in-house sandwich ELISA that was established by Jon Green (Green et al., 2005) and modified by using a polyclonal antibody (Ab 63) that was useful in predicting late embryonic mortality (Reese et al., 2018). PAG concentrations on day 7 were used to establish a baseline since depending on the days postpartum, a cow may have circulating PAGs from the previous pregnancy (Wallace et al., 2015). Results were analyzed using a Synergy™ HTX Multi-Mode Reader (BioTek® Instruments, Inc., Winooski, VT) with Gene5 Software at 405 nm, 450 nm, and 490 nm. Intraassay and interassay coefficients of variation (CV) for the in-house sandwich ELISA were 6.85% and 21.35%, respectively, over 28 assays. Assay sensitivity was 0.2 pg/mL.

A commercial ELISA (IDEXX Bovine Pregnancy; IDEXX Laboratories, Inc., Westbrook, ME) that detects circulating PAGs was used to diagnose pregnancy on day 30. Results from duplicates were analyzed using a Synergy™ HT Multi-Detection Microplate Reader (BioTek® Instruments, Inc., Winooski, VT) with Gen5 Software at 405 nm, 450 nm, and 490 nm. The preceding ELISA has a positive predictive value of 93% and a very good kappa score of 0.86 for detection of pregnancy around day 30 (Northrop et al., 2019). Intraassay and interassay coefficients of variation (CV) for the commercial ELISA (IDEXX) were 3.77% and 7.75%, respectively, over 28 assays. The same standard curve that was used with the in-house sandwich PAG ELISA was also used with the commercial ELISA (IDEXX) to obtain concentrations in addition to S-N values. The S-N value is the provided negative control absorbance (N value) subtracted from the sample absorbance (S value).

3.3.8 RNA isolation and ISG expression

Expression of ISG abundance on days 18 to 22 has been used to identify the presence of a bovine embryo (Wijma et al., 2016). In the present study, leukocytes (buffy coats) were collected from all cows on day 0 and 19 to quantitate ISG expression. ISG expression on day 0 served as a baseline for ISG expression on day 19. Total RNA was isolated from buffy coats collected following centrifugation of blood collected as described above. Buffy coats were transferred into a 1.5 mL centrifuge tube and mixed with approximately a 1:1 volumetric ratio of Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Tubes were vortexed thoroughly until Tri Reagent was mixed throughout each sample. Samples were placed in ice until stored at -80˚C. Samples remained at -80˚C until RNA was isolated. Total RNA was isolated by using the Direct-

zol RNA Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. RNA was treated with DNaseI to prevent contamination during RNA purification by using the RNase-Free DNase Set (Qiagen, Germantown, MD, USA). RNA was eluted using nuclease free water and concentrations along with purity was determined via a NanoDrop spectrophotometer (Fisher Scientific, Pittsburgh, PA, USA). RNA integrity was confirmed via a 2.5% agarose gel (Appendix Figure 2). Samples were diluted in nuclease free water to 500 ng/µL and reverse-transcribed using iScript Reverse Transcription Supermix for RT-qPCR (BIO-RAD, Hercules, California, USA) to produce complementary DNA (cDNA) using the following conditions: priming for 5 minutes at 25°C; reverse transcription for 20 minutes at 46°C; and inactivation for 1 minutes at 95°C. A negative control was produced from each sample via incubation without reverse transcriptase. The cDNA was stored at –20°C.

Expression of the interferon stimulated genes *ISG15*, *MX2*, *OAS1*, and the reference gene *RPL19* were measured using previously validated primers (Table 3.1) by RT-PCR using a CFX384 Touch Real Time System (Bio-Rad). Samples were run in triplicate in 10 µL reactions consisting of 0.5 µL forward PrimePCR primer, 0.5 µL reverse PrimePCR primer, 5 µL of SYBR Green Supermix, 1.5 µL of nuclease-free water, and 2.5 µL of cDNA (12.5 ng). Reactions without templates served as a negative control for each primer while reactions with template substituted by total RNA were used to verify possible genomic contamination. PCR conditions were 95°C for 2 minutes; 95°C for 5 seconds, and 60°C for 30 seconds. At the end of amplification, a melt curve was created to assess whether a single product was amplified. Samples with ≥ 36 CT were considered to have no expression. The ΔCT was estimated as the difference

Table 3.1. Genes, primer sequences, and primer locations for genes amplified during RT-PCR.

Gene	Primer	Primer sequence	NCBI sequence	Reference
ISG15	Forward	5 -GGTATCCGAGCTGAAGCAGTT-3	NM_174366.1	(Wijma et al., 2016)
	Reverse	5 -ACCTCCCTGCTGTCAAGGT-3		
MX2	Forward	5 -CTTCAGAGACGCCTCAGTCG-3	NM_173941.2	(Wijma et al., 2016)
	Reverse	5 -TGAAGCAGCCAGGAATAGTG-3		
OAS1	Forward	5 -ACCCTCTCCAGGAATCCAGT-3	NM_001040606	(Green et al., 2010)
	Reverse	5 -GATTCTGGTCCCAGGTCTGA-3		
RPL19	Forward	5 -ATCGATCGCCACATGTATCA-3	NM_001040516.1	(Wijma et al., 2016)
	Reverse	5 -GCGTGCTTCCTTGGTCTTAG-3		

between the cycle threshold (CT) for the gene of interest and the geometric mean of the CT for the reference gene (*RPL19*). The ΔΔCT was estimated as the difference between the day 19 ΔCT and the day 0 ΔCT day.

3.3.9 Transrectal ultrasonography

Pregnancy was determined via ultrasound (Aloka 500V) on day 55 or 58 for Group 2 and 1, respectively with a 5 MHz linear array probe. Pregnancy was based on the visualization of a fetus.

3.3.10 Criteria for Presence of conceptus and pregnancy diagnosis

A set of criteria (Figure 3.3) was developed to determine if there was evidence of a conceptus present on days 19 and 24 or if a cow was pregnant on days 30 and 55/58. Cows were classified retrospectively relative to pregnancy status and periods of loss, such that if a cow was determined to be pregnant on day 55/58, she was also considered to have been pregnant/have evidence of a conceptus present on days 19, 24, and 30. One cow was excluded from the analysis as the buffy coat collected on day 19 was of suboptimal quality; therefore, RT-PCR results were inconclusive. This cow was never classified to a Period of Loss nor was she pregnant on day 55/58 therefore making it impossible to determine if there was ever evidence of a conceptus present.

Pregnant (n = 87): A cow was considered pregnant on day 55/58 if a 55/58 day viable fetus was identified by ultrasonography, and pregnant cows were excluded from further classification (Period 1, 2, 3, or 4 Loss). For cows in which a viable fetus of the correct age was not identified by ultrasound on day 55/58, further classification to a

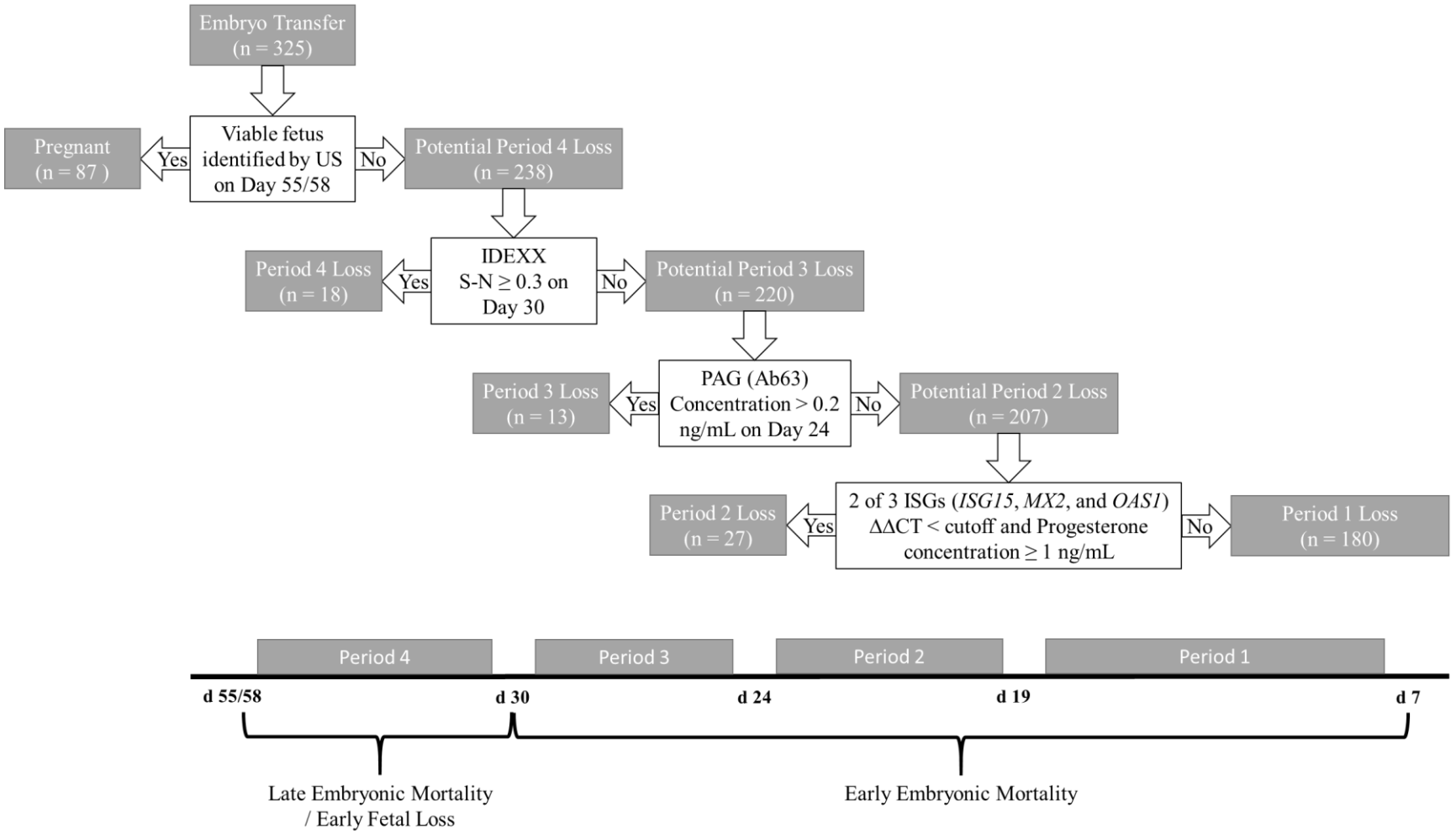


Figure 3.3. A total of 325 cows received ET on day 7 and thus were considered to have presence of an embryo present on day 7. All 325 cows were subjected to ultrasonography on day 55/58 and those with a 55/58 day viable fetus were considered pregnant (n = 87) and were not further classified. Cows without a 55/58 day viable fetus detected on day 55/58 (n = 238) were further classified by day 30 IDEXX S-N values, such that if a cow had an IDEXX S-N value ≥ 0.3 (n = 18), they were considered to have undergone Period 4

Loss and were excluded from further classification. If the IDEXX S-N value was < 0.3 (n = 220) they were further classified by day 24 PAG (Ab 63) concentrations, such that if they had a PAG (Ab 63) concentration > 0.2 ng/mL (n = 13), they were considered to have undergone Period 3 Loss and were not further classified. If the PAG (Ab 63) concentration was ≤ 0.2 ng/mL (n = 207), they were further classified by ISG ΔΔ CT values as well as circulating progesterone concentrations on day 19, such that if 2 of the 3 ISGs had a ΔΔ CT < their respective cutoff (Table 3.2) and circulating progesterone concentrations ≥ 1 ng/mL (n = 27), they were considered to have undergone Period 2 loss and were not further classified. Those cows that did not have 2 of the 3 ISGs ΔΔ CT less than their respective cutoff (Table 3.2) and circulating progesterone concentrations ≥ 1 ng/mL were considered to have undergone Period 1 Loss (n = 180). Loss that occurred during Periods 1 -3 would be considered Early Embryonic Mortality while loss that occurred in Period 4 is considered Late Embryonic Mortality/Early Fetal Loss.

Period of Loss occurred, with Period 1 being day 7 to 19; Period 2: day 19 to 24; Period 3: day 24 to 30; and Period 4: day 30 to 55.

Period 4 Loss (n = 18): A cow was considered to have undergone Period 4 Loss when 55/58 day viable fetus was not identified by ultrasound on day 55/58, but had an IDEXX S-N value ≥ 0.3 (as indicated by IDEXX) on day 30 (i.e. pregnant). These cows were excluded from any further classification. Cows in which a 55/58 day viable fetus was not identified by ultrasound and had an IDEXX S-N value < 0.3 were further assigned to a Period of Loss (1, 2, or 3) based on PAG (Ab 63) concentration values on day 24 or ISGs and progesterone on day 19.

Period 3 Loss (n= 13): A Youden index and its associated criterion was calculated from a receiver operating characteristic (ROC) curve for circulating concentrations of PAG (Ab 63) on day 24 (Figure 3.4). The associated criterion (> 0.2 ng/mL, Se = 55.10, Sp = 95.17) established a cutoff value for PAG (Ab 63) concentrations, on day 24, that provided evidence of the presence of a conceptus. Cows that had elevated (≥ 1ng/mL) day 7 PAG (Ab 63) concentrations and/or were ≤ 45 days postpartum (at day 0 of protocol) were excluded from ROC curve analysis (n = 21). Cows with an IDEXX S-N value ≥ 0.3 on day 30 provided data for positive pregnancy outcomes. Since there was not a group of cows that did not receive an embryo nor could be confirmed as definitely not pregnant on day 24, cows with an IDEXX S-N value < 0.3 on day 30 provided data for negative pregnancy outcomes. Cows with a PAG (Ab 63) concentration > 0.2 on day 24, but an IDEXX S-N < 0.3 on day 30 and a 55/58 day viable fetus was not identified on day 55/58, were considered to have undergone Period 3 Loss and were not further classified. Cows with PAG (Ab 63) concentrations ≤ 0.2 ng/mL

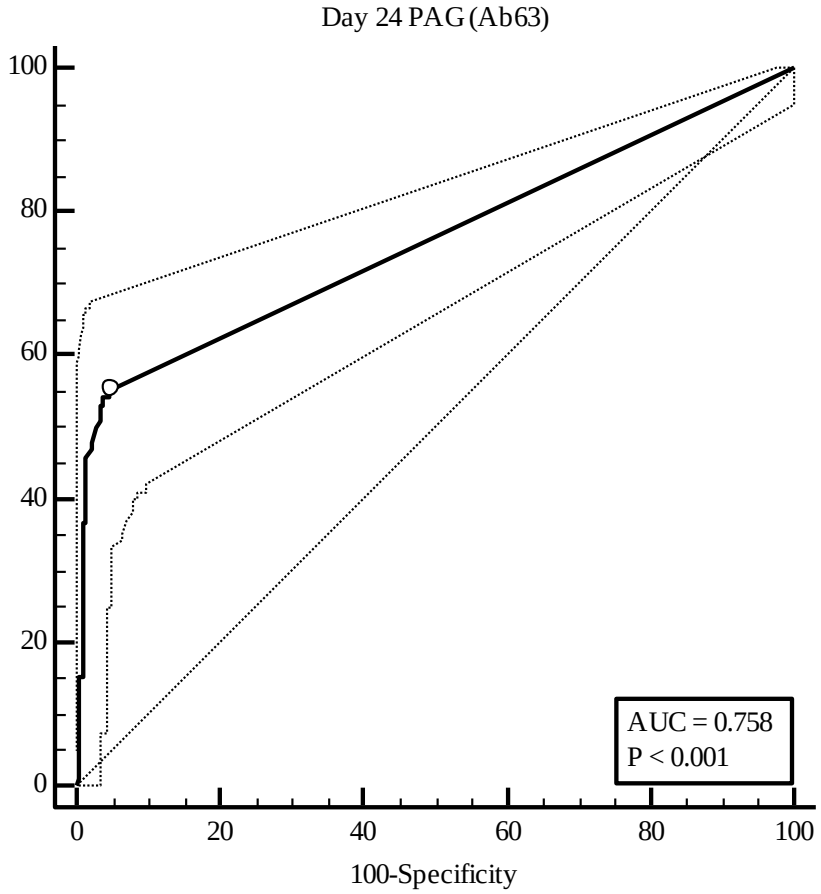


Figure 3.4. Receiver operating characteristic (ROC) curve utilizing day 24 circulating concentrations of PAGs determined by an in-house sandwich ELISA (Ab 63). The ROC curve graphically displays the relationship between the true positive rate (Sensitivity) and the false positive rate (100-Specificity) when an increasing cutoff for the PAG test was applied. When the true positive rate and the false positive rate both decrease as the cutoff value is increased this results in a diagonal line (represented by the 45˚ dotted line) through the center meaning the test is not predictive (50:50 probability). However, when the solid line is deflected to the left of center the test is useful since it has a relatively high true positive rate and a low false positive rate at a specific cutoff. The Youden index (0.5027; represented on the curve by ^{O}; $J = \max_c \{Se_c + Sp_c - 1\}$ where c ranges over all possible criterion values and J is the maximum vertical distance between the ROC curve and the 45˚ diagonal) and its associated criterion (0.2 ng/mL) were used to generate a predictive cutoff value for determining if there was evidence of a conceptus present on day 24 which resulted in a Sensitivity (Se; true positive rate) of 55.10 and Specificity (Sp; true negative rate) of 95.17. The 95% Confidence Interval is represented by the two dotted lines surrounding the solid ROC line. The ROC yielded an Area Under the Curve (AUC; represents the probability that a cow randomly selected from the cows diagnosed as pregnant on day 30 had PAG (Ab 63) concentration on day 24 that indicated greater suspicion than that of a cow randomly selected from the cows diagnosed as nonpregnant on day 30) of 0.758 that was significant ($P < 0.0001$).

were further classified based on expression of ISGs and serum concentrations of progesterone on day 19.

Period 2 Loss (n = 27): Since a specific criteria to determine the presence of an embryo based on ISG expression has yet to be established, a Youden index and its associated criterion, following receiver operating characteristic (ROC) curve (Figure 3.5, 3.6, 3.7) analysis, determined a cutoff value for *ISG15*, *MX2* and *OAS1* ΔΔCT values that provided evidence of the presence of a conceptus. Cows with an IDEXX S-N value ≥ 0.3 on day 30 provided data for positive pregnancy outcomes. Since there was not a group of cows that did not receive an embryo nor could be confirmed as definitely not pregnant on day 19, cows with an IDEXX S-N value < 0.3 on day 30 provided data for negative pregnancy outcomes. A cow was considered to have undergone Period 2 Loss when the following criteria were met: 1) 2 of the 3 ISG genes (*ISG15*, *MX2*, *OAS1*) had a ΔΔCT value less than its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2), 2) Day 19 progesterone concentration was ≥ 1 ng/mL, 3) PAG (Ab 63) concentration on day 24 was ≤ 0.2, and 4) the IDEXX S-N value on day 30 was < 0.3, and 4) a 55/58 day viable fetus was not identified via ultrasound on day 55/58. These cows were not further classified.

Period 1 Loss (n = 180): If a cow did not meet the criteria requirements to have undergone Period 2 Loss (see above for Period 2 Loss criteria) then she was considered to have undergone Period 1 Loss.

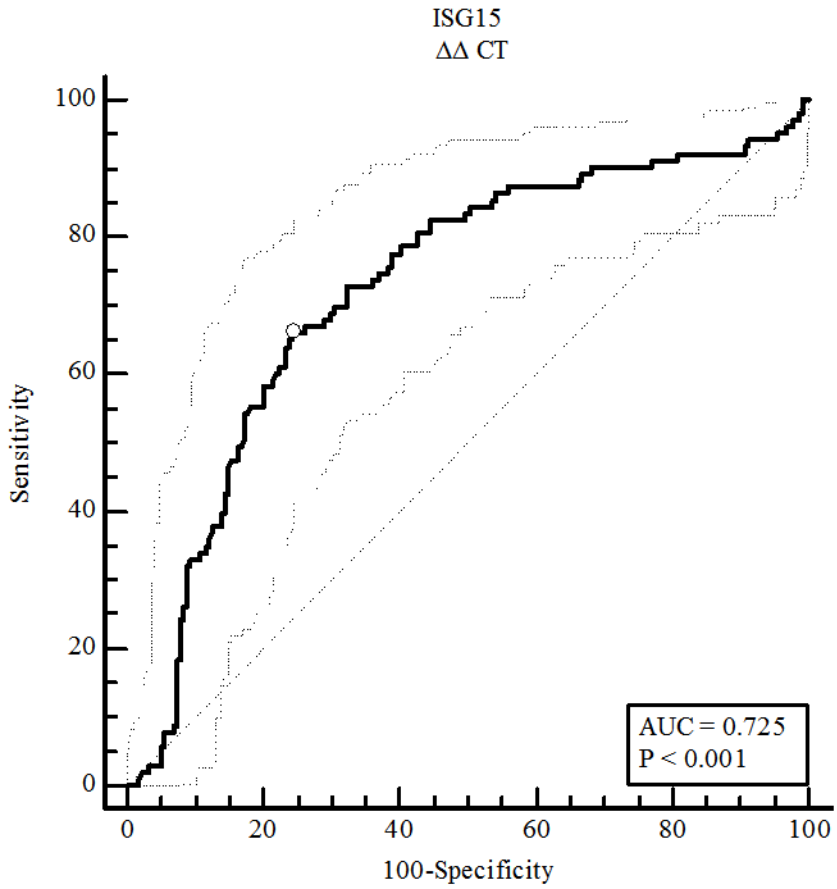


Figure 3.5. Receiver operating characteristic (ROC) curve utilizing day 19 *ISG15* ΔΔCT values determined by RT-PCR. The ROC curve graphically displays the relationship between the true positive rate (Sensitivity) and the false positive rate (100-Specificity) when an increasing cutoff for the PAG test was applied. When the true positive rate and the false positive rate both decrease as the cutoff value is increased this results in a diagonal line (represented by the 45˚ dotted line) through the center meaning the test is not predictive (50:50 probability). However, when the solid line is deflected to the left of center the test is useful since it has a relatively high true positive rate and a low false positive rate at a specific cutoff. The Youden index (0.4172; represented on the curve by ^{O}; $J = \max_c \{Se_c + Sp_c - 1\}$ where c ranges over all possible criterion values and J is the maximum vertical distance between the ROC curve and the 45˚ diagonal) and its associated criterion (< - 1.495) were used to generate a predictive cutoff value for determining if there was evidence of a conceptus present on day 19 which resulted in a Sensitivity (Se; true positive rate) of 66.02 and Specificity (Sp; true negative rate) of 75.70. The 95% Confidence Interval is represented by the two dotted lines surrounding the solid ROC line. The ROC yielded an Area Under the Curve (AUC; represents the probability that a cow randomly selected from the cows diagnosed as pregnant on day 30 had a *ISG15* ΔΔCT value on day 19 that indicated greater suspicion than that of a cow randomly selected from the cows diagnosed as nonpregnant on day 30) of 0.725 that was significant ($P < 0.0001$).

