ROLE OF PREOVULATORY ESTRADIOL IN UTERINE AND LUTEAL FUNCTION

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LIST OF ABBREVIATIONS

AI	artificial insemination
AP	alkaline phosphatase
C	control
CIDR	controlled internal drug release
CL	corpus luteum
cAMP	cyclic adenosine monophosphate
DMSO	dimethyl sulfoxide
E2	estradiol 17β
EB	estradiol benzoate
ECP	estradiol cypionate
ELISA	enzyme-linked immunosorbent assay
ESR	estradiol receptor
ET	embryo transfer
FTAI	fixed-time artificial insemination
GnRH	gonadotropin releasing hormone
GPCR	G-protein coupled receptor
GVB	germinal vesicle breakdown
ICM	inner cell mass

i.m.	intramuscular
IFI	intrafollicular injection
IHC	immunohistochemistry
IVM	in vitro maturation
mRNA	messenger ribonucleic acid
P4	progesterone
PAF	paraformadeyhde
PAGs	pregnancy associated glycoproteins
PBS	phosphate buffered saline
PGF	prostaglandin $F_{2\alpha}$
PR	Progesterone receptor
PRID	progesterone releasing intravaginal device
RIA	radioimmunoassay
RT	reverse transcription
RT-PCR	real time polymerase chain reaction
STAI	split time artificial insemination
ULF	uterine luminal fluid

ROLE OF PREOVULATORY ESTRADIOL IN UTERINE AND LUTEAL FUNCTION

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Abstract

In beef heifers, expression of estrus at fixed-time artificial insemination (FTAI) was associated with increased preovulatory circulating concentrations of estradiol (E2) and increased pregnancy rates compared to heifers that were not detected in estrus (Richardson et al., 2016). We hypothesized that increased preovulatory concentrations of estradiol would enhance conceptus elongation in beef heifers. To test the preceding hypothesis conceptus elongation on day 16 (day 0 = GnRH-induced ovulation) was examined in Angus heifers having low (5.0±0.6; pg/mL n=6) or high (10.2±1.0 pg/mL; n=6) preovulatory concentrations of E2 on day 0 (P< 0.0005). In vivo produced embryos (n=5 per heifer) were transferred on day 7, and reproductive tracts were recovered and flushed on day 16 of gestation. Mean (±SEM) conceptus number and length did not differ (P > 0.15) between heifers in the Low E2 (2.5±0.7; 7.1±1.7 cm) and High E2 (2.3±0.5; 5.2±1.3 cm) groups, respectively. Consequently, circulating concentrations of preovulatory E2 did not affect pre-implantation conceptus elongation. Therefore, a second experiment was conducted to examine embryonic loss in heifers exposed to low (3.7±0.3 pg/mL; n=19) or high (7.0±0.4 pg/mL; n=25) preovulatory concentrations of estradiol on day 0 (GnRH-induced ovulation)

between days 7 (embryo transfer) and 36 spanning a critical period for implantation. Pregnancy was monitored using circulating progesterone concentrations (days 16 to 24), pregnancy associated glycoproteins (days 22 to 36), and ultrasonography (days 30 and 36). Pregnancy rate did not differ between the Low and High E2 groups on days 22, 24, 30, or 36. A potential explanation for why there was no difference in pregnancy rate between the Low and High E2 groups, is that preovulatory estradiol in the Low E2 group was not as low as in other studies in which a difference in pregnancy rate was detected.

Since estradiol is reported to prepare granulosa for luteinization and postovulatory progesterone is an important factor affecting pregnancy rate in beef cows, a third experiment was conducted to determine if intrafollicular administration of an estradiol receptor (ESR) antagonist (ICI 182,780) would affect luteal progesterone secretion from the subsequent corpus luteum (CL). Ovulation was synchronized in nonlactating beef cows (n=35) and cows were allocated to three groups (control, vehicle injection, or ICI 182,780 injection) and balanced for dominant follicle size on day -2 (day 0 = GnRH-induced ovulation). Plasma concentrations of estradiol on days -2, -1 and 0, increased ($P< 0.0001$), but did not differ among groups ($P>0.5$). Furthermore, plasma concentrations of progesterone on days 0 through 20 were not affected by treatment ($P=0.86$). These results indicate that either estradiol is not required to prepare granulosa cells to luteinize during the preovulatory period or that this effect may not be mediated through the nuclear estradiol receptors (ESR1 and 2), which presumably was blocked. In summary, preovulatory estradiol did not affect

conceptus elongation or pregnancy rate on day 36 and antagonizing estradiol's genomic action within the preovulatory follicle did not affect circulating postovulatory progesterone.

Chapter One:

INTRODUCTION

Maximizing the proportion of beef heifers and cows that conceive early in the breeding season has a positive effect on weaning weights and subsequent reproductive performance (Schafer et al., 1990). The development of fixed-time artificial insemination (FTAI) protocols that precisely synchronize the time of ovulation in cycling and anestrous cattle has provided the opportunity to artificially inseminate at a predetermined time to genetically superior sires and achieve pregnancy rates at the start of the breeding season that frequently exceed 50%. Consequently, FTAI in beef cattle has emerged as a powerful tool for shortening the breeding season as well as genetic improvement. FTAI protocols currently do a good job of synchronizing ovulation in cycling and anestrous cattle; therefore, the focus of research is shifting to increasing the pregnancy rate to a single insemination.

At the time of FTAI, there are cows that have expressed estrus and those that have not. A meta-analysis of field trials involving 10,116 beef females reported a 27% increase in pregnancy rate to FTAI for animals that expressed estrus compared to those that did not express estrus (Richardson et al., 2015). Furthermore, extending the duration of proestrus (i.e. interval from luteolysis to onset of estrus) and, thereby, increasing circulating preovulatory concentrations of estradiol, increased pregnancy rates to FTAI in beef cattle (Bridges et al., 2010). Using path analysis, Atkins et al. (2013) reported that preovulatory

estradiol was positively associated with probability of fertilization, progesterone concentrations on day 7 following ovulation, and pregnancy success on day 27 of pregnancy following embryo transfer. Consequently, it has been hypothesized that elevated preovulatory concentrations of estradiol (E2) have an important role in the establishment and maintenance of pregnancy in cattle. Elucidating the physiological role of preovulatory estradiol in pregnancy establishment may result in the development of new approaches for increasing pregnancy rates to FTAI.

Preovulatory estradiol affects the vagina, cervix, uterine endometrium, granulosa cells, anterior pituitary, and hypothalamus via genomic receptors (ESR1 and 2; Hall et al., 2001). In addition, nongenomic actions of estradiol on reproductive tissues have been reported (Morley et al., 1992; Wang et al., 2007). Preovulatory estradiol has effects on the endometrium and granulosa cells. More specifically, administration of estradiol during the preovulatory period in ovariectomized beef cows was required to prevent premature luteolysis (Kieborz-Loos et al., 2003) and for the establishment of pregnancy (Madsen et al., 2015). The preceding observations may have been partially due to the induction of endometrial progesterone receptors (PGR) by estradiol (Zelinski et al., 1982) . Elevated preovulatory concentrations of estradiol, produced by a longer proestrus period, increased staining intensity for PGR in the deep glandular epithelium and *ESR1* mRNA in the uterine endometrium (Bridges et al., 2012). Other actions of estradiol on the bovine endometrium include increased expression of glucose transporters (SLC2A1 and SLC5A1, Northrop et al., 2018).

Estradiol may also have a direct effect on follicular cells. More specifically, estradiol inhibited apoptosis (Quirk et al., 2006), increased granulosa cell proliferation (Goldenberg et. al., 1972), promoted formation of gap junctions between granulosa cells (Merk et al., 1972), increased the stimulatory action of FSH on aromatase activity (Zhuang et al., 1982), regulated the expression of steroidogenic enzymes (i.e. 3B-HSD; Gore-Langton and Armstrong, 1994), had a positive feedback on aromatase mRNA expression (Luo and Wiltbank, 2006; Rovani et al., 2014), and enhanced progestin synthesis by rat granulosa cells following gonadotropin stimulation (Welsh et al., 1983). Therefore, the stimulatory effect of estradiol on subsequent progesterone synthesis could be due to increased granulosa cell number and(or) a direct effect on the ability of granulosa cells to luteinize. Experiments reported herein were designed to investigate the effect of low or high circulating concentrations of estradiol during the preovulatory period on conceptus elongation (Chapter 3; experiment 1); maintenance of pregnancy following embryo transfer (Chapter 3; experiment 2); and the effect of administration of an estradiol receptor antagonist into the preovulatory follicle on the preparation of granulosa cells for luteinization in cattle (Chapter 4).

We hypothesized that expression of estrus, via increased preovulatory concentration of estradiol, promotes pre-implantation conceptus elongation and(or) reduces embryonic mortality following maternal recognition of pregnancy in cattle. The objective of the first study in Chapter 3 was to determine the effect of preovulatory estradiol concentration on conceptus recovery rate and conceptus elongation. The second experiment was designed to characterize

embryo loss from day 22 through day 36 following embryo transfer. Embryo transfer (ET) occurred on day 7 and the presence or absence of an embryo was determined on day 22 to 24 (circulating progesterone), 30 (measurement of circulating concentrations of pregnancy associated glycoproteins (PAGs)) and on days 30 and 36 (PAGs and ultrasonography). The objective of the third study (Chapter 4) was to determine the effect of blocking estradiol binding to the nuclear receptor (ESR1 and 2) within the follicle (i.e. granulosa cells) on progesterone production by the corpus luteum following ovulation. We hypothesized that intrafollicular injection (IFI) of an estradiol receptor antagonist, ICI 182,780, would compromise luteinization following a gonadotropin releasing hormone (GnRH)-induced gonadotropin surge and decrease progesterone production by the subsequent corpus luteum.

Chapter Two:

LITERATURE REVIEW

2.1 Introduction

The development of fixed time artificial insemination (FTAI) protocols has allowed insemination of beef heifers and cows to a timed ovulation regardless of estrous expression. This technology can increase reproductive efficiency of a herd, as it allows 50 to 65% of females to conceive the first day of the breeding season to genetically superior bulls. Ovulation can be precisely synchronized in cycling and noncycling beef heifers and cows; therefore, the research focus is shifting to improving pregnancy rates to FTAI. Since fertilization rate is high in beef cattle (≈90%; reviewed by Sreenan and Diskin, 1986), the opportunity to increase pregnancy rates to a single insemination requires an understanding of the underlying biology of pregnancy establishment and development of management strategies to decrease embryonic mortality.

Expression of estrus and circulating concentrations of estradiol at FTAI were positively associated with pregnancy rates (Atkins et al., 2013; Jinks et al., 2013; Richardson et al., 2016). Preovulatory estradiol is critical for estrous expression, induction of the preovulatory gonadotropin surge, gamete transport (Hawk, 1983), proper timing of prostaglandin F2α (PGF) release by the bovine endometrium (Kieborz-Loos et al., 2003), and establishment of pregnancy in sheep and cattle (Miller et al., 1977; Madsen et al., 2015). This literature review

will focus on the role of preovulatory estradiol in the establishment and maintenance of pregnancy, with particular emphasis on the effects of estradiol on the oocyte, granulosa cells, and uterine environment in domestic ruminants.

2.2 Embryonic development and mortality

Following fertilization, the embryo remains in the oviduct until day 4, at which point it enters a uterine horn at approximately the 16-cell stage. The bovine embryo subsequently becomes a morula and then a blastocyst (Lonergan and Forde, 2015). Approximately 8 to 9 days after ovulation, the blastocyst hatches and the inner cell mass (ICM) develops into the pluripotent epiblast (Pedersen et al., 2017). Up to the blastocyst stage the bovine embryo is not dependent upon the maternal environment since embryos produced by in vitro fertilization develop normally to the blastocyst stage in culture. Furthermore, transfer of in vitro produced bovine embryos into recipient cows on day 7 post estrus results in pregnancy establishment. However, the uterine environment (e.g. histotroph) is essential for embryonic development beyond the hatched blastocyst stage since bovine embryos will not elongate in vitro or in vivo in a uterine gland knock out model (Gray et al., 2001; Lonergan and Forde, 2015).

Following hatching on day 8 to 9, the bovine embryo becomes ovoid (≈ day 13) then tubular, and eventually filamentous. Embryonic elongation occurs from day 12-19 in cattle (Spencer and Hansen, 2015) and can be quite variable within a bovine uterus when multiple embryos are transferred, suggesting that the response of the conceptus to the maternal environment has a role in elongation (Clemente et al., 2009). After day 19, the conceptus is adhered to the

luminal epithelium and begins placentation. From day 12 to 38 of pregnancy the ruminant trophoblast produces and secretes interferon tau (IFNT), a cytokine that is required for maternal recognition of pregnancy in cattle (Bazer, 2013). Expression of *IFNT* mRNA peaks on day 20 in cattle (Hansen et al., 1988; Stewart et al., 1989). IFNT prevents the pulsatile secretion of PGF by inhibiting the expression of the ESR1 in the ovine uterus (Bazer, 2013).

Maternal recognition of pregnancy in cattle (i.e. day 15 to 16) is followed by trophoblast apposition (≈day 19 to 20) and adhesion (≈ day 20 to 21). In sheep, apposition begins in the vicinity of the embryo and is characterized by close association of uterine microvilli and the trophoblast (reviewed by Guillomot, 1995). Apposition is closely followed by adhesion and is characterized by further penetration of uterine microvilli into folds of the trophoblast resulting in a close attachment between the two tissues. Migration of and fusion of trophoblastic binucleate cells with uterine epithelial cells results in formation of a syncytium (reviewed by Guillomot, 1995). Binucleate cells secrete pregnancy associated glycoproteins (PAGs) which can be used a marker of pregnancy and late embryonic mortality (see review by Wallace et al., 2015).

Fertilization rate following FTAI has varied from 75 to 100%, and, on average, appears to be lower in lactating animals (Santos et al., 2004); however, the proportion that calve to a single insemination is frequently 60% or lower. Consequently, there could be as much as a 30% pregnancy loss from fertilization to calving. Although fewer studies have been conducted in dairy cows,

fertilization rate may be lower compared to heifers and beef females (Sartori et al., 2002).

Embryonic loss can be divided into two periods, early and late embryonic loss. Early embryonic loss occurs between fertilization and day 28 of pregnancy, when ultrasound can be reliably used for pregnancy diagnosis. Major events occurring during this time period are maternal recognition of pregnancy, apposition, and adhesion of the extraembryonic membrane to the luminal epithelium. Late embryonic loss generally occurs from day 28 to 84 of pregnancy (Diskin and Morris, 2008), a time associated with initiation and completion of placentation. There is very little fetal loss after placentation is complete in beef cattle. Fertilization and embryonic loss rates differ based on parity, sire, lactational status, season, nutritional status, and breed (reviewed by Diskin and Morris, 2008; Diskin et al., 2012). Based on previous studies, Diskin et al. (2006) estimated that fertilization failure, early embryonic loss, and late embryonic/fetal loss accounts for 10, 40 and 7% reduction in pregnancy respectively, in lactating Holstein-Friesian cows. Although values differ among studies, early embryonic loss consistently accounts for the majority of embryonic loss. The incidence of early and late embryonic loss is shown in Table 2.1. These early embryonic losses could be due to failure of maternal recognition of pregnancy, due to inadequate IFNT production by the conceptus, or problems with apposition and adhesion of the extraembryonic membranes to the luminal epithelium of the endometrium.

Table 2.1: Incidence of early and late embryonic loss in cattle. (Adapted from Pohler, 2015).

Type of EM	Cattle Type	No. Studies	Specific Days of EM	Incidence of EEM Mean (Range)	Ref
Early	Beef Heifers	2	2 to 16	21.8% (4.5 to 43.7%)	1
	Dairy Heifers	1	2 to 6	28.9%	2
	Beef Cows	3	2 to 16	35.6% (10.5 to 70%)	3
	Dairy Cows (Lactating)	1	2 to 7	46.3%	4
	Dairy Cows (Nonlactating)	3	2 to 7	33.3% (13.8 to 46.9%)	5
Late	Beef Heifers	4	30-90	4.2% (4 to 5%)	6
	Dairy Heifers	2	30-80	5.4% (4 to 6 %)	7
	Beef Cows	3	25-65	10% (6.5 to 14%)	8
	Dairy Cows (Lactating)	11	27-60	14% (3.2 to 42.7%)	9

References 1=Maurer and Chenault, 1983; Dunne et al., 2000; 2= Sartori et al., 2002; 3= Maurer and Chenault, 1983; Ahmad et al., 1995; Breuel et al., 1993; 4= Sartori et al., 2002; 5= DeJarnette et al., 1992; Dalton et al., 2001; Sartori et al., 2002; 6= Lamb, 2002; Kill et al., 2013; 7= Dunne et al., 2000; Silke et al., 2002; 8= Stevenson et al., 2003; Perry et al., 2005; Pohler et al., 2013; 9= Vasconcelos et al., 1997; Cartmill et al., 2001a; Cartmill et al., 2001b; Cerri et al., 2003; Thompson et al., 2010; Ricci et al., 2015

2.3 Effect of expression of estrus and lengthening proestrus on pregnancy rate to FTAI

At FTAI in cattle, there normally are two groups of females, those that have and have not expressed estrus. A meta-analysis of field trials involving 10,116 beef females reported a 27% increase in pregnancy rate to FTAI for animals that expressed estrus compared to those that did not express estrus (Richardson et al., 2016). Furthermore, expression of estrus was associated with reduced pregnancy loss in lactating dairy cows (Pereira et al., 2013; Pereira et al., 2014).

Split-time AI (STAI) is a management strategy to increase pregnancy rates to FTAI in beef females that have not expressed estrus by the normal time of insemination (Thomas et al., 2014). This approach has been used with the Co-Synch CIDR or 14-day CIDR-PGF FTAI protocols in which females that have expressed estrus by the normal insemination time are inseminated; whereas, any females that have not expressed estrus by the first insemination are inseminated approximately 20 hours later. When this approach was employed in beef heifers, STAI increased AI pregnancy rate from 46 to 54%. Overall estrous expression in STAI was higher than conventional FTAI, due to delaying induction of ovulation in heifers that had not shown estrus by the normal insemination time. The STAI protocol was especially beneficial when using sex-sorted semen (Thomas et al., 2014). In the latter study, the benefit of STAI on pregnancy rates with sex-sorted semen may have been due to increased estrous expression in beef heifers and(or) depositing the sex-sorted semen closer to the time of ovulation.

Lengthening the period of proestrus is another approach that has been used to increase estradiol secretion during the preovulatory period to increase pregnancy rates to FTAI in beef cattle. Proestrus has been defined as the interval from luteolysis to the onset of estrus and is characterized by increased luteinizing hormone (LH) pulse frequency, a rapid increase in circulating concentrations of estradiol, estrogenic changes in the reproductive tract (e.g. cervix, uterus, and oviduct), and increased preovulatory follicular growth and maturation. Pregnancy rates following FTAI were positively associated with length of proestrus in beef (Mussard et al., 2007; Bridges et al., 2008; Bridges et al., 2010; Geary et al., 2013) and dairy (Santos et al., 2010) cattle. Bridges et al. (2010; 2012) investigated the effect of short (30 hours) and long (54 hours) proestrus on pregnancy rates to FTAI in beef cattle. Long proestrus resulted in a higher pregnancy rate to AI (50%) than short proestrus (2.6%) and the average ovulatory follicle size did not differ between groups. Circulating concentrations of preovulatory estradiol and postovulatory progesterone were greater in the long proestrus group. Furthermore, more cows in the short proestrus group experienced short luteal phases following ovulation, which could increase embryonic loss (Bridges et al., 2010). The preceding studies on the effect of the length of proestrus resulted in development of the 5 day CIDR-PGF protocol in beef cattle (Bridges et al., 2008; Kasimanickam et al., 2009).

The physiological basis for increased pregnancy rates following FTAI in cows that express estrus versus cows that don't express estrus is currently not known, but likely involves physiological pathways regulated by estradiol.

Circulating estradiol during the preovulatory period can induce behavioral estrus and may coordinate other physiological processes associated with the establishment of pregnancy. Atkins et al. (2013) performed a single ovulation reciprocal embryo transfer study to separate effects of the follicular microenvironment on oocyte competence from an effect on the maternal environment in postpartum beef cows. Cows were synchronized and induced to ovulate a single oocyte in donor and recipient postpartum beef cows. Animals were divided into treatment groups based on dominant follicle size at GnRH-induced ovulation and were classified as ovulating a large (>12.5mm) or small (<12.5mm) dominant follicle. Cows that expressed estrus were not included in this study. In the preceding study, path analysis was employed to identify potential cause and effect relationships among the measured variables. The authors reported a positive association between preovulatory estradiol concentration on day 0 and fertilization rate (donor cows), progesterone on day 7 (donor cows), and pregnancy rate on day 27 (reciepent cows; Atkins et al., 2013). In the preceding study, preovulatory concentrations of estradiol and postovulatory concentrations of progesterone emerged as the two most important factors affecting pregnancy rate in postpartum beef cows. While there was a positive association between preovulatory estradiol and pregnancy rate, the mechanism through which this effect was mediated is unknown. However, there are many possibilities as estradiol has been demonstrated to affect the ovary, oviduct, uterus, cervix, vagina, anterior pituitary, and hypothalamus.

2.4 Effect of estradiol on pregnancy establishment

The effect of estradiol on a target tissue can be mediated through the nuclear receptors 1 and 2 (ESR1, ESR2) and(or) nongenomic pathways (Freeman, 1985; Morley et al., 1992; Hall et al., 2001; Beker-van Woudenberg, 2004). The nongenomic action of estradiol occurs more quickly than the genomic action of this steroid. Both ESR1 and 2 bind estradiol with high affinity, but differ in expression throughout the reproductive tract (Stormshak and Bishop, 2008). ESR1 is the primary estradiol receptor in the oviduct, uterus, cervix, and vagina, while ESR2 is the primary estradiol receptor in the ovary, including the oocyte. (Hall et al., 2001; Beker-van Woudenberg, 2004; Binelli et al., 2018). Bovine granulosa cells express both ESR1 and 2 (Beker-van Woudenberg, 2004) and theca interna cells have weak staining for ESR2 (Schams and Berisha, 2002). How a genomic or nongenomic receptor for estradiol is capable of being regulated in the presence of high concentrations of estradiol in bovine follicular fluid (e.g. 1 µg/ml in a preovulatory follicle) is a mystery. Estradiol receptors (ESR1 and 2) are also present in the bovine corpus luteum. In luteal tissue, ESR1 was the primary receptor and decreased throughout the luteal phase; whereas, ESR2 was lowly expressed and did not change throughout the cycle (Schams and Berisha, 2002).

2.4.1 Estradiol's effect on the oocyte and cumulus cells

The cumulus-oocyte complex is exposed to high concentrations of estradiol within the antrum and bovine oocytes and cumulus cells express ESR1 (cumulus cells) and 2 (cumulus cells and oocyte; Beker-van Woudenberg, 2004). Although

effects of estradiol on the cumulus-oocyte complex have been reported, results to date have not been repeatable. For example, addition of estradiol to media during maturation of bovine oocytes had a detrimental effect (Beker-van Woudenberg, 2004), no effect (Beker-van Woudenberg et al., 2006), or a positive effect (Fukui et al., 1982) on embryonic development, in vitro. Addition of estradiol to IVM media, after germinal vesicle breakdown (GVB), significantly increased the percentage of nuclear aberrations. Since transcriptional activity ceases after the LH surge, this effect was likely nongenomically mediated (Beker-van Woudenberg, 2004). Blastocyst development rate was greater for bovine oocytes isolated from follicles with high compared to low intrafollicular estradiol concentrations after in vitro maturation (IVM), fertilization, and culture (Van De Leemput et al., 1999; Mermillod et al., 1999). However, this effect may be due to follicular maturity rather than a direct effect of estradiol.

In postpartum beef cows, Atkins and coworkers (2013) reported a positive association between preovulatory estradiol on day 0 (GnRH-induced ovulation) and fertilization rate that was independent of ovulatory size. However, no direct association between circulating estradiol concentration at GnRH induced ovulation and the viability of in vivo produced bovine embryos was detected in the preceding study. Consequently, a physiological role for estradiol in the bovine oocyte has not been determined.

There is bidirectional communication between the oocyte and surrounding cumulus cells that is required for acquisition of oocyte competence (Eppig, 2001). In ruminants, this communication can occur via gap junctions (e.g. cyclic

adenosine monophosphate [cAMP]; Thomas et al., 2004)), paracrine signaling (e.g. cumulus derived KIT ligand; Kidder and Vanderhyden, 2010; Lima et al., 2016), and transzonal processes (Macaulay et al., 2016). Consequently, effects of estradiol on the oocyte may be mediated by the cumulus cells, which express ESR1 and 2 (Beker-van Woudenberg, 2004).

2.4.2 Estradiol's effect on granulosa cells

Bovine granulosa cells synthesize estradiol via the Two Cell Two Gonadotropin Concept (Figure 2.1; see review by Fortune and Quirk, 1988) and contain ESR1 and 2 (Beker-van Woudenberg, 2004). Therefore, estradiol has an intracrine/autocrine action in granulosa cells in addition to endocrine effects on other tissues. Estradiol and FSH have been reported to induce LH receptor in rat granulosa cells (Richards et al., 1976). Furthermore, LHR mRNA expression was decreased following an intrafollicular injection of an estradiol receptor antagonist (ICI 182,780) into bovine antral follicles (Rovani et al., 2014). Estradiol inhibited apoptosis (Quirk et al., 2006), increased granulosa cell proliferation (Goldenberg et. al., 1972), promoted formation of gap junctions between granulosa cells (Merk et al., 1972), increased the stimulatory action of FSH on aromatase activity (Zhuang et al., 1982), regulated the expression of steroidogenic enzymes (Gore-Langton and Armstrong, 1994), and enhanced progestin synthesis following gonadotropin stimulation (Welsh et al., 1983).

