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# The Influence of Preovulatory Estradiol on Uterine Transcriptomics and Proteomics Around Maternal Recognition of Pregnancy in Beef Cattle

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THE INFLUENCE OF PREOVULATORY ESTRADIOL ON UTERINE  
TRANSCRIPTOMICS AND PROTEOMICS AROUND MATERNAL RECOGNITION  
OF PREGNANCY IN BEEF CATTLE

BY

EMMALEE J. NORTHROP

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Animal Science

South Dakota State University

2019

THE INFLUENCE OF PREOVULATORY ESTRADIOL ON UTERINE  
TRANSCRIPTOMICS AND PROTEOMICS AROUND MATERNAL RECOGNITION  
OF PREGNANCY IN BEEF CATTLE

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Animal Science degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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## ABSTRACT

THE INFLUENCE OF PREOVULATORY ESTRADIOL ON UTERINE  
TRANSCRIPTOMICS AND PROTEOMICS AROUND MATERNAL RECOGNITION  
OF PREGNANCY IN BEEF CATTLE

EMMALEE J. NORTHROP

2019

Preovulatory estradiol has been reported to play a crucial role in pregnancy establishment and maintenance, but the mechanism by which estradiol exerts its effects has not been well characterized. Specifically, the interactions between the maternal uterine environment and the developing conceptus can greatly impact pregnancy success or loss. The objective of this dissertation is to determine the effects of preovulatory estradiol exposure on uterine and trophectoderm transcriptomes, and uterine luminal fluid (ULF) protein composition. Beef cows/heifers were synchronized, artificially inseminated (d 0), and grouped into either high (highE2) or low (lowE2) preovulatory estradiol. Uteri were flushed to collect d16 conceptuses either nonsurgically or following slaughter, and endometrial biopsies (n=29) were collected from the ipsilateral uterine horn. Real-Time PCR (RT-PCR) was performed on trophectoderm (TE; n=21) RNA to measure the relative abundance of IFNT, PTGS2, TM4SF1, C3, FGFR2, and GAPDH. Total cellular RNA was extracted from endometrium for RNA sequencing. ULF pools (n=28) for the following groups: highE2/noconceptus, highE2/conceptus, lowE2/noconceptus, and lowE2/conceptus were analyzed using a 2D LC-MS/MS based 8plex iTRAQ method. RT-PCR data were analyzed using the MIXED procedure in SAS.

Transcript abundances in the endometrium were quantified using kallisto, differentially expressed genes (DEGs) were determined using DESeq2 (FDR <0.05, FC>2), and IPA was used for pathway analysis. Scaffold Q+ was used to quantitate peptide and protein identifications in the ULF. There were no differences in mRNA abundances in TE, but there were 432 DEGs among the highE2/conceptus versus lowE2/conceptus groups, 253 were downregulated (CR2, CDH4, TROAP, COL1A2) and 179 were upregulated (PRSS8, FABP3, IDO1, MUC13, CXCL10) in the highE2/conceptus group. There were 48 differentially expressed proteins (DEPs) among the highE2/conceptus and lowE2/conceptus groups (19 upregulated, 29 downregulated in the highE2/conceptus group), 6 of these were differentially expressed (FDR <0.10) at the mRNA level. Similar pathways for mRNA and proteins included: calcium signaling, protein kinase A signaling, and CRH signaling. These results demonstrate greater differences in uterine function than in conceptus developmental competence between highE2 and lowE2 animals on d16 of pregnancy.

CHAPTER I  
LITERATURE REVIEW  
INTRODUCTION

Embryonic mortality is a major factor that impacts production and economic efficiency in the cattle industry. Early embryonic death is costly to livestock producers, and leads to a decrease in herd productivity and an increase in calving interval. Possible factors that contribute to early embryonic loss include: genetic defects, reproductive diseases, heat stress, and nutrition (Bridges et al., 2012). Among cattle, fertilization rates are reported to be around 90%, while pregnancy loss ranges from 30 to 56%, with the majority of the losses occurring during the first month of pregnancy (Wiltbank et al., 2016). In the United States, early embryonic loss costs the beef industry approximately 1 billion dollars annually (Bellows et al., 2002). With a continuously growing world population, the demand for beef will increase, and improving reproductive efficiency in cattle will be critical to maximize beef production. If we could prevent embryonic loss in just five out of every 100 cows, we would wean an additional 2100 pounds per 100 cows (Geary, 2005).