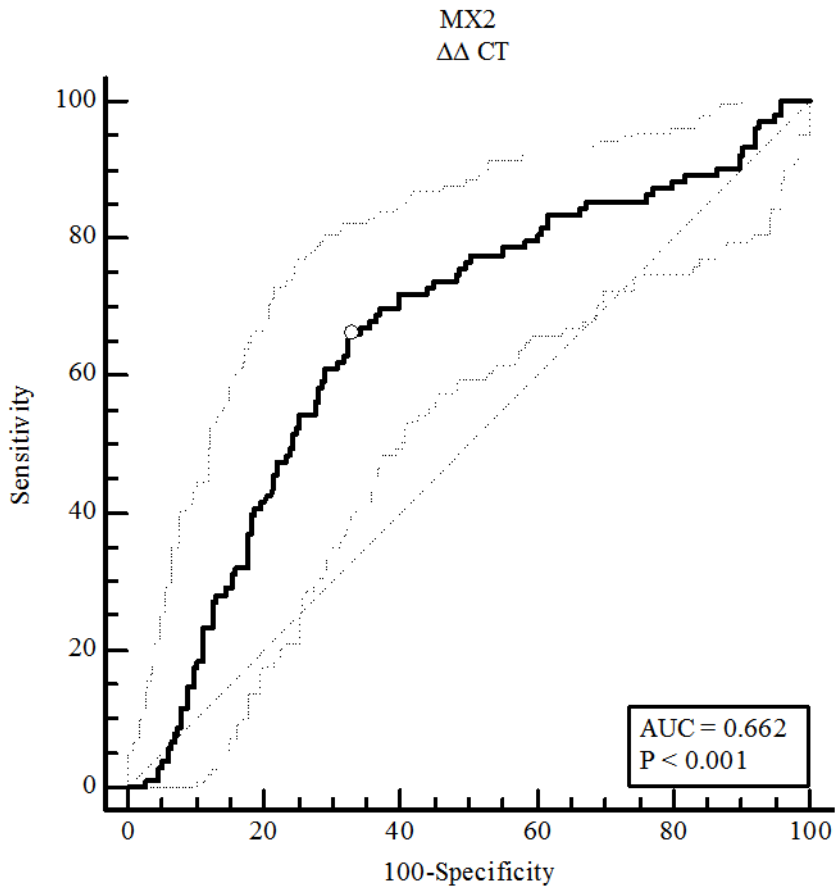


Figure 3.6. Receiver operating characteristic (ROC) curve utilizing day 19 *MX2* ΔΔCT values determined by RT-PCR. The ROC curve graphically displays the relationship between the true positive rate (Sensitivity) and the false positive rate (100-Specificity) when an increasing cutoff for the PAG test was applied. When the true positive rate and the false positive rate both decrease as the cutoff value is increased this results in a diagonal line (represented by the 45˚ dotted line) through the center meaning the test is not predictive (50:50 probability). However, when the solid line is deflected to the left of center the test is useful since it has a relatively high true positive rate and a low false positive rate at a specific cutoff. The Youden index (0.3331; represented on the curve by ^{O}; $J = \max_c \{Se_c + Sp_c - 1\}$ where c ranges over all possible criterion values and J is the maximum vertical distance between the ROC curve and the 45˚ diagonal) and its associated criterion (< - 1.045) were used to generate a predictive cutoff value for determining if there was evidence of a conceptus present on day 19 which resulted in a Sensitivity (Se; true positive rate) of 66.02 and Specificity (Sp; true negative rate) of 67.29. The 95% Confidence Interval is represented by the two dotted lines surrounding the solid ROC line. The ROC yielded an Area Under the Curve (AUC; represents the probability that a cow randomly selected from the cows diagnosed as pregnant on day 30 had a *MX2* ΔΔCT value on day 19 that indicated greater suspicion than that of a cow randomly selected from the cows diagnosed as nonpregnant on day 30) of 0.662 that was significant ($P < 0.0001$).

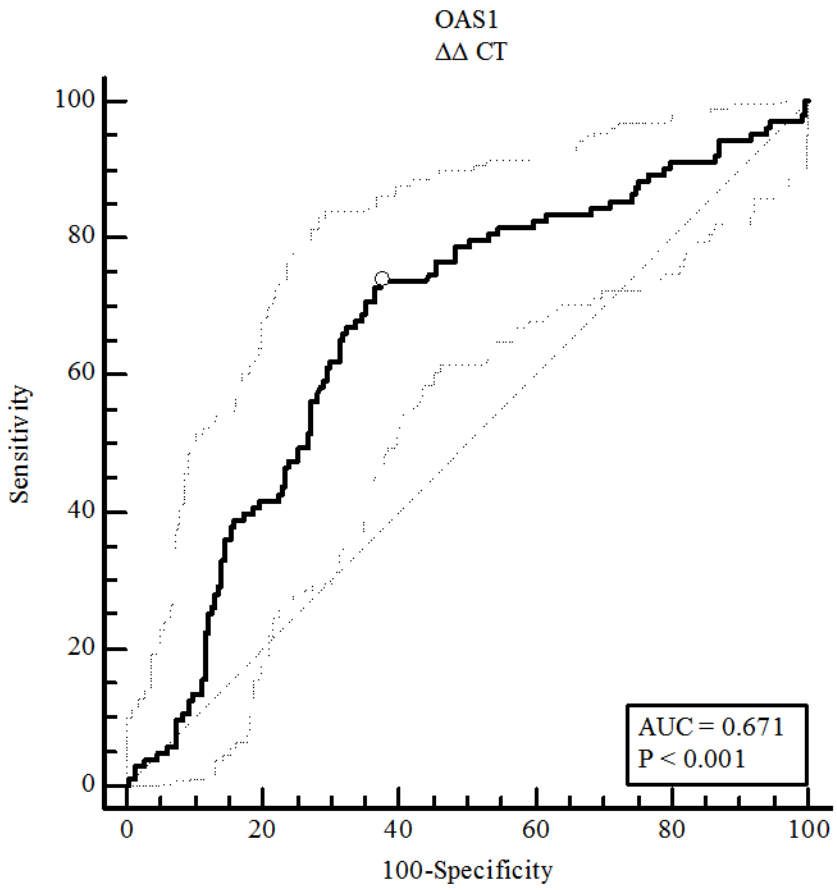


Figure 3.7. Receiver operating characteristic (ROC) curve utilizing day 19 *OAS1* ΔΔCT values determined by RT-PCR. The ROC curve graphically displays the relationship between the true positive rate (Sensitivity) and the false positive rate (100-Specificity) when an increasing cutoff for the PAG test was applied. When the true positive rate and the false positive rate both decrease as the cutoff value is increased this results in a diagonal line (represented by the 45˚ dotted line) through the center meaning the test is not predictive (50:50 probability). However, when the solid line is deflected to the left of center the test is useful since it has a relatively high true positive rate and a low false positive rate at a specific cutoff. The Youden index (0.3640; represented on the curve by ^{O}; $J = \max_c \{Se_c + Sp_c - 1\}$ where c ranges over all possible criterion values and J is the maximum vertical distance between the ROC curve and the 45˚ diagonal) and its associated criterion (< - 1.188) were used to generate a predictive cutoff value for determining if there was evidence of a conceptus present on day 19 which resulted in a Sensitivity (Se; true positive rate) of 73.79 and Specificity (Sp; true negative rate) of 62.62. The 95% Confidence Interval is represented by the two dotted lines surrounding the solid ROC line. The ROC yielded an Area Under the Curve (AUC; represents the probability that a cow randomly selected from the cows diagnosed as pregnant on day 30 had a *OAS1* ΔΔCT value on day 19 that indicated greater suspicion than that of a cow randomly selected from the cows diagnosed as nonpregnant on day 30) of 0.671 that was significant ($P < 0.0001$).

Table 3.2. Youden Index, Associated Criterion, Sensitivity, Specificity, Area Under the Curve, and *P* values for *ISG15*, *MX2*, and *OAS1*.

Gene	Youden Index (*J*)[1]	Associated Criterion[2]	Sensitivity (Se)[3]	Specificity (Sp)[4]	Area Under the Curve (AUC)[5]	*P* value
ISG15	0.4172	< -1.495	66.02	75.70	0.725	< 0.001
MX2	0.3331	< -1.045	66.02	67.29	0.662	< 0.001
OAS1	0.3640	< -1.1883	73.79	62.62	0.671	< 0.001

[1]$J = \max_c \{Se_c + Sp_c - 1\}$ where c ranges over all possible criterion values. *J* is the maximum vertical distance between the ROC curve and the 45˚ diagonal.

[2]Optimal criterion value that gives equal weight to sensitivity and specificity and is associated with *J*.

[3]Se = true positive rate, probability that a cow will be classified as having evidence of a conceptus present on day 19 when she was diagnosed pregnant on day 30.

[4]Sp = true negative rate, probability that a cow will be diagnosed classified as nonpregnant on day 19 when she was diagnosed nonpregnant on day 30.

[5]Probability that a cow randomly selected from the cows diagnosed as pregnant on day 30 had an ISG ΔΔCT value on day 19 that indicated greater suspicion than that of a cow randomly selected from the cows diagnosed as nonpregnant on day 30.

3.3.11 Statistical analyses

All statistical analyses were conducted using SAS 9.4 software. Herd was found to have a significant effect ($P < 0.05$) on all measured variables and therefore was included as a variable in the remainder of the analyses to control for the impact of herd. Differences among treatments in BCS, DPP, Wt, age, embryo stage, embryo grade, time from PG to d0h0, and d0h0 to d0h2 interval were analyzed by analysis of variance in SAS (PROC GLM). The statistical model consisted of the independent variables of treatment and herd and the dependent variables tested (BCS, DPP, Wt, age, embryo stage, embryo grade, time from PG to d0h0, and d0h0 to d0h2 interval). Mean separation was performed using least significant differences (Means ± SEM [standard error of the mean]; Snecdecor and Cochran 1991). Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$. All data are reported as LSmeans ± (SEM).

Presence of a conceptus/fetus as well as circulating concentrations of progesterone and estradiol 17-β at designated timepoints were analyzed by analysis of variance for repeated measures (PROC MIXED; Littell et al. 1998). The covariance structures used were ante-dependence (estradiol 17-β) and autoregressive (progesterone and presence of a conceptus/fetus). The statistical model consisted of the dependent variable tested (treatment, herd, presence of a conceptus/fetus), day, and their interactions unless the interaction was not significant ($P > 0.05$). Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$. All data are reported as LSmeans ± (SEM).

Change in estradiol 17-β from day -2 to 0h0 and day 0h0 to 0h2, change in progesterone from day -2 to 0 and from day 0 to 7, PAG concentrations on day 7 (In-house sandwich ELISA), 24 (In-house sandwich ELISA), and 30 (In-house sandwich ELISA and commercial IDEXX), and PAG S-N values (commercial IDEXX) were analyzed by analysis of variance in SAS (PROC GLM). The statistical model consisted of the independent variables (treatment, herd, pregnancy status on day 30) and the dependent variables being tested, and their interactions unless the interaction was not significant ($P > 0.05$). Mean separation was performed using least significant differences (Means ± SEM [standard error of the mean]; Snecdecor and Cochran 1991). Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$. All data are reported as LSmeans ± (SEM).

Estrual status, as determined by patch scores, on day 0, 7, 19, 24, and 30 and patch scores on day 7 as well as presence of a conceptus on day 19 and 24 or presence of a fetus on day 30 and 55/58 were analyzed with the GLIMMIX procedure in SAS (PROC GLIMMIX) using the binary distribution, denominator degrees of freedom KR (Estrual status on day 0, 7, 19, 24, and 30 and patch scores on day 7) or Residual (presence of a conceptus on day 19 and 24 or presence of a fetus on day 30 and 55/58) method. The statistical model included the independent variables of treatment, herd and the dependent variable of interest (estrual status on day 0, estrual status on day 7, estrual status on day 19, estrual status on day 24, estrual status on day 30, day 7 patch score 0, day 7 patch score 1, day 7 patch score 2, day 7 patch score 3, day 7 patch score 4, presence of a conceptus on day 19, presence of a conceptus on day 24, presence of a fetus on day 30, or presence of a fetus on day 55/58). The model also included any interactions that were

significant. Mean separation was performed using least significant differences (Means ± SEM [standard error of the mean]; Snecdecor and Cochran 1991). Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$. All data are reported as LSmeans ± (SEM).

Receiver Operating Characteristic (ROC) curves were made using MedCalc Version 19.1.7 software. ROC curves were made to acquire a Youden Index and associated criterion for the day 24 PAG concentration (In-house sandwich ELISA), *ISG15* ΔΔCT, *MX2* ΔΔCT, and *OAS1* ΔΔCT.

3.4 Results

3.4.1 General treatment groups

Cow age, weight, body condition score, and days postpartum did not differ ($P > 0.05$) among treatments (Table 3.3). Time from PG to d0h0 was 47.1 ± 0.007^{a}, 46.5 ± 0.007^{b}, and 46.6 ± 0.007^{b} h ($^{ab}P < 0.0001$) for the Positive Control, Estradiol, and Negative Control treatments, respectively (Table 3.4). The preceding differences in time from PG to d0h0 were minor, and likely not physiologically significant. The interval (h) from d0h0 to d0h2 did not differ ($P > 0.05$) among treatments. In addition, embryo stage (4.4 ± 0.67) and grade (1.2 ± 0.39) did not differ ($P > 0.05$) among treatment groups.

3.4.2 Estradiol

Circulating concentrations of estradiol 17-β 48 h prior to day 0 (day -2) were greater ($P < 0.0001$) in the Positive Control group than both the Estradiol and Negative Control groups. On day -2, plasma concentrations of estradiol 17-β tended to be higher

Table 3.3. LSmean ± (SEM) cow age, body weight (Wt), body condition score (BCS), and days postpartum (DPP) by treatment.

Treatment[1]	N	Age, yr	Wt[2], kg	BCS[3]	DPP[4]
Positive Control	105	4.6 ± 0.23	565 ± 7.38	5.0 ± 0.05	76 ± 1.73
Estradiol	109	5.0 ± 0.23	567 ± 7.15	4.9 ± 0.05	76 ± 1.68
Negative Control	112	5.1 ± 0.22	574 ± 7.05	4.9 ± 0.05	76 ± 1.66

[1]The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation prior to assignment of cows to treatment. The protocol required administration of GnRH (GnRH1) and CIDR® insertion on day -9. Administration of prostaglandin F2α and CIDR® removal occurred on day -2. On day 0 (d0h0; 48 h after PG and CIDR® removal), cows were assigned to one of the three following treatments with the Estradiol and Negative Control treatments randomly assigned on day 0: 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (Estradiol), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. For further description of the treatments see the Materials and Methods.

[2]Weight (kg) was obtained on d -9.

[3]Cow body condition scores, based on a 1 to 9 scale (1= emaciated; 9 = obese), were obtained on d -9.

[4]Days postpartum (DPP) from calving to GnRH2

Table 3.4. LSmean ± (SEM) interval from PG to d0h0 and d0h0 to d0h2 by treatment.

Treatment[1]	Time from PG to d0h0, h[2]	d0h0 to d0h2 interval[3], h
Positive Control	47.1 ± 0.07^{a}	1.96 ± 0.01
Estradiol	46.5 ± 0.07^{b}	1.94 ± 0.01
Negative Control	46.6 ± 0.07^{b}	1.96 ± 0.01

[1]The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation prior to assignment of cows to treatment. The protocol required administration of GnRH (GnRH1) and CIDR® insertion on day -9. Administration of prostaglandin F2α and CIDR® removal occurred on day -2. On day 0 (d0h0; 48 h after PG and CIDR® removal), cows were assigned to one of the three following treatments with the Estradiol and Negative Control treatments randomly assigned on day 0: 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (Estradiol), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. For further description of the treatments see the Materials and Methods.

[2]Interval in hours between PG administration on d -2 and d0h0.

[3]Interval in hours between d0h0 administration and the 2-hour blood sample collection on d 0.

Means having different superscripts are different ($^{ab}P < 0.05$)

($P = 0.0712$) in the Negative Control group compared to the Estradiol group. Furthermore, on d0h0 estradiol 17-β concentrations were similar among all groups ($P > 0.2565$). Estradiol 17-β concentrations in the Positive Control group were lower ($P = 0.0290$) than the Negative Control group on d0h2. However, cows in the Estradiol group had greater ($P < 0.0001$) estradiol 17-β concentrations on d0h2 than cows in both the Positive Control and Negative Control groups, as shown in Figure 3.8.

The change in estradiol 17-β from day -2 to d0h0 was lower ($P < 0.0001$) in the Positive Control (0.52 ± 0.265 pg/mL) group than both the Estradiol (2.59 ± 0.25 pg/mL) and Negative Control (2.49 ± 0.27 pg/mL) groups and there was no difference ($P = 0.7856$) between the Estradiol and Negative Control groups (Figure 3.9). The change in estradiol 17-β from d0h0 to d0h2 tended to differ ($P = 0.0787$) between the Positive Control (-0.64 ± 0.36 pg/mL) and Negative Control (0.27 ± 0.36 pg/mL) groups. However, the change in estradiol 17-β was greater ($P < 0.0001$) in the Estradiol (13.93 ± 0.35 pg/mL) group than both the Positive Control and Negative Control groups (Figure 3.9). Concentrations of estradiol 17-β did not differ ($P = 0.2261$) between pregnant and nonpregnant cows, within treatment, or at any time point based on pregnancy status on day 30 (IDEXX S-N ≥ 0.3) (Figure 3.10).

3.4.3 Patch scores

On d0h0, only cows in the Positive Control group had an activated Estrotect® patch (Patch score ≥ 3); whereas, none of the cows in the Estradiol and Negative Control groups had an activated patch at the time of GnRH2 injection. Cows in the Estradiol and Negative Control groups that had an activated patch by day 7 were considered to have

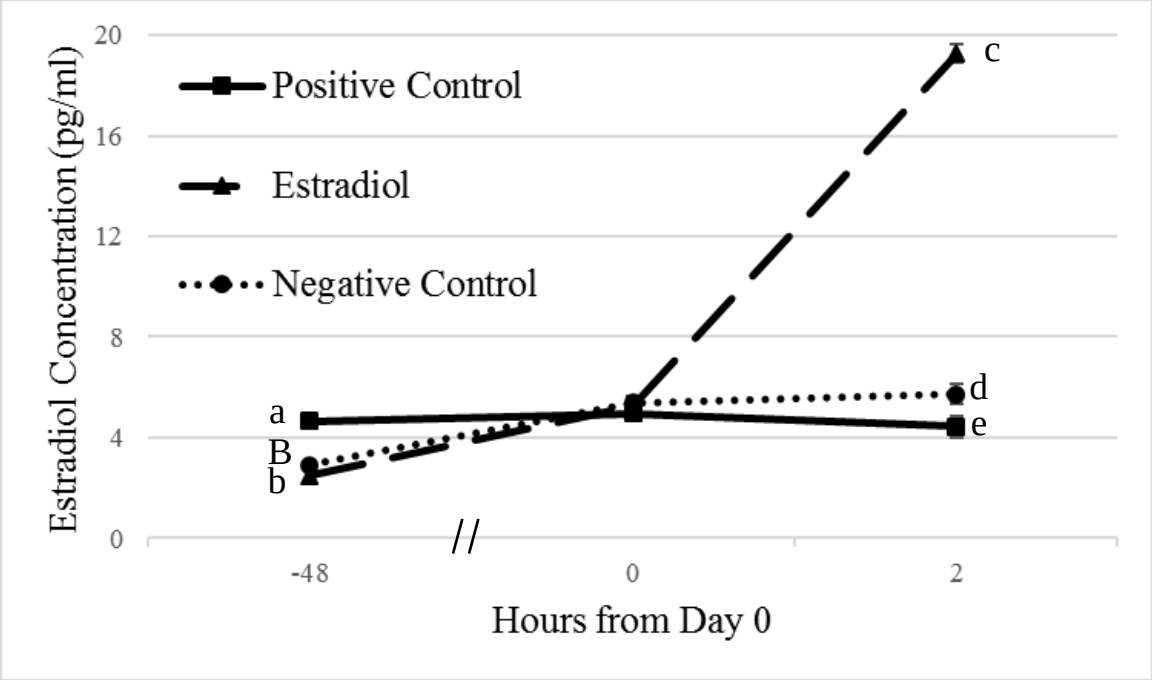


Figure 3.8. LSmean ± (SEM) circulating concentrations of estradiol (pg/mL) following PG-induced luteolysis on day -2 (-48 h) for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. Circulating concentrations of estradiol 48 h prior to day 0 were greater ($^{ab}P <$ 0.0001) in the Positive Control group than both the Estradiol and Negative Control groups. Estradiol concentrations at -48 h tended to differ between the Estradiol and Negative Control groups ($^{Bb}P = 0.0712$). On day 0 at h 0, estradiol concentrations were similar among all groups (P > 0.2565). On day 0 at h 2, estradiol concentrations in the Positive Control group were lower ($^{de}P = 0.0290$) than the Negative Control group. However, cows in the Estradiol group had greater ($^{cde}P < 0.0001$) estradiol concentrations on day 0 at h 2 than cows in both the Positive Control and Negative Control groups.