There is also evidence to support a nongenomic action of estradiol in granulosa cells. Morley and coworkers (1992) reported rapid release of calcium in response to addition of estradiol to either porcine or chicken granulosa cells in

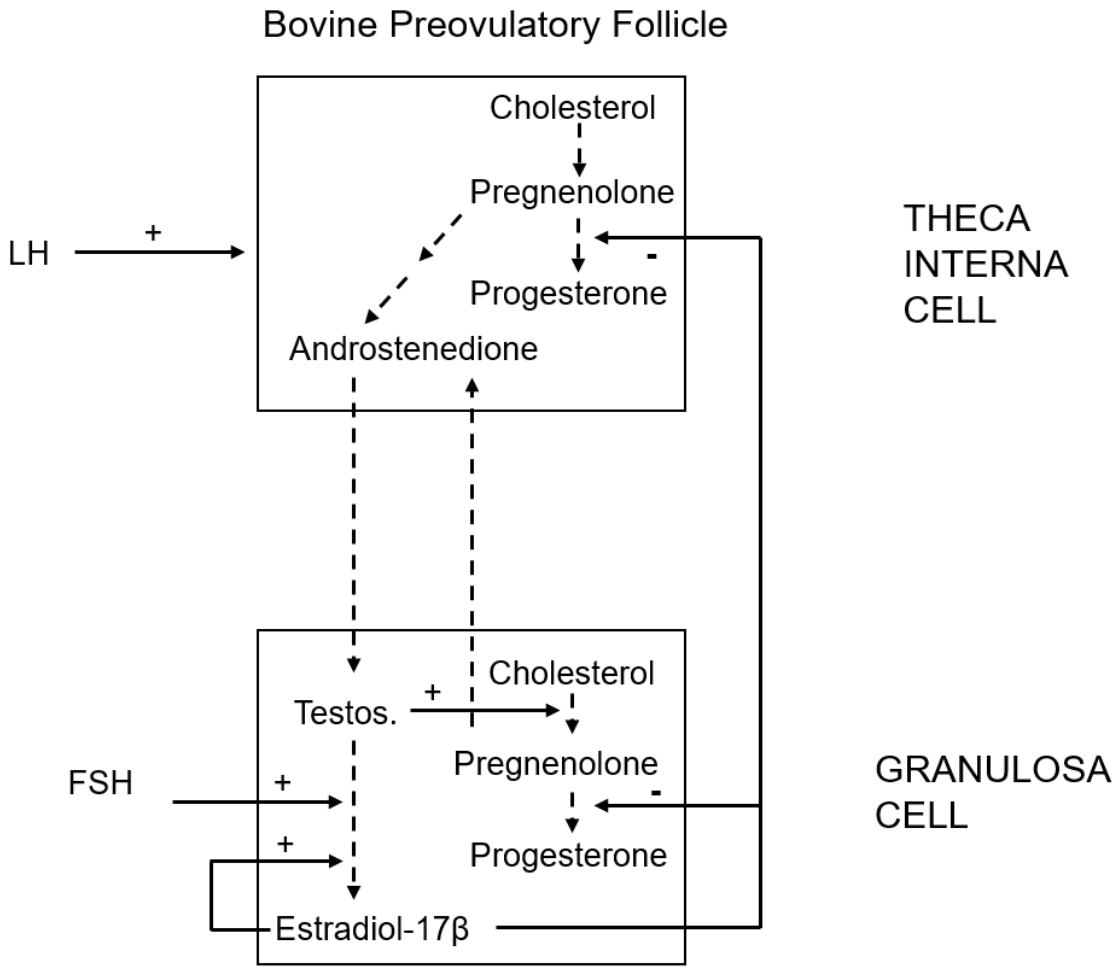

Figure 2.1: Proposed model for regulation of steroidogenesis in cattle (Two cell-two gonadotropin concept of estradiol production). In this model, thecal cells respond to LH to produce androstenedione and granulosa cells respond to FSH to convert androstenedione to estradiol via the delta 5 pathway of steroidogenesis. Plus sign (+) indicates a stimulatory effect and a negative sign (-) an inhibitory effect. Adapted from (Fortune and Quirk, 1988).

culture. Addition of an estradiol receptor antagonist, tamoxifen, failed to block this calcium release and it was specific to estrogens as progestins and androgens did not induce calcium release (Morley et al., 1992).

Finally, there is the potential for estradiol to have a direct effect on bovine steroidogenic luteal cells. Bovine large luteal cells are believed to originate from granulosa cells and produce more progesterone than small luteal cells, which are reported to originate from theca cells (Meidan et al., 1990). Interestingly, bovine luteal cells contain estradiol receptors (see previous section). In luteal tissue, ESR1 is the primary receptor and decreases throughout the luteal phase; whereas, ESR2 is lowly expressed and does not change throughout the cycle (Schams and Berisha, 2002). Expression of estrus affected gene expression in the subsequent corpus luteum on day 19 of pregnancy. More specifically, genes associated with apoptosis (*BAX)*, progesterone (P4) synthesis (*CYP11A*) and prostaglandin receptor (*FPr*) were decreased on day 19 of pregnancy in animals that expressed estrus compared to those that did not express estrus prior to insemination (Davoodi et al., 2016). Consequently, estradiol may decrease apoptosis and prostaglandin sensitivity after luteinization in bovine granulosa cells.

As the corpus luteum is an extension of follicular maturation, the microenvironment of the preovulatory follicle may affect the preparation of granulosa and theca cells for luteinization. For example, a transient decrease in circulating FSH during the follicular phase of the menstrual cycle resulted in luteal phase defects following ovulation (Sherman and Korenman, 1974). In

sheep, GnRH induction of ovulation at 12 hr versus 36 hr after PGF-induced luteolysis decreased progesterone production by the subsequent CL (Murdoch and Van Kirk, 1998). In the preceding study, administration of progesterone on day 4 after insemination marginally improved pregnancy rates in sheep in which ovulation was induced prematurely. The authors proposed that premature induction of ovulation compromised estrogen mediated granulosa cell proliferation and led to formation of an incompetent CL that had fewer large steroidogenic cells and produced less progesterone. Furthermore, these effects extended to the uterus as ewes induced to ovulate early had fewer endometrial glands (Murdoch and Van Kirk, 1998).

In cattle, circulating estradiol on day 0 (GnRH-induced ovulation) was positively correlated with circulating progesterone on day 7, regardless of ovulatory follicle size (Atkins et al., 2013), suggesting that preovulatory estradiol may affect subsequent luteinization. Alternatively, larger follicles generally produce larger CLs, which, subsequently, secrete more progesterone, perhaps due to increased granulosa cell number (Binelli et al., 2001; Vasconcelos et al., 2001). Granulosa cells are believed to differentiate into large steroidogenic luteal cells (Alila and Hansel, 1984) that produce more progesterone than small steroidogenic luteal cells (Meidan et al., 1990). Therefore, it is unclear whether an effect of preovulatory concentrations of estradiol on circulating concentrations of progesterone, during the subsequent luteal phase, is due to better preparation of granulosa cells for luteinization and(or) is a reflection of a larger preovulatory follicle that produced a large CL capable of producing more progesterone. There

is evidence to support estradiol acting both to prepare granulosa cells for luteinization and to stimulate their proliferation (Goldenberg et al., 1972).

Luteinization is initiated by the preovulatory gonadotropin surge and morphological and physiological evidence of luteinization is present even before follicular rupture in several species, including cattle (Smith et al., 1994). There is evidence, in vitro, for a role of estradiol in preparation of granulosa cells for luteinization. Human granulosa cells collected from follicles that had higher follicular fluid concentrations of estradiol produced more progesterone following luteinization, in vitro (McNatty et al., 1979). Furthermore, administration of estradiol to bovine granulosa cells, in culture, initially inhibited, but subsequently stimulated progesterone production compared to granulosa cells not treated with estradiol (Fortune and Hansel, 1979). The initial inhibitory effect could be due to estradiol inhibiting conversion of pregnenolone to progesterone, which would provide more pregnenolone as a substrate for conversion to androstenedione by theca cells (see Figure 2.1; Fortune and Quirk, 1988). In the preceding review, the authors hypothesized that the increase in progesterone secretion following removal of estradiol from the culture media may be mediated through an inhibitory effect on 3β hydroxyl steroid dehydrogenase (3B-HSD) by estradiol. Interestingly, estradiol was reported to be a competitive inhibitor of 3B-HSD in cells from a Leydig Cell tumor at a Ki of 1.8 μM (Freeman, 1985).

2.4.2 Estradiol's effects on gamete transport

Fertilization requires the lifespan of the sperm (≈24 hr for frozen/thawed semen) and oocyte (8 to 12 hr) to overlap within the oviduct. Preovulatory

secretion of estradiol has an essential role in coordinating the transport of both gametes to accomplish fertilization. After ovulation, the oocyte is directed into the oviductal infundibulum and transported by ciliary beats and muscular contractions, through the ampulla to the ampullary-isthmic junction where fertilization occurs (Overstreet and Blazak, 1983). After fertilization, the bovine embryo remains in the oviduct until day 4 and where it is exposed to oviductal secretions. Porcine and ovine embryo development was improved after culture in the presence of oviductal fluid (Binelli et al., 2018).

The oviduct undergoes cyclic changes in response to estradiol and progesterone. These changes are mediated by both genomic and non-genomic mechanisms and include changes in gene expression, ciliary beat frequency, smooth muscle contractions, and oviductal secretions (reviewed by Binelli et al. 2018). During estrus, the number and degree of folding of the ampulla is increased and proteins such as, heat shock proteins, mucins, and redox enzymes were more abundant. Estradiol increases oviductal secretions and ESR and PR staining (Binelli et al., 2018).

To optimize fertilization rate, sperm must be readily transported within the uterus. Sperm are transported to the oviducts through contractions and ciliary movement (Hawk, 1983). Ovariectomized ewes treated with progesterone followed by estradiol benzoate (EB) had more sperm in the cervix, following artificial insemination (AI), than those not treated with estradiol (Allison and Robinson, 1972). At the beginning of estrus in sheep, contractions start at the uterine body and move towards the uterine horns; however, after estrus,

contractions move from the oviduct towards the uterus. In the latter case, administration of estradiol prevented this change in direction of uterine contractions. Furthermore, in ovariectomized ewes, administration of estradiol induced uterine contractions towards the uterine horns (Hawk, 1975). These data suggest that estradiol controls the direction of uterine contractions required for sperm transport and that increased circulating concentrations of estradiol could increase fertilization rate by prolonging uterine contractions towards the uterine horns.

Sperm transport is also a function of sperm motility which may be dependent on uterine pH. In cattle, there is a transient decrease in uterine pH that appears to be mediated by estradiol (Perry and Perry, 2008a). More specifically, cows that expressed estrus had decreased uterine pH (pH ≈6.72) compared to cows that did not show estrus (pH≈ 7.0; Perry and Perry, 2008a). Furthermore, uterine pH of cows treated with estradiol cypionate, but did not express estrus, was intermediate compared to control cows that did or did not express estrus. Motility of bovine sperm was reduced following a decrease in pH but increased at a higher pH (Goltz et al., 1988). Therefore, an estradiol mediated decrease in uterine pH may transiently decrease sperm motility and extend sperm lifespan (Jones and Bavister, 2013). This may be particularly important for FTAI protocols in which before insemination there are cows that have and have not expressed estrus. In cattle, inseminated at a fixed time, there was an inverse relationship between uterine pH and pregnancy rate to FTAI (Lares et al., 2008). Therefore, expression of estrus and the associated elevated

concentrations of circulating estradiol decreased uterine pH which may increase sperm lifespan and enhance fertilization rate.

2.4.4 Estradiol's effects on the uterus

In cattle, administration of estradiol cypionate 24 hr before GnRH-induced ovulation and artificial insemination in postpartum beef cows significantly increased pregnancy rates on day 28 in postpartum beef cows that ovulated a follicle smaller than 12.2 mm (Jinks et al., 2013). In the preceding study, the beneficial effect of estradiol may have been on gamete transport and(or) on uterine function. In a reciprocal embryo transfer study, embryos were transferred from postpartum beef cows with low or high estradiol on day 0 (GnRH-induced ovulation) into recipient cows with low or high estradiol on day 0 (Jinks et al., 2013). Pregnancy rate was improved when the recipient cow had high estradiol on day 0 regardless of the donor cow's estradiol status on day 0. Thus, the increase in pregnancy rate in recipients, due to higher preovulatory estradiol, was likely mediated through the uterus. In the preceding study, preovulatory estradiol and postovulatory progesterone were the most important factors affecting pregnancy rate in postpartum beef cows.

NW Moore and colleagues conducted an elegant series of experiments in which they employed steroid administration to ovariectomized ewes to elucidate the physiological role of estradiol and progesterone in the establishment of pregnancy following embryo transfer (Figure 2.2; Miller et al., 1977). Ewes were ovariectomized and treated with the following steroid treatments to simulate

	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 2.2: Effect of administration of ovarian steroids on the establishment of pregnancy in ovariectomized ewes (Adapted from Miller et al., 1977). Administration of different combinations of progesterone (priming and maintenance progesterone) and estradiol (preovulatory estradiol) was performed to determine the relative importance of the preceding steroids for pregnancy establishment. In this study, administration of estradiol during the preovulatory period was required for pregnancy.

changes in progesterone and estradiol during an estrous cycle: 1) Priming progesterone on days 3 to 14 to simulate circulating concentrations of progesterone prior to estrus, 2) Estradiol treatment, in the absence of progesterone, on days 15 and 16 to mimic changes in estradiol during the preovulatory period, and 3) Maintenance progesterone on days 18 to 33 to simulate circulating concentrations of progesterone during a cycle in which ewes received an embryo. Ovariectomized ewes treated with priming progesterone followed by maintenance progesterone and no estradiol failed to maintain pregnancy (pregnancy rate = 0%) following embryo transfer. The pregnancy rate in ewes that received estradiol injections followed by maintenance progesterone was 16%. However, when ewes were treated with priming progesterone, estradiol, and maintenance progesterone the pregnancy rate increased to 77.8% These data indicate that exposure of the uterus to estradiol is required for the establishment of pregnancy and that progesterone priming before estradiol treatment further increased pregnancy rate in ovariectomized ewes. Omitting estradiol from the preceding experiment decreased protein content in the uterine lumen, rate of protein synthesis, uterine weight, and number of progesterone and estradiol receptors (Miller et al., 1977).

Short luteal phases normally occur following the first ovulation in postpartum beef cows and are due to an advance in the timing of uterine secretion of PGF (Garverick et al., 1992). Progestogen pretreatment is required for normal luteal lifespan following the first ovulation in sheep and cattle (Lishman and Inskeep, 1991). A series of experiments were conducted to examine whether

progestogen pretreatment has a direct or indirect effect on the timing of PGF secretion by the endometrium in cattle. Progestogen pre-treatment may affect the timing of PGF secretion indirectly by increasing preovulatory concentrations of estradiol. For example, preovulatory concentrations of estradiol were higher preceding a normal versus a short luteal phase (Garverick et al., 1988). Furthermore, increased circulating concentrations of preovulatory estradiol in postpartum beef cows was associated with increased progesterone receptor binding on day 6 of the subsequent luteal phase compared to cows with lower circulating concentrations of preovulatory estradiol (Zollers et al., 1993). However, progestogen pretreatment may have a direct effect on uterine PGF secretion, since Cooper et al. (1991) reported increased PGF secretion by the uterus following the first exposure postpartum.

Kieborz-Loos et al. (2003) examined the effect of progesterone alone or progesterone and estradiol on oxytocin-induced PGF release in postpartum beef cows. Anestrous postpartum beef cows were ovariectomized and treated with different combinations of progesterone and estradiol to simulate estrous cycles of short or normal duration. Progesterone priming (7 days) followed by estradiol (2 days) was required to prevent an advance in the timing of PGF secretion. The current concept regarding steroid regulation of endometrial PGF secretion in ruminants is as follows: 1) Preovulatory estradiol induces endometrial progesterone receptors and progesterone binding to its receptor inhibits estradiol receptor expression in the endometrium, 2) Increased circulating concentrations of progesterone downregulates the genomic progesterone receptor in the luminal

and superficial epithelium of the uterus, 3) Downregulation of the endometrial progesterone receptor permits expression of the estradiol receptor, 4) Binding of estradiol, presumably of ovarian origin, induces endometrial oxytocin receptors, and 5) Oxytocin binding to its receptor initiates the luteolytic cascade (Kotwica et al., 1999). In the pregnant ewe, IFNT inhibits endometrial estradiol receptor expression thereby preventing the induction of oxytocin receptors and pulsatile PGF secretion (see review by Spencer and Hansen, 2015).

Ovarian steroids have a critical role in the regulation of uterine function in ruminants, including induction of endometrial progesterone receptors (Zelinski et al., 1982), sperm transport (Hawk, 1975), production of histotroph (reviewed by Spencer et al., 2016), regulation of uterine pH (Perry and Perry, 2008a), and regulation of the timing of luteolytic pulses of PGF (Kieborz-Loos et al., 2003). However, little is known regarding the action of estradiol in uterine gene expression. An experiment was conducted to examine the effect of estrous expression on uterine gene expression. Estrous expression affected endometrial, luminal, and conceptus gene expression in Nelore beef cows. Endometrial transcripts upregulated by estrous expression were mainly involved in the immune system and adhesion molecule family (*MX1, MX2, MYL12A, MMP19, CXCL10, IGLL1, and SLP1*) and prostaglandin synthesis (*OTR and PTGS2*). These changes favorably influence the receptivity of the endometrium by suppressing the maternal immune response, and partially inhibiting the PGF synthesis pathway (Davoodi et al., 2016). Furthermore, preovulatory estradiol may affect glucose transport, which could enhance conceptus development by

increasing availability of nutrients to the conceptus. Specifically, high estradiol animals had increased mRNA abundance of glucose transporters, SLC2A1 and SLC5A1, in caruncular endometria compared to low estradiol animals, but there was no difference in glucose concentration of uterine luminal fluid (ULF) among groups (Northrop et al., 2018). Estradiol may stimulate gene expression in the uterine epithelium to directly influence uterine receptivity, increasing pregnancy rate.

2.5 Postovulatory effects of progesterone on embryo development

Progesterone is required for the establishment and maintenance of pregnancy in mammals. Increased concentrations of progesterone during the postovulatory period have been associated with increased conceptus elongation and IFNT production, advancement of endometrial gene expression , and higher pregnancy rates (Mann and Lamming, 1999; Mann and Lamming, 2001; Forde et al., 2009). In cattle, the effect of progesterone appears to be on the maternal environment (e.g. uterus) rather than the embryo. Bovine progesterone receptor mRNA expression was detected in the immature oocyte and early embryo through the blastocyst stage, with the exception of the morula stage. However, there was no direct effect of progesterone on bovine embryo development, in vitro, through the blastocyst stage (Clemente et al., 2009). More specifically, addition of progesterone to culture media did not affect cleavage rate or blastocyst development in vitro. Furthermore, embryos produced in vitro in the presence of progesterone and subsequently transferred to synchronized

recipients did not yield a greater recovery rate or embryo elongation on day 14 of pregnancy.

Administration of exogenous progesterone during the early postovulatory period increased conceptus elongation (reviewed by Lonergan and Forde, 2015). Administration of 100 mg of progesterone on days 1 through 4 of pregnancy increased circulating progesterone on days 2 through 5 and increased bovine conceptus elongation by day 14 (Garrett et al., 1988). Recipients primed with a progesterone releasing intravaginal device (PRID), inserted on day 3 after ovulation, produced significantly longer embryos by day 14 after embryo transfer on day 7 compared to recipients that were not treated with progesterone. As circulating concentrations of progesterone did not differ between treatment groups on day 7, these results indicate the increased elongation was mediated through an effect of progesterone on the endometrium (Clemente et al., 2009). Administration of progesterone also advanced embryonic development in sheep and these effects could be blocked by administering the progesterone receptor antagonist, RU486 (Satterfield et al., 2006).

Progesterone stimulates changes in the endometrial transcriptome that are required for uterine receptivity to the embryo. These changes affect the composition of the histotroph, secreted by the uterine glands to support embryo elongation (Spencer et al., 2016). Uterine glands and their secretions are imperative for embryonic development as evidenced by the inability of uterine gland knockout (UGKO) sheep to support development past the blastocyst stage (Gray et al., 2001; Spencer et al., 2007). Advancing the early luteal phase

increase in progesterone, through insertion of a progestin called controlled internal drug release (CIDR) on day 3 after GnRH induced ovulation, increased endometrial *DGAT2* expression on day 7 compared to non-supplemented heifers This is noteworthy because *DGAT2* catalyzes the final step in production of acyl coenzyme A, an enzyme required for production of triglyceride, which is an energy source for developing conceptuses (Forde et al., 2009).

Since progesterone treatment during the early luteal phase advanced conceptus elongation and IFNT production, there have been numerous attempts to increase pregnancy rates in cattle with progesterone supplementation (Lonergan and Forde, 2015). However, attempts to increase pregnancy by providing supplementary progesterone after ovulation have produced mixed results (Clemente et al., 2009). Methods to provide supplemental progesterone include exogenous progesterone administration via intravaginal administration or injection, formation of an accessory CL during the luteal phase, or increasing luteal progesterone secretion by increasing preovulatory follicle size or providing exogenous luteotropic support (e.g. human chorionic gonadotropin, review by Lonergan and Forde, 2015). One of the challenges in providing supplemental progesterone is that timing of the luteolytic secretion of PGF can be advanced thereby inducing luteolysis and decreasing interestrous interval (Ginther, 1970; Garrett et al., 1988, Lonergan and Forde, 2015).

Chapter Three:

EFFECT OF PREOVULATORY ESTRADIOL ON CONCEPTUS ELONGATION AND EMBRYONIC LOSS IN BEEF HEIFERS

3.1 Abstract

In beef cattle, expression of estrus at FTAI is associated with increased preovulatory circulating concentrations of estradiol (E2) and increased pregnancy rates on day 28 of gestation compared to females not detected in estrus. In a reciprocal single-ovulation embryo transfer study, donor cows with greater circulating concentrations of E2, at gonadotropin releasing hormone (GnRH)-induced ovulation (day 0), were more likely to yield an embryo than an unfertilized oocyte, and recipient cows with greater estradiol at GnRH-induced ovulation had an increased pregnancy rate on day 27. We hypothesized that increased preovulatory estradiol would increase pre-implantation conceptus elongation by day 16 in beef heifers. Ovulation was synchronized in Angus heifers (n=35) as follows: GnRH and an intravaginal progesterone implant (CIDR insert) were administered on day -9; CIDR was removed and prostaglandin $F_{2\alpha}$ (PGF) administered on day -2; and GnRH was injected on day 0. Heifers were separated into a High E2 (n=6) or Low E2 (n=6) group based on circulating E2 on days -2, -1, and 0 as determined by radioimmunoassay. Mean (±SEM) plasma

concentrations of E2 (High E2, 10.2±1.0 pg/mL vs Low E2, 5.0±0.6 pg/mL) and mean (±SEM) dominant follicle diameter (High E2, 13.6±0.6 mm vs Low E2, 11.4±0.6 mm) on day 0 were increased ($P < 0.02$ and 0.05, respectively) in High E2 compared to the Low E2 group. In vivo produced embryos (n=5 per heifer) were transferred on day 7, and reproductive tracts were recovered on day 16 of gestation. The uterus was gently flushed to recover conceptuses. Only one heifer (Low E2) did not have a conceptus on day 16. Mean (±SEM) conceptus number and length did not differ ($P > 0.15$) between heifers in the High E2 (2.3±0.5; 5.2±1.3 cm) and Low E2 (2.5±0.7; 7.1±1.7 cm) groups, respectively. These results do not support the hypothesis that increased circulating concentrations of E2 during the preovulatory period in beef heifers promotes pre-implantation conceptus elongation. Therefore, a second experiment was conducted to examine pregnancy loss between days 7 (transfer of a single embryo) and 36 in heifers exposed to low (3.7±0.3 pg/mL; n=19) or high (7.0±0.4 pg/mL; n=25) preovulatory concentrations of estradiol on day 0 (GnRH-induced ovulation). Heifers allotted to the High E2 group had greater estrous expression, higher ($P <0.0005$) estradiol concentration on day 0, and a larger ($P < 0.05$) ovulatory follicle on day 0 compared to the Low E2 group. Pregnancy was determined on day 22 (circulating progesterone), day 24 (circulating PAGs), day 30 and 36 (ultrasonography). Pregnancy rates did not differ between the Low and High E2 groups on days 22, 24, 30, or 36. Pregnancy rate for the combined Low and High E2 groups was significantly decreased from 100% (ET) by day 22 ($P<0.05$). In

summary, there was no difference between the Low and High E2 groups in conceptus elongation on day 16 or pregnancy rate from days 22 to 36.

3.2 Introduction

Maximizing the proportion of beef heifers and cows that conceive early in the breeding season has a positive effect on weaning weights and subsequent reproductive performance (Schafer et al., 1990). The development of FTAI protocols that precisely synchronize the time of ovulation has provided an effective management tool for accomplishing the preceding goal. At the time of FTAI there are cows that have expressed estrus and those that have not. A meta-analysis of field trials involving 10,116 beef females reported a 27% increase in pregnancy rate to FTAI for animals that expressed estrus compared to those that did not express estrus (Richardson et al., 2016). Estrous expression has generally been positively associated with increased circulating concentrations of estradiol during the preovulatory period and larger ovulatory follicle size (Perry et al., 2005; Perry and Perry, 2008b; Jinks et al., 2013). Furthermore, extending the duration of proestrus and thereby increasing circulating concentrations of preovulatory estradiol increased pregnancy rates to FTAI in beef cattle (Bridges et al., 2010). Consequently, it has been hypothesized that elevated preovulatory concentrations of estradiol (E2) have an important role in the establishment and maintenance of pregnancy in cattle. Identifying the physiological role of preovulatory estradiol in pregnancy establishment may result in new approaches for increasing pregnancy rates to FTAI.

To date, the physiological mechanism by which estradiol/estrous expression results in increased pregnancy rates is unclear. Circulating concentrations of estradiol during the preovulatory period are reported to induce endometrial progesterone receptors in several species, including cattle (Zelinski et al., 1982; Xiao and Goff, 1999), and help establish the time of the luteolytic release of PGF in cattle (Kieborz-Loos et al., 2003). Furthermore, increased preovulatory estradiol increased expression of glucose transporters, SLC2A1 and SLC5A1 in the endometrium of cattle (Northrop et al., 2018). Alternatively, estradiol may have an effect on the preparation of granulosa cells for luteinization and subsequent luteal progesterone secretion (McNatty, 1979). We hypothesized that increased preovulatory concentrations of estradiol would promote conceptus elongation by day 16 (day 0 = estrus) and (or) reduce the incidence of embryonic loss between conceptus elongation (i.e. maternal recognition of pregnancy) and day 36. The objective of the first study was to determine the effect of preovulatory estradiol concentration (high vs. low) on bovine conceptus elongation, and uterine gene expression on day 16 after embryo transfer. The second study was designed to characterize embryo loss through day 36 of gestation, following embryo transfer on day 7, in heifers that had low or high preovulatory circulating concentrations of estradiol.

3.3 Materials and Methods

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

Experiment 1

The purpose of experiment 1 was to determine if there is an effect of low or high circulating concentrations of estradiol during the preovulatory period on embryo elongation on day 16 following GnRH-induced ovulation (day 0).

Animal handling. Pubertal Angus and Angus cross heifers (n=35) were divided into two replicates (n=15 and 20) and ovulation was synchronized as follows: Administration of gonadotropin releasing hormone [GnRH1; Cystorelin, 100 µg intramuscularly (i.m.)] and application of progesterone via a controlled internal drug release (CIDR, 1.38 g progesterone) on day -9 followed by prostaglandin $F_{2\alpha}$ injection (PGF, Lutalyse, 25 mg i.m.) and CIDR removal on day -2 and GnRH (GnRH2; 100 µg i.m.) on day 0 (Figure 3.1).

Estrous detection. Heifers were visually observed for signs of behavioral estrus three times daily from PGF injection (d -2) to GnRH2 (d 0). Estrotect patches (Western Point Inc., Apple Valley, MN) were applied at PGF to assist in estrous detection. Heifers were classified as having expressed estrus if >50% of the gray coating on the patch was removed or if they were observed to stand to be mounted for at least 3 seconds.

Ovarian ultrasonography. Ovarian structures were visualized and measured via transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz transducer; SonoSite Inc.) on day -2 (PGF), day 0 (GnRH2), and day 7 [embryo transfer (ET)]. On days -2 and 0, diameter and location of the largest follicle on either ovary was recorded. On day 7, diameter of the corpus luteum and its cavity, if present, were measured and recorded for each heifer. Follicle diameter

was calculated as the average diameter taken at the widest point and perpendicular to the widest measurement. Corpus luteum volume was calculated as a sphere ($4/3\pi r^3$) and the volume of the luteal cavity, if present, was subtracted from the volume of the CL.

Blood Collection and Hormone Quantification. Blood was collected via coccygeal venipuncture into 10 mL vacutainer tubes containing K_3EDTA (Fisher Scientific, Pittsburgh, PA) and centrifuged at 1,200 x g for 20 minutes at 4℃. Plasma was harvested and stored in 2 mL aliquots at -20℃ until RIA. In experiment 1, blood was collected on days -2 ($PGF_{2\alpha}$), -1, 0 (GnRH2), 4, 7 (ET), 12, and 16 (conceptus collection). Plasma concentrations of estradiol-17β were quantified in both experiments as described previously (Kirby et al., 1997). Sensitivity of the estradiol assay was 0.5 pg/mL. Intraassay and interassay coefficients of variation were 5.8 and 5.4%, respectively. Plasma concentrations of progesterone were quantified in both experiments using the MP Biomedical Progesterone RIA kit as described and validated previously (Pohler et al., 2016). Sensitivity of the assay was 0.05 ng/mL. Intraassay and interassay coefficients of variation were 1.5 and 10.6%, respectively. Heifers in each replicate were allotted to a low (n=6) or high (n=6) estradiol group based on circulating concentrations of estradiol on days -2, -1 and 0. Animals that had decreasing estradiol across all three samples were excluded.

Embryo transfer. To more rigorously test the hypothesis that preovulatory concentrations of estradiol promote conceptus elongation, 5 embryos were transferred to each heifer on day 7. Transfer of multiple bovine embryos into the

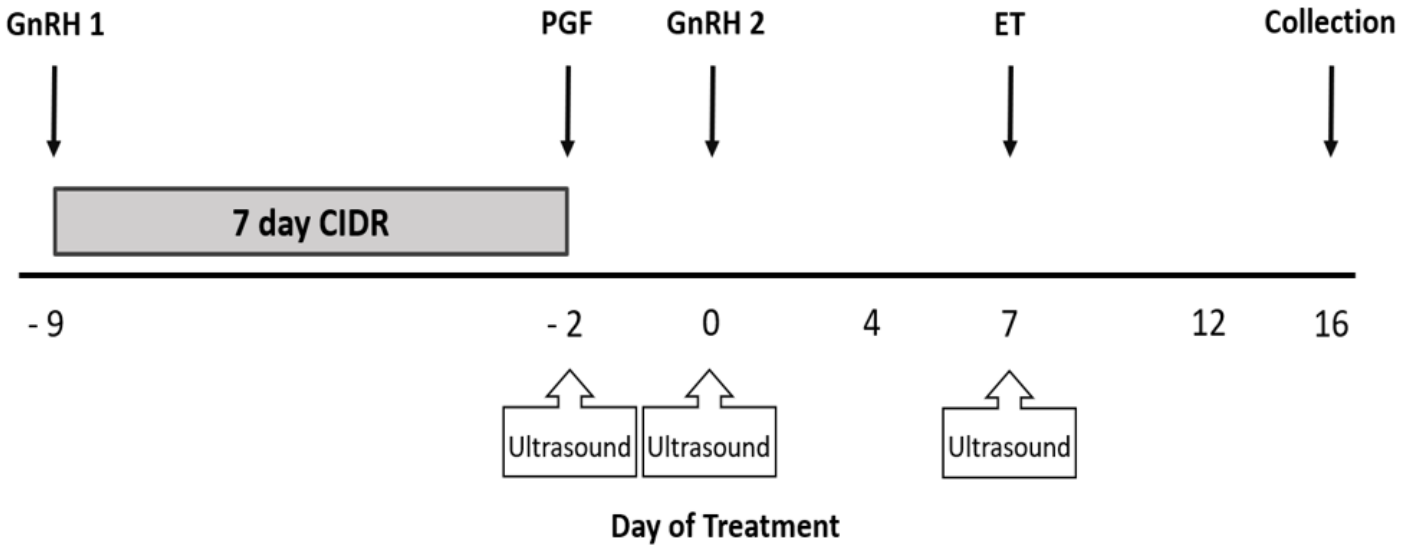

Figure 3.1: Protocol for synchronization of ovulation, blood sample collection, embryo transfer (ET), and conceptus collection for experiment 1. Heifers were assigned to the High or Low E2 groups based on circulating concentrations of estradiol on day 0 (GnRH 2 injection). See materials and methods for further detail regarding the synchronization of ovulation, blood sample collection, examination of ovaries by ultrasonography, embryo transfer, and conceptus collection.

uterus is an experimental approach that has been used by other laboratories (Clemente et al, 2009). Prior to transfer, embryos were balanced across treatment groups for sire, dam, stage of embryonic development, and quality grade. Frozen embryos were thawed in a water bath at 28-32℃ for at least 30 seconds, and washed three times in holding medium (SYNGRO, Agtech, Manhattan, KS, USA). Five embryos in holding media were loaded into a 0.25 mL straw (Agtech) and transferred on the same day. Embryos were transferred on day 7 to the uterine horn ipsilateral to the CL, as previously described (Geary et al., 2016).

Collection of endometrium and conceptuses. Following harvest of the heifers at the University of Missouri abattoir, the uteri were collected and transported to the laboratory. The mean (±SD) time from collection of the uteri to conceptus recovery was 27 ± 11 min. The uterine horn ipsilateral to the CL was flushed with 20 mL of sterile filtered phosphate buffered saline (PBS) to collect conceptuses. Following measurement of individual conceptus length, conceptuses were snap frozen individually in liquid nitrogen, and stored at -80℃. Sections of the uterine horn were collected and fixed in 4% paraformaldehyde (PAF). Uterine endometrium was collected and snap frozen in liquid nitrogen at -80℃. Luteal tissue was collected, weighed, snap frozen in liquid nitrogen, and stored at -80℃.

RNA Extraction and RT-PCR. Endometrium from the ipsilateral horn was homogenized and lysed in Trizol per the manufacturer's instructions (Invitrogen) to extract RNA. Total RNA was extracted using the RNeasy Mini Kit (Qiagen,

Valencia, CA) according to the manufacturer's protocol. The Rneasy MinElute Cleanup kit (Qiagen) containing Dnase I was used to eliminate DNA contamination. Concentrations of extracted total RNA were determined with a Qubit fluorimeter using the RNA reagent kit (Invitrogen) and integrity was verified using 1% agarose gel electrophoresis. Total RNA (500 ng) was reverse transcribed using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). The cDNA was stored at -20℃ until use.

Real-time PCR was performed in a CFX384 Touch Real Time System (Bio-Rad) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Each sample was run in triplicate in 10 µL reactions consisting of 0.5 µL of PrimePCR primer, 5 µL of SYBR Green Supermix, 3.5 µL nuclease-free water, and 1 µL of cDNA. Cycling conditions were as follows: 2 min at 95℃, 5 seconds at 95℃, and 30 seconds at 60.

Commercially validated primers for selected bovine interferon stimulated genes (*ISG15, MX2),* progesterone-stimulated genes*, (NID2, DKK1, PRSS23, RBP4, MEP1B, CTGF, FABP3, SGLT, GRP, IGFBP1, LGAL),* and genes associated with prostaglandin synthesis *(OXTR, SGLT)* were acquired from Bio-Rad (PrimePCR, BioRad). Forward and reverse primers were designed and validated for bovine *SERPINA14* (IDT Technologies, Coralville, IA, USA, Appendix table A.1). *GAPDH* was used as the reference gene for all genes. In addition to *GAPDH*, *SDHA* was used as a housekeeping gene for *ISG15* and *MX2.* The two following controls were performed; 1) Negative control - Primers for *GAPDH* in the absence of template and 2) Reverse transcriptase (RT)

negative control – Addition of water in place of RT followed by PCR with *GAPDH* primers to identify possible genomic contamination. The ΔCT was calculated as the difference between the cycle threshold (CT) for the gene of interest and the CT for *GAPDH*. Fold change was calculated relative to the reference genes ($2^{\Delta CT}$).

Immunohistochemistry. Transverse sections of the uterine horn ipsilateral to the CL were excised and fixed in 4% paraformaldehyde (PAF) for 24 hours followed by rinses with 70% ethanol. Sections were stored at room temperature in 70% ethanol until paraffin embedding. Antigen retrieval was accomplished by microwaving for 10 minutes in a 0.01 M citrate buffer (pH 6). Progesterone receptor was localized using a monoclonal rabbit antibody (1:750 dilution, rabbit monoclonal IgG Clone SP2, Invitrogen, Carlsbad, CA, USA). Estradiol receptor (ESR1) was localized using a monoclonal mouse antibody (1:50 dilution, mouse monoclonal IgG_{2a} Clone 311, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies were detected using Vectastain ABC kits (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin (blue) and a cover slip placed over the stained section (Wang et al., 2018). Positive staining appeared brown.

Quantification of staining intensity was completed using the histogram function of ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/). Images were converted to 8-bit, and a threshold was set by isolating positive and negative cells and measuring minimum and maximum gray value. For measurements of glandular epithelium, a box was placed around at least 5

glands, minimizing the amount of stroma around the glands. For the luminal epithelium, a section of unfolded luminal epithelium was isolated using the freeform function. Values were converted to a weighted average with 100 being the highest intensity and 1 being the lowest positive staining intensity.

Experiment 2

The purpose of experiment 2 was to determine if there was an effect of low or high circulating concentrations of preovulatory estradiol on embryonic mortality from days 7 (embryo transfer) to 36 following GnRH-induced ovulation (day 0). Heifers were allotted to a low or high estradiol group based on circulating concentrations of estradiol on days -2, -1, and 0. Animals that had decreasing estradiol across all three samples were excluded. Three replicates were completed (n= 16, 14, and 14 per replicate).

Synchronization of ovulation, blood collection, and hormone quantification. Synchronization of ovulation and estrous detection were conducted as described in experiment 1 and are shown in (Figure 3.2). A second Estrotect patch was placed on each heifer on day 16 and heifers were observed daily from day 17 to 36 for evidence of estrous activity. Plasma was collected and processed as described in experiment 1 on days -2, -1, 0, 4, 7, 12, and every other day from day 16 to 36. Estradiol (days -2, -1, and 0) and progesterone (all days) were analyzed by radioimmunoassay as described above.

Embryo Transfer. As in experiment 1, embryos were balanced across the treatment groups for sire, dam, stage of embryonic development, quality,

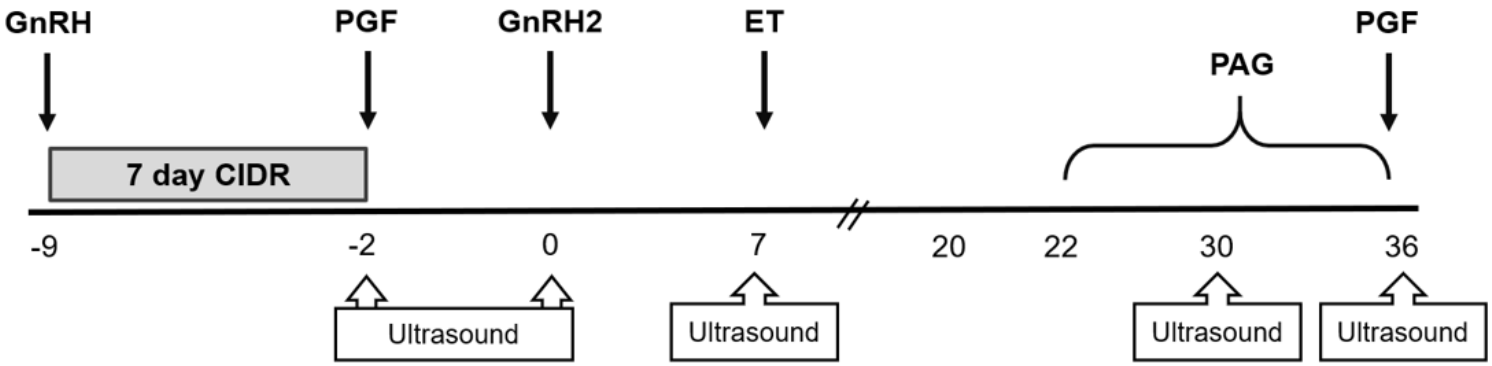

Figure 3.2: Protocol for synchronization of ovulation, embryo transfer, and pregnancy diagnosis for Experiment 2. Heifers were assigned to the Low or High E2 groups based on circulating concentrations of estradiol on day 0. See materials and methods for further detail regarding the synchronization of ovulation, blood sample collection, embryo transfer, measurement pregnancy associated glycoproteins (PAG), and examination of ovaries and uterus by ultrasonography.

and grade. Frozen embryos were thawed and washed as described for experiment 1. One embryo in holding media (Biolife Holding Media; Agtech Inc., Manhattan, KS) was loaded into a 0.25 mL straw and transferred on the same day. Embryos were transferred on day 7 to the uterine horn ipsilateral to the CL. Embryos were thawed and transferred directly without placement in media in replicate C.

Pregnancy diagnosis. In experiment 2, transrectal ultrasonography was used to assess fetal presence and viability (heartbeat) on days 30 and 36 (SonoSite EDGE equipped with a L52 10.0-5.0 MHz transducer; SonoSite Inc.).

Pregnancy associated glycoprotein ELISA. Circulating concentrations of pregnancy associated glycoproteins were used to monitor the presence or absence of an embryo on days 22 to 36 as described below. In addition, circulating PAG concentrations have been used to identify animals experiencing embryonic mortality after day 28 (Pohler et al., 2013).

Quantification of pregnancy associated glycoproteins (PAG) was conducted on plasma samples collected every other day from days 22 to 36 from all pregnant and aborting heifers in both treatments using a sandwich enzyme linked immunosorbent assay (ELISA) previously described by (Green et al., 2005). Each well in a 96 well plate was coated with 100 µL of 10 µg/mL sheep anti-mouse antibody diluted in 0.1 M sodium bicarbonate overnight. The sheep anti-mouse antibody was directed toward the Fc region to help position the three monoclonal antibodies mentioned below. All wells were then blocked with 200 µL of 0.1 M sodium bicarbonate, 2 % nonfat dry milk, 0.02% sodium azide solution

for one hour at room temperature. Wells were incubated with 100 µL of monoclonal antibody mixture containing 50 ng each of three monoclonal antibodies (A6, J2 and L4) directed against bovine PAG and diluted with tris buffered saline with 0.05% tween (TBST) for one hour at room temperature. The preceding solution was removed from the plate by inverting, shaking and patting each plate dry. To keep wells moist, 50 µL of TBST with a 1/20 dilution of blocking proteins (2% ovine albumin, 1% dry nonfat milk) was added to all wells. The standard curve consisted of eleven standards (ranging from 0.01 to 10 ng/mL) produced by a 1:2 serial dilution of the stock PAG concentration (10 ng/mL). For each standard, 50 µL was diluted with 50 µL of steer or nonpregnant heifer serum (NPHS). Each plasma sample or standard (100 µL) was run in duplicate. Plates were incubated at 4℃ overnight. Plasma was removed from the plate by inverting and shaking the plate and the plate was washed with 0.15 M NaCl, 0.05% Tween-20 with a 96-well platewasher (Elx405, BioTek, Winooski, VT, USA) 8 times, turning the plate 180 degrees after 4 washes. All wells were incubated with 100 µL of 1 µg/mL of polyclonal antibody 63 diluted in TBST and 2% NPHS for one hour at room temperature. Fluid was removed by inverting and shaking and the plate was washed 4 times. Then, 100 µL of alkaline phosphatase (AP) conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted to 1:2000 in TBST was added to all wells. Fluid was removed by shaking and the plate was washed 8 times, turning 180 degrees after 4 washes. Then 100 µL of 1 mg/mL p-nitrophenyl phosphate (PNPP) was added to all wells as quickly as possible. Immediately after, the plate was

covered to block exposure to light. After 30 minutes of incubation at room temperature plates were read using an EL808 plate reader (Bio-Tek, Winooski, VT, USA) to measure the absorbance at 405 nm in the wells.

Quantification of pregnancy associated glycoproteins (PAGs) was also conducted on the same plasma samples using the IDEXX ELISA (Idexx Laboratories Inc.; Westbrook, ME) according to manufacturer's instructions (Pohler et al., 2016).

Statistical Analysis. Differences in plasma concentrations of estradiol, progesterone, and PAG were analyzed by analysis of variance for repeated measures in SAS (Proc Mixed; Littell et al., 1998). Differences in follicle size, CL weight, CL volume, conceptus number, and conceptus length were analyzed with an ANOVA (Proc GLM). Differences in pregnancy rate were analyzed using a generalized linear model (Proc Glimmix), using a binomial distribution and the link function of logit. Differences in gene expression and staining intensity for steroid receptors were analyzed using an ANOVA (Proc GLM). Pregnancy associated glycoprotein data was square root transformed to account for heterogeneity of variation between treatment groups. Differences in PAG were analyzed by analysis of variance for repeated measures in SAS (Proc Mixed; Littell et al., 1998). Finally, an F test was used to compare the variances in concentration of PAGs on days 22 to 36 for the Low and High E2 groups.

3.4 Results

Experiment 1

Circulating estradiol increased in both groups from days -2 to 0 and was

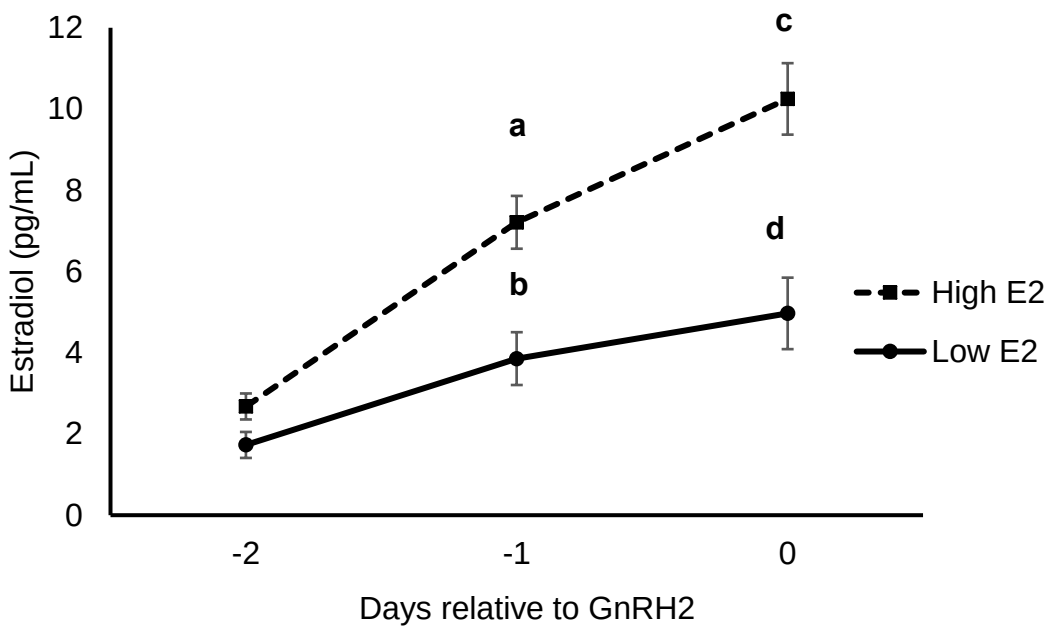

Figure 3.3: Mean (±SEM) circulating concentrations of estradiol on days -2 (PGF), -1 and 0 (GnRH2; see Figure 3.1), as determined by RIA, for heifers assigned to the Low and High E2 groups in experiment 1. Means having different superscripts within day differ ($^{ab}P < 0.02$; $^{cd}P < 0.0005$). (Treatment, P = 0.002; Day, P<0.0001; and Treatment x Day, P = 0.0007).

significantly greater in the High E2 compared to the Low E2 group on days -1 and 0 (Figure 3.3). All heifers in the High E2 group expressed standing estrus; whereas, none expressed estrus in the Low E2 group. Mean dominant follicle diameter was greater on day 0, but not day -2 for the High compared to the Low E2 group (Table 3.1; $P<0.05$) and corpus luteum weight on day 16 tended to be increased in the High E2 group (Table 3.1, $P<0.1$). Mean circulating concentrations of progesterone was similar on days 0, 4, and 16 for both groups, but increased in the High versus Low E2 group on days 7 and 12 (Figure 3.4, $P<0.005$).

Conceptus recovery rate did not differ between groups, nor did mean conceptus length (Table 3.1; Figure 3.5). Length of conceptuses recovered varied greatly within treatment (Figure 3.5) and within recipient. For example, in one heifer conceptus length ranged from 0.2 to 15.4 mm. No embryos were recovered from one heifer and tissue from this heifer was not included in subsequent analysis (Low E2 group). A picture of representative conceptuses collected from the Low and High E2 groups is shown in Appendix Figure A.1.

In both the Low and High E2 groups, progesterone and estradiol receptor (ESR1) were localized to the nuclei of endometrial stromal cells and nuclei of the glandular superficial and deep epithelium on day 16 (Figures 3.6, 3.7, 3.8, 3.9). Estradiol receptor but not progesterone receptor was detected in the luminal epithelium (Figure 3.6 and 3.7). Staining intensity for progesterone (superficial glandular epithelium plus surrounding stroma and deep glandular epithelium plus surrounding stroma) and estradiol receptor (luminal epithelium, superficial

Table 3.1: Mean ± SEM (range) dominant follicle diameter at day -2 (PGF) and 0 (GnRH2), luteal weight, conceptus number, and conceptus length on day 16 for the Low and High E2 groups.