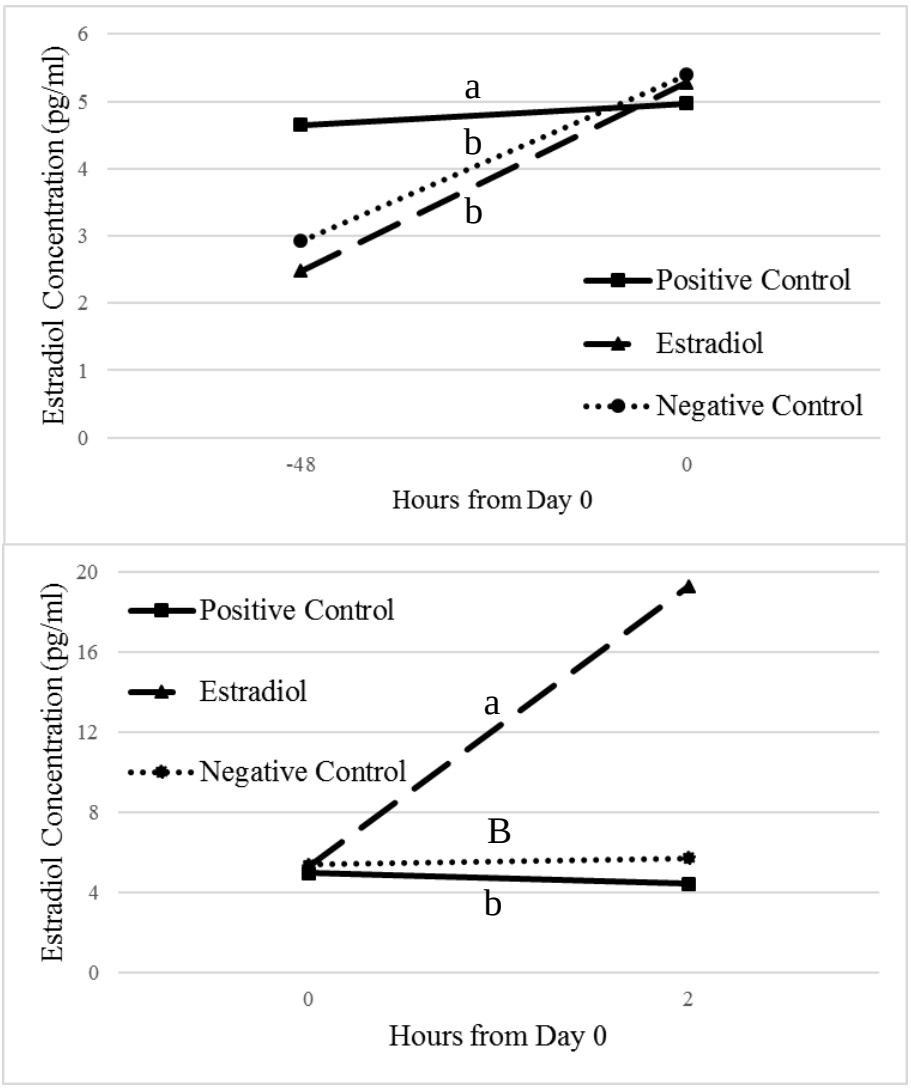


Figure 3.9. Change in circulating concentrations of estradiol (pg/mL) from PG-induced luteolysis on day -2 (-48 h) to day 0h0 (treatment assignment; *Panel A)* and from day 0h0 (treatment assignment) to day 0h2 *(Panel B)* for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. In *Panel A*, LSmean ± (SEM) for change in estradiol was lower ($^{ab}P < 0.0001$) in the Positive Control (0.52 ± 0.265 pg/mL) group than both the Estradiol (2.59 ± 0.25 pg/ml; $P < 0.0001$) and Negative Control (2.49 ± 0.27 pg/mL; $P < 0.0001$) groups. LSmean ± (SEM) for change in estradiol did not differ ($P = 0.7856$) between the Estradiol and Negative Control groups. In *Panel B*, LSmean ± (SEM) for change in estradiol tended to differ ($^{Bb}P = 0.0787$) between the Positive Control (-0.64 ± 0.36 pg/mL) and Negative Control (0.27 ± 0.36 pg/mL) groups. However, LSmean ± (SEM) change in estradiol was greater ($^{aBb}P < 0.0001$) in the Estradiol (13.93 ± 0.35 pg/mL) group than both the Positive Control and Negative Control groups.

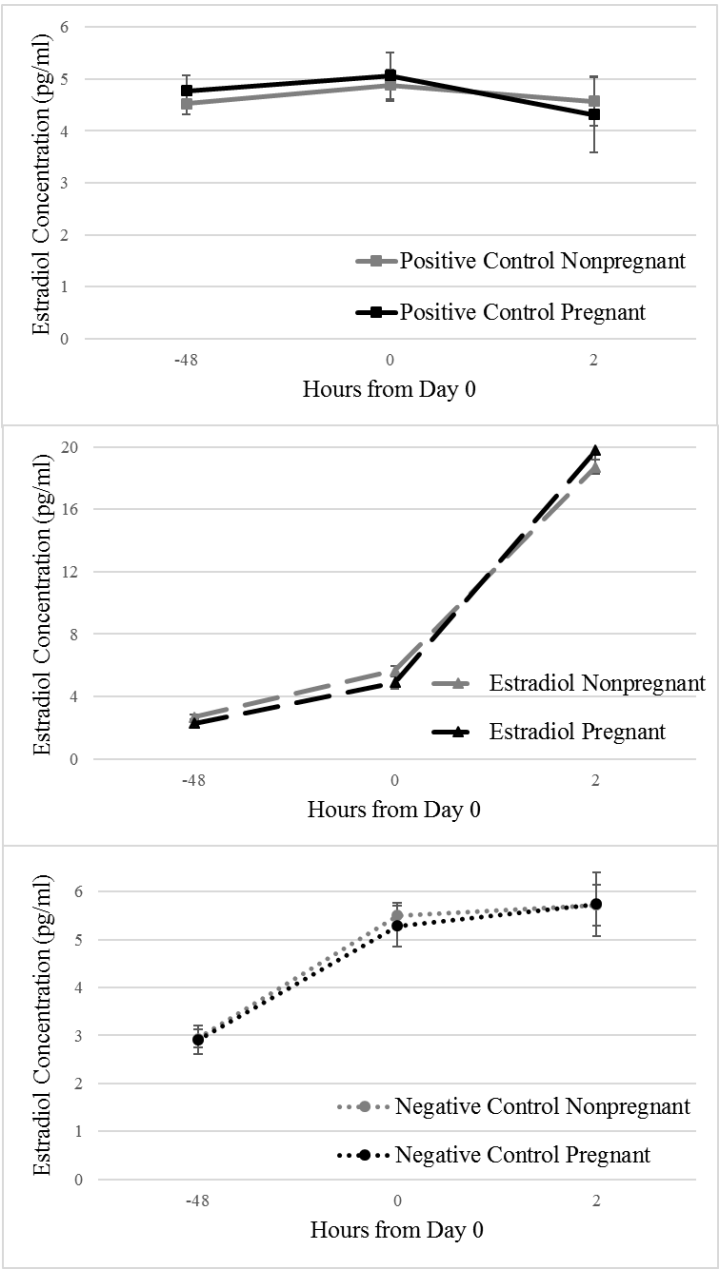


Figure 3.10. LSmean ± (SEM) circulating concentrations of estradiol (pg/mL) following PG-induced luteolysis on day -2 (-48 h) for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously (*Panel A*); 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-*β* (E2) (*Panel B*), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2 (*Panel C*). In *Panel A to C*, concentrations of estradiol did not differ ($P = 0.2261$) between pregnant and nonpregnant cows at any timepoint when day 30 (commercial IDEXX S-N ≥ 0.3) pregnancy status was used.

expressed estrus on day 0 or shortly thereafter rather than having had a short cycle between GnRH2 injection and day 7. This assumption is based on all cows in these groups having received an injection of GnRH on d0h0 and having an observable corpus luteum based on ultrasonography on day 7. By day 7, the proportion of cows classified as estrual, by patch score, differed ($P < 0.0001$) among treatment groups with a greater proportion of the Positive Control group (97.1 ± 0.02%) having expressed estrus than both the Estradiol (83.0 ± 0.04%) and Negative Control groups (47.5 ± 0.05%; Table 3.5). Importantly, the proportion of cows in the Estradiol group that expressed estrus by day 7 was greater ($P < 0.0001$) than in the Negative Control group. The proportion of cows with patch scores of 0, 1, 2, 3, or 4 by day 7 are shown in Table 3.6.

3.4.4 Progesterone

Concentrations of progesterone on day -2 were decreased ($P < 0.0001$) in the Positive Control group compared to both the Estradiol and Negative Control groups. There was a tendency ($P = 0.0987$) for concentration of progesterone to differ between the Estradiol and Negative Control groups on day -2, with the Estradiol group having higher concentrations than the Negative Control group. Concentrations of progesterone did not differ ($P > 0.9000$) among treatments on day 0. On day 7, the Positive Control group had greater ($P < 0.05$) concentrations of progesterone than both the Estradiol and Negative Control groups and concentrations of progesterone did not differ ($P = 0.9439$) between the Estradiol and Negative control groups (Figure 3.11).

There was no difference in circulating concentrations of progesterone on day 0; however, the change in circulating progesterone from day -2 to day 0 was lower ($P < 0.0001$) in the Positive Control group (-1.60 ± 0.295 ng/mL) than both the Estradiol

Table 3.5. LSMEAN ± (SEM) proportion of cows classified as having an activated Estrotect® patch[2] by different time points by treatment.

Treatment[1]	d7	d19	d24	d30
Positive Control	97.1 ± 0.02[a]	43.1 ± 0.05	51.8 ± 0.05	11.9 ± 0.03
Estradiol	83.0 ± 0.04[b]	36.9 ± 0.05	57.3 ± 0.05	9.36 ± 0.03
Negative Control	47.5 ± 0.05[c]	40.9 ± 0.05	51.6 ± 0.05	11.8 ± 0.03

[1]The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation prior to assignment of cows to treatment. The protocol required administration of GnRH (GnRH1) and CIDR® insertion on day -9. Administration of prostaglandin F2α and CIDR® removal occurred on day -2. On day 0 (d0h0; 48 h after PGF and CIDR® removal), cows were assigned to one of the three following treatments with the Estradiol and Negative Control treatments randomly assigned on day 0: 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. For further description of the treatments see the Materials and Methods.

[2]A Patch score (0= missing, 1= 0-25% activated, 2= 25-50% activated, 3= 50-75% activated, 4= 75-100% activated; Pohler et al. 2016) was recorded for all cows on day 0, 7, 19, 24, and 30. Females with a patch score ≥ 3 or 0 were considered to have an activated patch. Cows with a missing or activated patch at an earlier date received a new patch on day 7, 19, and 24.

Means having different superscripts are different ($^{abc}P < 0.05$)

Table 3.6. LSMEAN ± (SEM) proportion of cows with patch scores[1] of 0, 1, 2, 3, or 4 by treatment on day 7.

Treatment[2]	0	1	2	3	4
Positive Control	10.7 ± 0.03	3.0 ± 0.02	0.0 ± 0.00	0.0 ± 0.0	86.5 ± 0.03
Estradiol	2.7 ± 0.02	16.1 ± 0.04	0.9 ± 0.01	3.7 ± 0.02	76.3 ± 0.04
Negative Control	1.8 ± 0.01	47.0 ± 0.05	5.4 ± 0.02	3.6 ± 0.02	41.6 ± 0.05

[1]All cows were observed to see if they had expressed estrus by day 7 with the aid of Estrotect® patches. Patches read on day 7 were applied on day -2. Patch scores (0= missing, 1= 0-25% activated, 2= 25-50% activated, 3= 50-75% activated, 4= 75-100% activated; Pohler et al. 2016) were recorded and those females with a patch score ≥ 3 or 0 were classified as having expressed estrus.

[2]The 7-day CO-Synch + CIDR® protocol was used to synchronize estrus/ovulation prior to assignment of cows to treatment. The protocol required administration of GnRH (GnRH1) and CIDR® insertion on day -9. Administration of prostaglandin F2α and CIDR® removal occurred on day -2. On day 0 (d0h0; 48 h after PGF and CIDR® removal), cows were assigned to one of the three following treatments with the Estradiol and Negative Control treatments randomly assigned on day 0: 1) Positive control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative control: Nonestrual cows that were induced to ovulate with GnRH2. For further description of the treatments see the Materials and Methods.

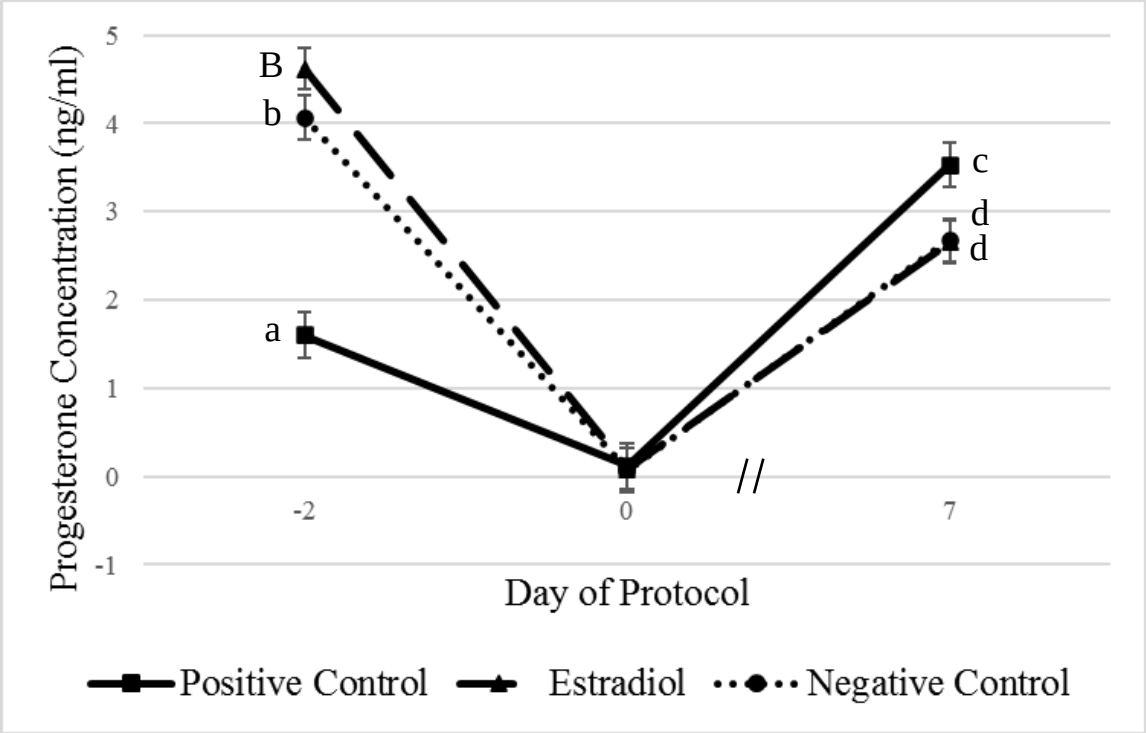


Figure 3.11. LSmean ± (SEM) circulating concentrations of progesterone (ng/mL) following PG-induced luteolysis on day -2 for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. Concentrations of progesterone on day -2 were decreased ($^{aBb}P < 0.0001$) in the Positive Control group compared to both the Estradiol and Negative Control groups. There was a tendency ($^{Bb}P = 0.0987$) for concentration of progesterone to differ between the Estradiol and Negative Control groups on day -2. Concentrations of progesterone did not differ ($P > 0.9000$) among treatments on day 0. On day 7, the Positive Control group had greater ($^{cd}P < 0.05$) concentrations of progesterone than both the Estradiol and Negative Control groups. Concentrations of progesterone did not differ ($P = 0.9439$) between the Estradiol and Negative Control groups on day 7.

(-4.70 ± 0.278 ng/mL) and Negative Control (-3.94 ± 0.294 ng/mL) groups. The Estradiol group tended ($P = 0.0621$) to have a greater change in circulating progesterone from day -2 to 0 than the Negative Control group. The change in circulating progesterone from day 0 to 7 was greater ($P < 0.05$) in the Positive Control (3.32 ± 0.166 ng/mL) group than both the Estradiol (2.52 ± 0.157 ng/mL) and Negative Control (2.54 ± 0.165 ng/mL) groups and Estradiol and Negative Control groups did not differ ($P = 0.9504$; Figure 3.12). When treatment groups were subdivided by pregnancy status on day 30 (IDEXX S-N ≥ 0.3 = pregnant), there were no treatment differences ($P > 0.2530$) after day 7 (Figure 3.13, Appendix Figure 3).

3.4.5 PAGs

There was no effect of treatment ($P = 0.2111$) nor a treatment by pregnancy interaction ($P = 0.5160$) for circulating concentrations of PAGs (Ab 63; ng/mL) on day 24. However, circulating concentrations of PAGs (Ab 63) were greater ($P < 0.0001$) in pregnant cows compared to nonpregnant cows regardless of treatment (Figure 3.14). On day 30 (Figure 3.15), concentrations of PAGs (Ab 63) were greater ($P < 0.05$) in pregnant cows in the Positive Control group than pregnant cows in the Estradiol group. Cows in the pregnant Negative Control group tended ($P = 0.0630$) to have higher PAG concentrations (Ab 63) than pregnant Positive Control cows. Pregnant cows in the Negative Control group had greater ($P < 0.0001$) circulating concentrations of PAGs than pregnant cows in the Estradiol group. IDEXX S-N values (see Materials and Methods) did not differ ($P = 0.2969$) among treatment groups. Concentrations of PAGs (IDEXX) were greater ($P < 0.05$) in pregnant cows in the Estradiol group than pregnant cows in both the Positive Control and Negative Control groups. Concentrations of PAGs

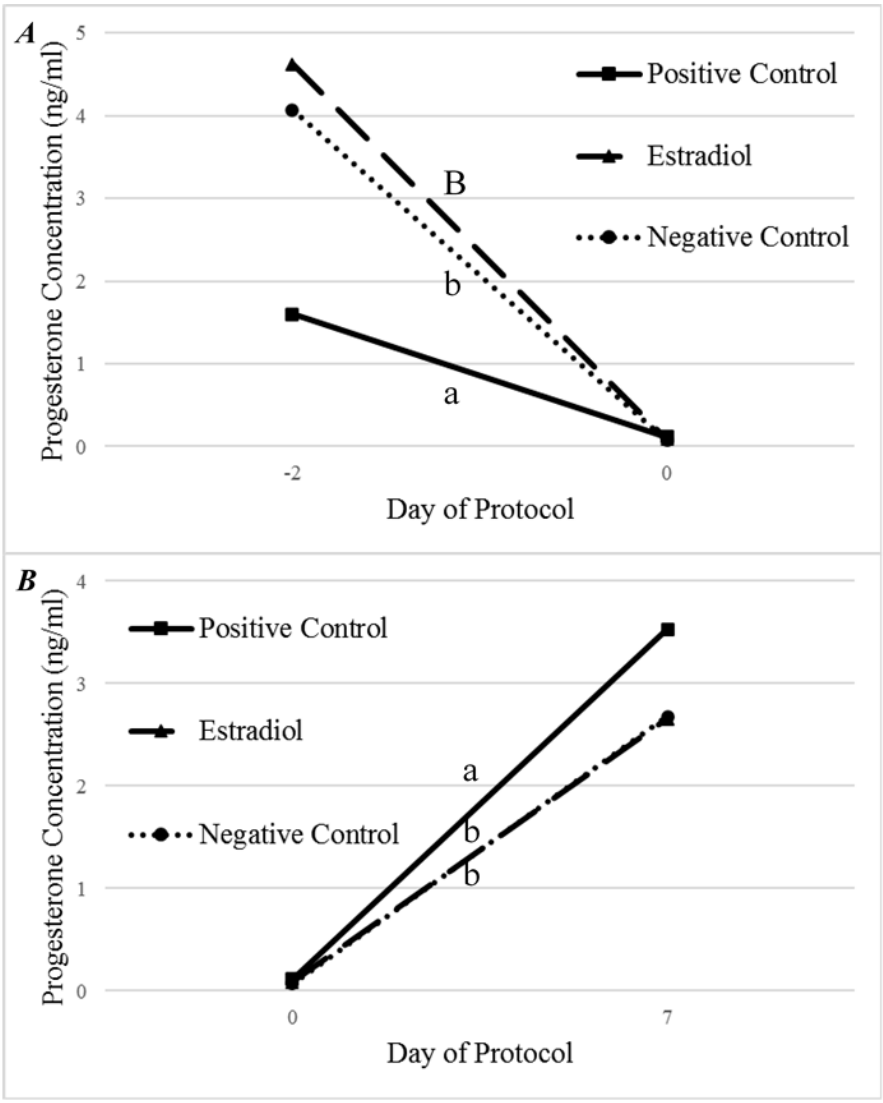


Figure 3.12. Change in circulating concentrations of progesterone from PG-induced luteolysis on day -2 to day 0 (treatment assignment; *Panel A)* and from day 0 (treatment assignment) to day 7 *(*embryo transfer; *Panel B)* for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. In *Panel A*, LSmean ± (SEM) for change in circulating progesterone was less ($^{aBb}P < 0.0001$) in the Positive Control group (-1.60 ± 0.295 ng/mL) than both the Estradiol (-4.70 ± 0.278 ng/mL) and Negative Control (-3.94 ± 0.294 ng/mL) groups. The Estradiol group tended ($^{Bb}P = 0.0621$) to have a greater change in circulating progesterone from day -2 to 0 then the Negative Control group. In *Panel B*, LSmean ± (SEM) for change in circulating progesterone from day 0 to 7 was greater ($^{ab}P < 0.05$) in the Positive Control (3.32 ± 0.166 ng/mL) group than both the Estradiol (2.52 ± 0.157 ng/mL) and Negative Control (2.54 ± 0.165 ng/mL) groups. Estradiol and Negative Control groups did not differ ($P = 0.9504$).

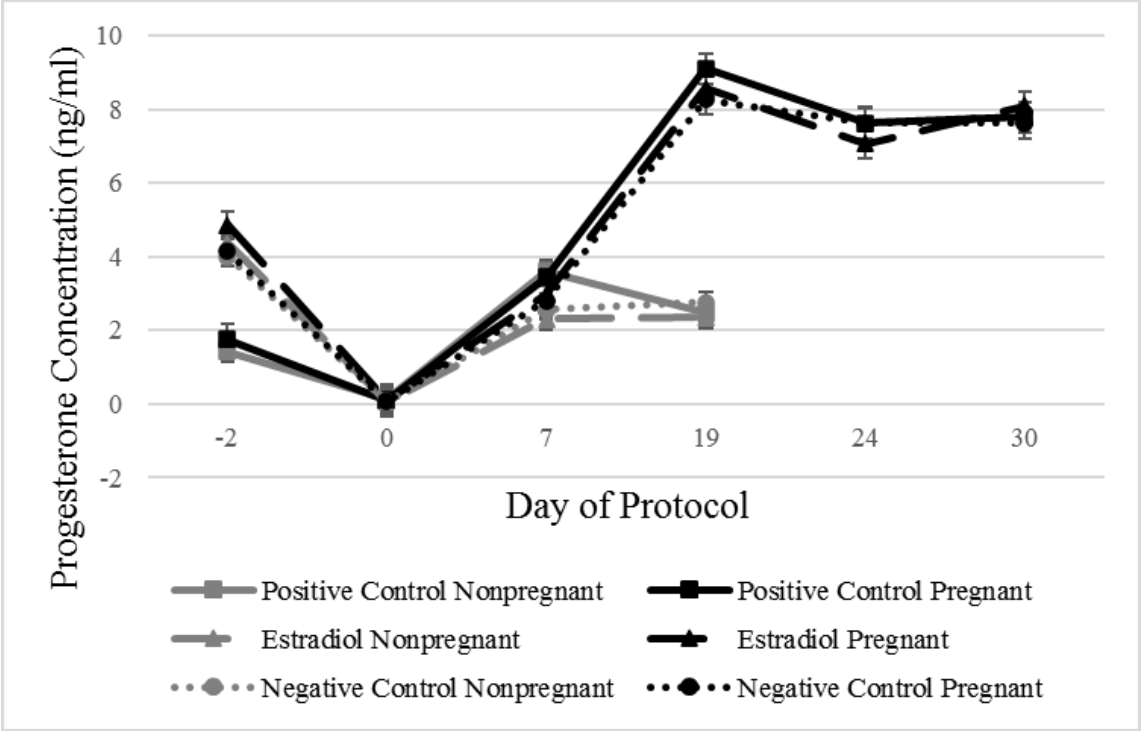


Figure 3.13. LSmean ± (SEM) circulating concentrations of progesterone (ng/mL) following PG-induced luteolysis (d -2) for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. Treatment groups were subdivided by day 30 (commercial IDEXX S-N $\geq$ 0.3) pregnancy status. There were no treatment differences ($P > 0.2530$) after day 7.