Treatment Group	Follicle Diameter (mm)		Luteal weight (g)	Average conceptus number	Average conceptus length (cm)
	Day -2	Day 0	Day 16	Day 16	Day 16
Low E2	8.6 ± 0.7[c] (7.0-11.2)	11.4 ± 0.6[ad] (9.5 – 13.2)	4.0 ± 0.3[e] (2.8 – 5.1)	3.0 ± 1.3 (0 – 5)	7.1 ± 1.7 (0.2 – 15.9)
High E2	10.6± 0.7[c] (7.1-13.0)	13.6 ± 0.6[bd] (11.4 – 16.0)	4.8 ± 0.4[f] (3.8 – 5.9)	2.2 ± 0.9 (1 – 4)	5.2 ± 1.3 (0.8 – 16)

Superscripts that differ within a row are different ([c,d]P<0.0005)

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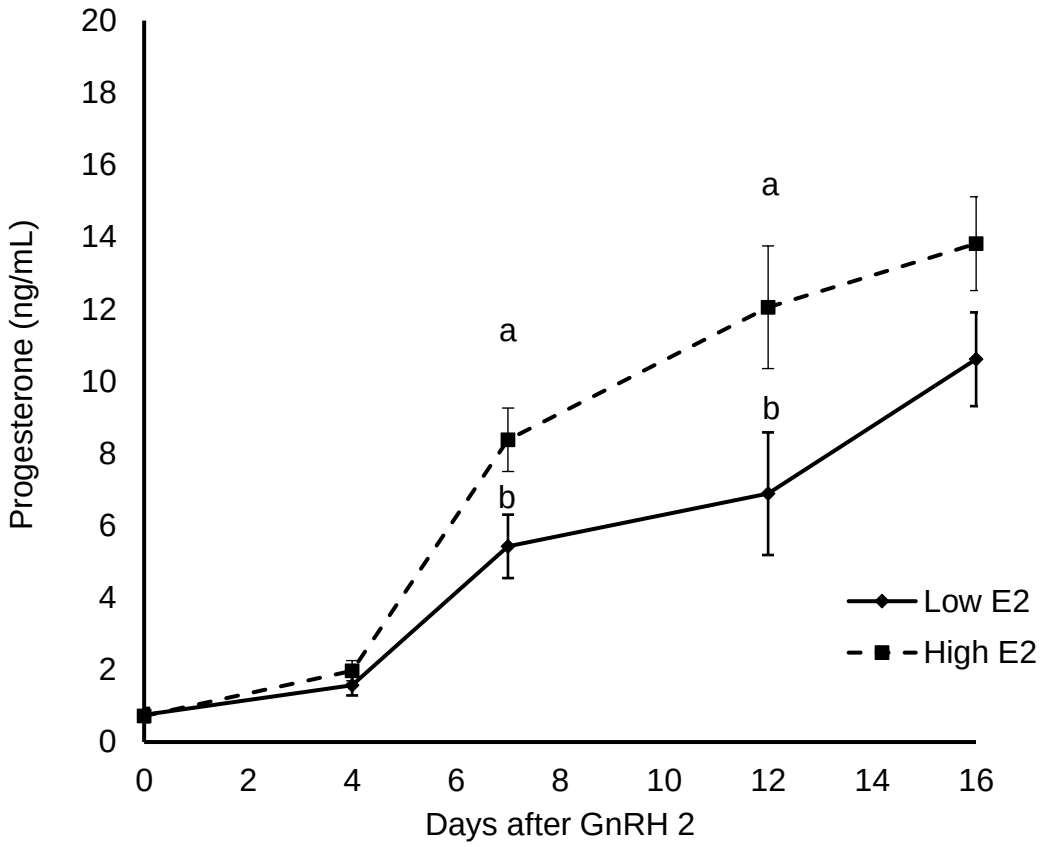

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Figure 3.4: Mean (± SEM) circulating concentrations of progesterone on days 0 (GnRH2), 4, 7, 12, and 16, as determined by RIA, for heifers assigned to the Low and High E2 groups in experiment 1. Concentrations of progesterone differed significantly between groups on day 12 ($^{ab}P < 0.005$). (Treatment, P = 0.16; Day, P<0.0001; and Treatment x Day, P = 0.01).

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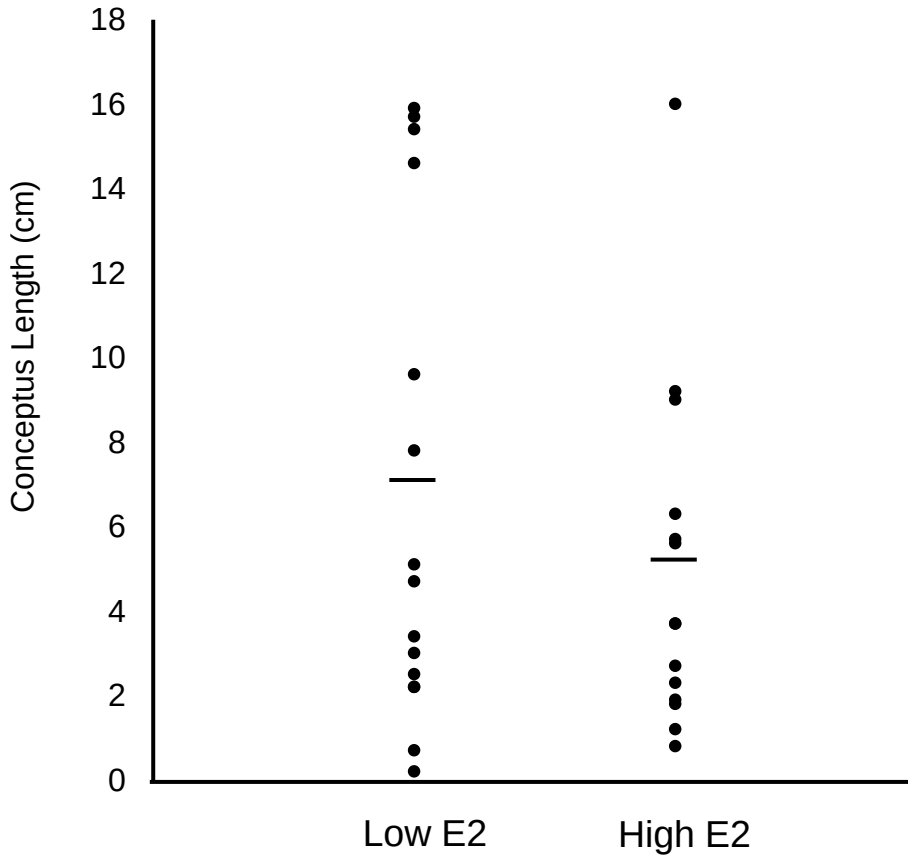

Figure 3.5: Individual conceptus length recovered from each heifer for the low and high E2 groups in experiment 1. The horizontal lines represent mean conceptus length for each group which were 7.1 ± 1.7 and 5.2 ± 1.3, respectively (P=0.4).

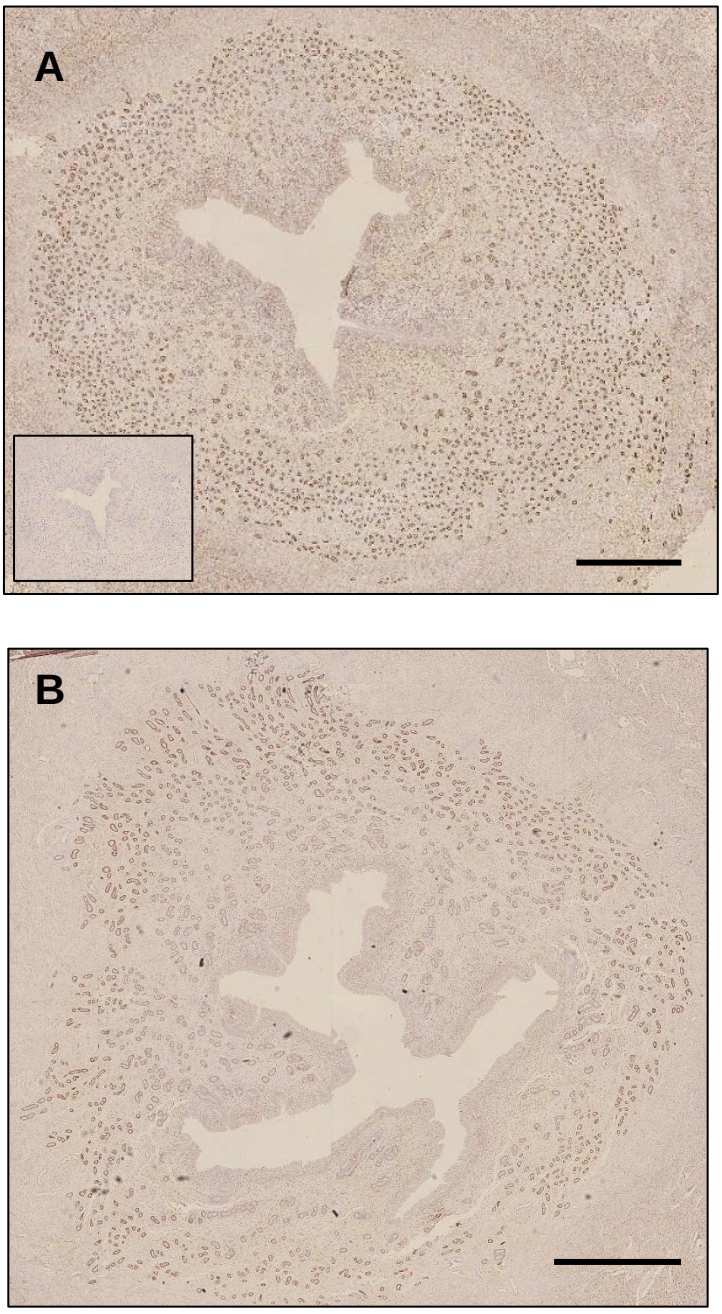

Figure 3.6: Immunolocalization of progesterone receptor (PR; brown staining) for heifers in the High E2 (A) and Low E2 (B) groups. Tissue sections were counterstained with hematoxylin (blue). Note that staining for PR was absent from the luminal epithelium and present in the deep glandular epithelium and stroma of the endometrium. Negative control in inset. Scale bars 1 mm.

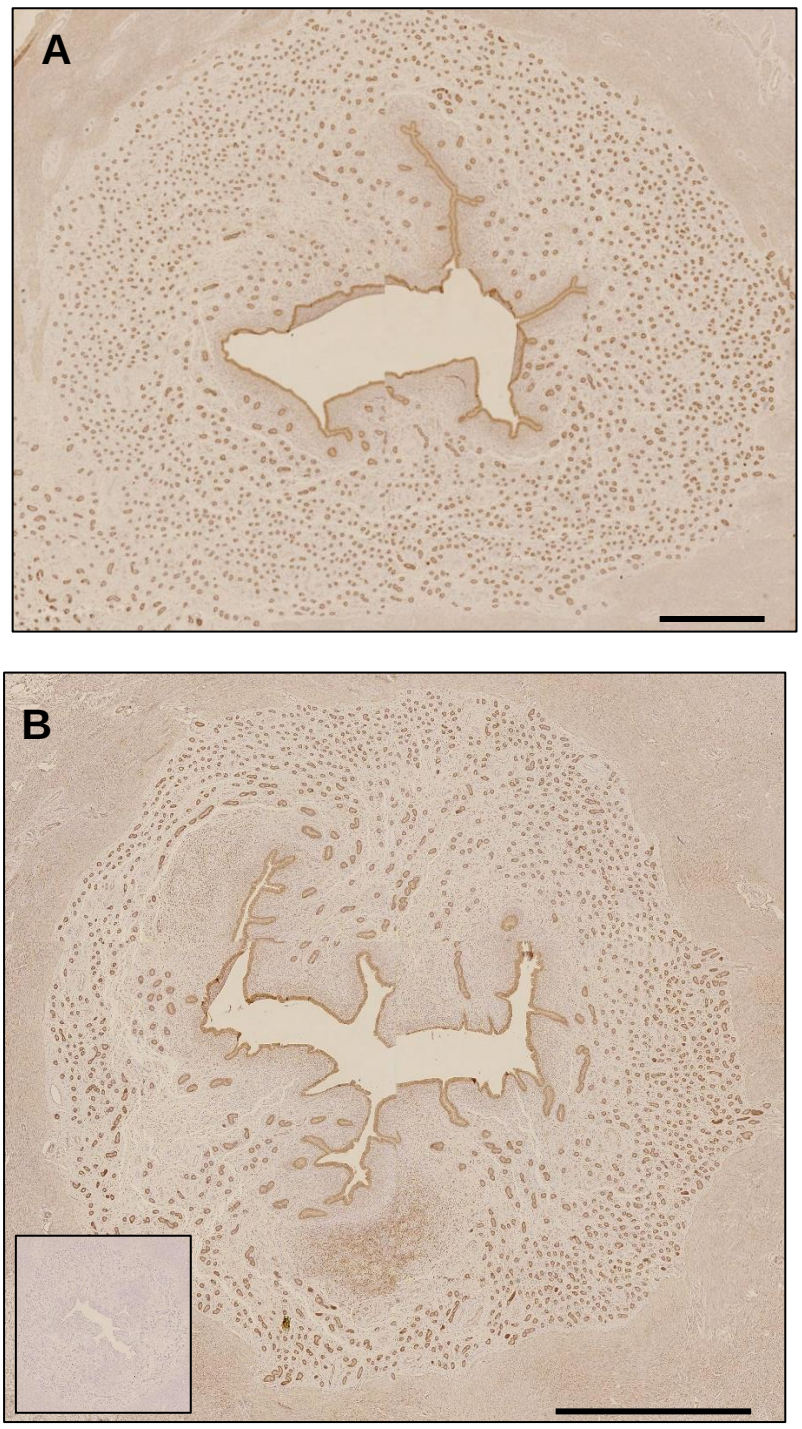

Figure 3.7: Immunolocalization of estradiol receptor (ESR1; brown staining) for heifers in the High E2 (A) and Low E2 (B) groups. Tissue sections were counterstained with hematoxylin (blue). Note that staining for ER was present in the luminal epithelium, superficial glandular epithelium, deep glandular epithelium and stroma of the endometrium. Negative control in inset. Scale bars 1mm.

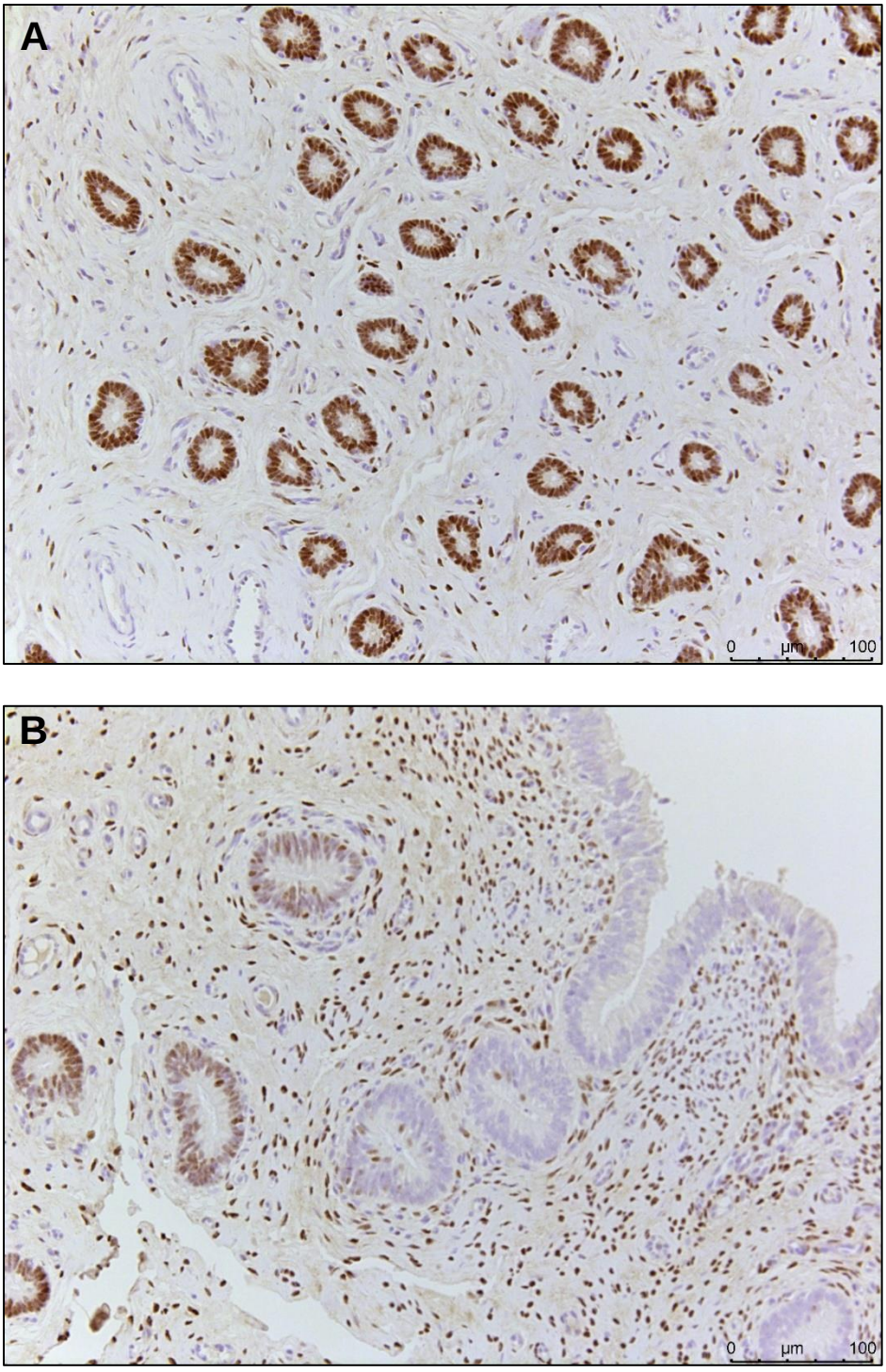

Figure 3.8: Immunolocalization of progesterone receptor (PR, brown staining) in heifers for deep glandular epithelium (A) and superficial glandular and luminal epithelium (B) groups. Tissue sections were counterstained with hematoxylin (blue). Scale bar in the bottom right.

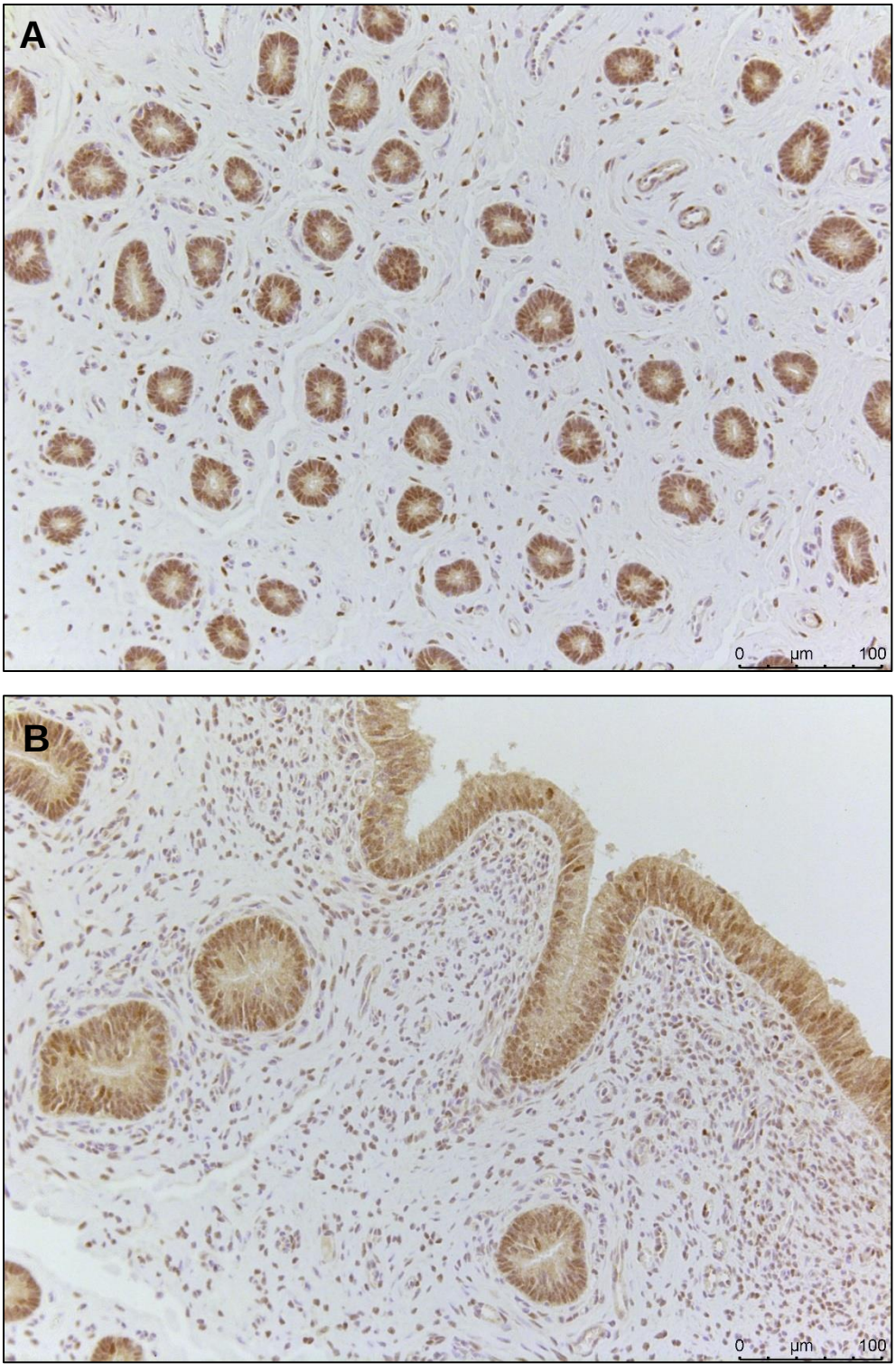

Figure 3.9: Immunolocalization of estradiol receptor 1 (ESR1 brown staining) in heifers for deep glandular epithelium (A) and superficial glandular and luminal epithelium (B) groups. Tissue sections were counterstained with hematoxylin (blue). Scale bar in the bottom right.

glandular epithelium plus stroma, deep glandular epithelium plus stroma, and stroma only) did not differ (P > 0.25) between the High and Low E2 groups (Figures 3.10, 3. 11). Staining intensity for progesterone receptor was greater (P < 0.0001) in the deep glandular epithelium than superficial glandular epithelium in both treatment groups (Figure 3.10). Regardless of treatment group, staining intensity for ESR1 was greater (P < 0.05) in the deep glandular epithelium compared to the superficial glandular epithelium and luminal epithelium. Staining intensity for ESR1 in stroma was lower (P< 0.05) than all the other tissue types regardless of treatment (Figure 3.11).

To determine if preovulatory estradiol affected endometrial gene expression, selected interferon-stimulated genes (ISGs), progesterone-induced genes, and two genes associated with luteolysis were examined. As expected. expression of ISGs was increased in the endometrium of all heifers from which conceptuses were collected compared to the one heifer from which a conceptus was not recovered. For all the genes interrogated, mRNA expression was highly variable within and between the Low and High E2 groups and no significant differences were detected for any of the genes examined (Table 3.2).

Experiment 2

Circulating estradiol increased from days -2 to 0 for the High but not the Low E2 group and was greater in the High compared to the Low E2 group on day 0 (Figure 3.12, P<0.0005). In addition, estrous expression was greater in High E2 versus the Low E2 group (71%, 29%, respectively). Dominant follicle size increased (P< 0.0005) from day -2 to 0 in both High and Low E2 groups, and

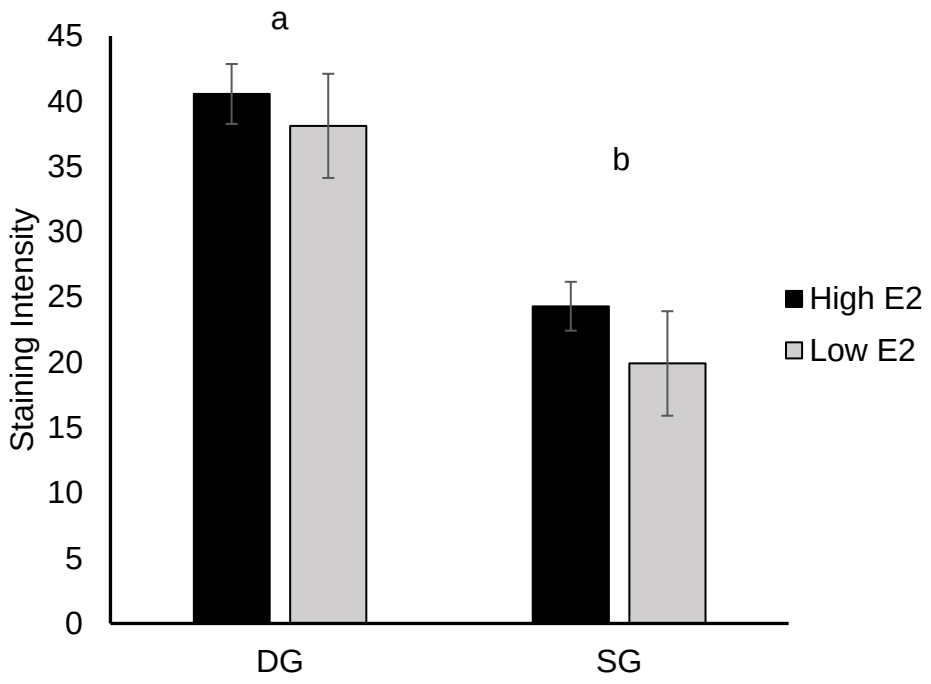

Figure 3.10: Mean ± SEM staining intensity for estradiol receptor 1 (ESR1) in deep glandular epithelium plus stroma (DG) and superficial glandular epithelium plus stroma (SG) in uterine sections for High and Low E2 groups. There were no differences between the low and high E2 groups for any of the tissue types; however, there were differences in staining intensity among the tissue types. Means having different superscripts differ ($^{ab}P<0.0001$).

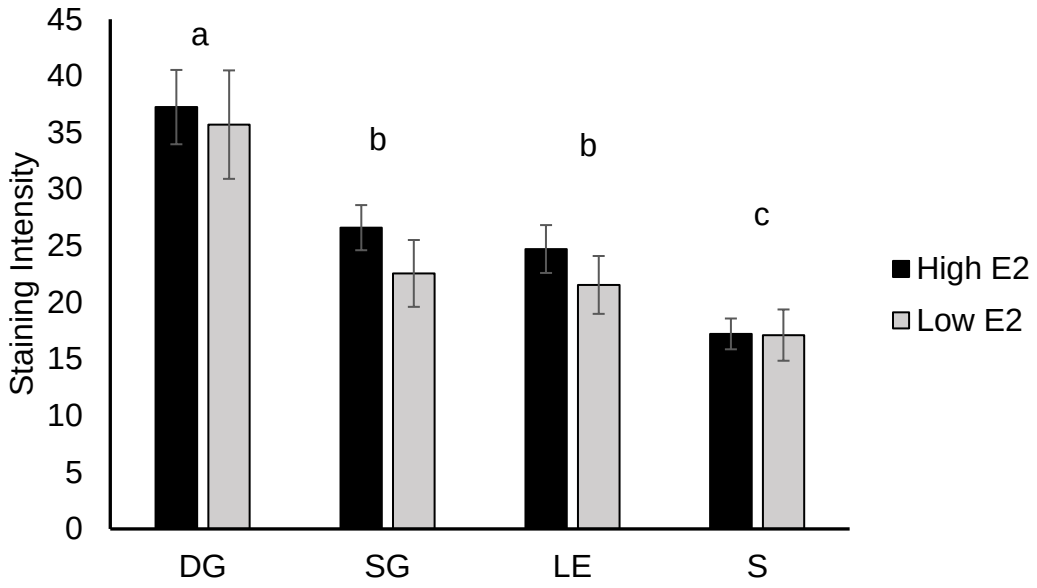

Figure 3.11: Mean ± SEM staining intensity for estradiol receptor 1 (ESR1) in deep glandular epithelium plus stroma (DG), superficial glandular epithelium plus stroma (SG), luminal epithelium (LE), and stroma (S) in uterine sections for High and Low E2 groups. There were no differences between the low and high E2 groups for any of the tissue types; however, there were differences in staining intensity among the tissue types. Means having different superscripts differ ($^{abc}P<0.05$).

Table 3.2: Mean ± SEM fold change in endometrial gene expression relative to housekeeping genes for Low and High E2 groups at collection (day 16).

Gene		Low E2	High E2
IFNT stimulated genes			
	ISG15	74.3 ± 31.7	33.0 ± 18.5
	MX2	2.7 ± 0.8	1.0 ± 0.6
Progesterone stimulated genes			
	NID2	0.7 ± 0.4	1.2 ± 0.6
	DKK1	2.1 ± 0.6	2.5 ± 0.8
	PRSS23	0.4 ± 0.1	0.8 ± 0.7
	RBP4	6.8 ± 0.9	5.2 ± 0.3
	MEP1B	2.2 ± 0.8	1.7 ± 0.2
	CTGF	4.8 ± 1.0	3.2 ± 0.6
	FABP3	5.3 ± 2.4	3.7 ± 1.4
	GRP	719.6 ± 198.4	780.4 ± 788.5
	IGFBP	8.4 ± 1.8	9.0 ± 2.1
	LGALS9	0.2 ± 0.03	0.2 ± 0.04
	SERPINA14	0.9 ± 0.3	0.7 ± 0.2
	SLC5A1	0.3 ± 0.2	0.3 ± 0.3
PGF synthesis			
	OXTR	0.01 ± 0.06	0.01 ± 0.1
	PTGS2	0.3 ± 0.2	0.08 ± 0.1

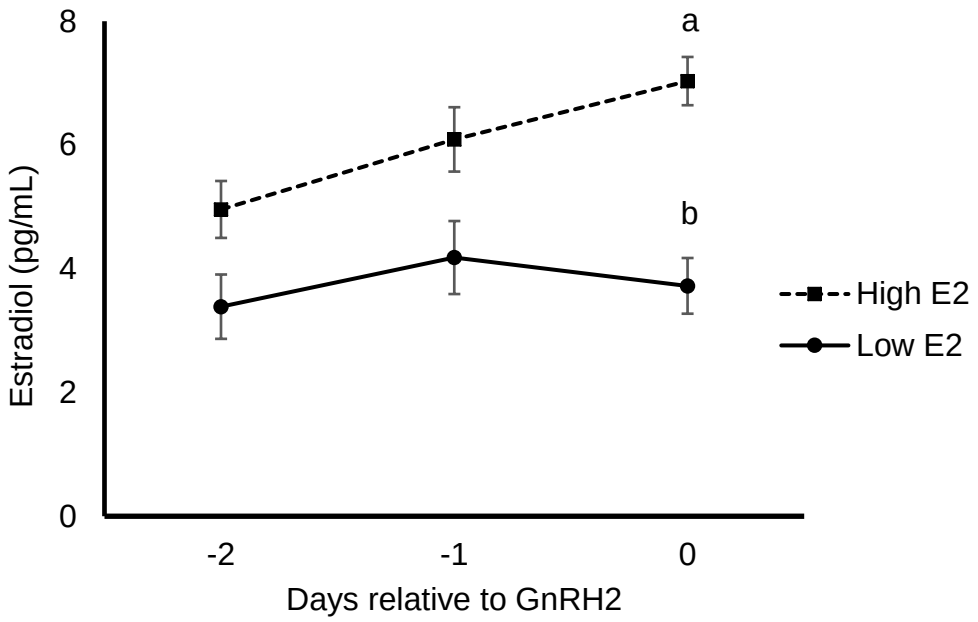

Figure 3.12: Mean (±SEM) circulating concentrations of estradiol on days -2 (PGF), -1 and 0 (GnRH2; see Figure 3.2) as determined by RIA, for heifers assigned to the Low and High E2 groups in experiment 2. Means having different superscripts differ ($^{ab}P<0.0001$). (Treatment, P=0.0006; Day, P<0.0001; and Treatment x Day, P = 0.0042).

dominant follicle size on day 0 was greater (P< 0.05) in the High compared to the Low E2 group. In addition, corpus luteum volume was increased (P< 0.05) in the High E2 group (Table 3.3).

Heifers that experienced a short cycle (n = 3), as determined by P4 <1 ng/mL on day 12, were removed from the study. The rational for removing them from the study is that maternal recognition of pregnancy occurs on days 15 to 16 in cattle and our primary objective was to measure pregnancy loss between maternal recognition of pregnancy and day 36.

Mean circulating progesterone for nonpregnant heifers was similar to pregnant heifers from day 0 to day 18, at which point progesterone decreased rapidly (Figure 3.13). Heifers were classified as pregnant on day 24 if they had circulating progesterone concentrations of >1 ng/mL on days 7 through 24. Circulating progesterone for pregnant heifers was greater in Low E2 compared to High E2 animals on day -2 (Figure 3.14, P<0.0005) and day 32 (P<0.05), but similar between groups on all other days.

Circulating concentrations of PAGs from days 22 to 36 were used to detect the presence of a conceptus between day 24 and ultrasonography on days 30 and 36. Pattern of circulating PAGs (day 22 to 36) did not differ between the IDEXX ELISA and the in house PAG ELISA. Both assays accurately identified heifers as not pregnant, pregnant, or aborting on day 30, as determined by ultrasonography. All heifers designated as being pregnant on day 30 and 36 had evidence of a heartbeat. The first detectable increase (P< 0.0001) in circulating PAGs occurred on day 24 and continued to increase to day 30 to 36

Table 3.3: Mean ± SEM dominant follicle diameter at day -2 and 0 (GnRH2) and luteal volume on day 7 for the High and Low E2 groups (experiment 2).

	Follicle Diameter (mm)		CL volume (cm^3)
	day -2	day 0	day 7
High E2	10.4 ± 0.4^{a}	13.1 ± 0.3^{bc}	3.6 ± 0.3^{c}
Low E2	9.8 ± 0.5^{a}	11.7 ± 0.5^{bd}	2.5 ± 0.3^{d}

Superscripts that differ within a row are significantly different ($^{a,b}P<0.0005$)

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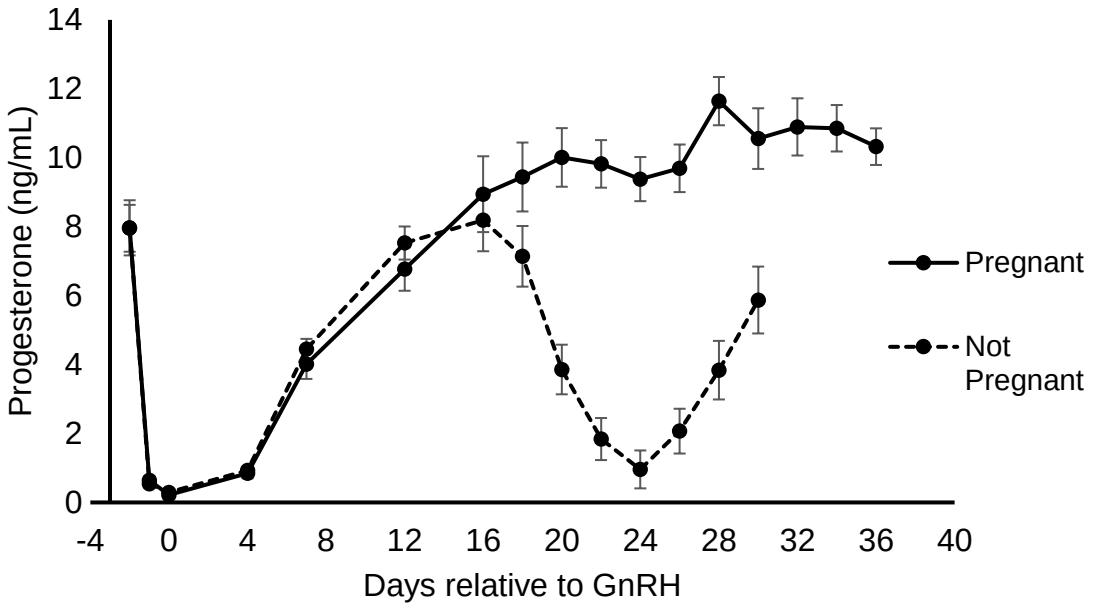

Figure 3.13: Mean (±SEM) circulating concentrations of progesterone, as determined by RIA, for heifers determined to be pregnant (n=15) or not pregnant (n=22) via ultrasound on day 30 following transfer of a single embryo on day 7. Means from days 18 through 30 were different between groups($^{\&}P<0.05$).

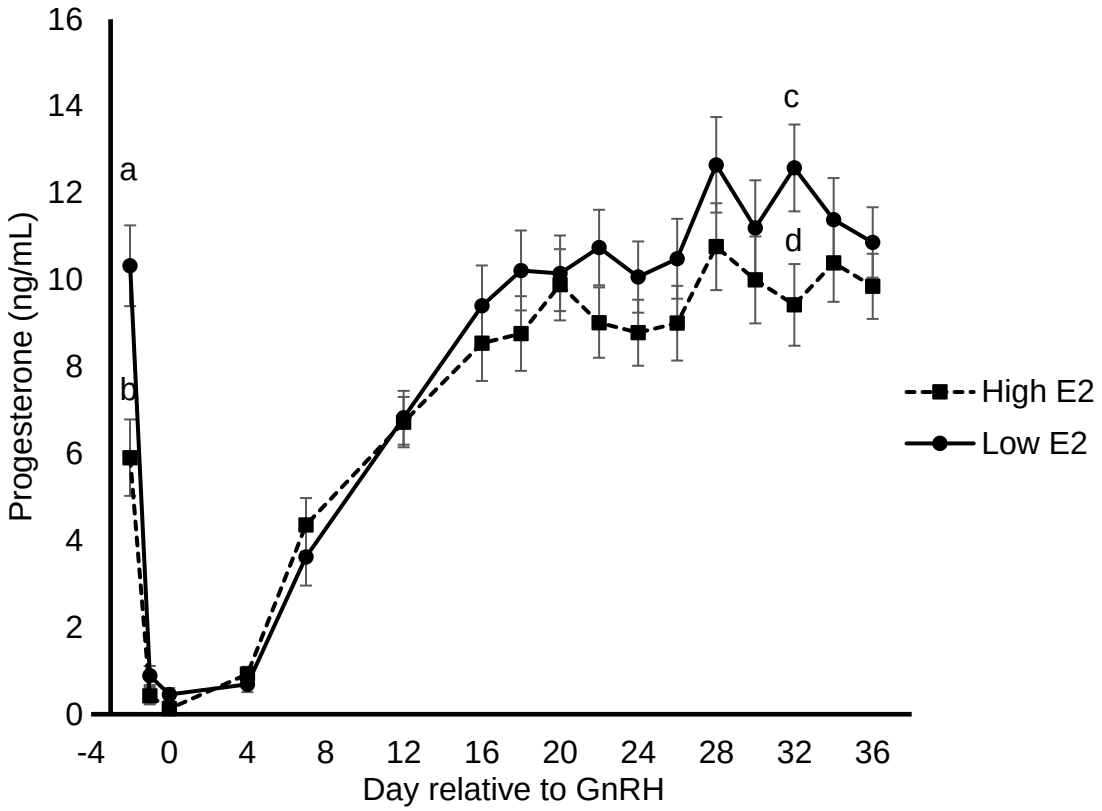

Figure 3.14: Mean (±SEM) circulating concentrations of progesterone from day -2 to day 36 (day 0 = GnRH2), as determined by RIA, for heifers assigned to the Low and High E2 groups (n=8 and 7, respectively) that were determined to be pregnancy via ultrasound on day 30. Means having different superscripts differ ($^{ab}P<0.0005$, $^{cd}P<0.05$). (Treatment, P = 0.14; Day, P<0.0001; and Treatment x Day, P = 0.0098).

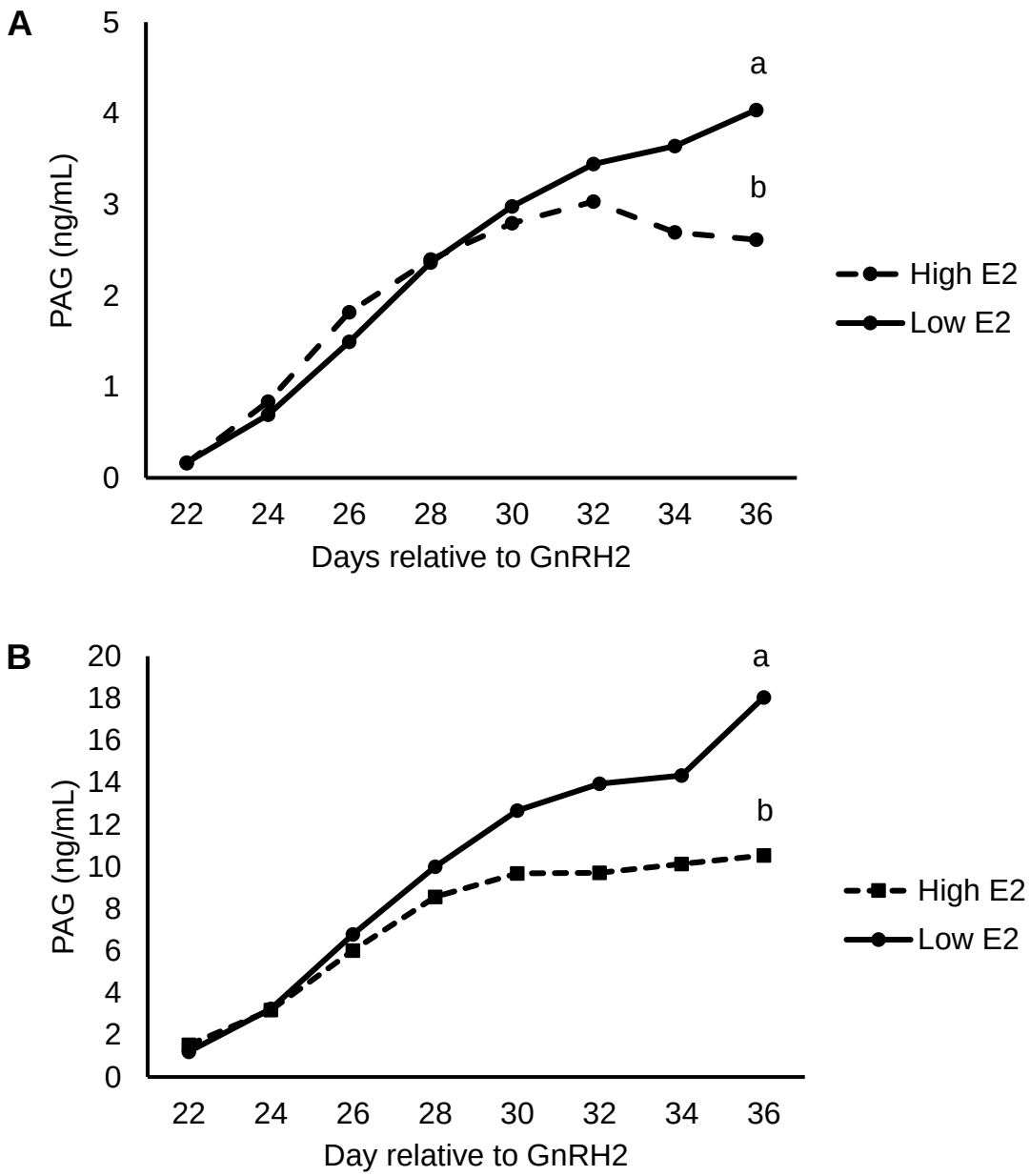

Figure 3.15: Comparison of mean circulating concentrations of PAGs as determined by IDEXX (A) and an in house (B) ELISA for the Low and High E2 groups that were pregnant on day 36 post GnRH2**.** Means having different superscripts differ ($^{ab}P < 0.05$). Due to heterogeneity of variance, the data were transformed prior to analysis; therefore, SEMs are not displayed.

(Figure 3.15). Circulating concentrations of PAGs increased similarly for pregnant heifers in both the Low and High E2 groups until day 36, when they were higher for the Low compared to the High E2 heifers (Figure 3.15, $P<0.05$).

There were four heifers that experienced late embryonic mortality by day 30 (n=3) or day 36 (n=1) based on no evidence of a heartbeat at the time of ultrasonography. Heifers that experienced late embryonic loss by day 30 had elevated circulating concentrations of PAGs on day 30 and evidence of fluid in the uterine lumen and placental membrane fragments, as determined by ultrasonography. One heifer that experienced embryonic mortality had evidence of an embryo with a heartbeat on day 30 but no heartbeat on day 36.

The four heifers that experienced late embryonic mortality by day 36 had decreased PAGs ($P < 0.05$) on day 34 and 36 compared to pregnant heifers (Figure 3.16). In three of four heifers that experienced embryonic mortality, circulating concentrations of PAGs decreased after it was determined the embryo did not have a heartbeat on day 30 (Figure 3.17; Panel A and B) or 36 (Figure 3.17; Panel D). There was one heifer in which the embryo did not have a heartbeat on day 30, but circulating PAGs remained elevated through day 36, at which time no evidence of an embryo or extraembryonic membranes were observed by ultrasonography. In 3 of the 4 aborting heifers, circulating progesterone decreased prior to circulating PAG decrease.

One heifer in both the Low and High E2 groups had detectable, but low circulating concentrations of PAGs from day 22 to 28 and was detected in estrus on day 28 or 21, respectively (Figure 3.18). It is not known whether the

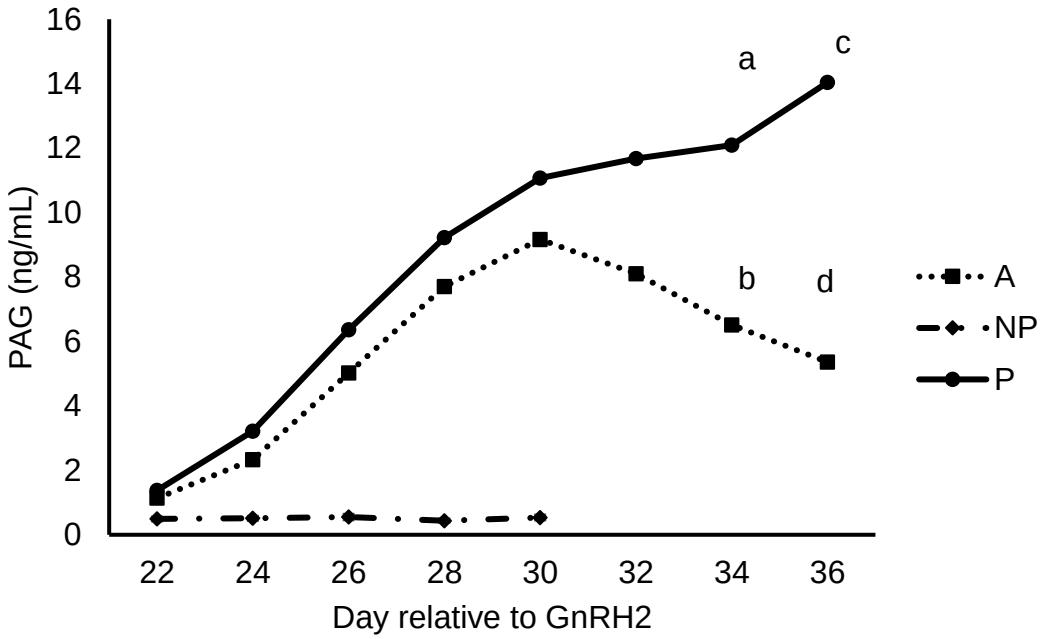

Figure 3.16: Mean circulating concentrations of PAGs, as determined by in house ELISA, for heifers determined to be pregnant (P; n=15), aborting (A; n=4) or not pregnant (NP; n=16) via ultrasound on day 30. Means (P and A groups) having different superscripts differ ($^{ab}P < 0.05$; $^{cd}P < 0.005$).

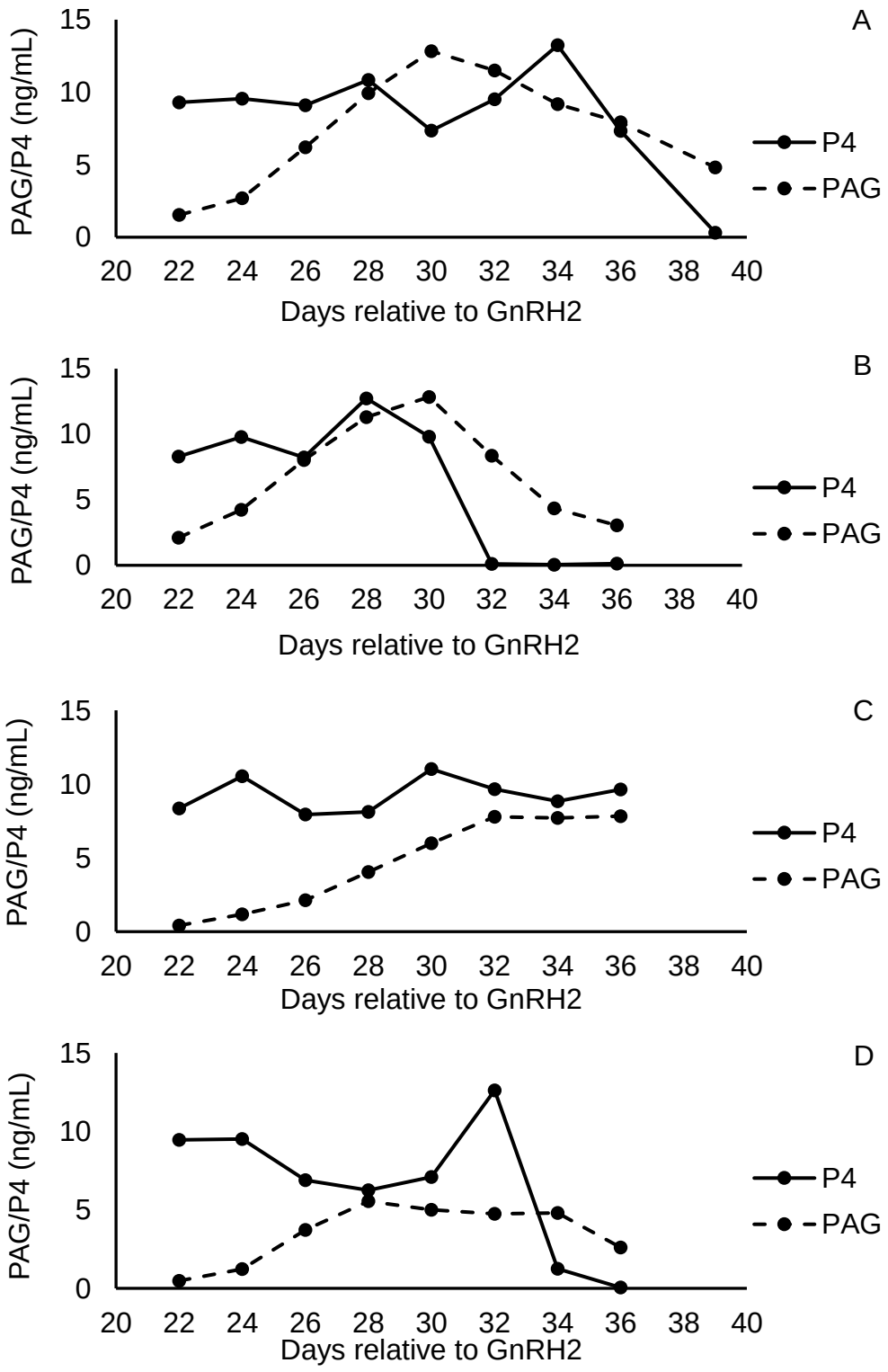

Figure 3.17: Circulating concentrations of PAGs and progesterone for individual heifers that experienced embryonic mortality as determined by increased circulating concentrations of PAGs after day 22 and no evidence of a heartbeat on day 30 (Panel A, B, and C) or on day 36 (Panel D).