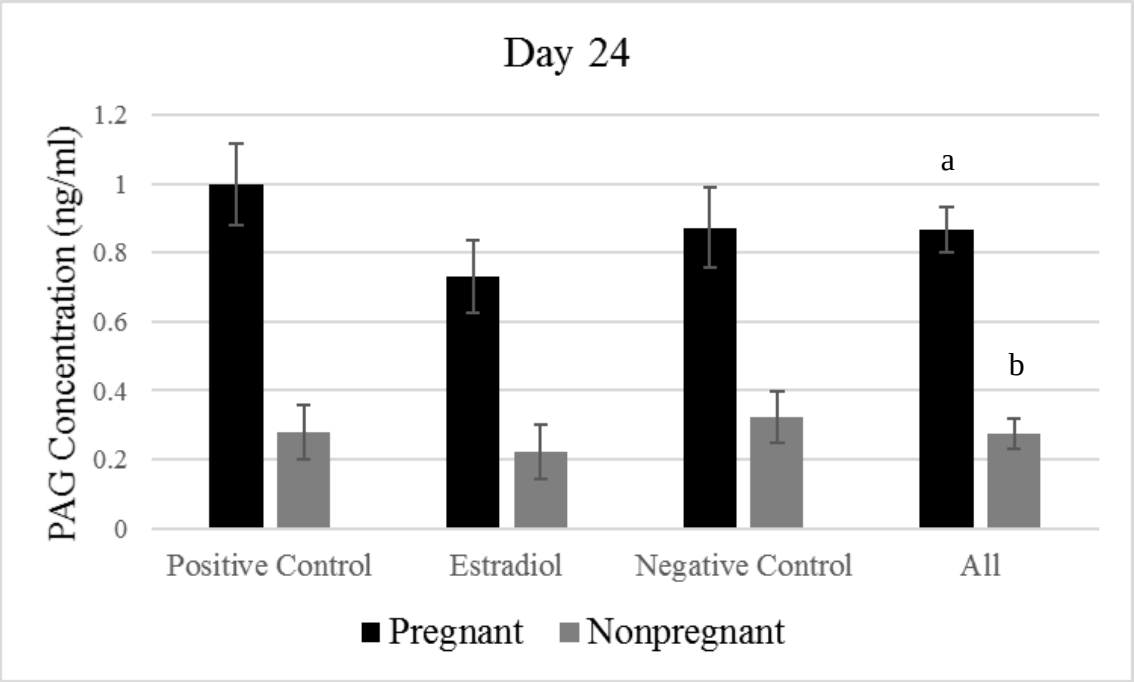


Figure 3.14. LSmean ± (SEM) circulating concentrations of PAGs (Ab 63; ng/mL) on day 24 for pregnant and nonpregnant cows, based on day 30 (commercial IDEXX S-N ≥ 0.3) pregnancy status, assigned to 1) Positive Control: Estrual cows that ovulated spontaneously, 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2, and 4) All: All cows included in the study regardless of treatment. There was no treatment by pregnancy interaction ($P = 0.5160$), nor treatment effect ($P = 0.2111$). However, circulating concentrations of PAGs were greater ($^{ab}P < 0.0001$) in pregnant compared to nonpregnant cows.

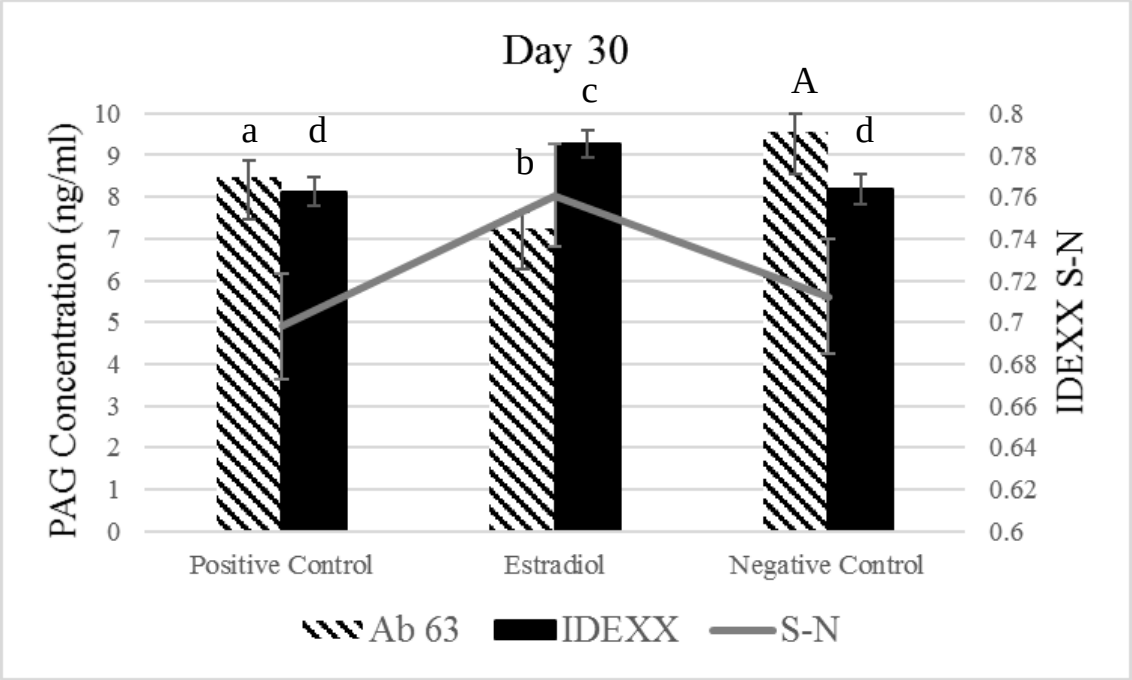


Figure 3.15. LSmean ± (SEM) circulating concentrations of PAGs (ng/mL) and commercial IDEXX S-N values on day 30 for pregnant (day 30 commercial IDEXX S-N ≥ 0.3) cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously, 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. Commercial IDEXX S-N values represent the absorption of the sample minus the absorption value of the negative control. A commercial IDEXX S-N value of ≥ 0.3 is indicative of pregnancy. Concentrations of PAGs (Ab 63) were greater ($^{ab}P < 0.05$) in pregnant Positive Control cows than pregnant Estradiol cows. Concentrations of PAGs (Ab 63) tended to differ ($^{aA}P = 0.0630$) between pregnant Positive Control cows and pregnant Negative Control cows. Pregnant Negative Control cows had greater ($^{Ab}P < 0.0001$) circulating concentrations of PAGs than pregnant Estradiol cows. Commercial IDEXX S-N values did not differ ($P = 0.2969$) among treatment groups. Concentrations of PAGs (commercial IDEXX) were greater ($^{cd}P < 0.05$) in pregnant Estradiol cows than both pregnant Positive Control cows and pregnant Negative Control cows. Concentrations of PAGs (commercial IDEXX) did not differ ($P = 0.9176$) between pregnant Negative Control cows and pregnant Positive Control cows.

(IDEXX) did not differ ($P = 0.9176$) between pregnant cows in the Negative Control and Positive Control groups. Embryo grade and stage had no effect on circulating concentrations of PAGs (Ab 63) on day 24, circulating concentrations of PAGs (Ab 63 and IDEXX) on day 30, nor S-N values (IDEXX) on day 30.

3.4.6 Evidence of a conceptus/fetus and periods of loss including all timepoints.

The GLIMMIX procedure revealed no treatment effect on the proportion of cows considered to have evidence of a conceptus present on day 19 ($P = 0.7378$) or day 24 ($P = 0.6835$). The proportion of cows considered pregnant on day 30 ($P = 0.3871$) and on day 55/58 ($P = 0.4510$) were also not affected by treatment.

The repeated measures analysis of variance revealed no effect of a treatment by day interaction ($P = 0.9919$) on pregnancy rates/evidence of a conceptus, nor was there a main effect of treatment ($P = 0.1144$); however, there was a main effect of day ($P < 0.0001$). Regardless of treatment, the proportion of cows that had an embryo on day 7 after ET (i.e. 100%) was greater ($P < 0.0001$) than the proportion of cows that had evidence of a conceptus present on day 19 and 24 as well as the proportion of pregnant cows on day 30 and 55/58. The proportion of cows that provided evidence of a conceptus present on day 19 was greater ($P = 0.0094$) than on day 24. The proportion of cows that had evidence of a conceptus present on day 24 did not differ ($P = 0.2070$) from the proportion diagnosed pregnant on day 30. Pregnancy rates tended to differ ($P = 0.0856$) between day 30 and 55/58; however, the proportion of cows that had evidence of a conceptus present on day 24 was greater than pregnancy rates on day 55/58 ($P = 0.0029$) as shown in Table 3.7.

Table 3.7. Percent[1] of Cows with Evidence of Conceptus Present and Pregnant to ET on Days 7, 19, 24, 30, and 55/58 by treatment.

	ET	Evidence of a Conceptus		Percent Pregnant	
Treatment[2]	Day 7[3]	Day 19[4]	Day 24[5]	Day 30[6]	Day 55/58[7]
Positive Control	100 ± 0.04	47 ± 0.04	38 ± 0.04	34 ± 0.04	29 ± 0.04
Estradiol	100 ± 0.04	44 ± 0.04	37 ± 0.04	34 ± 0.04	28 ± 0.04
Negative Control	100 ± 0.04	42 ± 0.04	33 ± 0.04	27 ± 0.04	22 ± 0.04
Total	100 ± 0.02[a]	44 ± 0.02[b]	36 ± 0.02[c]	32 ± 0.02[c,d]	26 ± 0.02[D]

[1]Presented as LSMEAN x 100 ± SEM

[2]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2.

[3]All cows received an embryo by ET on day 7.

[4]Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone ≥ 1 ng/mL on day 19; or 2) Had PAG (Ab 63) concentrations > 0.2 on day 24, or 3) Had an IDEXX S-N value ≥ 0.3 on day 30, or 4) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[5]Evidence of a conceptus was considered to be present on day 24 when a cow had 1) a PAG (Ab 63) concentration ≥ 0.2 on day 24; or 2) An IDEXX S-N ≥ 0.3 on day 30; or 3) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[6]Cows were considered pregnant on day 30 when they had an IDEXX S-N value ≥ 0.3 on day 30 or a 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[7]Cows were considered pregnant on day 55/58 if a 55/58 day viable fetus of the correct age was identified by ultrasonography.

Different letters in the same row indicate a difference $^{a,b,c,d}P < 0.05$

Same letters with different capitalization indicate a tendency $^{D,d}P = 0.0856$

There was no treatment by period interaction for the proportion of embryonic/early fetal loss from day 7 to 55/58; however, there was a main effect of period. The proportion of loss that occurred in Period 1 (day 7 to 19) was greater ($P < 0.0001$) than the loss that occurred in Period 2 (day 19 to 24), but the loss that occurred in Period 4 (day 30 to 55/58) was similar ($P = 0.1603$) to Period 2 (day 19 to 24). The proportion of loss that occurred in Period 3 (day 24 to 30) was less than ($P = 0.0238$) the loss that occurred in Period 2 (day 19 to 24) but similar ($P = 0.1603$) to the loss that occurred in Period 4 (day 30 to 55/58) as shown in Table 3.8.

3.4.7 Evidence of a conceptus/fetus and periods of loss without day 24 data

Due to the low sensitivity (55.10; true positive rate) that resulted from the ROC curve analysis of the day 24 PAG (Ab 63) concentrations, the presence of a conceptus/pregnancy and pregnancy loss data were analyzed without using the day 24 PAG (Ab 63) concentration criterion (Figure 3.16). In this analysis, a cow was considered pregnant on day 55/58 if a viable fetus of the correct age was identified by ultrasonography and was then excluded from any further classification (Day 19 or 30). A cow was considered to be pregnant on day 30 when a 55/58 day viable fetus was not identified by ultrasound on day 55/58, but she had an IDEXX S-N value ≥ 0.3 on day 30. Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2) and day 19 progesterone concentration was ≥ 1 ng/mL, or 3) the IDEXX S-N value on day 30 was < 0.3, or 4) there was a 55/58 day viable fetus identified via ultrasound on day 55/58. In

Table 3.8. Percent[1] Loss within Periods[2] 1, 2, 3, and 4 by Treatment.

Treatment[3]	Period 1[4]	Period 2[5]	Period 3[6]	Period 4[7]
Positive Control	53 ± 0.05	9 ± 0.03	3 ± 0.02	6 ± 0.02
Estradiol	56 ± 0.05	7 ± 0.03	3 ± 0.02	5 ± 0.02
Negative Control	58 ± 0.05	9 ± 0.03	6 ± 0.02	5 ± 0.02
Total	56 ± 0.03[a]	8 ± 0.02[b]	4 ± 0.01[c,d]	6 ± 0.01[b,d]

[1]Presented as LSMEAN x 100 ± SEM

[2]Period 1: day 7 to 19; Period 2: day 19 to 24; Period 3: day 24 to 30; Period 4: day 30 to 55/58.

[3]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. All cows received and embryo by ET on day 7.

[4]Cows receiving an embryo on day 7 were considered to have undergone Period 1 loss when on day 19, 2 of the 3 ISG genes (*ISG15*, *MX2*, *OAS1)* had a ΔΔCT value ≥ its respective associated criterion (Figure X and Table Y) and circulating progesterone < 1 ng/mL on day 19.

[5]A cow was considered to have undergone Period 2 Loss when the following criteria were met: 1) 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion on day 19 (Figure 3.5, 3.6, 3.7 and Table 3.2), 2) Day 19 circulating progesterone concentration was ≥ 1 ng/mL, 3) PAG (Ab 63) concentration on day 24 was < 0.2, and 4) the IDEXX S-N value on day 30 was < 0.3, and 4) nor was a 55/58 day viable fetus identified via ultrasound on day 55/58.

[6]Cows were considered to have undergone Period 3 loss when PAG (Ab 63) concentration on day 24 was ≥ 0.2, but the IDEXX S-N value on day 30 was < 0.3 and no 55/58 day viable fetus was identified on day 55/58.

[7]A cow was considered to have undergone Period 4 Loss when a 55/58 day viable fetus was not identified by ultrasound on day 55/58 but she had an IDEXX S-N value ≥ 0.3 on day 30 (i.e. pregnant).

Different letters in the same row indicate a difference [a,b,c,d]$P < 0.05$

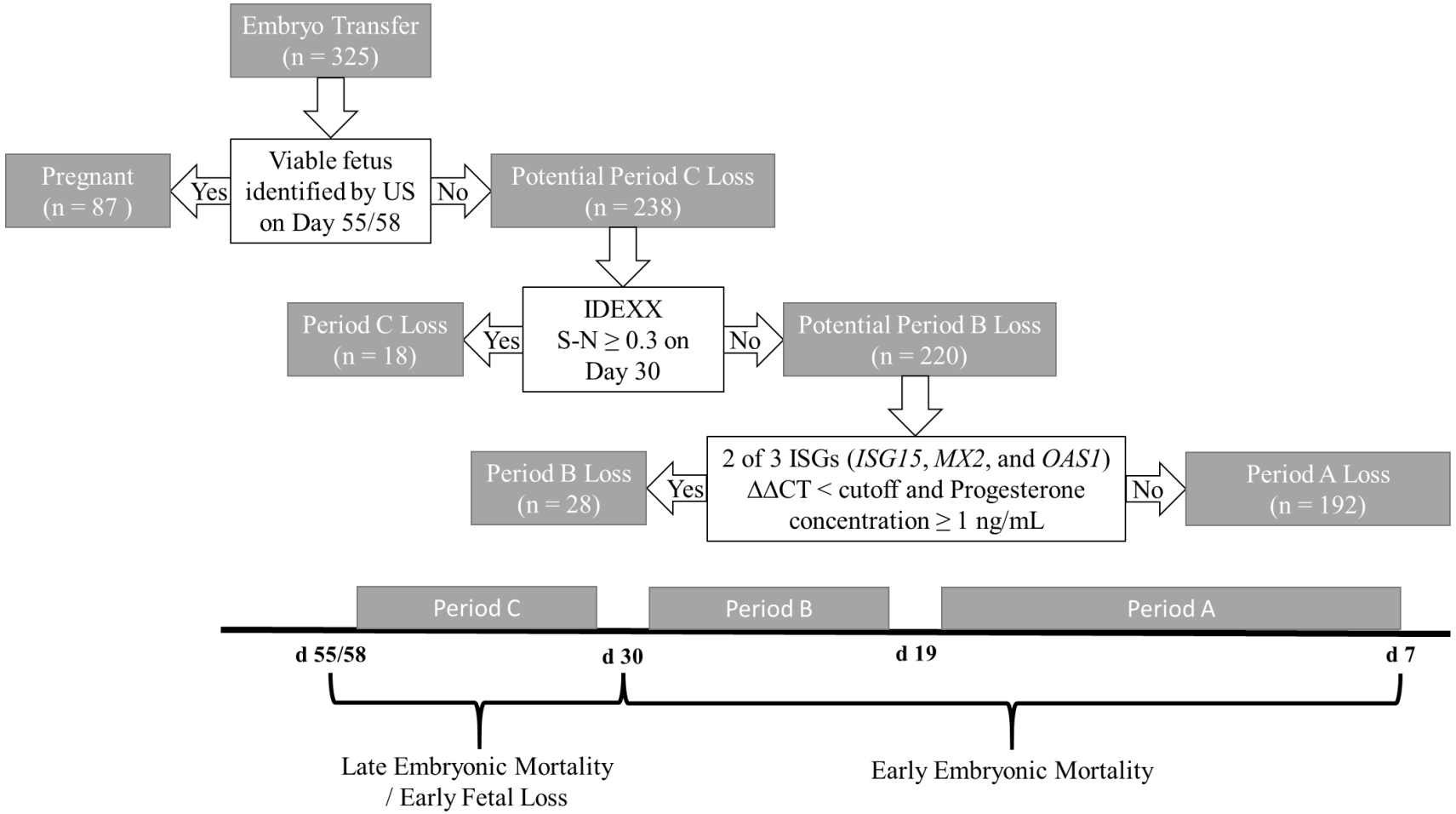


Figure 3.16. A total of 325 cows received ET on day 7 and thus were considered to have presence of an embryo present on day 7. All 325 cows were subjected to ultrasonography on day 55/58 and those with a 55/58 day viable fetus were considered pregnant (n = 87) and were not further classified. Cows without a 55/58 day viable fetus detected on day 55/58 (n = 238) were further classified by day

30 IDEXX S-N values, such that if a cow had an IDEXX S-N value ≥ 0.3 (n = 18), they were considered to have undergone Period C Loss and were excluded from further classification. If the IDEXX S-N value was < 0.3 (n = 220) they were further classified by ISG ΔΔ CT values as well as circulating progesterone concentrations on day 19, such that if 2 of the 3 ISGs had a ΔΔ CT < their respective cutoff (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone concentrations ≥ 1 ng/mL (n = 28), they were considered to have undergone Period B loss and were not further classified. Those cows that did not have 2 of the 3 ISGs ΔΔ CT < their respective cutoff (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone concentrations ≥ 1 ng/mL were considered to have undergone Period A Loss (n = 192). Loss that occurred during Periods A and B would be considered Early Embryonic Mortality while loss that occurred in Period C is considered Late Embryonic Mortality/Early Fetal Loss.

(Figure 3.5, 3.6, 3.7 and Table 3.2) and day 19 regard to analyzing pregnancy loss during specific periods, Period A included days 7 to 19; Period B: days 19 to 30; and Period C: days 30-55/58. A cow was considered to have undergone Period C Loss when a 55/58 day viable fetus was not identified by ultrasound on day 55/58 but she had an IDEXX S-N value ≥ 0.3 on day 30. A cow was considered to have undergone Period B Loss when the following criteria were met: 1) 2 of the 3 ISG genes (*ISG15*, *MX2*, *OAS1*) had a ΔΔCT value < its respective associated criterion progesterone concentration was ≥ 1 ng/mL, but 3) the IDEXX S-N value on day 30 was < 0.3, and 4) a 55/58 day viable fetus was not identified via ultrasound on day 55/58. A cow was considered to have experienced Period A loss if she did not meet the criteria for a conceptus to be present on day 19, as previously described.

The GLIMMIX procedure revealed no treatment effect on the proportion of cows considered to have evidence of a conceptus present on day 19 ($P = 0.4515$), the proportion of cows considered pregnant on day 30 ($P = 0.3871$) or on day 55/58 ($P = 0.4510$). In regards to presence of a conceptus/pregnancy on specific days, there was no treatment by day interaction ($P = 0.9400$), however; there was a main effect of day ($P < 0.0001$) and there was a tendency for an effect of treatment ($P = 0.0800$). Independent of treatment, the proportion of cows that were considered to have a viable embryo on day 7 (i.e. 100%) was greater ($P < 0.0001$) than the proportion of cows that were considered to have evidence of a conceptus present on day 19 and the proportion of cows considered pregnant on day 30 and 55/58. The proportion of cows that were considered to have evidence of a conceptus present on day 19 was greater than those considered pregnant on day 30 ($P = 0.0047$) as well as on day 55/58 ($P < 0.0001$; Table 3.9). Pregnancy rates on

Table 3.9. Percent[1] of Cows with Evidence of Conceptus Present and Pregnant to ET on days 7, 19, 30, and 55/58 by treatment.

	ET	Evidence of a Conceptus	Percent Pregnant	
Treatment[2]	Day 7[3]	Day 19[4]	Day 30[5]	Day 55/58[6]
Positive Control	100 ± 0.04	44 ± 0.04	35 ± 0.04	29 ± 0.04
Estradiol	100 ± 0.04	42 ± 0.04	33 ± 0.04	28 ± 0.04
Negative Control	100 ± 0.04	36 ± 0.04	27 ± 0.04	22 ± 0.04
Total	100 ± 0.02[a]	40 ± 0.02[b]	32 ± 0.02[c,d]	26 ± 0.02[D]

[1]Presented as LSMEAN x 100 ± SEM

[2]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2.

[3]All cows received ET on day 7.