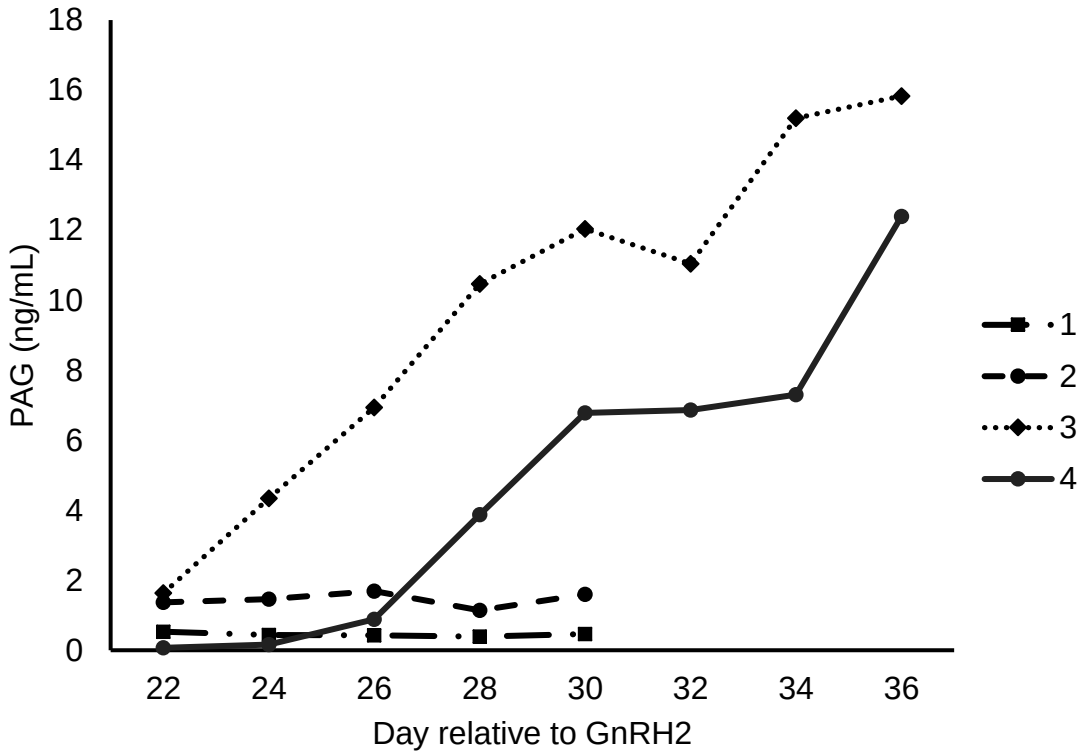

Figure 3.18: An example of circulating concentrations of PAG, as determined by in house sandwich ELISA, for the following heifers: 1) a non-pregnant heifer, 2) a heifer that had detectable PAGs in circulation but expressed estrus on day 28 after GnRH, 3) a pregnant heifer in which PAG increased rapidly (3), or slowly (4).

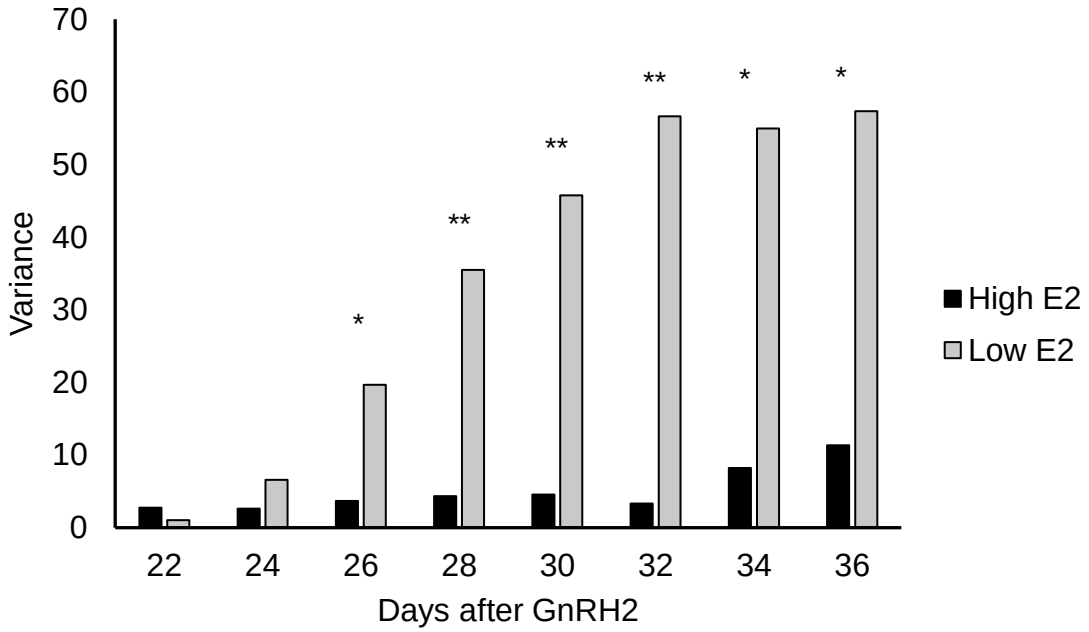

Figure 3.19: Variances associated with circulating PAGs on days 22 to 36 for heifers that were pregnant on day 36 for the Low (n=7) and High E2 (n=8) groups. Variances differ between E2 groups ($^{*}P<0.05$, $^{**}P<0.01$).

preceding heifers experienced embryonic mortality or were not pregnant by day 24. There was considerable variation in circulating concentrations of PAGs among some of the heifers classified as pregnant on day 30 and 36. For example, there was a rapid increase in PAGs beginning on day 24 in some heifers, but others did not increase above 1 ng/mL until day 28 (Figure 3.18). A potentially important observation was the increased ($P<0.05$) variance associated with circulating concentrations of PAGs in the Low versus the High E2 group on days 26 to 36 (Figure 3.19). Pregnancy rate on day 24 (elevated progesterone from day 16 to 24), day 30 (PAGs), and day 36 (Ultrasonography) did not differ between the Low and High E2 groups (Table 3.4, $P> 0.1$). As pregnancy rate did not differ between groups, the data were pooled, and the first significant pregnancy loss occurred by day 22, based on circulating concentrations of progesterone. No statistical difference in pregnancy loss was detected between day 22 and 36 based on circulating PAGs (days 22 to 36) and ultrasonography (days 30 and 36; Table 3.4).

3.5 Discussion

Higher pregnancy rates to FTAI in females that express estrus by the time of FTAI, compared to nonestrous females, has been a consistent observation in cattle (Richardson et al., 2016). Expression of estrus in cattle is positively associated with circulating preovulatory concentrations of estradiol during the preovulatory period (Perry and Perry, 2008b). In the present study, heifers in the High E2 group had a higher incidence of estrous expression and larger dominant follicle size at GnRH-induced ovulation (day 0) in both experiments 1 and 2.

Table 3.4: Pregnancy rate over time as determined by circulating progesterone concentration, pregnancy associated glycoprotein (PAG), and ultrasound.

	ET[1]	Progesterone[2]					PAG[4]	Ultrasound[5]	
Day	7	16	18	20	22	24[3]	30	30	36
High E2	24/24 (100)	24/24 (100)	20/24 (83)	16/24 (67)	14/24 (58)	12/24 (50)	11/24 (46)	8/24 (33)	8/24 (33)
Low E2	17/17 (100)	16/17 (94)	14/17 (82)	12/17 (71)	10/17 (59)	9/17 (53)	8/17 (47)	8/17 (47)	7/17 (41)
Total[6]	41/41[a] (100)	40/41[a] (98)	34/41[a] (83)	28/41[ab] (68)	24/41[b] (59)	21/41[b] (51)	19/41[b] (46)	16/41[b] (39)	15/41[b] (37)

[1] Single embryo was transferred into each heifer on day 7

[2] Proportion (%) of heifers that had plasma concentrations of P4 >1 ng/mL on days 16 to 24.

[3] Heifers that had elevated P4 on days 16 through 24 were considered to be pregnant on day 24.

[4] Heifers that had a PAG concentration >5 ng/mL on day 30 were designated as pregnant

[5] Heifers that had membranes, fluid, and an embryo with a heartbeat on days 30 and 36, were designated as pregnant

[6] Pregnancy rate from day 7 to 36 did not differ between the High and Low E2 groups ($P>0.05$); when the data were pooled (Total) there was a decrease in pregnancy rate on day 22. Superscripts that differ within a row are significantly different ($^{a,b}P<0.05$).

Larger ovulatory follicle size was positively associated with estradiol at GnRH-induced ovulation and pregnancy rates to FTAI in beef heifers (Perry et al., 2007) and postpartum beef cows (Perry et al., 2005). Higher pregnancy rates following GnRH-induced ovulation of large compared to small dominant follicles in beef heifers and cows may be due to an effect of the follicular microenvironment on acquisition of oocyte competence and (or) the maternal environment (reviewed by Pohler et al., 2012, Geary et al., 2013). However, preovulatory estradiol and postovulatory progesterone were the most important factors affecting the establishment of pregnancy in postpartum beef cows following GnRH-induced ovulation (Atkins et al., 2013, Jinks et al., 2013).

Since preovulatory estradiol can induce endometrial progesterone receptors in cattle (Zelinski et al., 1982; Xiao and Goff, 1999) and circulating concentrations of progesterone can affect conceptus elongation (Lonergan and Forde 2015), we examined the effect of low or high preovulatory concentrations of estradiol on conceptus elongation. In ruminants, the rapid elongation of the conceptus coincides with trophoblast production of interferon tau (IFNT), the primary conceptus signal for maternal recognition of pregnancy (Spencer and Hansen, 2015). In sheep, conceptus elongation on day 14 is relatively uniform (Rowson and Moor, 1966; Wales and Cuneo, 1989; TE Spencer, personal communication) and a delay in conceptus elongation resulting in inadequate production of IFNT is considered a cause of early embryonic mortality. Bovine conceptus elongation begins between days 12 to 14 and continues throughout the period of maternal recognition of pregnancy (days 15 to 16). Considerable

variation in the degree of bovine conceptus elongation occurs by the time of maternal recognition of pregnancy (Berg et al., 2010; Geary et al., 2016; Moraes et al., 2018). In the present study, conceptus length was highly variable within recipient and between the Low and High E2 groups on day 16 and there was no effect of preovulatory concentration of estradiol on the number or length of conceptuses on day 16 after GnRH2. These results are consistent with previous studies that examined the effect of preovulatory estradiol on conceptus elongation in cattle (Bridges et al., 2012; Northrop et al., 2018).

It is possible that preovulatory concentrations of estradiol are a reflection of the size or maturity of the ovulatory follicle and that there is an indirect effect of estradiol on the uterus via postovulatory progesterone and (or) induction of endometrial progesterone receptors. In postpartum beef cows, there was a positive correlation between ovulatory follicle size and circulating concentrations of estradiol (Jinks et al., 2013) and preovulatory estradiol had a positive effect on postovulatory progesterone, that was independent of ovulatory size (Atkins et al., 2013). In the present study, circulating concentrations of progesterone were increased on days 7 and 12 in experiment 1 but not in experiment 2. In addition, CL weight on day 16 (experiment 1) and volume on day 7 (experiment 2) tended to be larger in the High compared to the Low E2 group. Increased preovulatory concentrations of progesterone during the early luteal phase following GnRH-induced ovulation may be due to larger ovulatory follicle size leading to increased luteal volume and(or) an effect of the follicular microenvironment (e.g. estradiol) on the preparation granulosa cells for luteinization (see Chapter 4)

Although elevated circulating progesterone during the early luteal phase increased conceptus elongation in cattle (reviewed by Lonergan and Forde, 2015), there was no increase in conceptus elongation when circulating progesterone was elevated on days 7 and 12 in the High compared to the Low E2 group (experiment 1). Circulating concentrations of progesterone were greater in pregnant cows compared to nonpregnant cows on day 6 (Mann and Lamming, 1999) or 9 (Perry et al., 2005); however, there was no difference in circulating progesterone between pregnant and nonpregnant cows from day 0 to 18 (experiment 2)

Interferon tau is produced by the trophoblast of bovine embryos around the time of maternal recognition of pregnancy and induces expression of interferon stimulated genes in the endometrium including *ISG15* and MX2(see review by Spencer and Hansen, 2015). Although there was no difference in endometrial gene expression between pregnant and nonpregnant cows up to the time of IFNT production, there were pronounced differences after that time (Forde et al., 2011). In experiment 1, interferon stimulated genes (i.e. *ISG15* and *MX2*) were highly expressed in the endometrium of heifers from which embryos were collected, but not from the heifer that did not have any embryos. There was no effect of low versus high preovulatory concentrations of estradiol on progesterone or IFNT stimulated genes in this study. Since multiple embryos (n = 5) were transferred into each heifer and no difference was detected in conceptus recovery and length, it is not surprising that IFNT simulated gene expression was similar between the High and Low E2 groups.

Estradiol reportedly induces endometrial progesterone receptors in numerous species including cattle (Zelinski et al., 1982; Xiao and Goff, 1999) and progesterone receptor binding was increased on day 6 postestrus in postpartum beef cows that had high versus low preovulatory concentrations of estradiol (Zollers et al., 1993). Therefore, we investigated whether preovulatory estradiol had an effect on progesterone and estradiol receptor staining intensity, by immunohistochemistry, in the endometrium on day 16. Although Bridges and coworkers (2012) found that staining intensity for progesterone receptor was greater in the High E2 than Low E2 group in the deep glandular epithelium on day 15.5 of pregnancy, no such difference was found in this study.

Progesterone is required for establishment and maintenance of pregnancy in mammals and in cattle the effect of progesterone is mediated through the endometrium rather than a direct effect on the bovine embryo (Clemente et al., 2009). Therefore, we investigated the effect of low or high preovulatory concentrations of estradiol on several progesterone-stimulated genes in the endometrium known to be mitogens (*GRP and CTGF*), promote migration/attachment (*IGFBP1, RBP4, and LGALS9*), or that have been associated histotroph (*SLC5A1 and FABP3*) to determine if preovulatory estradiol could influence subsequent uterine receptivity. However, there was considerable variation in expression of each gene within and between treatments and no difference between the Low E2 and High E2 groups was detected.

In postpartum beef cows, a short versus long luteal phase was associated with an advance in the luteolytic secretion of PGF (reviewed by Garverick et al.,

1992). It was further determined that a short luteal phase in cattle was preceded by decreased preovulatory concentrations of estradiol and on day 6 of the subsequent short luteal phase there was a decrease in endometrial progesterone receptor binding and an increase in endometrial oxytocin receptor binding compared to cows having a normal luteal phase (Zollers et al., 1993). Preovulatory estradiol is required for establishing the timing of PGF secretion during a normal estrous cycle in postpartum beef cows (Kieborz-Loos et al., 2003). In addition, INFT inhibits luteolysis around the time of maternal recognition of pregnancy in sheep by inhibiting expression of ESR1, which is required for estradiol to induce endometrial oxytocin receptors in the endometrium in sheep (reviewed by Spencer and Hansen, 2015). Davoodi and coworkers (2016) conducted a study to evaluate the effect of estrous expression on endometrial gene expression in cattle. As mentioned previously, estrous expression in cattle is positively correlated with preovulatory concentrations of estradiol (Perry and Perry, 2008b). The authors reported that endometrial mRNA expression of oxytocin receptor and prostaglandin synthase-2 was decreased on day 19 (day 0 = estrus) in pregnant cows that expressed estrus at artificial insemination compared to pregnant cows that did not express estrus (Davoodi et al., 2016). However, in the present study, there was no effect of Low or High E2 on expression of oxytocin receptor or prostaglandin synthase on day 16.

Due to the lack of difference in conceptus recovery rate, conceptus elongation, intensity of progesterone and estradiol receptor staining, and endometrial gene expression on day 16 between heifers in the Low E2 versus

High E2 groups (experiment 1), a second experiment investigated embryonic mortality from day 7 (embryo transfer) through day 36. Madsen and coworkers (2015) found the majority of embryo loss in cattle occurred between maternal recognition of pregnancy (day 17-21) and day 28 of pregnancy following transfer of embryos into ovariectomized cows that received either no estradiol, estradiol benzoate, or estradiol cypionate during a simulated preovulatory period. In the preceding study pregnancy rates were 4 and 25% for control and estradiol treated (estradiol benzoate or cypionate), respectively. A likely explanation for the lower pregnancy rate in the control group compared to the estradiol treated groups is that estradiol is required to induce progesterone receptors in the endometrium and program the normal time of PGF release in a nonpregnant heifer or cow (Kieborz-Loos et al., 203). The present study failed to support the hypothesis that preovulatory estradiol improves pregnancy rate on day 36 following embryo transfer. There was no difference in the timing or extent of embryonic mortality between the High and Low E2 groups on days 22 determined by circulating progesterone); 24 (determined by circulating progesterone), 30 (determined by PAGs and ultrasonography), and 36 (determined by ultrasonography).

Pregnancy-associated glycoproteins are abundantly expressed products of the placenta of species within the *Cetartiodactyla* order (even-toed ungulates; (Szafranska et al., 1995; Xie et al., 1997; Garbayo et al., 2000; Green et al., 2000; Brandt et al., 2007). Although, no clear function for PAGs has been identified, their accumulation in maternal blood of ruminant ungulates has

become a useful tool for monitoring pregnancy. Indeed, a majority of the published work on PAGs has focused on development of a reliable tool for diagnosing pregnancy in ruminant species, including cattle, sheep, goats, bison, moose and elk (Sasser et al., 1986; Szafranska et al., 2006; Wallace et al., 2015). In addition to serving as an accurate tool for diagnosing pregnancy in ruminants, PAGs can serve as a marker for monitoring embryonic/fetal viability and placental function (Pohler et al., 2013). Consistent with previous studies, mean circulating concentrations of PAGs were elevated on day 24 for pregnant compared to nonpregnant heifers and concentration of PAGs continued to increase from day 24 to days 30 to 36, as reported by others (Wallace et al., 2015). There was variation among pregnant heifers in when circulating PAGs first increased with some heifers increasing (>1 ng/mL PAGs) on day 22 while others were not elevated until day 28. The increased variance associated with concentrations of PAGs on days 26 to 36 in the Low E2 compared to High E2 group was an unexpected finding. The physiological significance of these results is not clear and requires future investigation. Perhaps the increased variance associated with circulating PAGs in the Low versus High E2 group is a reflection of increased variation in number of giant binucleated trophoblast cells, endometrial environment, or placentation.

Reese and coworkers (2018) demonstrated that low circulating PAGs on day 24 was predictive of embryonic loss. In the current study, circulating PAGs were significantly lower in 3 of 4 heifers on days 34 and 36 that experienced late embryonic mortality compared to heifers that maintained pregnancy to day 36.

The first significant decrease in pregnancy rate for the pooled data from experiment 2 (Combination of Low and High E2 heifers) occurred by day 22, which is similar to what others have reported regarding the timing of embryonic loss in cattle (reviewed by Ayalon, 1978; Sreenan and Diskin, 1986). However, the lack of effect of low versus high preovulatory estradiol on pregnancy rate by day 36 in the current study was surprising. Numerous studies in cattle from various laboratories in the USA and Brazil have reported positive associations among preovulatory estradiol, ovulatory follicle size, expression of estrus, and pregnancy rate (reviewed by Pohler et al., 2012; Geary et al., 2013; Richardson et al, 2016).

One of the studies that provided a strong rational for conducting experiments 1 and 2 was reported by Jinks et al. (2013). A single ovulation, reciprocal embryo transfer study was conducted in which embryos were transferred from donor cows with either high (>8.4 pg/mL) or low (<8.4 pg/mL) estradiol into recipient cows with either high or low estradiol on day 0. Pregnancy rates were increased when embryos were transferred from the low into high and high into high groups (65, 61%, respectively) compared to the low into low and high into low groups (45, 43%, respectively). The preceding results provide strong evidence for an effect of increased preovulatory estradiol on pregnancy rate mediated through the uterus and (or) postovulatory progesterone. A large-scale embryo transfer study (>700 cows), by our laboratories, is currently underway to separate the effects of preovulatory estradiol and postovulatory progesterone in postpartum beef cows. Thus far, the data indicate that

pregnancy rate increased as circulating estradiol on day 0 (GnRH-induced ovulation) and change in estradiol from PGF-induced luteolysis to day 0 increased, but no data on the effect of postovulatory progesterone on pregnancy rate is currently available (Ciernia et al., 2018).

The reasons for the lack of effect of preovulatory estradiol are not clear. It is possible that the difference in circulating estradiol between the Low and High E2 groups, while statistically significant, was not sufficient to result in differences in pregnancy rate by day 36. In the study by Ciernia et al. (2018), pregnancy rate following embryo transfer in postpartum beef cows on day 30 to 34 for cows with low (2.4±0.7 pg/mL), middle (4.0±0.5 pg/mL), and high (5.8±1.0 pg/mL) preovulatory estradiol were 28^{a}, 42^{b}, and 55^{b}%, respectively, ($^{ab}P < 0.05$). In the current study, preovulatory estradiol was 3.7 and 7.0 pg/mL in the low and high E2 groups compared to 2.4, 4.0, and 5.8 pg/ml in the low, middle, and high E2 groups in the study by Ciernia et al. (2018). For the preceding study, it is noteworthy that pregnancy rate did not differ between the middle (4.0 pg/mL) and high (5.8 pg/mL) E2 groups, indicating that the low level of estradiol in the current study, may not have been low enough to produce a difference in pregnancy rate. Alternatively, the effect of preovulatory estradiol on pregnancy rate may not be the same as in postpartum beef cows. The majority of the data generated on the relationship between estrus, preovulatory estradiol, ovulatory follicle size, and subsequent pregnancy rate has been in cows and not heifers. Physiological differences between postpartum beef cows (e.g. lactational status) and heifers

may be a contributing factor. In summary, preovulatory estradiol did not affect conceptus elongation or pregnancy rate on day 36.

Chapter Four:

EFFECT OF INTRAFOLLICULAR ADMINISTRATION OF AN ESTRADIOL RECEPTOR ANTAGONIST INTO A DOMINANT FOLLICLE DURING THE PREOVULATORY PERIOD ON SUBSEQUENT LUTEAL PROGESTERONE SECRETION IN BEEF COWS

4.1 Abstract

Luteinization is initiated following the preovulatory gonadotropin surge and prior to follicular rupture. Human granulosa cells collected from follicles with high follicular fluid concentrations of estradiol produced more progesterone, *in vitro*, than cells from follicles with low follicular fluid concentrations of estradiol. Furthermore, addition of estradiol to granulosa cells in culture resulted in an initial inhibitory effect on progesterone production and a subsequent stimulatory effect after removal of estradiol. We hypothesized that preovulatory, intrafollicular administration of an estradiol receptor antagonist, ICI 182,780, would decrease subsequent luteal progesterone secretion. The objective was to determine the effect of blocking estradiol's action through the genomic receptor in granulosa cells of the preovulatory follicle, *in vivo*, on progesterone secretion from the subsequent corpus luteum (CL). Ovulation was synchronized in nonlactating beef cows (n=35) as follows: administration of gonadotropin releasing hormone (GnRH) and a CIDR on d-7, CIDR removal and PGF injection on day -2, and GnRH injection on day 0. Cows were allocated to one of three groups (control; n

= 10, vehicle injection; n = 10, and ICI 182,780 injection n = 10) and balanced for dominant follicle size on day -2. Intrafollicular injection of vehicle (100 µl) or the antagonist (25 µg in 100 µl) into the largest follicle on either ovary occurred on day -2. Plasma samples were collected on days -2, -1, and 0 (GnRH2) and every other day from day 2 to 20. Plasma concentrations of estradiol on days -2, -1 and 0 increased ($P< 0.0001$), and did not differ among treatment groups. Furthermore, the dominant follicle increased in diameter from day -2 to 0 and was similar among groups. Plasma concentrations of progesterone did not differ ($P>0.5$) among treatment groups for any days sampled and luteal volume was similar on days 8 and 14. These results do not support the hypothesis that estradiol, acting on the genomic receptor, is required to prepare granulosa cells to luteinize during the preovulatory period and therefore produce more progesterone during the subsequent luteal phase.

4.2 Introduction

The corpus luteum is an extension of follicular maturation; therefore, physiological changes during the development of a preovulatory follicle may affect subsequent progesterone secretion. Premature induction of ovulation in ewes with GnRH was associated with luteal insufficiency that may have resulted from a reduced number of follicular cells and/or inadequate preparation of follicular cells for luteinization and subsequent secretion of progesterone (Murdoch and Van Kirk, 1998). Atkins et al. (2013) reported that circulating concentrations of estradiol at GnRH-induced ovulation was positively associated with circulating progesterone seven days later and that this effect was

independent of ovulatory follicle size in postpartum beef cows. The ability of luteinized human granulosa cells to secrete progesterone increased when the cells were collected from follicles having increased follicular fluid concentrations of estradiol (McNatty et. al., 1979). In addition, ewes treated with an aromatase inhibitor prior to induced ovulation had a delayed rise in serum progesterone (Benoit et al., 1992). Furthermore, administration of estradiol to granulosa cells, in culture, resulted in an initial inhibitory effect on progesterone production, but a subsequent stimulatory effect on progesterone after removal of estradiol (Fortune and Hansel, 1979). The preceding inhibitory effect of intrafollicular estradiol may have been mediated by inhibition of conversion of pregnenolone to progesterone (3β Hydroxysteroid Dehydrogenase; 3β-HSD) to provide more pregnenolone as a substrate for androstenedione biosynthesis in thecal cells via the delta 5 pathway of steroidogenesis (Fortune and Quirk, 1988). Removal of estradiol from the media may have increased conversion of pregnenolone to progesterone by luteinizing granulosa cells.