[4]Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone ≥ 1 ng/mL on day 19; or 2) Had an IDEXX S-N value ≥ 0.3 on day 30, or 3) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[5]Cows were considered pregnant on day 30 when they had an IDEXX S-N value ≥ 0.3 on day 30 or a 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[6]Cows were considered pregnant on day 55/58 if a 55/58 day viable fetus was identified by ultrasonography.

Different letters in the same row indicate a difference [a,b,c,d]$P < 0.05$

Same letters with different capitalization indicate a tendency [D.d]($P = 0.0709$)

day 30 and 55/58 tended (P = 0.0709) to differ. When the tendency for a main effect of treatment was further evaluated, the overall proportion of cows considered pregnant was similar (P = 0.7095) between the Positive Control group and the Estradiol group. The Positive Control group had a greater (P = 0.0388) overall proportion of cows considered pregnant than the Negative Control group while the Estradiol group tended (P = 0.0786) to have a greater proportion of cows considered pregnant than the Negative Control group (Figure 3.17).

There was no treatment by Period of Loss interaction (P = 0.9690), nor a main effect of treatment (P = 1.000). There was a main effect of Period of Loss (P < 0.0001) such that a greater (P < 0.0001) amount of loss occurred in Period A then both Period B and C. The amount of loss that occurred in Periods B and C were similar (P = 0.1441) as shown in Table 3.10.

3.5 Discussion

The development of effective FTAI protocols have provided producers the opportunity to access a higher quality genetic pool and afford every female an opportunity to conceive on the first day of breeding season. Most current FTAI protocols synchronize follicular waves and control luteal lifespan by administering GnRH, a progestin (CIDR® or MGA), and PG. The final GnRH injection of a FTAI protocol can induce ovulation of a wide range of dominant follicle sizes that represent different stages of physiological maturity, including sub-optimal follicles with reduced estradiol secretion

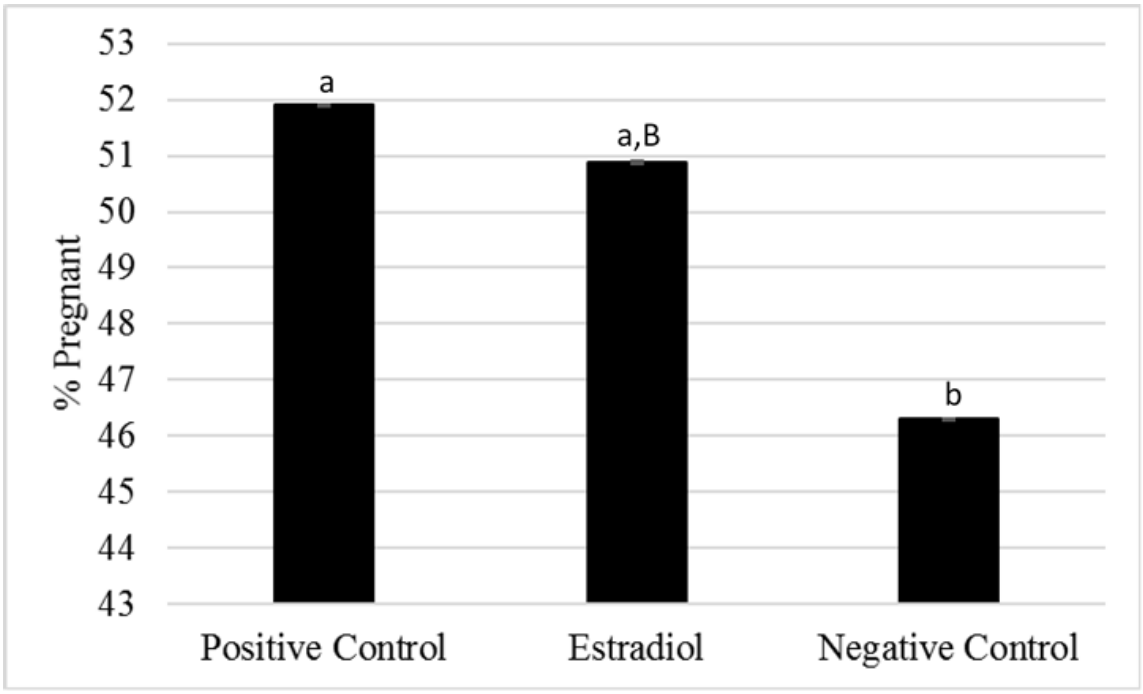


Figure 3.17. Overall pregnancy rates (Days 7 [ET] to Day 55/58) for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2. When the day 24 data were excluded, treatment tended (P = 0.0800) to have a main effect on overall pregnancy rates. More specifically, pregnancy rates were similar (P = 0.7095) between the Positive Control and Estradiol groups. The Negative Control group had a lower (P = 0.0388) pregnancy rate than the Positive Control group, but only tended (P = 0.0786) to differ from Estradiol group.

Table 3.10. Percent[1] Loss within Periods[2] A, B, and C by Treatment.

Treatment[3]	Period A[4]	Period B[5]	Period C[6]
Positive Control	78 ± 0.05	13 ± 0.04	9 ± 0.03
Estradiol	80 ± 0.05	12 ± 0.04	8 ± 0.03
Negative Control	81 ± 0.04	12 ± 0.04	7 ± 0.03
Total	78 ± 0.02[a]	12 ± 0.02[b]	8 ± 0.02[b]

[1]Presented as LSMEAN x 100 ± SEM
[2]Period A: day 7 to 19; Period B: day 19 to 30; Period C day 30 to 55/58
[3]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2.
[4]If a cow did not meet the criteria requirements indicating evidence of a conceptus present on day 19 (Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone was ≥ 1 ng/mL; or 2) Had an IDEXX S-N value ≥ 0.3 on day 30, or 3) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.) then she was considered to have undergone Period A Loss.
[5]A cow was considered to have undergone Period B Loss when the following criteria were met: 1) 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2), 2) Day 19 circulating progesterone concentration was ≥ 1 ng/mL, and 3) the IDEXX S-N value on day 30 was < 0.3, 4) nor was there a 55/58 day viable fetus identified via ultrasound on day 55/58.
[6]A cow was considered to have undergone Period C Loss when no 55/58 day viable fetus was identified by ultrasound on day 55/58 but she had an IDEXX S-N value ≥ 0.3 on day 30.
Different letters in the same row indicate a difference [a,b]$P < 0.0001$.

(Perry et al., 2005). Beef heifers and cows that express estrus by the time of FTAI have a higher pregnancy rate than females that do not express estrus (Richardson et al., 2016). Expression of estrus was positively associated with preovulatory secretion of estradiol (Coe & Allrich, 1989; Fields et al., 2012), ovulatory follicle size (Atkins et al., 2013), and is a marker of the physiological maturity of a dominant follicle (Perry et al., 2005). In postpartum beef cows that had not expressed estrus, preovulatory estradiol had a positive effect on pregnancy rate following embryo transfer (Atkins et al., 2013; Jinks et al., 2013; Ciernia, 2018), suggesting that preovulatory estradiol has a direct or indirect effect on uterine receptivity. Since fertilization rate is relatively high in beef females (80-90%; Reese et al., 2020), we hypothesized that the beneficial effect of preovulatory estradiol on pregnancy rate is likely mediated by increasing embryo/early fetal survival at physiologically important time points (e.g. maternal recognition of pregnancy and(or) placentation).

In this study, the Positive Control group had higher estradiol 17-β concentrations on day -2 than the Estradiol and Negative Control groups, suggesting the presence of a more physiologically mature follicle that produced more estradiol 17-β at CIDR® removal. Lopes et al., (2007) reported that larger preovulatory follicles were associated with greater preovulatory estradiol 17-β secretion. Fields et al. (2012) reported that cows that spontaneously expressed estrus following the CO-Synch protocol had larger ovulatory follicles than cows that did not express estrus. Absence of a primary and(or) accessory CL during CIDR® treatment would create a low progesterone environment resulting in low amplitude, high frequency LH pulsatility which is favorable to estradiol 17-β production. Potentially, a subset of the cows in the Positive Control group could

have been at a stage in their follicular wave (recruitment to selection) on day -9, in which they were incapable of responding to GnRH1 and forming an accessory CL or potentially a primary CL. Furthermore, they could have undergone spontaneous luteolysis prior to CIDR® removal. This scenario may be one factor contributing to the low circulating progesterone and elevated estradiol at CIDR® removal compared to the other groups. Interestingly, circulating concentrations of estradiol 17-β at CIDR® removal (day -2) in the Positive Control group (4.6 pg/mL) was similar to that reported for persistent follicles (3 to 6 pg/mL) in postpartum beef cows (Perry et al., 2002; Risley, 2009). Another subset of the Positive Control cows could have developed a persistent follicle, which forms in cycling cattle due to a prolonged increase in LH pulse frequency (Duffy et al., 2000). A proportion of cows in the Positive Control group (n = 15) had estradiol 17-β concentrations > 6 pg/mL on day -2, while that was the case for only 1 and 9 cows in the Estradiol and Negative Control groups, respectively. Rhodes et al. (1997) reported that, despite no differences in the incidence of persistent follicles, anestrous postpartum dairy cows had increased LH pulse frequency in response to progesterone treatment compared to a control group. In a postpartum anestrous cow, increased LH pulse frequency may allow estradiol 17-β concentrations to become elevated. Therefore, it is possible that a third subset of the Positive Control group could have been anestrual and therefore had elevated concentrations of estradiol 17-β on day -2.

Circulating concentrations of estradiol 17-β approached 20 pg/mL in the Estradiol group on d0h2 and were higher than the other two groups. This response is consistent with the results of the preliminary experiment (Figure 3.2). Therefore, it is expected that the Estradiol group experienced peak estradiol 17-β concentrations between 1- and 2-

hours following administration of 0.1 mg estradiol 17-β and that estradiol 17-β concentrations returned to baseline within 8 hours. The preceding peak concentration of estradiol is similar to that reported during the preovulatory period in cattle (Lemon et al., 1975; Wolfenson et al., 2004). In the preliminary study, concentrations of estradiol remained elevated for 8 hours, which is shorter than that reported (Chenault et al., 1975; Glencross et al., 1981; Perry et al., 2014) for endogenous secretion of estradiol 17-β in cattle during the preovulatory period. It is not clear whether a physiological increase of estradiol for 8 h is adequate to have a positive effect on the uterus for the establishment of pregnancy. However, administration of estradiol 17-β increased the proportion of cows that expressed estrus after d0h0 compared to the Negative Control group. Hillegass et al. (2008) also reported an increase in the proportion of females that expressed estrus after administering 1 mg of estradiol cypionate 24 hours following the final PG injection in the CO-Synch protocol compared to controls. Ciernia (2018) reported that postpartum beef cows that had not expressed estrus but had high preovulatory concentrations of estradiol 17-β (> 5.8 pg/mL) had a greater pregnancy rate following embryo transfer compared to cows with lower estradiol 17-β, indicating an effect of preovulatory estradiol on uterine receptivity. There may be a threshold concentration of preovulatory estradiol that is required to optimize pregnancy rate. Alternatively, the magnitude of the change in estradiol, during the preovulatory period, maybe what is important. For example, Rozell and Keisler (1990) reported that the rate of change in estradiol controlled the time of the LH surge in ovariectomized ewes (Rozell & Keisler, 1990).

Both the Negative Control and Estradiol groups had a greater change in estradiol 17-β from day - 2 to 0 than the Positive Control group. A number of cows in the Positive

Control group may have already undergone luteolysis or may not have had any luteal tissue producing progesterone on day -2; whereas, PG may have induced luteolysis on day -2 in a number of cows in both the Negative Control and Estradiol groups. Therefore, in the Estradiol and Negative Control groups there would have been a greater decrease in progesterone from day -2 to 0 compared to the Positive Control group. This may explain why both the Negative Control and Estradiol groups had a greater positive change in estradiol 17-β from day -2 to 0 than the Positive Control group. Atkins et al. (2013) reported that progesterone concentrations on day -2 had a positive effect on day 0 estradiol 17-β concentrations.

Circulating concentrations of progesterone were lower on day -2 in the Positive Control group compared to both Negative Control and Estradiol groups. These results along with the Positive Control group having higher estradiol 17-β concentrations on day -2 indicate that at least a portion of this group of cows did not respond to GnRH1 and therefore underwent follicular development in a low progesterone environment that may have contributed to greater estradiol 17-β production. The Positive Control group had higher circulating concentrations of progesterone on day 7 compared to the Estradiol and Negative Control group. The Positive Control cows probably ovulated a larger dominant follicle, and since follicular cells differentiate into luteal cells, the resulting CL would have been larger leading to greater production of progesterone (Fields et al., 2012).

In cattle, elevated progesterone during the early luteal phase increased conceptus elongation (reviewed by Lonergan & Forde, 2015). Although the bovine embryo contains progesterone receptors, in vitro exposure to progesterone did not affect blastocyst rate, blastocyst cell number, nor post hatching elongation when transferred into a recipient.

However, a fourfold increase in conceptus length was observed when in vitro derived blastocyst were transferred into a progesterone primed uterine environment (Clemente et al., 2009). Thus, progesterone indirectly acts on the conceptus through the endometrium to alter the intrauterine milieu and alter histotroph production in such a way that it is conducive to blastocyst growth and embryo elongation (Brooks et al., 2014). However, as discussed below, the pregnancy rate in this study was not greater at any individual time point in the Positive Control group compared to the other groups.

The Estradiol and Negative Control groups had similar progesterone concentrations on day 7, which suggests that administration of 0.1 mg estradiol 17-β on day 0 did not have an effect on progesterone production on day 7. Estradiol 17-β is thought to play a role in preparing granulosa cells for luteinization (McNatty, 1979), proliferation (Goldenberg et al., 1972), enhancing progestin synthesis following gonadotropin stimulation (Welsh et al., 1983), and promoting gap junction formation between granulosa cells (Merk et al., 1972). Furthermore, Atkins et al. (2013), through path analysis, reported a beneficial effect of elevated preovulatory estradiol 17-β concentrations on progesterone concentrations on day 7 in postpartum beef cows that was independent of preovulatory follicle size. However, McLean et al. (2019) reported that when estrual cows were grouped according to low or high preovulatory concentrations of estradiol 17-β concentrations, subsequent progesterone concentrations were not affected. McLean et al. (2018) further reported that estradiol 17-β did not work through the genomic receptor to prepare granulosa cells for luteinization during the preovulatory period. More specifically, CL volume and circulating progesterone concentrations did not differ among treatments (control: no intrafollicular injection; vehicle: intrafollicular

injection of vehicle; or antagonist: intrafollicular injection of an estradiol receptor antagonist [Fluvestrant]). Irrespective of whether or not preovulatory concentrations of estradiol are required to prepare granulosa cells for luteinization, exogenous administration of a physiological dose of estradiol 17-β is unlikely to affect follicular fluid concentrations of estradiol 17-β to cause an effect.

Progesterone concentrations, in cows diagnosed pregnant on day 30 (IDEXX S-N $\geq$ 0.3), did not differ among treatments after day 7 indicating that estradiol 17-β concentration on day 0 did not elicit an effect after day 7, again reiterating that exogenous administration of a physiological dose of estradiol 17-β is unlikely to affect follicular fluid concentrations of estradiol 17-β to cause an effect on subsequent CL formation and progesterone production.

Detection of pregnancy/presence of a conceptus prior to day 30 not only has practical applications to improve reproductive efficiency but also is critical for understanding when losses are occurring. Interval from AI to next estrus, circulating concentrations of progesterone, circulating concentrations of PAGs, or combinations thereof have all been used to estimate the presence of an embryo prior to day 30 (Breukelman et al., 2012; Gábor et al., 2007; Grimard et al., 2006). However, each of these endpoints have their limitations. For example, using PAGs as early as day 24 to identify the presence of a conceptus could result in underestimation of pregnancy losses, whereas, using circulating progesterone around the expected time of luteolysis or interval from AI to next estrus could potentially overestimate embryonic mortality. Thus, techniques to detect presence of a conceptus earlier than feasible with transrectal ultrasonography have been developed with specific emphasis placed on changes in the

pattern of gene expression in circulating immune cells in response to interferon-tau (IFNT) around the time of maternal recognition of pregnancy (Gifford et al., 2007; Green et al., 2010; Han et al., 2006; Stevenson et al., 2007). On day 14 of gestation, the ruminant trophoblast begins to produce IFNT (Thatcher et al., 1995), which can act on leukocytes and cause expression of ISGs. IFNT has a transitory secretion pattern where maximum secretion by the bovine embryo occurs by day 20 to 24 but is absent by day 30 of gestation (Roberts, 2007). Expression of ISG abundance on days 18 to 22 has been used to identify the presence of a bovine embryo (Wijma et al., 2016). Haq et al. (2016) reported increased levels of *ISG15* in pregnant cows compared to non-pregnant cows. Similarly, Madsen et al., (2015) reported that on days 17, 19, and 21 cows that were diagnosed pregnant by ultrasound on day 29 had elevated expression of *ISG15*, *MX2*, and *OAS1*. Han et al. (2006) reported that when blood samples were collected on days 15 - 21, 25, and 32, low progesterone profiles (< 2.0 ng/mL) in combination with low *ISG15* mRNA (< -7.0 arbitrary units relative to reference gene) were 100% accurate in identifying nonpregnant cows as nonpregnant (true negative). They also reported, from serial blood collections collected on days 15-21, 25, and 32, the positive predictive value (probability that a cow is truly pregnant if the diagnosis is pregnant) of *ISG15* mRNA expression was 78% accurate for identifying presence of a conceptus while progesterone was only 58% accurate. When only the day 18 values were used, the negative predictive value (probability a cow is truly not pregnant if the diagnosis is nonpregnant) for progesterone and *ISG15* mRNA was 100% and 89% accurate, respectively. The positive predictive value accuracy was 47% and 62% for progesterone and *ISG15* mRNA, respectively. While expression of ISGs in circulating immune cells have predictive value

for assessing the reproductive status of a cow prior to day 30 of gestation, they are still not 100% accurate in detecting the presence of a conceptus.

In ruminants, PAGs are a product of binucleated trophoblast cells that enter the maternal circulation via exocytosis of secretory granules following the interdigitation of microvilli on fetal and maternal membrane ("Synepitheliochorial Placentation : Ruminants [Ewe and Cow]," 2008; Wooding & Wathes, 1980) and have been previously reported to accurately (95-98%) diagnose pregnancies between days 28-30 of gestation in cattle (Breukelman et al., 2012; Pohler et al., 2013; Romano & Larson, 2010; Silva et al., 2007). Currently, the earliest time that pregnancy can accurately be detected in beef cattle is 28-30 days of gestation. Due to the negative economic impact of maintaining a nonpregnant cow as well as the economic significance of generating the most pregnancies possible as early in the breeding season, emphasis has been placed on development of techniques to detect pregnancy prior to day 28 with the goal of identifying nonpregnant cows earlier and improving reproductive efficiency. Pohler et al. (2013), reported that the first increase in circulating PAGs in beef cows occurred on day 24 of gestation, by using breakpoint analysis. PAG concentrations continued to increase until day 36 of gestation. Filho et al. (2020) reported that pregnant beef cows had higher circulating concentrations of PAG on day 24 compared to nonpregnant females and that cows experiencing late embryonic/early fetal mortality had lower concentrations of PAG on day 24 compared to females that maintained their pregnancy. Similarly, higher circulating concentrations of PAG on day 24 were reported in pregnant dairy cows compared to nonpregnant dairy cows (Reese et al., 2018).

In this study, cows considered to be pregnant on day 30 (IDEXX S-N ≥ 0.3) had greater concentrations of PAGs (Ab 63) on day 24 than cows with an IDEXX S-N < 0.3, however there was no treatment effect. The low sensitivity (55.10; true positive rate) that resulted from the ROC curve analysis of the day 24 PAG (Ab 63) concentrations warranted concern. The Youden Index that resulted from ROC curve analysis had an associated criterion of 0.2 ng/mL, which coincides with the sensitivity of the PAG ELISA that was used to measure circulating concentrations of PAGs on day 24. The high quantity of cows with an IDEXX S-N value < 0.3 provided values for the negative outcomes (i.e. not pregnant) and the large number of nonpregnant cows may have contributed to the low sensitivity of the cutoff value. Only 59, out of 105, cows (56%) that were diagnosed as pregnant on day 30 (IDEXX S-N ≥ 0.3) were also determined to have a conceptus present on day 24 based on PAG (Ab 63) concentrations that were > 0.2. However, there were also 46 cows (44%) diagnosed as pregnant on day 30 (IDEXX S-N ≥ 0.3) that were not considered to have a conceptus present on day 24 (PAG [Ab 63] concentration ≤ 0.2 ng/mL) and therefore were classified as false negatives. There were 13 cows that were classified as Period 3 Loss (day 24 to 30) as they had an IDEXX S-N value < 0.3 on day 30 but a PAG (Ab 63) concentration > 0.2 on day 24, however this value may be an underestimation of the actual loss that occurred in Period 3 due to inadequate assay sensitivity and(or) the variation in when PAGs first increase (Table 3.11). Therefore, the data were analyzed with and without the day 24 PAG (Ab 63) concentrations. Those results are discussed later.

Pohler (2013), reported the absence of a relationship between PAG concentrations and estradiol on day 0, ovulatory follicle size, progesterone on day 7, and embryo grade

Table 3.11. Diagnosis of presence of a conceptus as detected by PAGs (Ab63) on day 24 and pregnancy (IDEXX S-N) on day 30.

	PAG (Ab 63) Concentration[1]	
	0.2 ng/mL	> 0.2 ng/mL
Nonpregnant[2]	208	13
Pregnant[3]	46	59

[1]A Youden index and its associated criterion was calculated from a receiver operating characteristic (ROC) curve for circulating concentrations of PAG (Ab 63) on day 24 (Figure 3.4). The associated criterion (> 0.2 ng/mL, Se = 55.10, Sp = 95.17) established a cutoff value for PAG (Ab 63) concentrations, on day 24, that provided evidence of the presence of a conceptus.
[2]Cows with an IDEXX S-N value < 0.3 were considered nonpregnant.
[3]Cows with an IDEXX S-N value ≥ 0.3 were considered pregnant.

and stage. This is consistent with the results from this study where the Positive Control (greater concentrations of progesterone on day 7), Estradiol (greater concentrations estradiol 17-β on day 0), and Negative Control treatments did not have differing concentrations of PAG on day 24. Also consistent with Pohler (2013), embryo grade and embryo stage did not affect PAG concentrations on day 24 (Ab 63), day 30 (Ab 63 and IDEXX) nor IDEXX S-N values on day 30.

Perry (2005) reported a tendency for cows to have lower PAG concentrations on day 27 when late embryonic mortality occurred due to suboptimal embryo development, suboptimal uterine environment and(or) insufficient placental development during the attachment period of placentation. In this study, pregnant cows (IDEXX S-N value $\geq$ 0.3) had greater concentrations of PAG (Ab 63 and IDEXX) as well as greater IDEXX S-N values than nonpregnant cows on day 30. Among cows considered pregnant on day 30 (IDEXX S-N value $\geq$ 0.3), IDEXX S-N values on day 30 did not differ among treatments. The Estradiol group had lower concentrations of PAG (Ab 63) but greater concentrations of PAG (IDEXX) on day 30 than the Positive Control and Negative Control groups. The bovine PAG family contains > 20 members and the time these members are expressed varies throughout gestation (Touzard et al., 2013; Wallace et al., 2015). Gatea et al. (2018) reported that the ability to predict embryonic loss differed among PAG antibody combinations, which may explain why the Estradiol group had higher IDEXX concentrations, but lower Ab 63 concentrations on day 30, when compared to the Positive Control and Negative Control groups. The monoclonal antibodies (A6, J2, L4) used in the in-house sandwich ELISA (Ab 63) bind to different but specific members of the PAG family (Green et al., 2005), such that antibody A6 binds PAG 4, 6, 7, and 16; antibody J2

binds PAG 20; and antibody L4 binds PAG 4, 6, 9, and 21. The polyclonal antibody, Ab 63, is directed towards PAGs 4 and 6 which are both secreted up to day 250 of gestation; however; PAG 4 is first secreted on day 25 while PAG 6 is not secreted until day 45 of gestation. PAG 7 has a similar pattern of secretion throughout gestation as PAG 6 while PAG 9 is secreted from day 25 to 250 of gestation (Green et al., 2000). The IDEXX antibody is directed towards a mixture of PAGs (e.g. PAGs 4, 6, 9, 16, 18, 19; described in US Patent no. 7,604,950B2) Thus, the Estradiol group may have been secreting a profile of PAGs that differed from the Negative Control and Positive Control cows.

Estradiol supplementation, during the preovulatory period, as a means of increasing pregnancy rates has been studied in both dairy and beef cattle; however, the results have been variable. As mentioned above, Hillegass et al. (2008) reported an increase in the proportion of females that expressed estrus after administration of 1 mg of estradiol cypionate 24 hours following the final PG injection in the CO-Synch protocol compared to controls. However, despite an increase in the proportion of females that expressed estrus, no improvements in fertility were seen when estradiol cypionate was administered. Conversely, when estradiol cypionate (0.5mg, IM) was administered 24 hours prior to AI to postpartum beef cows, exposed to the CO-Synch + CIDR® protocol, females that were induced to ovulate follicles < 12.2 mm had greater pregnancy rates following ECP treatment compared to control cows. However; females induced to ovulate larger follicles did not benefit from ECP treatment (Jinks et al., 2013). During the follicular phase, it is thought that GnRH- induced ovulation, before the endogenous preovulatory gonadotropin surge, may cause estradiol secretion to decrease earlier than normal; therefore, fertility may be adversely affected. ECP was incorporated into the CO-

Synch protocol by administering 0.25 mg of ECP at the second GnRH injection in an attempt to determine if fertility could be increased in crossbred beef cows that were presynchronized with a single injection of PG. Cows were randomly assigned to either receive ECP at the second GnRH or GnRH alone. When females were immediately artificially inseminated following treatment, pregnancy rates at 35 and 70 days did not differ between treatments (Howard et al., 2007). However, when ECP was administered to females at the second GnRH injection in the Ovsynch protocol and artificially inseminated 10 hours later, those receiving ECP tended to have higher conception rates (68%) than females only receiving GnRH (57.5%) (Ahmadzadeh et al., 2003). As shown above by the previous studies, incorporating estradiol into synchronization protocols results in conflicting results which may partially be explained by the timing of insemination following ECP treatment as well as differences in the physiological maturity of the follicles being induced to ovulate.

Madsen et al. (2015) reported a lack of a difference in expression of *ISG15*, *MX2*, and *OAS1* on days 17, 19, 21, and 28 among treatments when cows received ECP 36 h, EB 12 h, or no estradiol before a GnRH induced LH surge. A binary pregnancy prediction factor was also generated for each cow using ISG expression on day 17, 19, 21, and 28. Again, treatment failed to affect the pregnancy prediction factor on any of the previously mentioned days. Thus, preovulatory estradiol may not elicit its effects by the time of maternal recognition of pregnancy. These results are similar to this study as there was no treatment effect on the proportion of cows that were classified as having evidence of a conceptus present on day 19. Madsen et al. (2015) also measured PSPB from days 22 to 28 and reported that on day 24, pregnancy rates (based on PSPB concentration) were

greater in cows that received estradiol treatment, however pregnancy rates, as determined by PSPB, did not differ from days 25-28. The authors concluded that preovulatory estradiol 17-β may be needed for embryo attachment or growth after day 21 of gestation since this corresponds to an active period of placentation in cattle (Assis Neto et al., 2010). In this study, there were no differences in the proportion of cows considered to have evidence of a conceptus present on day 24 among treatments. However, part of the criteria to classify cows as having evidence of a conceptus present on day 24 was PAG (Ab 63) concentrations and as mentioned above, the low sensitivity (55.10; true positive rate) that resulted from the ROC curve analysis of the day 24 PAG (Ab 63) warranted concern. Therefore, the proportion of cows considered to have evidence of a conceptus present on day 24 but diagnosed nonpregnant (IDEXX S-N < 0.3) on day 30 could have been underestimated. Madsen et al. (2015) reported greater pregnancy rates in estradiol treated cows on day 29 compared to controls and concluded that estradiol treated cows had greater maintenance of pregnancy to day 30. In this study, treatment had no effect on day 30 pregnancy rates nor Period 3 Loss (day 24 to 30). Perhaps most of the differences observed between the two studies is due to the possible underestimation of loss that occurred during Period 3.

Reese et al. (2020) performed a meta-analysis (Reese et al., 2020) that characterized pregnancy loss in beef cattle in which gestation was divided into the following periods: 1) Fertilization and pre-blastocyst period (days 1 to 7 of gestation), 2) Early embryo period (days 7 to 32), and 3) Late embryo/early fetal period (days 32 to 100). Approximately 28.4% of loss occurred during the fertilization and pre-blastocyst period with 23% of loss occurring by day 4. Approximately 3.9% of females experienced

reproductive failure from day 7 to 16 of gestation while 15.6% of females experienced loss from day 16 to 32. After the first month of gestation < 6% of females experienced reproductive failures. In the present study, there was no treatment by Period of Loss interaction indicating that there was not a greater amount of loss during a specific Period of Loss in one treatment compared to another. However, there was a main effect of Period of Loss. The percent loss that occurred in Period 1 (days 7 to 19) was 56% which is higher than what was reported in the meta-analysis mentioned above and unexpected. There are no clear conclusions on why the loss in the present study was greater than expected, however; there are several pivotal events that must occur during Period 1, including emergence from the zona pellucida, elongation and secretion of IFNT (Wiltbank et al., 2016), for an embryo and conceptus to continue development past day 19. Thus, the loss that occurred during Period 1 could have been related to disruptions in any of these processes.

Period 2 encompassed days 19 to 24, Period 3: days 24 to 30, and Period B: days 19 to 30. The loss that occurred in Period 1, 2 and B was 8%, 4% and 12%, respectively. This is in agreement with what Reese et al. (2020) reported (Days 16 to 32 = 15.6%). During this time period of gestation the amnion, yolk sac, and circulatory system develops and there is a transition of nutritional source to choriovitelline (yolk sac placenta) type nutrition, as the conceptus previously had histotroph type nutrition (Wiltbank et al., 2016).

There was approximately a 6% pregnancy loss that occurred during Period 4 (days 30 to 55/58). This is again in agreement with Reese et al. (2020). Interestingly, Reese et al. (2020) reported that pregnancies that resulted from ET experienced 10.2%

loss after 1 month of gestation, while the reproductive failures from pregnancies resulting from artificial insemination was only 4.9%. During this stage of gestation there is adherence and attachment of the maternal and allantoic membranes, development of placentomes, and another switch in nutritional source. Loss during this period is usually due to improper placentation or disrupted transition from amniotic to allantoic nutrition (Wiltbank et al., 2016). In this study, there was no treatment by Period of Loss interaction indicating that there was not a greater amount of loss during a specific Period of Loss in one treatment than another. Lopes et al. (2007) reported that cows diagnosed pregnant on day 29 that then became nonpregnant by day 43 or 64, or those that remained pregnant until day 64 had similar estradiol 17-β at time of AI. The lack of a treatment by Period of Loss interaction is potentially due to the unexpectedly low overall pregnancy rates and therefore potentially a second replicate will provide further insight as to when preovulatory estradiol 17-β may be eliciting its effect on the establishment and maintenance of pregnancy.

When the pregnancy data were analyzed using repeated measures analysis of variance and the day 24 data were included, treatment had no effect on the overall pregnancy rate nor the pregnancy rates on a given day. These results were unexpected since Ciernia (2018) reported that postpartum beef cows that had not expressed estrus but had high preovulatory concentrations of estradiol 17-β (> 5.8 pg/mL) had a greater pregnancy rate following embryo transfer compared to cows with lower estradiol 17-β. These data indicate an effect of preovulatory estradiol on uterine receptivity. Lopes et al. (2007) also reported a beneficial effect of estradiol at the time of AI on day 29 pregnancy. However, when the day 24 pregnancy data were excluded from the analysis,

in the present study, treatment tended to have a main effect on overall pregnancy rates. More specifically, the Positive Control group had greater overall pregnancy rates over time than the Negative Control group while the Estradiol group only tended to have greater overall pregnancy rates than the Negative Control group. Meanwhile, the Positive Control group and the Estradiol group had similar overall pregnancy rates. It is likely that the Estradiol group only tended to have greater overall pregnancy rates then the Negative Control group because there was not enough power to detect a significant difference due to the low overall number of cows that were considered pregnant. Perhaps after a second replicate and additional animal numbers, there will be enough power to detect a significant difference between the Estradiol group and the Negative Control group.

CHAPTER 4

EVALUATION OF LATER TIMEPOINTS FOR SPLIT-TIME ARTIFICIAL INSEMINATION WHEN USING SEX-SORTED SEMEN AMONG BEEF HEIFERS FOLLOWING THE 14-D CIDR®-PG PROTOCOL

4.1 Abstract

An experiment was designed to evaluate later insemination timepoints when using Split-Time AI, with the hypothesis that later timing of AI may improve estrous response and pregnancy rates when using sex-sorted semen. Estrus was synchronized in 794 heifers across 4 locations with the 14-d CIDR®-PG protocol. Heifers were administered a 1.38 g progesterone insert (CIDR®) on day 0, which was removed on day 14. On day 30, 25 mg dinoprost tromethamine (PG) was administered intramuscularly and estrus detection aids were applied. Split-Time AI was performed based on estrous status. Within location, heifers were blocked based on breed composition, source, sire, RTS, and BW and assigned within block to one of two experimental approaches: Approach 66: heifers that were estrual prior to 66 h after PG administration were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h), with 100 μg gonadorelin acetate (GnRH) administered intramuscularly to heifers that were nonestrual by this time; or Approach 72: heifers that were estrual prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that were nonestrual by this time. Within approach, heifers were preassigned to receive either SexedULTRA 4M™ sex-sorted or conventional semen. Overall estrous response did not differ between approaches. However, the proportion of heifers that were estrual

by the first timepoint (66 h or 72 h following PG administration) was greater ($P <$ 0.0001) with Approach 72 (76%; 302/395) compared to Approach 66 (61%; 242/399). Pregnancy rates to STAI differed ($P = 0.0005$) between semen type but were not affected by an approach x semen type interaction or by approach. Conventional semen (59%; 240/404) pregnancy rates to STAI were greater than sex-sorted semen (48%; 187/390). Among heifers that were estrual by Timepoint 1, pregnancy rates tended ($P = 0.08$) to differ between semen type (Conventional: 62% [174/280]; Sex-sorted: 55% [146/264]). Pregnancy rates of nonestrual heifers were reduced ($P < 0.01$) with sex-sorted semen (25%; 15/61) compared to conventional semen (41%; 23/56). In summary, when using sex-sorted or conventional semen for STAI in heifers following the 14-d CIDR®-PG protocol, pregnancy rates did not differ when timing of STAI was delayed by 6 hours. However, the proportion of estrual heifers prior to the first timepoint for STAI was greater when using the later timepoints.

4.2 Introduction

A survey conducted by the USDA in 2007-08 reported less than 10% of U.S. beef producers use artificial insemination (AI) or estrus synchronization. The most common reasons producers gave for not utilizing estrus synchronization and AI were labor and time, followed by cost of AI (National Animal Health Monitoring System, 2008). In an effort to reduce the labor and time associated with implementing an estrus synchronization and AI program, fixed-time artificial insemination (FTAI) protocols were developed. These protocols allow a producer to inseminate all females at a single predetermined timepoint while still achieving acceptable pregnancy rates to AI.

The commercialization of sex-sorted semen represents a promising technology that could help cattle producers become more productive and efficient (Garner & Seidel, 2008). Sex-sorted semen allows producers to alter the proportion of male or female calves born across the herd, as well as produce progeny of a specific sex in individual planned matings. While this technology has the potential to have a large impact on both the beef and dairy industries, its adoption, in the beef industry, has been slow. Not only is sex-sorted semen generally more expensive than conventional semen, but lower pregnancy rates are obtained when sex-sorted semen is used for FTAI (Filho et al., 2012; Hall & Glaze, 2014; Thomas et al., 2014b). Since pregnancy rates with sex-sorted semen are especially poor among females that fail to express estrus prior to FTAI (Colazo et al., 2018; Thomas et al., 2014b), it is generally not recommended that FTAI be performed in conjunction with sex-sorted semen.

In an attempt to address this challenge, a new timed AI approach was developed, in which females that fail to express estrus prior to FTAI are afforded an additional 20-24 h to express estrus prior to insemination (Thomas et al., 2014a). This approach, termed "split-time AI", improves pregnancy rates in beef heifers following a 14-d CIDR®-PG protocol (Thomas et al., 2014a) or an MGA-PG protocol (Knickmeyer et al., 2018) by increasing the proportion of heifers allowed to express estrus before AI. Expression of estrus prior to timed AI is associated with greater pregnancy rates (Richardson et al., 2016), and expression of estrus prior to insemination appears to be particularly critical when using sex-sorted semen. Split-time AI (STAI) provides producers a management option to utilize sex-sorted semen effectively, while decreasing the amount of labor and time required compared to estrus detection.

It is not clear whether increased pregnancy rates with STAI are due primarily to increased estrus expression, deposition of sex-sorted semen closer to the time of ovulation, or a combination of those factors. However, implementing a STAI program allows producers to inseminate a larger proportion of females at a more optimal time and affords a greater proportion of females time to express estrus. Examination of the distribution of estrus onset times (Figure 4.1) following the 14-d CIDR®-PG protocol (Leitman et al., 2009a; Leitman et al., 2009b; Mallory et al., 2010) raises questions as to whether results with sex-sorted semen might be improved through use of a later set of time points for STAI. Therefore, the following experiment was designed to test the hypothesis that pregnancy rates to sex-sorted semen may be improved if using a later set of timepoints for STAI, 6 hours later than the timepoints currently recommended for use with conventional semen.

4.3 Materials and Methods

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

4.3.1 Animals and estrus synchronization

The 14-d CIDR®-PG protocol was used to synchronize estrus among Bos taurus (Angus and Angus-cross) yearling heifers (n = 794) across four locations. Reproductive tract score (RTS; 1-5 scale) (Anderson et al., 1991; Holm et al., 2009; Rosenkrans & Hardin, 2003) and body weight (BW) was recorded for each heifer on day 0. Heifers that were pregnant, underdeveloped, or had an abnormally small pelvic area were not included in

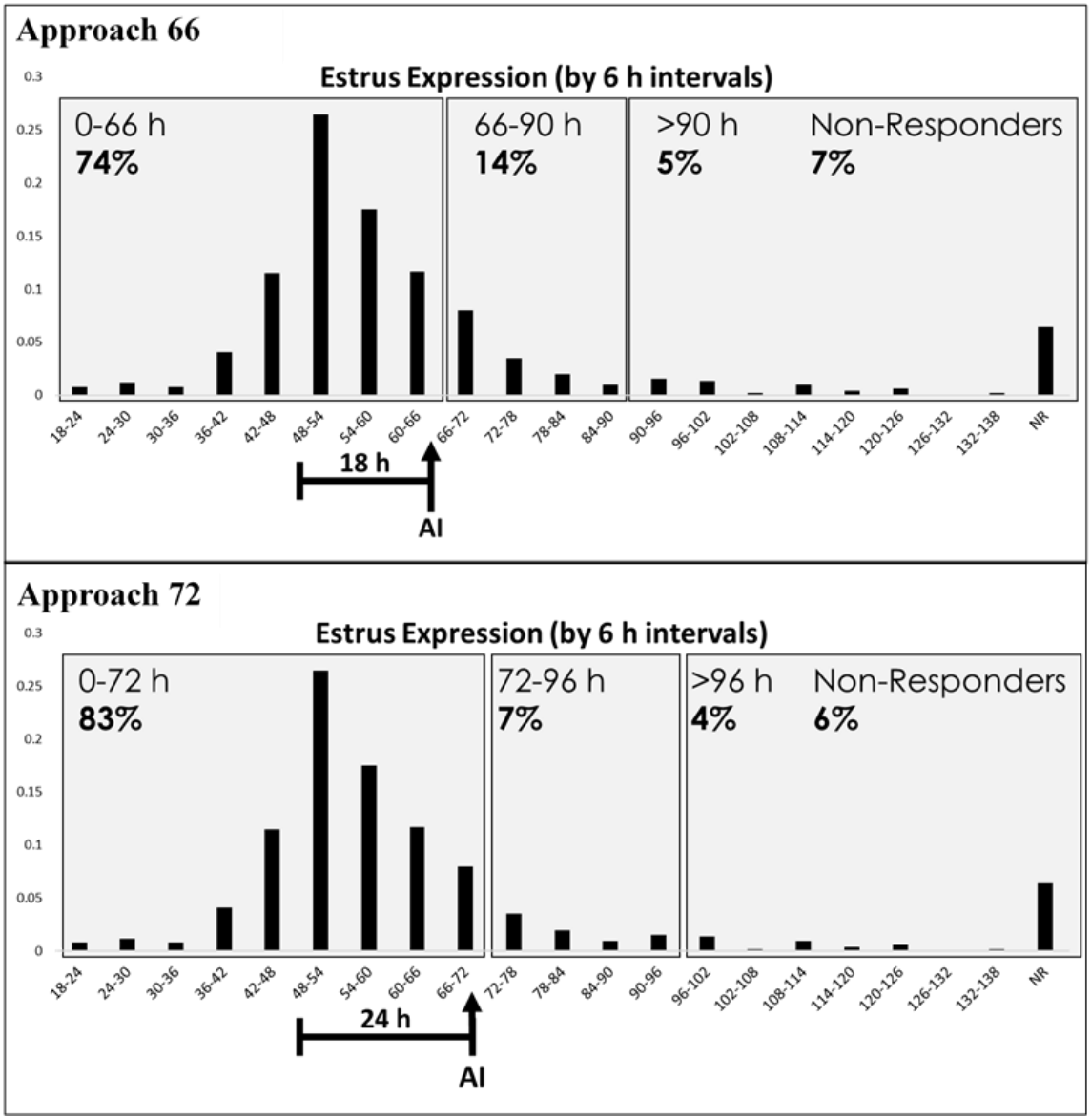


Figure 4.1. ***Approach 66.*** Proportion of anticipated distribution of estrus onset times, by 6 h intervals, following 14-d CIDR®-PG protocol with AI occurring at 66 h. Heifers that experienced onset of estrus 48-54 h following PG administration would be inseminated approximately 18 h following the onset of estrus. ***Approach 72.*** Proportion of anticipated distribution of estrus onset times, by 6 h intervals, following 14-d CIDR®-PG protocol with AI occurring at 72 h. Heifers that experienced onset of estrus 48-54 h following PG administration would be inseminated approximately 24 h following the onset of estrus. Figure adopted and modified from Leitman et al., 2009a; Leitman et al., 2009b; Mallory et al., 2010.

the experiment (Location 1: n = 0; Location 2: n = 0; Location 3: n = 6; Location 4: n = 3). Heifers were administered a 1.38 g progesterone intravaginal insert (CIDR®; Zoestis, Madison, NJ) on day 0, which was then removed on day 14. On day 30, 16 days after CIDR® insert removal, 25 mg dinoprost tromethamine (PG; Lutalyse®; Zoetis) was administered intramuscularly.

4.3.2 Estrus detection

Estrus detection aids (Estrotect®; Estrotect Inc., Spring Valley, WI) were applied on day 30 concurrent with PG administration. Patch scores (0 = missing, 1 = 0-25% activated, 2 = 25-50% activated, 3 = 50-75% activated, 4 = 75-100% activated; (Pohler et al., 2016) were recorded when animals were handled for STAI. Estrus detection aids remained attached to heifers that had not expressed estrus by the first timepoint (66 h or 72 h, based on treatment), and patch scores were recorded again at the second AI timepoint (90 h or 96 h, based on treatment). Estrus expression was defined as removal of greater than 50% of the scratch-off coating on the Estrotect (Patch Score 3 and 4). Heifers missing patches (Patch Score 0) were considered to have expressed estrus (n = 80).

4.3.3 Semen collection

Both conventional and sex-sorted semen, from five different bulls, passed the standard quality control criteria used for the respective semen types. Equal proportions of conventional and sex-sorted semen were used at each location. Units of sex-sorted semen were generated via SexedULTRA™ Genesis III sorting technology with 4.0 x 10^6 live cells per 0.25 mL straw prior to freezing, and units of conventional semen were generated with 25.0 x 10^6 live cells per 0.25 mL straw prior to freezing. Sex-sorted units were

sorted to contain either X (Bull A, B, D, and E) or Y chromosome-bearing (Bull C) sperm cells at an accuracy level of > 90%. Location 1 used Bull A exclusively. Location 2 used Bulls B and C, while Location 3 used Bulls B and D. Location 4 used Bull E exclusively. Within each location, a single sire was used among heifers of the same breed composition, with breed/sire then used for blocking prior to treatment assignment within location. In Location 1, semen from Bull A was used to inseminate commercial Angus heifers (n = 84). At Location 2, semen from Bull B was used to inseminate commercial Angus heifers (n = 212) while semen from Bull C was used to inseminate purebred Angus heifers (n = 69). In location 3, semen from Bull B was used to inseminate commercial Angus and Angus-cross heifers (n = 135) and semen from Bull D was used to inseminate Red Angus heifers (n = 101). At Location 4, semen from Bull E was used to inseminate commercial Angus heifers (n = 193).