Estradiol has also been shown to inhibit apoptosis (Quirk et al., 2006), increase granulosa cell proliferation (Goldenberg et. al., 1972), promote formation of gap junctions between granulosa cells (Merk et al., 1972), increase the stimulatory action of FSH on aromatase activity (Zhuang et al., 1982), regulate the expression of steroidogenic enzymes (Gore-Langton and Armstrong, 1994), and enhance progestin synthesis following gonadotropin stimulation (Welsh et al., 1983). Therefore, the stimulatory effect of estradiol on subsequent

progesterone synthesis could be due to increased granulosa cell number and(or) a direct effect on the ability of granulosa cells to luteinize.

Two genomic estradiol receptors (ESR1 and 2) have been localized to bovine granulosa cells (Beker-van Woudenberg, 2004). ICI 182,780 blocks dimerization of the estradiol receptor (ESR) complex and the subsequent genomic action of ESR1 and ESR2 (Wakeling et al., 1991). We hypothesized that intrafollicular injection of an estradiol receptor antagonist, ICI 182,780, into a dominant follicle during the preovulatory period, would decrease subsequent luteal progesterone concentrations following GnRH-induced ovulation but have no effect on corpus luteum lifespan. Therefore, this study examined the effect of antagonizing ESR1 and 2, *in vivo*, following intrafollicular injection of ICI 182,780 on subsequent corpus luteum lifespan, luteal volume, and function (i.e. progesterone secretion).

4.3 Materials and Methods

Animal Handling: All protocols and procedures were approved by the Fort Keogh Livestock and Range Research Laboratory Animal Care and Use Committee (IACUC approval number 022014-2). The experimental protocol is shown in Figure 4.1.

Synchronization of ovulation: Follicular waves were presynchronized in multiparous nonlactating beef cows (n=35) as follows: a CIDR was inserted and an injection of estradiol 17β (E2; 2.5 mg) and progesterone (P4; 2.5 mg E2, 50 mg P4, i.m.) was administered on day -19. CIDR removal and PGF (Lutalyse, 25 mg, i.m.) administration occurred on day -9. On day -7 a second CIDR was

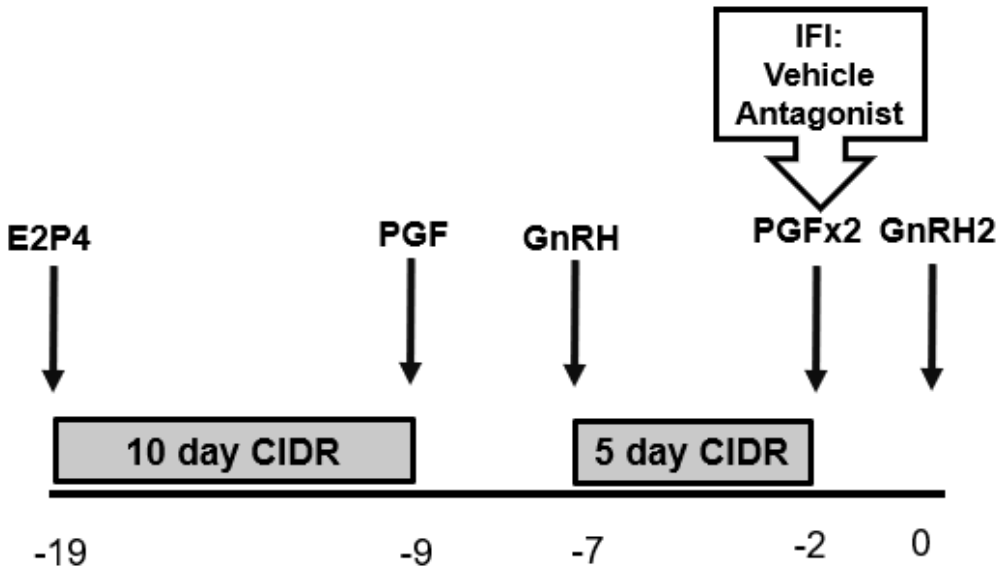

Figure 4.1: Protocol for synchronization of dominant follicle emergence prior to intrafollicular injection (IFI) on day -2. Cows were assigned to treatment groups that were balanced for dominant follicle size, cow weight and age on day -2.

inserted and GnRH (GnRH1; Factrel, 100 μg, i.m.) was administered, on day -2 the CIDR was removed and PGF (50 mg, i.m.) was administered, and on day 0 GnRH (GnRH2; Factrel, 100 μg, i.m.) was administered to induce ovulation. Cows were allotted into one of three treatments: control (no intrafollicular injection; n=10), intrafollicular injection of vehicle (n=10) or intrafollicular injection of an estradiol receptor antagonist (ICI 182,780; n=10) following measurement of a dominant follicle on day -2. Treatment groups were balanced for cow age, weight, and follicle size on day -2. If there were multiple follicles which could be dominant, the cow was allocated to the control group. Expression of estrus was monitored by visual observation twice daily following application of an Estrotect patch at PGF injection (day -2) until the end of the experiment.

Transrectal Ultrasonography: Ovaries of all animals were examined with transrectal ultrasonography using an Aloka 500V ultrasound with a 7.5-MHz linear probe (Aloka, Wallingford, CT) to determine the presence and size of a dominant follicle on days -2 and 0. On days 2, 8, and 14 ovaries were examined to determine if cows had ovulated and formed a CL. Cows were determined to have ovulated if the dominant follicle could no longer be visualized on day 2 and a CL was present in the same location on day 8 and 14. Corpus luteum volume was calculated using $4/3\pi r^3$ (r= average radius) and when a luteal cavity was present the volume of the cavity was subtracted from the volume of the CL. Luteal volume was determined on days 8 and 14 post GnRH2 (day 0).

Blood collection: Blood samples were collected daily from day -2 to day 0 and every other day from day 0 to 20. Blood was collected via coccygeal

venipuncture into 10 mL Vacutainer tubes containing K_3EDTA (Fisher Scientific, Pittsburgh, PA) and centrifuged at 1,200 x g for 20 minutes at 4℃. Plasma was harvested and stored at -20℃ until RIA. Plasma concentrations of estradiol-17β were quantified for samples collected on days -2, -1 and 0 by RIA as described previously (Kirby et al., 1997). Sensitivity of the estradiol assay was 0.5 pg/mL. Intraassay coefficient of variation were 4.4%. Plasma concentrations of progesterone for all samples were determined by RIA (Engel et al., 2008). Sensitivity of the progesterone assay was 0.4 ng/mL. Interassay and intraassay coefficients of variation were 13.3 and 4.4% respectively.

Preparation of solutions for injection: The amount of estradiol receptor antagonist (ICI 182,780, Imperial Chemical Industries; Abcam, Cambridge, MA) that was injected was determined as described below and further described in appendix Figure A.4. Based on previous published studies, the anticipated follicular fluid concentrations of estradiol were estimated to be 500 to 1000 ng/ml (Martin et al., 1991). The goal was to inject the antagonist at 27 times the estimated total amount of estradiol in follicular fluid (≈ 925 ng), which is equivalent to approximately 25 μg per follicle. This number was based on the antagonist's relative binding affinity compared to estradiol, which is reported to be 37.5% (Blair et al., 2000). Follicles were expected to have a maximum diameter of 12 mm on day -2 and calculations were based on an antrum volume associated with a follicle of this diameter. Thus, we anticipated we would provide a 10-fold excess of inhibition compared to estradiol in the follicular fluid.

A stock solution of ICI 182,780 was prepared by dissolving 5 mg in 111 µL of dimethyl sulfoxide (DMSO) and stored at -20℃. The day before the vehicle or antagonist was to be injected, the vehicle and ICI 182,780 treatment solutions were prepared by diluting DMSO to 0.56% in sterile physiological saline. DMSO has been reported to have cytotoxic properties at a concentration of 1% (Timm et al., 2013); however, in this study the final concentration of DMSO in follicular fluid following injection was calculated to not exceed 0.4%. In addition, there was no evidence of a cytotoxic effect of DMSO or the antagonist since there was no difference in dominant follicle growth rate (day -2 to day 0), circulating concentrations of estradiol (day -2 to day 0), or proportion of cows that ovulated in response to GnRH among the three groups (see Results section).

On the day of injection, the solutions were warmed to 37℃. Immediately before injections, solutions were loaded into 0.25 mL straws which were then stored in an incubator at 37℃. Straws were loaded with vehicle or antagonist using a 1 mL tuberculin syringe. First, a small amount of saline was loaded, which was used to activate the plug. Next, a bubble of approximately 50 µL of air was loaded which was necessary to expel the solution from the needle. Using a pipette, 100 µL of solution was loaded into the straw, followed by a small bubble and approximately 50 µL of saline to flush the needle prior to puncturing the follicle. All ICI treated follicles received 25 µg of antagonist dissolved in 100 µL saline containing 0.56% DMSO. Vehicle treated follicles were injected with 100 µL of saline containing 0.56% DMSO.

Intrafollicular injection of estradiol antagonist: Intrafollicular injection procedure was similar to a previously published procedure for transvaginal injection into the antrum of a dominant follicle (Peters et al., 2004). On day -2, prior to injection, cows were divided into three groups that were balanced for dominant follicle size as determined by transrectal ultrasonography Cows received one of three treatments: no injection (control; n = 10), vehicle injection (100 µL of saline containing 0.56% DMSO; n = 10), or injection of the estradiol receptor antagonist ICI 182,780 (100 µL of 0.412 mM ICI 182,780 dissolved in saline containing 0.56% DMSO; n = 10).

To reduce chances of infection from transvaginal injection, the vagina was rinsed with 2% chlorhexidine solution after the removal of the CIDR. A caudal lidocaine block (2-4 mL of 2% lidocaine hydrochloride) was performed immediately prior to transvaginal injection. The dominant follicle was visualized via an ultrasound transducer mounted on a probe. The needle was advanced through the vaginal wall and the saline bubble was used to rinse the needle prior to puncturing the follicular wall. The follicle wall was punctured, and the solution slowly injected into the follicle. The needle was slowly removed from the follicle and observed for leakage. Leakage of the dominant follicle was characterized as follows: immediate visual decrease in follicle size and/or marked decrease in follicle size on day 0. There were four cows that appeared to have leakage following intrafollicular injection and were excluded from the study.

Statistical analysis:

A power of the test was conducted prior to the experiment to determine the minimum number of animals per treatment required to detect a difference in serum concentrations of progesterone of 1 ng/ml with a power of 0.9 at an alpha of .05. This test indicated that a minimum of ten animals per treatment would be required to detect a 1 ng/mL difference ($P<0.05$) in circulating concentrations of progesterone. However, after excluding animals from the study that had a ruptured follicle, that had incomplete luteolysis, or that did not ovulate within 2 days after GnRH2, the number of cows in the control, vehicle, and estradiol receptor antagonist group were 5, 3, and 4, respectively. Differences in plasma concentrations of estradiol and progesterone were analyzed by analysis of variance for repeated measures in SAS (Proc Mixed; Littell et al., 1998). Differences in follicle size, CL volume, cow age and weight were analyzed by one-way ANOVA (Proc GLM).

4.4 Results

Mean cow age and weight did not differ among groups at the start of the project (Table 4.1; $P>0.2$). Half of the animals in the study appeared to have experienced incomplete luteolysis following administration of PGF on day -2 (n = 9), failed to ovulate in response to GnRH administration on day 0 (n = 2), and(or) experienced damage to the follicle during the intrafollicular injection procedure (n = 4; Figure 4.2). Evidence of incomplete luteolysis was based on the following criteria: 1) circulating concentrations of progesterone greater than 1 ng/mL on day 0, 2) Failure to observe a new CL on day 8 by ultrasonography, and 3) low circulating concentrations of progesterone on days 6 through 10. Cows that had

Table 4.1: Mean ± SEM for cow age and weight by treatment group (n=10 per group).

Group	Age (years)	Weight (kg)
Control	4.7 ± 0.8	582 ± 23
Antagonist	4.3 ± 0.8	567 ± 32
Vehicle	3.8 ± 0.8	540 ± 20

Cow age and weight did not significantly differ among groups ($P>0.2$)

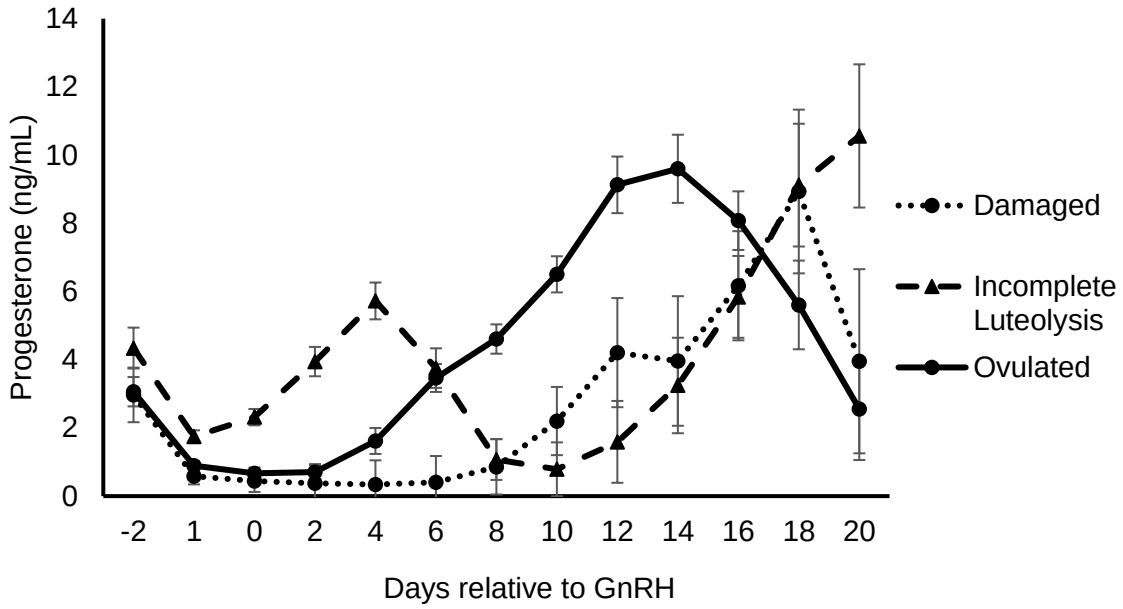

Figure 4.2: Mean ± SEM circulating concentrations of progesterone for cows representing different patterns of luteal function following intrafollicular injection: 1) Heifers in which the dominant follicle was damaged during the injection process and failed to ovulate (Damaged; n = 4), 2) Cows that had circulating progesterone >1 ng/ml on day 0 (Incomplete Luteolysis; n = 7), and 3) Cows that experienced GnRH-induced ovulation (Ovulated; n = 13).

incomplete luteolysis were removed from the study, since it is unclear how elevated progesterone at GnRH-induced ovulation might affect subsequent luteinization. Cows which did not form a CL following injection of the dominant follicle (2 vehicle, 1 antagonist), as evidenced by low progesterone (<1ng/mL) following PGF administration until days 10 and 12, were also removed from the study. The number of animals that remained in the control, vehicle, and antagonist groups was 5, 4, and 4, respectively.

Mean circulating estradiol increased across days -2, -1 and 0 ($P<0.0001$), and did not differ among groups ($P=0.47$; Figure 4.2). Furthermore, there was no treatment by day interaction for circulating concentrations of estradiol ($P=0.48$; Figure 4.2). Mean ovulatory follicle diameter on day -2 (IFI) and day 0 (GnRH) did not differ ($P>0.25$) among control, antagonist, and vehicle groups. However, ovulatory follicle diameter did increase significantly from days -2 to 0 for all treatment groups. Corpus luteum volume did not differ among groups on days 8 and 14 ($P>0.05$) (Table 4.2). Mean circulating concentrations of progesterone did not differ among groups from day 0 to 20 (Figure 4.3; $P=0.5$). Although there was an effect of day on circulating concentrations of progesterone ($P<0.0001$), there was no treatment by day interaction ($P=0.95$).

4.5 Discussion

Circulating concentrations of estradiol during the preovulatory period coordinate several physiological events that are important for the establishment of pregnancy, including induction of estrous behavior, induction of the preovulatory gonadotropin surge, gamete transport, and induction of endometrial

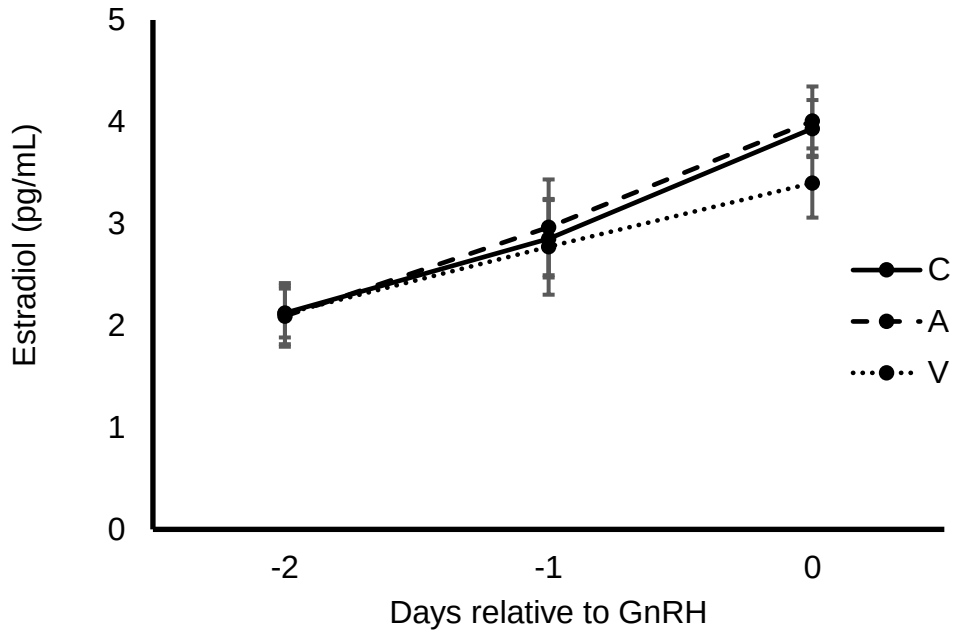

Figure 4.3: Mean ± SEM circulating concentrations of estradiol on days -2 (PGF), -1 and 0 (GnRH; see Figure 1) for the control (C), antagonist (A), and vehicle (V) groups as determined by RIA. Cows that did not undergo complete luteolysis or ovulate following GnRH were excluded (Treatment, P = 0.86; Day, P<0.0001; and Treatment x Day, P = 0.56).

Table 4.2: Mean ± SEM follicle diameter on day -2 and 0, and CL volume on day 8 and 14.

Group[1]	**Follicle diameter (mm)**		**CL Volume (cm^3)**[2]	
	day -2	day 0	day 8	day 14
Control	8.7±0.8[a]	12.4±0.7[b]	4.8±1.1	5.2±0.9
Antagonist	8.7±0.8[a]	11.5±0.7[b]	4.6±1.2	5.3±1.0
Vehicle	8.2±0.8[c]	10.3±0.7[d]	3.4±1.2	4.3±1.0

[1]Cows that did not undergo complete luteolysis ovulate in response to GnRH (day 0) were excluded from the analysis.

[2]CL volume did not differ among groups or days measured (P>0.05)

Superscripts that differ within a row are significantly different ([a,b]P<0.005, [c,d]P<0.01)

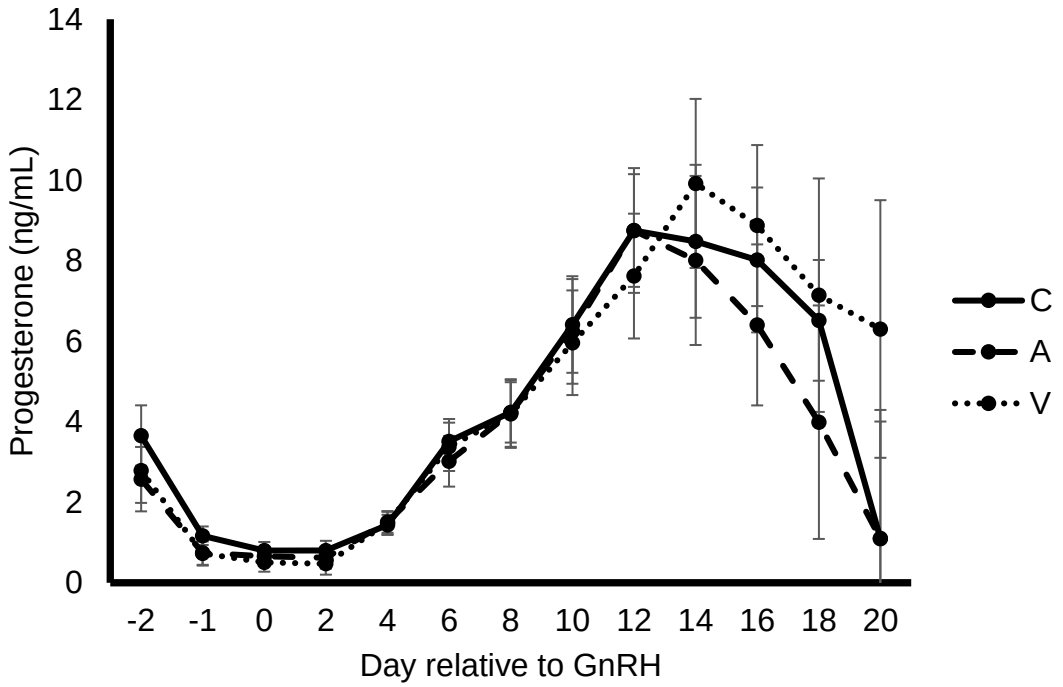

Figure 4.4: Mean (± SEM) circulating concentrations of progesterone, as determined by RIA for cows in the control (C; n=5), antagonist (A; n=4) and vehicle groups (V; n=4) that ovulated in response to GnRH (Treatment, P = 0.79; Day, P<0.0001; and Treatment x Day, P = 0.99).

progesterone receptors. In cattle, the presence of ESR 1 and 2 in granulosa cells (Schams and Berisha, 2002) and the oocyte (Beker van Woudenberg et al., 2004) imply that estradiol may have an important intrafollicular role. Furthermore, increased preovulatory concentrations of progesterone was independent of ovulatory follicle size (Atkins et al., 2013).

The role of estradiol in the oocyte is not clear; however, there is evidence for a role of preovulatory estradiol in the preparation of granulosa cells for luteinization (see review by Pohler et al., 2012). For example, in culture, human granulosa cells from follicles that contained higher estradiol concentrations produced more progesterone during luteinization, compared to granulosa cells from follicles that had lower intrafollicular concentrations of estradiol (McNatty, 1979). Furthermore, ewes treated with an aromatase inhibitor prior to induced ovulation had a delayed rise in serum progesterone (Benoit et al., 1992). administration of an aromatase inhibitor.

However, when estradiol receptors within a preovulatory follicle were antagonized by ICI 182,780 in the present study, no difference was found in circulating progesterone from days 0 to 20 compared to control or vehicle injected animals. These results fail to support the hypothesis that estradiol, acting via a genomic receptor, is required for adequate luteinization and luteal progesterone secretion. Although it is possible that the relationship between preovulatory estradiol and postovulatory progesterone (Atkins et al., 2013; Jinks et al., 2013) is merely correlative, there are other potential explanations for the observed lack of difference in luteal progesterone secretion. One possibility is

that the antagonist did not adequately inhibit signaling via ESR1 and 2 in granulosa cells due to an inadequate concentration of antagonist administered, rapid metabolism of the antagonist in follicular fluid, and(or) binding or inactivation of the antagonist in follicular fluid making it unavailable to the receptor. However, these possibilities seem unlikely since effects of the antagonist were reported after continuous infusion into the uterus of sheep after only 14 hours at a concentration of 0.1 μM (Robertson et al., 2001). In addition, administration of the antagonist (3 μM for 5 hr) to bovine granulosa cells in culture, in the presence of 30 ng/mL of estradiol, decreased expression of *CYP19A1* (Luo and Wiltbank, 2006). Finally, bovine granulosa cells isolated from follicles injected in vivo with the antagonist (100 μM, ICI 182,780) 12 hours beforehand had decreased expression of *CYP19A1* (Rovani et al., 2014). In this experiment, all follicles were injected with the same amount of antagonist and the minimum final concentration of antagonist in the follicle was 41 μM.

Another possibility is that by the time the estradiol receptor antagonist was administered (48 hr before GnRH-induced ovulation), estradiol had completed its role in preparing granulosa cells for luteinization. During a follicular wave in cattle, aromatase expression increases in granulosa cells at follicular recruitment (Bao et al., 1997) and at selection of the future dominant follicle estradiol concentration in follicular fluid is increased (Fortune et al., 2004). Therefore, granulosa cells in a future dominant follicle would be exposed to elevated concentrations of estradiol for several days during a follicular wave before

injection of the antagonist. In fact, bovine follicles in the 7 to 8 mm range have been reported to have 100 ng/ml of estradiol in follicular fluid (Martin et al., 1991).

It is puzzling how the genomic estradiol receptor is capable of being regulated in the presence of such high concentrations of estradiol in follicular fluid. However, as previously mentioned (Rovani et al., 2014), intrafollicular administration of the antagonist, in vivo, was associated with a decrease in *CYP19A1* expression in the presence of high concentrations of estradiol in bovine follicular fluid and follicular fluid estradiol concentration tended to be lower (P=0.08) in antagonist treated follicles compared to the control group (124.3±74.6 ng/mL vs. 402.3±98.8 ng/mL, respectively; Gasperin et al., 2014).

It is conceivable that the antagonist may not block all estradiol signaling within the follicle. The antagonist (ICI 182,780) inhibits dimerization of the nuclear estradiol receptor, localization of the receptor to the nucleus, and accelerates degradation of ESR protein (Osborne et al., 2004). However, estradiol can also signal through membrane receptors and G protein coupled receptors, which the antagonist does not affect. Morley and coworkers (1992) demonstrated estradiol's ability to induce a rapid release of calcium in porcine granulosa cells. Estradiol can activate the G-protein coupled receptor, GPR30, to phosphorylate Erk -1/-2. Not only would the antagonist fail to block this signaling pathway, but, in some studies, ICI 182,780 activated GPR30 (Filardo et al., 2000). This G-protein coupled receptor was found in the theca and granulosa cells of hamsters and the protein decreased after the LH surge and was not present in luteal cells (Wang et al., 2007). If estradiol's preparation of granulosa cells is mediated

through membrane or G-protein coupled receptors, not only would the antagonist not block signaling, it may enhance it.

As mentioned earlier, administration of estradiol to bovine granulosa cells, in culture, resulted in an initial inhibitory effect on progesterone production, but a subsequent stimulatory effect after estradiol removal (Fortune and Hansel, 1979). Fortune and Quirk (1988) hypothesized that the increase in progesterone secretion following removal of estradiol from the culture media may have had an inhibitory effect of estradiol on 3B-HSD. Interestingly, estradiol was reported to be a competitive inhibitor of 3B-HSD in cells from a Leydig Cell tumor with a Ki of 1.8 μM (Freeman, 1985). In the current study, the minimum final concentration of estradiol in the follicle was estimated to be 1.83 μM; therefore, the high follicular fluid concentrations of estradiol in follicular fluid may inhibit progesterone production by a nongenomic action.

Although studies indicate that estradiol, acting through its nuclear receptor, can increase expression of *CYP19A1* (Luo and Wiltbank, 2006; Rovani et al., 2014), no difference in circulating estradiol was observed among groups in the present study 4). This could indicate that treatment with the antagonist was ineffective, however, it is also possible that concentrations of follicular estradiol were sufficient to maintain elevated circulating estradiol from PGF-induced luteolysis until GnRH-induced ovulation. Furthermore, the enzymatic activity of aromatase may be sufficient to produce enough estradiol, even if the expression of the enzyme is inhibited. Lack of difference in follicle size on day 0 among groups, indicates that blocking estradiol's genomic receptor did not affect follicle

growth rate. This contradicts previous data in which intrafollicular injection of ICI 182,780 decreased follicle size over 72 hours, and *CYP19A1* and *LHCGR* mRNA expression after only 12 hours (Rovani et al., 2014).

One of the key requirements proposed for adequate luteal function is having sufficient granulosa cells at the time of ovulation since proliferation ceases at the LH surge. However, the lack of difference in CL volume, fails to support the hypothesis that estradiol, acting via a genomic receptor, enhances hypertrophy of granulosa cells prior to luteinization.

An unexpected finding was that incomplete luteolysis occurred in 23% of the cows in this study. Normally with the 5-day CIDR protocol, two injections of PGF are administered 8±2 hr apart with the first injection at CIDR removal. However, in the present study, both doses of PGF (50 mg of Lutalyse) was administered at CIDR removal. Pregnancy rate and estrous expression rate following the 5 day CIDR protocol was greater when two doses of PGF were administered 6 hr apart compared to both doses administered at the same time (Kasimanickam et al., 2009; Peterson et al., 2011), possibly due to decreased luteolytic response.

In conclusion, although no significant differences were observed in these studies, this does not disprove the theory that estradiol prepares granulosa cells to luteinize. This study provided evidence that genomic estradiol signaling does not significantly affect postovulatory progesterone production. However, the possibility remains that estradiol may facilitate preparation of granulosa cells for luteinization via a nongenomic mechanism.

APPENDIX

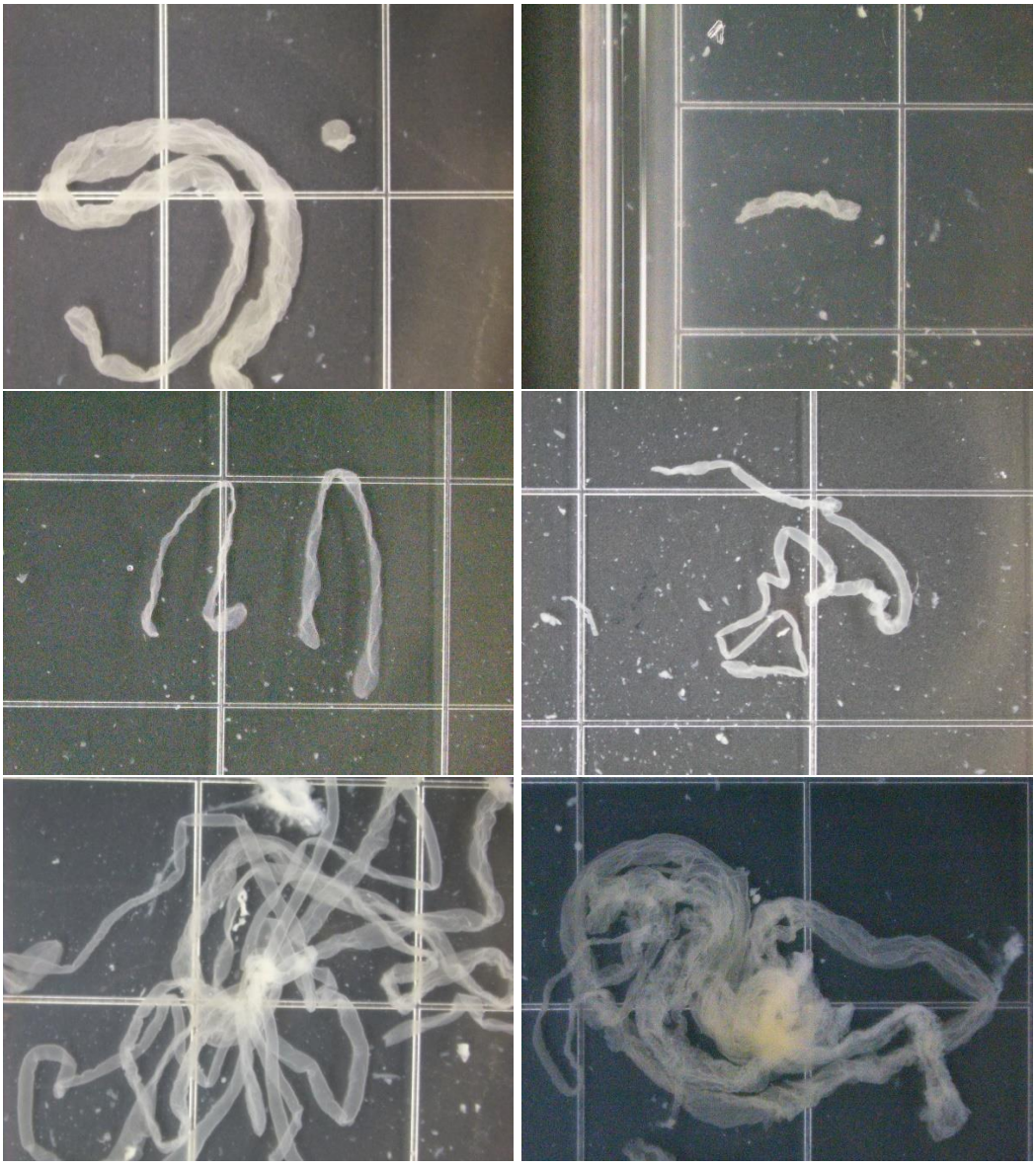

Figure A.1: Images of conceptuses recovered on day 16 after GnRH2. Each picture represents conceptuses flushed from a different heifer (n = 6). Conceptuses ranged in length from 0.2 cm to 16 cm on day 16 after GnRH2. All images at same magnification.

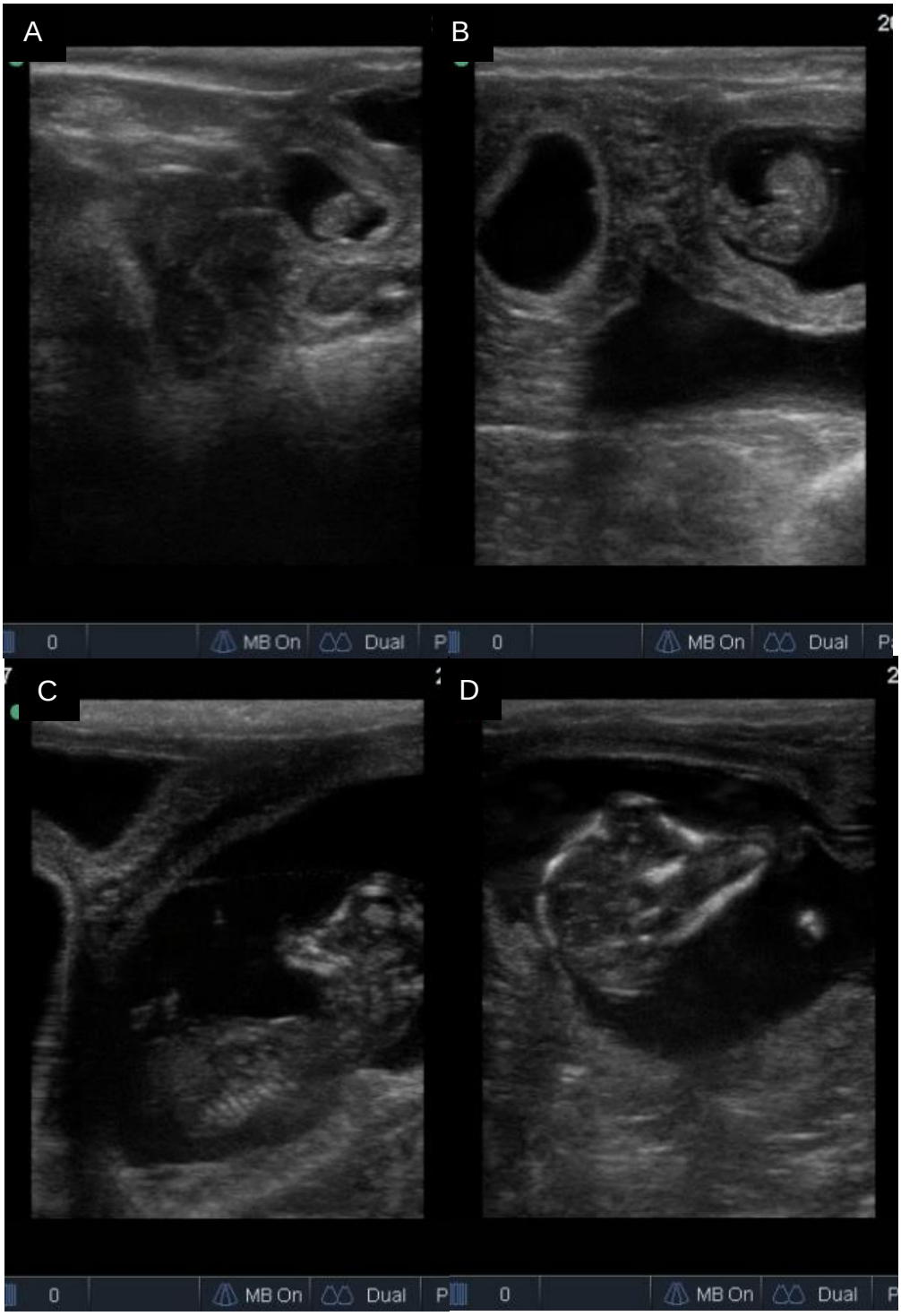

Figure A.2: Ultrasound images at different various days of gestation (A: day 30, B: day 36, C: day 57, D: day 75)

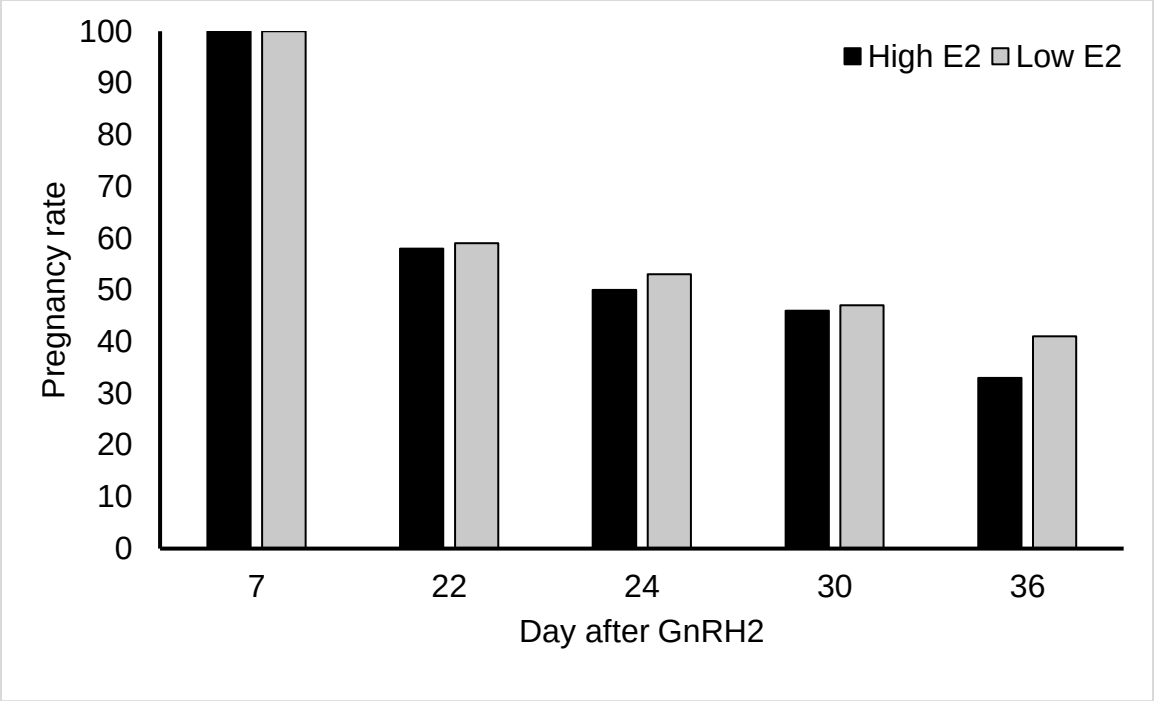

Figure A.3: Pregnancy rate following embryo transfer (day 7) in the Low and High E2 groups as determined by maintenance of circulating progesterone >1ng/mL from day 7 to 22 or 24, pregnancy associated glycoproteins (>5 ng/mL; day 30), and ultrasonographic visualization of an embryo (day 36). See Table 3.4.

Table A.1: Forward and reverse primer sequences for SERPINA14

Gene name	Primer sequences (5’ to 3’)
SERPINA14	F:GGGAATGGACATCCAGATGATT R: GGGTTCAGGTCGGTGATTAAG

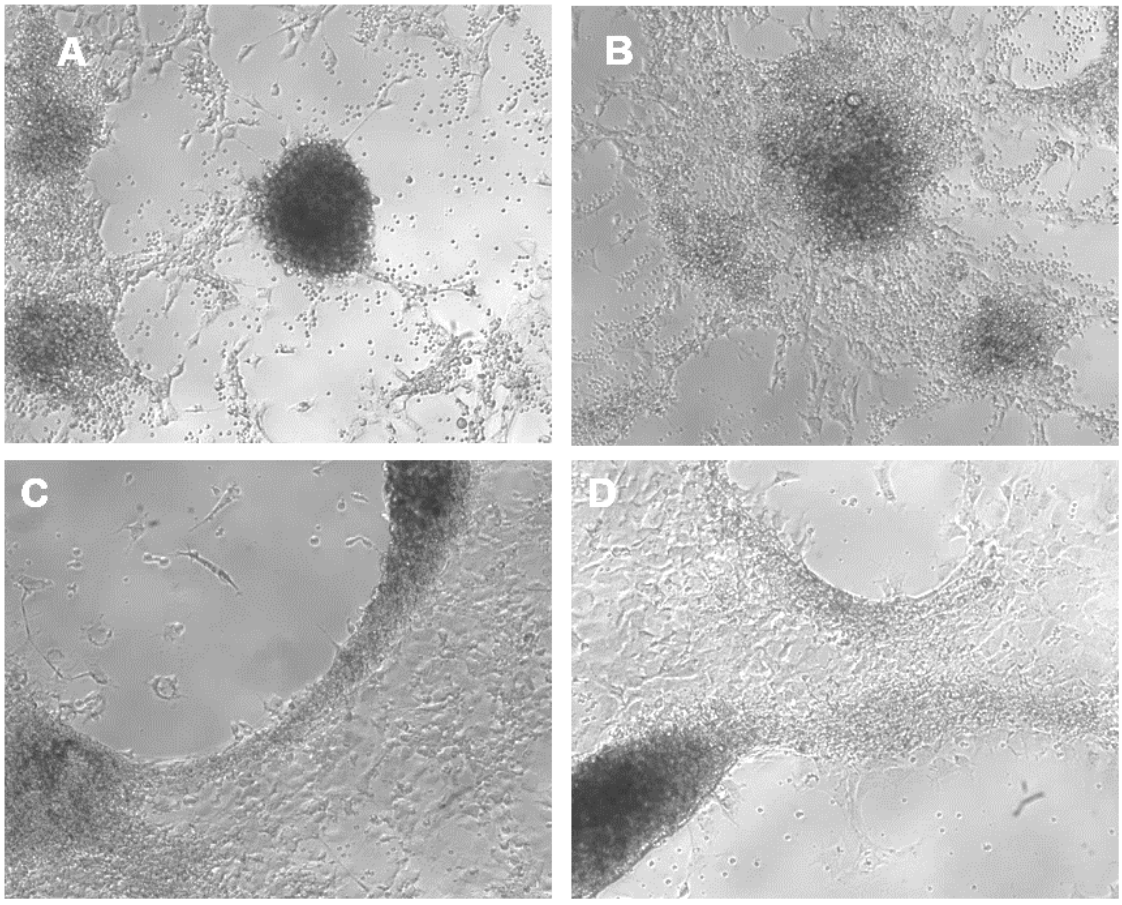

Figure A.4: Images of bovine granulosa cells in culture for 48 (Panels A and B) or 72 hrs (C and D). Panels A and B represent granulosa cells after 48 in culture with estradiol (1 µg/mL E2) and either vehicle (Panel A) or ICI 182,780 (Panel B; 10 µg/mL). Panels C and D represent granulosa cells that were incubated for 48 hr with estradiol (1 µg/mL E2) and either vehicle (Panel C) or ICI 182,780 (Panel D; 10 µg/mL), stimulated to luteinize with Forskolin (10 µg/mL) at 48 hr and cultured for a further 48 hours (Panels C and D).

Figure A.5: Calculations to determine desired concentration of antagonist

Assumptions:

Maximum expected follicle diameter = 12 mm

Estradiol concentration in follicular fluid = 1 µg/mL

Total estradiol mass in follicular fluid= 0.9 µg

Relative binding affinity (RBA) = 37.5% (Blair et al., 2000),

Desired excess of antagonist relative to estradiol = 10 fold

Volume of vehicle or vehicle plus antagonist injected per follicle = 100 µL

Desired concentration of antagonist:

Volume of a 12 mm (1.2 cm) follicle: $\frac{4}{3}\pi r^3 = \frac{4}{3}\pi(.6)^3 = 0.9\ cm^3 = 0.9\ mL$

10/(RBA) = 10/0.375 = **26.6 fold ICI to estradiol**

0.9 µg estradiol x 27 = 24.3 µg = **25 µg of ICI 182,780 per follicle**

Stock ICI 182,780 preparation:

5 mg antagonist was diluted in 111 µL DMSO:

$$5\ \text{mg} \div 111\ \mu\text{L} = 45\frac{\text{mg}}{\text{mL}}\ \text{stock}$$

$$25\ \mu\text{g} \div 45\frac{\text{mg}}{\text{mL}} = 0.56\ \mu\text{L stock per follicle}$$

To make 15 injections worth: 8.5 µL of stock solution + 1.492 mL of saline

$$45\frac{\text{mg}}{\text{mL}}\ x\ 8.5 = 382.8\ \mu\text{g of antagonist}$$

$$382.8\ \mu\text{g} \div 1.5\ mL = 257\ \mu\text{g/mL x .1 mL} = 25.7\ \mu\text{g antagonist per follicle}$$

Note on making solutions for injection:

- Make final solutions on the day of injection several hours beforehand
- Warm all fluids to 37℃
- Mix, then keep at 37℃

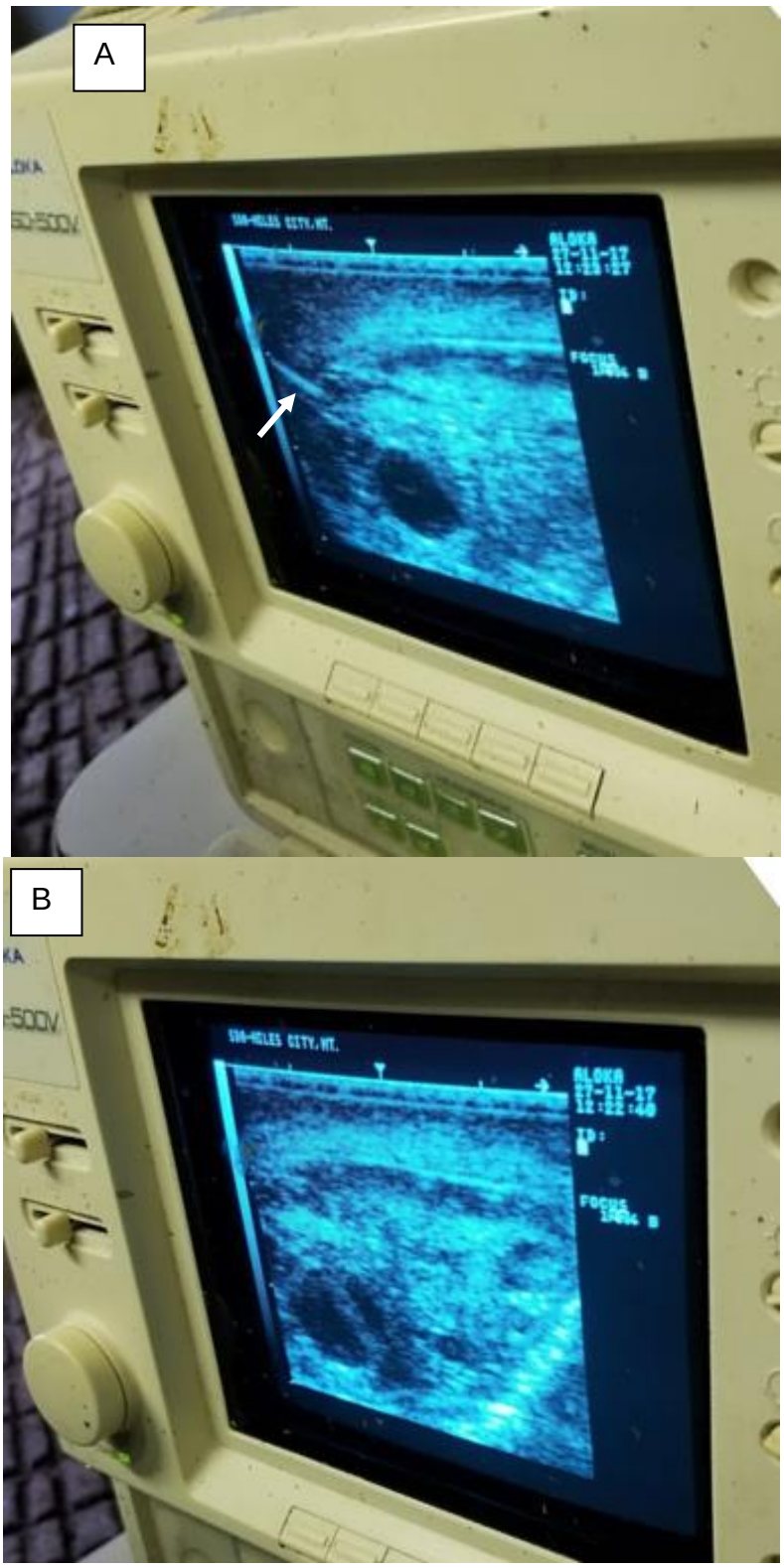

Figure A.6: Images of ultrasound screen during intrafollicular injection. A) Needle, indicated by an arrow, was advanced through the stroma into the follicle, B) Follicle became cloudy as solution was injected.

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Vita

Megan Kelsey McLean was born in Bethesda, Maryland to Duncan and Kathleen McLean on August 10, 1994. She was raised in Alexandria, Virginia. In her senior year of high school, she began volunteering at George Washington's Mount Vernon Estate where she gained experience with many livestock species including: cattle, sheep, pigs, chickens, and horses. Following her freshman year in college, she returned to Mount Vernon as a livestock caretaker. Although starting as a Chemistry major at Virginia Polytechnic Institute and State University, her interest in Animal Science, piqued by her work at Mount Vernon, led her to complete a Bachelor of Science Degree in Animal Science with a Minor in Chemistry in May 2016. In August 2016, she began a Master of Science program with Drs. Thomas Spencer and Michael Smith in the Division of Animal Sciences of the College of Agriculture, Food, and Natural Resources. This thesis is a result of research completed as part of that program and was defended in July of 2018.