4.3.4 Artificial insemination

Within each location, heifers were blocked based on breed composition (purebred Angus, commercial Angus, Red Angus, and Angus-cross), source, sire, RTS, and BW. Within block, heifers were then assigned to one of two approaches (Figure 4.2): 1) 66: heifers that expressed estrus prior to 66 h were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h), with 100 μg gonadorelin acetate (GnRH; Fertagyl®, Merck Animal Health, Madison, NJ) administered intramuscularly to heifers that failed to express estrus by this time or 2) 72: heifers that expressed estrus prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that failed to express estrus by this time. Within approach,

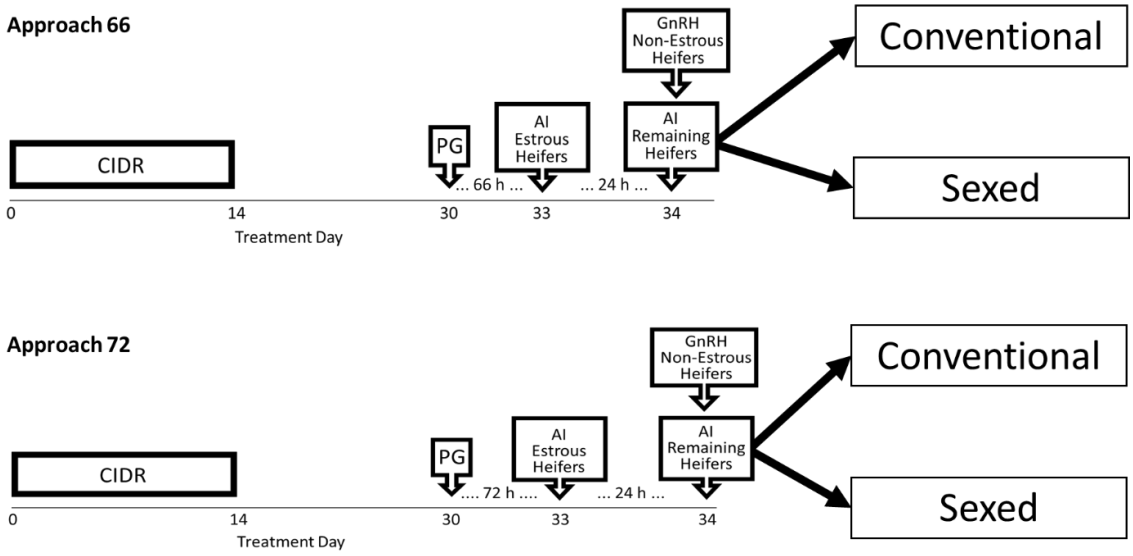


Figure 4.2. Experimental design evaluating either Approach 66 and Approach 72 with conventional or sexed semen. Heifers were administered 1.38 g progesterone insert (CIDR®; Zoestis, Madison, NJ) on Day 0, which was then removed on Day 14. On Day 30, 16 days after CIDR® insert removal, 25 mg dinoprost tromethamine (PG; Lutalyse®; Zoetis) was administered intramuscularly. Estrus detection aids (Estrotect; Estrotect Inc., Spring Valley, WI) were applied on Day 30 concurrent with PG administration. Heifers were then assigned to one of two approaches 1) 66: heifers that expressed estrus prior to 66 h were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h), with 100 μg gonadorelin acetate (GnRH; Fertagyl®, Merck Animal Health, Madison, NJ) administered intramuscularly to heifers that failed to express estrus by this time, or 2) 72: heifers that expressed estrus prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that failed to express estrus by this time. Heifers were also assigned to receive either conventional or sex-sorted semen for both Approach 66 and Approach 72.

heifers were also blocked based on the same criteria and preassigned to receive either sex-sorted or conventional semen.

4.3.5 Pregnancy diagnosis

Pregnancy rate to AI was determined via transrectal ultrasonography (Aloka 500V equipped with a 5.0-MHz linear-array transducer; Aloka, Wallingford, CT) 77 to 85 d after FTAI.

4.3.6 Statistical analyses

Potential differences among treatments in weight, RTS, and time interval from PG administration to AI were analyzed using the GLM Procedure of SAS (SAS Inst. Inc., Cary, NC). Potential differences among treatments in estrous response and pregnancy rates to STAI were analyzed with the GLIMMIX Procedure of SAS (SAS Inst. Inc., Cary, NC) using the binomial distribution, link logit function. Variables tested for inclusion in the mixed model for estrous response were weight, RTS, approach (66 or 72), semen type (conventional or sex-sorted), location, breed composition (purebred Angus, commercial Angus, Red Angus and Angus-cross), and all two-way interactions. Interactions that did not significantly ($P > 0.10$) affect estrous response were removed from the model. The final model for estrous response included weight, RTS, approach, semen type, breed composition, and the approach x semen type interaction as fixed effects and location as a random effect. Variables tested for inclusion in the mixed model for pregnancy to STAI were weight, RTS, approach (66 or 72), semen type (conventional or sex-sorted), location, breed composition (purebred Angus, commercial Angus, Red Angus and Angus-cross), bull, and all two-way interactions. Interactions that did not significantly (*P*

> 0.10) affect pregnancy to STAI were removed from the model. The final model for pregnancy rate to STAI included weight, RTS, approach, semen type, and the approach x semen type interaction as fixed effects, as well as location and bull as random effects.

4.4 Results

Heifer weight, RTS, and interval from PG to AI are reported in Table 4.1. Heifer RTS and weight did not differ based on treatment. For heifers assigned to Approach 66, the PG to AI interval for those heifers that expressed estrus prior to 66 h was 65.6 ± 1.0 h for conventional semen and 65.4 ± 1.1 h for sex-sorted semen. Heifers that failed to express estrus prior to 66 h had a PG to AI interval of 89.0 ± 1.2 h and 89.1 ± 0.9 h for heifers inseminated with conventional and sex-sorted semen respectively. Heifers assigned to Approach 72 had a PG to AI interval of 71.5 ± 1.2 h for conventional semen and 71.7 ± 1.1 h for sex-sorted semen when heifers expressed estrus prior to 72 h. Heifers failing to express estrus prior to 72 h had a PG to AI interval of 94.9 ± 0.9 h and 94.8 ± 1.0 h when inseminated with conventional and sex-sorted semen respectively.

Pregnancy rates to AI did not differ between Bull A (59%; 41/69), B (54%; 188/347), C (59%; 41/69), D (57%; 58/101), or E (49%; 94/193) nor was there a significant interaction of bull x approach.

Estrous response based on treatment and timepoint are shown in Table 4.2. The overall total estrous response did not differ ($P = 0.38$) between approaches. However, the proportion of heifers that expressed estrus by the first timepoint (66 h or 72 h following PG administration) was greater ($P < 0.0001$) when using Approach 72 (76%; 302/395) compared to Approach 66 (61%; 242/399). Correspondingly, fewer ($P = 0.01$) heifers in

Table 4.1. Mean heifer weight (Wt), reproductive tract score (RTS), and Prostaglandin $F_{2\alpha}$ (PG) to Artificial Insemination Interval (PG to AI Interval) by approach and semen type.

Approach[1]	Semen Type[2]	N	Wt mean ± SD, kg	RTS[3] mean ± SD	PG to AI Interval[4] mean ± SD, h	
					Timepoint 1	Timepoint 2
66	Conventional	203	388 ± 35	4.5 ± 0.8	65.6 ± 1.0	89.0 ± 1.2
	Sex-sorted	196	390 ± 35	4.5 ± 0.8	65.4 ± 1.1	89.1 ± 0.9
72	Conventional	201	391 ± 33	4.5 ± 0.8	71.5 ± 1.2	94.9 ± 0.9
	Sex-sorted	194	391 ± 34	4.5 ± 0.8	71.7 ± 1.1	94.8 ± 1.0

[1]Heifers were administered 1.38 g progesterone insert (CIDR®; Zoestis, Madison, NJ) on Day 0, which was then removed on Day 14. On Day 30, 16 days after CIDR® insert removal, 25 mg dinoprost tromethamine (PG; Lutalyse®; Zoetis) was administered intramuscularly. Estrus detection aids (Estrotect; Estrotect Inc., Spring Valley, WI) were applied on Day 30 concurrent with PG administration. Heifers were then assigned to one of two approaches 1) 66: heifers that expressed estrus prior to 66 h were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h), with 100 μg gonadorelin acetate (GnRH; Fertagyl®, Merck Animal Health, Madison, NJ) administered intramuscularly to heifers that failed to express estrus by this time, or 2) 72: heifers that expressed estrus prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that failed to express estrus by this time.

[2]Units of sex-sorted semen were generated via SexedULTRA™ Genesis III sorting technology with 4.0 x 10^6 live cells per 0.25 mL straw prior to freezing. Units of conventional semen were generated with 25.0 x 10^6 live cells per 0.25 mL straw prior to freezing.

[3]RTS (1-5 scale, with 1 = immature and 5 = estrous cycling, luteal phase) of the heifers, evaluated on Day 0 at CIDR® insertion.

[4]Interval from PG administration to AI among heifers receiving AI at Timepoint 1 (66 or 72 h) or Timepoint 2 (90 or 96 h).

Table 4.2. Estrous response by timepoint and approach.

	Overall Estrous Response[2]		Timepoint 1 Estrous Response[3]		Timepoint 2 Estrous Response[4]	
Approach[1]	Proportion	%	Proportion	%	Proportion	%
66	336/399	84	242/399	61[a]	94/399	24[c]
72	342/395	87	302/395	76[b]	40/395	10[d]

[ab]Estrous response by Timepoint 1 (66 h or 72 h) was greater ($P < 0.0001$) among heifers assigned to Approach 72 then Approach 66.
[cd]Estrous response by Timepoint 2 (90 h or 96 h) was greater ($P = 0.01$) among heifers assigned to Approach 66 then Approach 72.

[1]Heifers were administered 1.38 g progesterone insert (CIDR®; Zoestis, Madison, NJ) on Day 0, which was then removed on Day 14. On Day 30, 16 days after CIDR® insert removal, 25 mg dinoprost tromethamine (PG; Lutalyse®; Zoetis) was administered intramuscularly. Estrus detection aids (Estrotect; Estrotect Inc., Spring Valley, WI) were applied on Day 30 concurrent with PG administration. Heifers were then assigned to one of two approaches 1) 66: heifers that expressed estrus prior to 66 h were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h with 100 μg gonadorelin acetate (GnRH; Fertagyl®, Merck Animal Health, Madison, NJ) administered intramuscularly to heifers that failed to express estrus by this time, or 2) 72: heifers that expressed estrus prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that failed to express estrus by this time.
[2]Overall estrous response regardless of when estrus occurred.
[3]Expression of estrus by 66 h (Approach 66) or 72 h (Approach 72) following PG administration, with estrus defined as an estrus detection aid (Estrotect) patch score of 0 or ≥ 3.
[4]Expression of estrus between 66 h and 90 h (Approach 66) or 72 h and 96 h (Approach 72) following PG administration, as determined by a patch score of 1 or 2 at 66 h or 72 h but a patch score of 0 or ≥ 3 at 90 h or 96 h respectively.

Approach 72 (10%; 40/395) expressed estrus after Timepoint 1 and prior to Timepoint 2 (90 h or 96 h following PG administration) when compared to Approach 66 (24%; 94/399).

Pregnancy rates to STAI (Table 4.3) were not affected by an approach x semen type interaction ($P = 0.30$) or by approach ($P = 0.24$). Pregnancy rates to STAI differed ($P = 0.0005$) between semen types. Heifers inseminated with conventional semen (59%; 240/404) had greater pregnancy rates to STAI than those inseminated with sex-sorted semen (48%; 187/390). Among heifers that were estrual by Timepoint 1, pregnancy rates tended ($P = 0.08$) to differ by semen type (Conventional: 62% [; 174/280]; Sex-sorted: 55%; [146/264]). Among heifers that were estrual by Timepoint 2, insemination with conventional semen resulted in greater ($P = 0.04$) pregnancy rates (63%; 43/68) than insemination with sex-sorted semen (40%; 26/65). Across approaches, pregnancy rates among nonestrual heifers were reduced ($P < 0.01$) with sex-sorted semen (25%; 15/61) compared to conventional semen (41%; 23/56).

4.5 Discussion

Sex-sorted semen is generally associated with increased cost. This cost is comprised of not only the direct cost of purchasing sex-sorted semen but also the indirect cost of overall lower pregnancy rates to FTAI (Filho et al., 2012; Hall & Glaze, 2014; Thomas et al., 2014b). For dairy producers, lower pregnancy rates among heifers may manifest as more days on feed during the development and breeding phase (Claypool et al., 2019), whereas for beef producers, lower pregnancy rates of heifers manifest as revenue reductions due to decreased average age of calves at weaning, decreased

Table 4.3. Pregnancy rates to STAI[1] by approach, semen type, and estrous response.

Semen Type[2]	Overall		Estrual by Timepoint 1[4]		Estrual by Timepoint 2[5]		Nonestrual[6]	
Approach[3]	Proportion	%	Proportion	%	Proportion	%	Proportion	%
Conventional	240/404	59[a]	174/280	62[C]	43/68	63[e]	23/56	41[g]
66	125/203	62	80/122	66	32/49	65	13/32	41
72	115/201	57	94/158	59	11/19	58	10/24	42
Sex-sorted	187/390	48[b]	146/264	55[c]	26/65	40[f]	15/61	25[h]
66	91/196	46	63/120	53	19/44	43	9/32	28
72	96/194	49	83/144	58	7/21	33	6/29	21

[ab]Estrual heifers inseminated with conventional semen, regardless of when they expressed estrus, had higher (P = 0.0005) pregnancy rates to STAI then estrual heifers inseminated with sex-sorted semen.
[Cc]Heifers that were estrual by Timepoint 1 (66 h or 72 h) and were inseminated with conventional semen tended (P = 0.0765) to have greater pregnancy rates to STAI then heifers inseminated with sex-sorted semen.
[ef]Heifers that were nonestrual at 66 h or 72 h but became estrual by 90 h or 96 h and were inseminated with conventional semen had higher pregnancy rates to STAI (P = 0.0418) then heifers inseminated with sex-sorted semen.
[gh]Heifers that were nonestrual at 90 h or 96 h but were administered GnRH and conventional semen at 90 h or 96 h had higher pregnancy rates (P = 0.0088) to STAI then heifers inseminated with sex-sorted semen.

[1]Pregnancy rate to AI was determined via transrectal ultrasonography 77-85 d following STAI.
[2]Units of sex-sorted semen were generated via SexedULTRA™ Genesis III sorting technology with 4.0 x 10^6 live cells per 0.25 mL straw prior to freezing. Units of conventional semen were generated with 25.0 x 10^6 live cells per 0.25 mL straw prior to freezing.
[3]Heifers were administered 1.38 g progesterone insert (CIDR®; Zoestis, Madison, NJ) on Day 0, which was then removed on Day 14. On Day 30, 16 days after CIDR® insert removal, 25 mg dinoprost tromethamine (PG; Lutalyse®; Zoetis) was administered intramuscularly. Estrus detection aids (Estrotect; Estrotect Inc., Spring Valley, WI) were applied on Day 30 concurrent with PG administration. Heifers were then assigned to one of two approaches 1) 66: heifers that expressed estrus prior to 66 h were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h), with 100 µg gonadorelin acetate (GnRH; Fertagyl®, Merck Animal Health, Madison, NJ)

administered intramuscularly to heifers that failed to express estrus by this time, or 2) 72: heifers that expressed estrus prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that failed to express estrus by this time.

[4]Expression of estrus by 66 h (Approach 66) or 72 h (Approach 72) following PG administration, with estrus defined as an estrus detection aid (Estrotect) patch score of 0 or ≥ 3. Insemination occurred either 66 h or 72 h following PG administration.

[5]Expression of estrus between 66 h and 90 h (Approach 66) or 72 h and 96 h (Approach 72) following PG administration, as determined by a patch score of 1 or 2 at 66 h or 72 h but a patch score of 0 or ≥ 3 at 90 h or 96 h respectively. Insemination occurred 90 h or 96 h following PG administration.

[6]Failure to express estrus by 90 h (Approach 66) or 96 h (Approach 72), as defined as a patch score of 1 or 2 at both 66 h and 90 h (Approach 66) or 72 h and 96 h (Approach 72) following PG administration.

longevity of cows in the herd, and fewer lifetime pounds of calf weaned per heifer retained (Cushman et al., 2013). Other costs producers may need to take into account are the additional time and labor associated with the use of sex-sorted semen. Use of FTAI is generally not recommended with sex-sorted semen due to nonestrual females having further decreased pregnancy rates (Colazo et al., 2018; Thomas et al., 2014b). As an alternative to estrus detection-based programs, a STAI approach may be employed to maximize total estrous response prior to one of two potential timepoints for timed AI (Thomas et al., 2014b). This alleviates some time and labor challenges associated with estrus detection as well as reduces pharmaceutical cost when compared to a FTAI protocol.

It is not clear whether increased pregnancy rates with STAI are primarily attributed to factors associated with increased estrous response or the fact that deposition of sex-sorted semen presumably occurs closer to the time of ovulation. Delaying insemination with sex-sorted semen may improve pregnancy rates. Flow cytometric sorting and subsequent freeze-thaw results in precapacitation of sex-sorted sperm cells and potentially reduced sperm cell lifespan within the reproductive tract (Mocé et al., 2006). Sales et al. (2011) reported higher pregnancy rates to sex-sorted semen when time of FTAI was delayed from 54 to 60 h following progestin implant removal. These authors also reported that pregnancy rates to sex-sorted semen were increased when FTAI was performed closer to ovulation. Pregnancy rates were greatest for those females that were inseminated 0-12 h before ovulation (37.9%) while pregnancy rates of females that were inseminated either 12.1-24 h (19.4%) or > 24 h (5.8%) before ovulation were significantly reduced (Sales et al., 2011). Dairy heifers inseminated with sex-sorted

semen 16.1 to 24 h after onset of estrus had greater pregnancy rates (51.8%) than heifers inseminated 12 to 16 h after the onset of estrus (37.7%). However, the pregnancy rates for dairy heifers in which insemination was delayed to 24.1 to 30 h after onset of estrus did not differ from either of the previous insemination time points (45.5%) (Sá Filho et al., 2010b). When sex-sorted semen was used to inseminate superstimulated Nelore cows, fewer transferable embryos were recovered when insemination occurred 12 and 24 h after administration of porcine LH (2.4 +/- 1.8) than when insemination was delayed to 18 and 30 h (4.5 +/- 3.0) (Soares et al., 2011). Conversely, Hall et al. (2017) reported that despite more cows exhibiting estrus before FTAI, there was no difference in pregnancy rates to sex-sorted semen when insemination was delayed from 72 to 80 h following PG-induced luteolysis and CIDR® removal with a 5-day Co-Synch + CIDR® protocol in postpartum cows (Hall et al., 2017).

In the present study, exact timing of onset of estrus was not characterized for individual animals; rather, estrual status was assessed at the appointed times based on a patch score. Therefore, it is not possible to evaluate the effect of interval from insemination relative to the predicted time of ovulation in this study. However, examination of previously published distributions of estrus onset times (Figure 4.1) following the 14-d CIDR®-PG protocol (Leitman et al., 2009a; Leitman et al., 2009b; Mallory et al., 2010) indicates that by delaying insemination to 72 h, not only would a greater proportion of heifers be estrual by 72 h when compared to 66 h, but the interval from onset of estrus to insemination could be more optimal for a greater proportion of heifers. The data from this study indicate that, while use of a later set of timepoints for STAI did not alter the overall proportion of heifers that became estrual, a greater

proportion of heifers were estrual when using 72 versus 66 h as the first timepoint for potential AI. Despite a greater proportion of heifers being estrual by Timepoint 1 when assigned to Approach 72, pregnancy rates to AI for those heifers estrual by Timepoint 1 did not differ from heifers assigned to Approach 66, for both conventional and sex-sorted semen. In fact, overall pregnancy rates and pregnancy rates at either timepoint did not differ between approach, for conventional nor sex-sorted semen.

The lack of an observed difference in pregnancy rates between approaches for both conventional and sex-sorted semen at Timepoint 1 raises additional questions relative to FTAI strategies for effective use of sex-sorted semen. One recommendation for use of sex-sorted semen in FTAI is to restrict use of sex-sorted semen only to those heifers expressing estrus prior to the FTAI timepoint. While pregnancy rates in this experiment did not differ between approaches, the proportion of heifers expressing estrus by Timepoint 1 was greater when using the later timepoint of 72 h compared to 66 h. Based on these data, performing FTAI at 72 h rather than 66 h after the 14-d CIDR®-PG protocol would offer producers the opportunity to service more heifers with sex-sorted semen, thereby achieving a greater number of sex-selected pregnancies per animal. This assumes that pregnancy rate to AI, following estrus synchronization, was not compromised due to the altered timing of AI. Producers seeking to further reduce the labor and time associated with use of sex-sorted semen could simply perform FTAI at 72 hours following PG administration using the 14-d CIDR®-PG protocol, inseminating estrual heifers with sex-sorted semen and nonestrual heifers with conventional semen. This strategy would reduce the time and labor associated with sex-sorted semen in comparison to split-time AI, as it would allow producers to increase the number of

heifers that are inseminated with sex-sorted semen without requiring an additional day in the treatment schedule.

This potential FTAI approach for sex-sorted semen (Figure 4.3) merits further investigation as a commercially viable strategy. However, a much larger pool of data would be needed to determine equivalence: whether pregnancy rates with sex-sorted semen among estrous heifers are similar when AI is performed at 72 h rather than 66 h following PG using the 14-d CIDR®-PG protocol. Although we observed numerically greater pregnancy rates among estrous heifers inseminated with sex-sorted semen at 72 h (58%; 83/144) compared to 66 h (53%; 63/120), the confidence intervals around these pregnancy rates are too large at this sample size to conclude noninferiority of 72 h as a FTAI timepoint. For example, based upon this data and our model, the lower limit of a 90% confidence interval for the pregnancy rate of estrual heifers inseminated at 72 h with sex-sorted semen is 48% as compared to an observed pregnancy rate of 53% at 66 h. Of course, a pregnancy rate this low would result in a net reduction in the number of sex-selected pregnancies obtained, despite the fact that more heifers would be serviced with sex-sorted semen due to the higher estrous response observed. Therefore, based on these data alone, it would be premature to make a conclusive recommendation that FTAI be performed at 72 h following the 14-d CIDR®-PG if using sex-sorted semen.

In conclusion, use of later timepoints for STAI following the 14-d CIDR®-PG protocol results in a greater proportion of heifers having expressed estrus prior to the first timepoint for potential AI. This may have implications for FTAI approaches using sex-sorted semen; however, overall rates of estrous response were similar if using STAI. Pregnancy rates to STAI did not differ between approaches at this sample size. Future

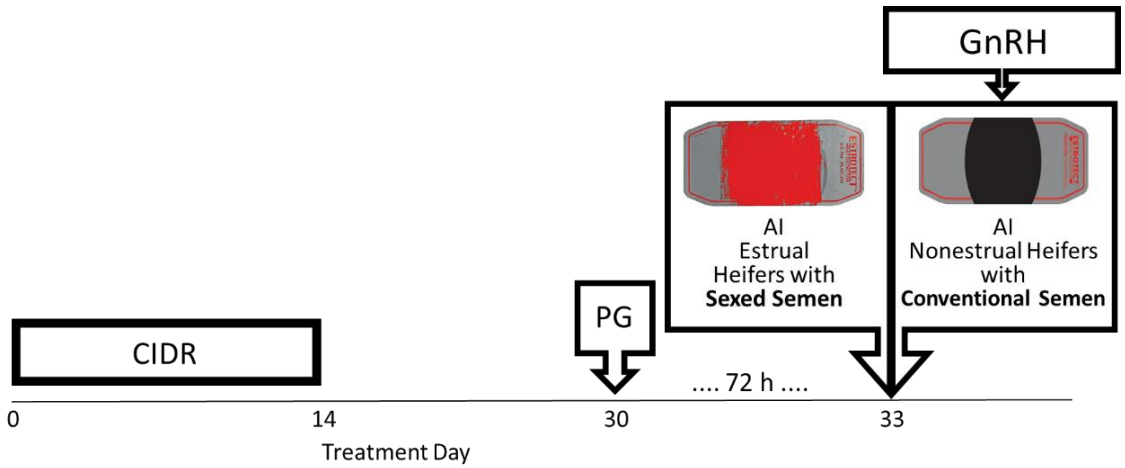
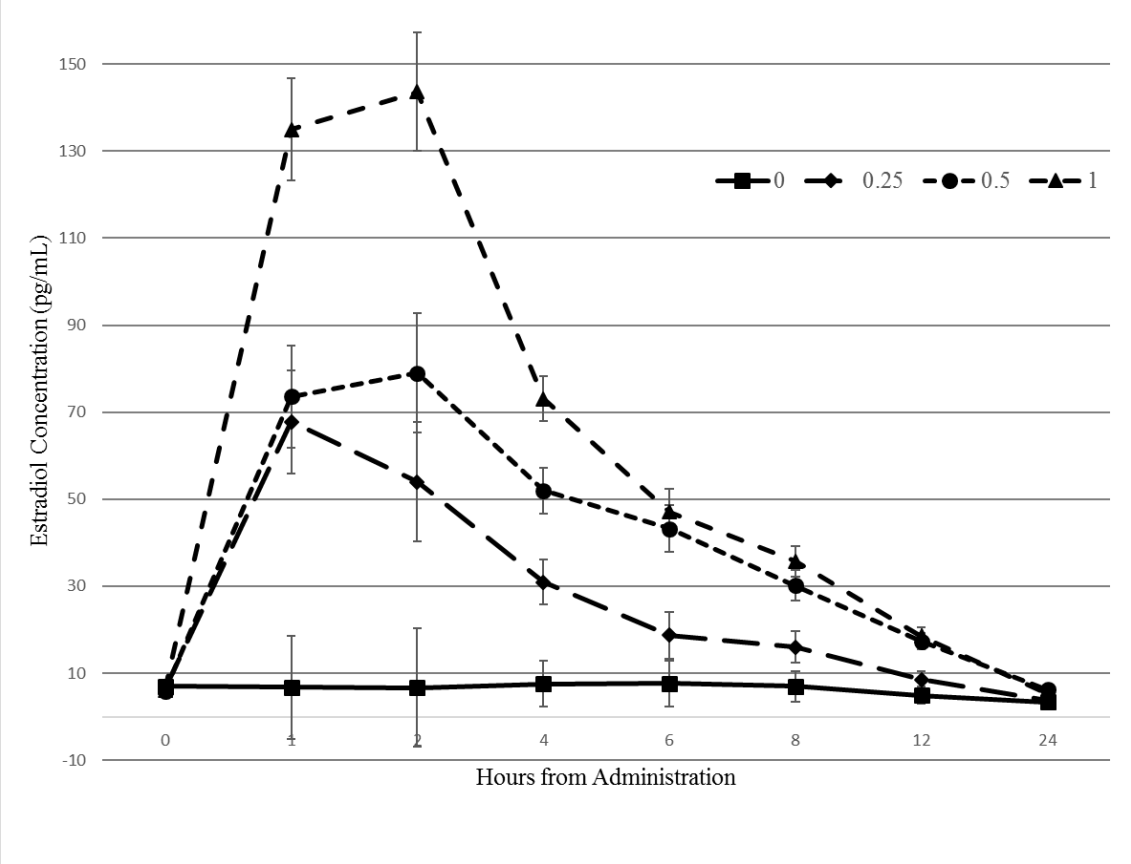


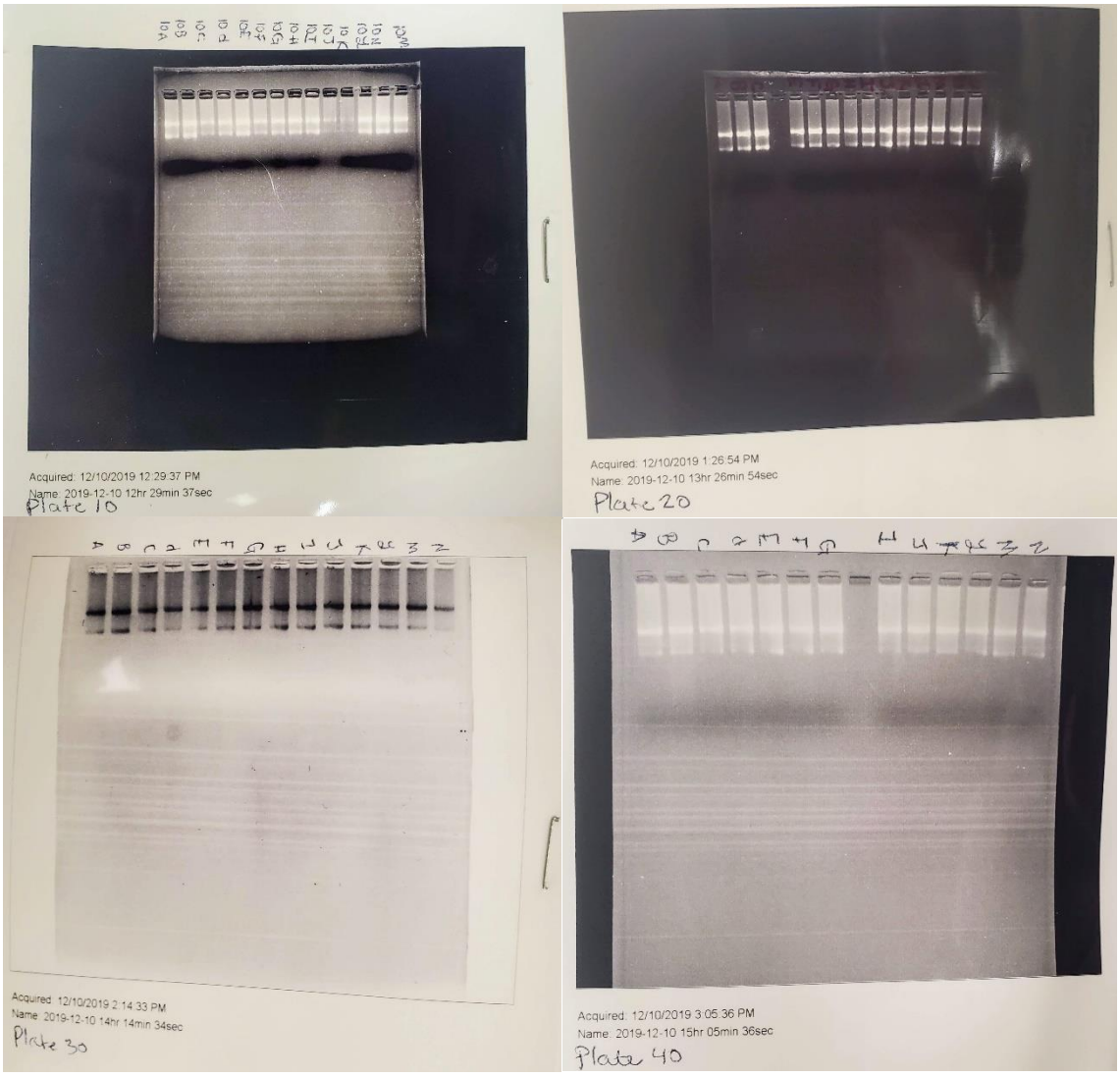
Figure 4.3. A potential fixed-time AI protocol for use of sex-sorted semen following the 14-d CIDR®-PG protocol. On Day 0, a 1.38 g progesterone intravaginal insert (CIDR®) is administered to heifers on Day 0, then removed on Day 14. On Day 30, 16 days after CIDR® insert removal, PG is administered. Estrus detection aids (Estrotect; Estrotect Inc., Spring Valley, WI) are applied on Day 30 coincident with PG administration. Approximately 72 h after PG administration, estrual heifers (Patch Score ≥ 3 or = 0) are inseminated with sex-sorted semen and nonestrual heifers (Patch Score = 1 or 2) are inseminated with conventional semen. At this time, gonadotropin-releasing hormone (GnRH) is administered to nonestrual heifers.

efforts may be warranted to evaluate later timing of FTAI if using sex-sorted semen following the 14-d CIDR®-PG protocol.

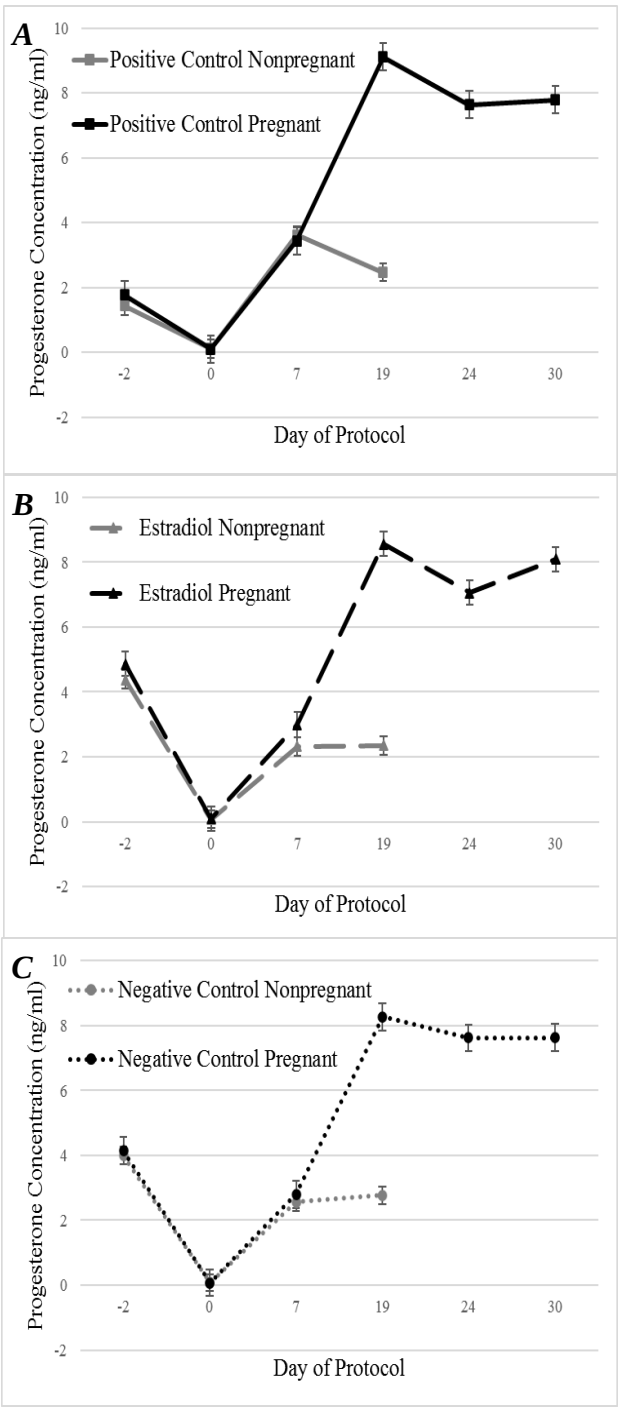
APPENDIX



Appendix Figure 1. LSmean ± (SEM) circulating concentrations of estradiol following administration (IM) of 0 mg, 0.25 mg, 0.5 mg, or 1.0 mg of estradiol 17-β.



Appendix Figure 2. Examples of RNA integrity that was confirmed via a 2.5% agarose gel.



Appendix Figure 3. LSmean ± (SEM) circulating concentrations of progesterone (ng/mL) following PG-induced luteolysis (d -2) for cows assigned to 1) Positive Control: Estrual cows that ovulated spontaneously (*Panel A*); 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2) (*Panel B)*, and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2 (*Panel C)*. Each treatment was subdivided by day 30 (commercial IDEXX S-N $\geq$ 0.3) pregnancy status.

Appendix Table 1. Proportion of Cows with Evidence of a Conceptus Present and Pregnant to ET on Days 19, 24, 30, and 55/58 by treatment.

	Evidence of a Conceptus				Pregnancy Rates			
	Day 19[1]		Day 24[2]		Day 30[3]		Day 55/58[4]	
Treatment[5]	Proportion	%	Proportion	%	Proportion	%	Proportion	%
Positive Control	44/104	42%	35/104	34%	31/104	30%	25/104	24%
Estradiol	52/109	48%	43/109	39%	40/109	37%	34/109	31%
Negative Control	50/112	45%	40/112	36%	34/112	30%	28/112	25%
Total	145/325	45%	118/325	36%	105/325	32%	87/325	27%

[1]Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone ≥ 1ng/mL on day 19; or 2) Had PAG (Ab 63) concentrations > 0.2 on day 24, or 3) Had an IDEXX S-N value ≥ 0.3 on day 30, or 4) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[2]Evidence of a conceptus was considered to be present on day 24 when a cow had 1) a PAG (Ab 63) concentration ≥ 0.2 on day 24; or 2) An IDEXX S-N ≥ 0.3 on day 30; or 3) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[3]Cows were considered pregnant on day 30 when they had an IDEXX S-N value ≥ 0.3 on day 30 or a 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[4]Cows were considered pregnant on day 55/58 if a 55/58 day viable fetus was identified by ultrasonography.

[5]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2.

Appendix Table 2. Proportion of Cows with Evidence of a Conceptus Present and Pregnant to ET on days 19, 30, and 55/58 by treatment.

	Evidence of a Conceptus		Pregnancy			
	Day 19[1]		Day 30[2]		Day 55/58[3]	
Treatment[4]	Proportion	%	Proportion	%	Proportion	%
Positive Control	44/104	42%	31/104	30%	25/104	24%
Estradiol	53/109	49%	40/109	37%	34/109	31%
Negative Control	50/112	45%	34/112	30%	28/112	25%
Total	145/325	45%	105/325	32%	87/325	27%

[1]Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone ≥ 1ng/mL on day 19; or 2) Had an IDEXX S-N value ≥ 0.3 on day 30, or 3) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[2]Cows were considered pregnant on day 30 when they had an IDEXX S-N value ≥ 0.3 on day 30 or a 55/58 day viable fetus was identified by ultrasonography on day 55/58.

[3]Cows were considered pregnant on day 55/58 if a 55/58 day viable fetus was identified by ultrasonography.

[4]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2.

Appendix Table 3. Proportion of Loss within Periods[1] 1, 2, 3, and 4 and Final Pregnancy Rates by Treatment.

	Period 1[2]		Period 2[3]		Period 3[4]		Period 4[5]		Pregnant[6]	
Treatment[7]	Proportion	%	Proportion	%	Proportion	%	Proportion	%	Proportion	%
Positive Control	60/104	58	9/104	9	4/104	4	6/104	6	25/104	24
Estradiol	58/109	53	8/109	7	3/109	3	6/109	6	34/109	31
Negative Control	62/112	55	10/112	9	6/112	5	6/112	5	28/112	25
Total	180/325	55	27/325	8	13/325	4	18/325	6	87/325	27

[1]Period 1: day 7 to 19; Period 2: day 19 to 24; Period 3: day 24 to 30; Period 4: day 30 to 55/58.

[2]If a cow did not meet the criteria requirements indicating evidence of a conceptus present on day 19 (Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone ≥ 1ng/mL on day 19; or 2) Had PAG (Ab 63) concentrations > 0.2 on day 24, or 3) Had an IDEXX S-N value ≥ 0.3 on day 30, or 4) A 55/58 day viable fetus was identified by ultrasonography on day 55/58) then she was considered to have undergone Period 1 Loss.

[3]A cow was considered to have undergone Period 2 Loss when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2), 2) Day 19 circulating progesterone concentration was ≥ 1 ng/mL, 3) PAG (Ab 63) concentration on day 24 was < 0.2, and 4) the IDEXX S-N value on day 30 was < 0.3, and 4) nor was a 55/58 day viable fetus identified via ultrasound on day 55/58.

[4]Cows with a PAG (Ab 63) concentration ≥ 0.2 on day 24, but an IDEXX S-N < 0.3 on day 30 and no 55/58 day viable fetus identified on day 55/58, were considered to have undergone Period 3 Loss.

[5]A cow was considered to have undergone Period 4 Loss when no 55/58 day viable fetus was identified by ultrasound on day 55/58 but she had an IDEXX S-N value ≥ 0.3 on day 30 (i.e. pregnant).

[6]A cow was considered pregnant on day 55/58 if a 55/58 day viable fetus was identified by ultrasonography and was then excluded from any further classification (Period 1, 2, 3, or 4 Loss).

[7]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2.

Appendix Table 4. Percent Loss within Periods[1] A, B, and C and Final Pregnancy Rates by Treatment.

	Period A[2]		Period B[3]		Period C[4]		Pregnant[5]	
Treatment[6]	Proportion	%	Proportion	%	Proportion	%	Proportion	%
Positive Control	64/104	62	9/104	9	6/104	6	25/104	24%
Estradiol	60/109	55	9/109	8	6/109	6	34/109	31%
Negative Control	68/112	61	10/112	9	6/112	5	28/112	25%
Total	192/325	59	28/325	9	18/325	6	87/325	27%

[1]Period A: day 7 to 19; Period B: day 19 to 30; Period C day 30 to 55/58

[2]If a cow did not meet the criteria requirements indicating evidence of a conceptus present on day 19 (Evidence of a conceptus was considered to be present on day 19 when the following criteria were met: 1) When 2 of the 3 ISG genes (*ISG15*, *MX2*, *OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2) and circulating progesterone ≥ 1 ng/mL on day 19; or 2) Had an IDEXX S-N value ≥ 0.3 on day 30, or 3) A 55/58 day viable fetus was identified by ultrasonography on day 55/58.) the she was considered to have undergone Period A Loss.

[3]A cow was considered to have undergone Period B Loss when the following criteria were met: 1) 2 of the 3 ISG genes (*ISG15, MX2, OAS1*) had a ΔΔCT value < its respective associated criterion (Figure 3.5, 3.6, 3.7 and Table 3.2), 2) Day 19 circulating progesterone concentration was ≥ 1 ng/mL, and 3) the IDEXX S-N value on day 30 was < 0.3, and 4) nor was a 55/58 day viable fetus identified via ultrasound on day 55/58.

[4]A cow was considered to have undergone Period C Loss when no 55/58 day viable fetus was identified by ultrasound on day 55/58 but she had an IDEXX S-N value ≥ 0.3 on day 30.

[5]A cow was considered pregnant if a 55/58 day viable fetus was identified by ultrasonography on day 55/58 and was then excluded from any further classification (Period A, B, or C Loss).

[6]Cows were assigned to 1) Positive Control: Estrual cows that ovulated spontaneously; 2) Estradiol: Nonestrual cows that on day 0 were induced to ovulate with GnRH (GnRH2) and administered (IM) 0.1 mg estradiol 17-β (E2), and 3) Negative Control: Nonestrual cows that were induced to ovulate with GnRH2.

Appendix Table 5. Pregnancy rates[a] to split-time AI (STAI) by semen type, bull, and approach.

Semen Type	Approach[b]				Overall	
	66		72			
Bull	Proportion	%	Proportion	%	Proportion	%
Conventional	125/203	62	115/201	57	240/404	59
A	11/23	48	13/21	62	24/44	55
B	53/89	60	47/86	55	100/175	57
C	13/18	72	12/17	71	25/35	71
D	19/25	76	18/26	69	37/51	73
E	29/48	60	25/51	49	54/99	55
Sex-sorted	91/196	46	96/194	49	187/390	48
A	8/20	40	14/20	70	22/40	55
B	47/86	55	41/86	48	88/172	51
C	5/17	29	11/17	65	16/34	47
D	10/25	40	11/25	44	21/50	42
E	21/48	44	19/46	41	40/94	43

[a]Pregnancy rate to AI was determined via transrectal ultrasonography 77-85 d following STAI.

[b]Heifers were administered 1.38 g progesterone insert (CIDR®; Zoestis, Madison, NJ) on Day 0, which was then removed on Day 14. On Day 30, 16 days after CIDR® insert removal, 25 mg dinoprost tromethamine (PG; Lutalyse®; Zoetis) was administered intramuscularly.

Estrus detection aids (Estrotect; Estrotect Inc., Spring Valley, WI) were applied on Day 30 concurrent with PG administration. Heifers were then assigned to one of two approaches 1) 66: heifers that expressed estrus prior to 66 h were inseminated at 66 h, and remaining heifers were inseminated 24 h later (90 h), with 100 μg gonadorelin acetate (GnRH; Fertagyl®, Merck Animal Health, Madison, NJ) administered intramuscularly to heifers that failed to express estrus by this time, or 2) 72: heifers that expressed estrus prior to 72 h were inseminated at 72 h, and remaining heifers were inseminated 24 h later (96 h), with GnRH administered to heifers that failed to express estrus by this time.

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VITA

Jaclyn Nicole Ketchum was born and raised in southeast Montana on her family's ranch where she was involved in the day to day activities of managing a registered Red Angus cattle herd. While remaining involved on the ranch throughout her school years, Jaclyn also actively participated in rodeo, basketball, 4-H, FFA, the National Honor Society, science club, math club, band, choir, Montana Junior Red Angus Association, and Junior Red Angus Association of America. After graduating from Carter County High School, Jaclyn attended Kansas State University where she worked in Dr. David Grieger's lab, became involved in the Kansas State University Block and Bridle and Collegiate Cattlemen's Club, and remained involved in the Junior Red Angus Association of America. During the summers, Jaclyn would return to the family ranch to work for her family's custom beef artificial insemination business. While Miss Ketchum always knew she had a passion for cattle, her first realization that she also enjoyed research came from the experience she gained in a high school science research class taught by Mrs. Linda Rost. Her interest in research continued to grow through the opportunities that Dr. Grieger (Kansas State University) provided her by working in his lab. Jaclyn followed her passion to pursue research by attending graduate school at the University of Missouri-Columbia under the guidance of Dr. Michael Smith. Miss Ketchum's next step is to continue her education with the goal of obtaining a Ph.D. under the guidance of Dr. George Perry (Texas A&M University).