Previous research indicated that estradiol leading up to breeding may be a critical factor for the establishment and maintenance of a successful pregnancy (Perry et al., 2005). Initiation of estrus occurs due to increased circulating estradiol at a time when progesterone is not present (De Silva et al., 1981). In the absence of progesterone, estradiol acts on the hypothalamus to induce behavioral estrus and an LH surge resulting in ovulation (Chenault et al., 1975). Preovulatory estradiol has been reported to impact follicular growth, oocyte maturation, sperm transport, uterine environment, and embryo

survival (Pohler et al., 2012). Animals that exhibit estrus prior to fixed-time AI have increased fertilization success, embryo quality and survival (Atkins et al., 2013), and pregnancy maintenance (Perry et al., 2005).

In cattle, the embryo/conceptus is free floating in the uterus until attachment (day 20). During this time, it is relying on the mother's uterine secretions (uterine histotroph) for growth and survival. The uterine histotroph is composed of a mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, and glucose (Gao et al., 2009). Some critical periods of development occur at hatching (day 5 after fertilization), elongation (day 10 after fertilization), maternal recognition of pregnancy (day 16 after fertilization), and attachment (day 20 after fertilization).

Maternal recognition of pregnancy is the physiological process that is defined as the requirement for the conceptus to produce and secrete a signal that acts on the uterus and ovary to ensure the maintenance of a functional corpus luteum so progesterone production and pregnancy can be maintained (Bazer, 2013). In cattle, this signal is interferon tau (IFNT). This conceptus signal is critical for the prevention of the pulsatile release of PGF2 $\alpha$  and the promotion of uterine gland secretions. Previous research determined that there were no differences in conceptus survival around the time of maternal recognition of pregnancy between highE2 and lowE2 animals based on conceptus recovery rates, apoptosis in the trophoctoderm, and IFNT concentration within the uterine luminal fluid (ULF). Glucose transporter expression in the endometrium, and glucose and protein concentrations in the ULF were influenced by conceptus presence and preovulatory estradiol (Northrop et al., 2018). This dissertation will focus on the impact of preovulatory estradiol during a fixed-time AI protocol on

uterine/trophoblast gene expression and uterine luminal fluid proteomics during this critical time point in pregnancy.

## BOVINE ESTROUS CYCLE

Heifers reach puberty when gonadotropin-releasing hormone (GnRH), a decapeptide, is secreted from the hypothalamus at a sufficient amplitude and frequency to stimulate a surge of luteinizing hormone (LH) from the anterior pituitary (Karsch et al., 1997). Within the anterior pituitary, GnRH binds to its G protein coupled, 7 transmembrane domain receptors (Sealfon et al., 1997) located on the gonadotropes. This causes the secretion of follicle stimulating hormone (FSH) and LH, which then bind to their specific receptors on ovarian follicles (Brinkley, 1981). A lesser pulse frequency of GnRH preferentially leads to FSH release, while a greater GnRH pulse frequency most often leads to the release of LH (Molter-Gerard et al., 1999). The frequency and amplitude of these gonadotropins depends on stage of the estrous cycle. Both estradiol and progesterone act as regulators for the release of FSH and LH (Walters et al., 1981). Elevated progesterone concentrations negatively feedback and cause a reduction in LH pulse frequency (Ireland and Roche, 1982). Additionally, elevated progesterone has also been reported to decrease GnRH receptors on the anterior pituitary.

Once a female attains puberty she enters a period of reproductive cyclicity. A normal estrous cycle is defined as the recurrent set of physiological and behavioral changes that occur from one period of estrus to another (Senger, 2003). The length of the estrous cycle varies among species. The average length of the estrous cycle in cattle is 21



days. An estrous cycle can be divided into the follicular phase (proestrus and estrus) and the luteal phase (metestrus and diestrus).

*Follicular phase:*

There are two stages in the follicular phase, proestrus and estrus. Proestrus (day 17-20) begins when progesterone decreases following luteolysis, it is characterized by a significant increase in estradiol production by the developing follicles. Estrus (day 0) is the most widely recognized stage due to the visual signs associated with the period of sexual receptivity. Follicle stimulating hormone is the protein hormone that stimulates the recruitment of follicular waves (Adams et al., 1992). During the follicular phase, preovulatory follicles grow and develop and begin to secrete estradiol (Echternkamp and Hansel, 1973).

*Recruitment:*

Follicle stimulating hormone concentrations peak about one day before follicular wave emergence (Ginther et al., 1996), which leads to the emergence of a cohort of follicles taken from the follicular pool (Driancourt, 2001). These follicles are dependent on FSH for growth and development. Usually an average of seven follicles develop to 5mm in diameter during a single follicular wave (Ginther et al., 2001). These follicles have FSH receptors on their granulosa cells, and LH receptors on their theca cells, but lack LH receptors on their granulosa cells (Richards, 1994).

*Selection:*

One follicle is then selected to outgrow other follicles in the cohort and establish dominance (Ginther et al., 2001). This deviation in growth occurs when the selected

follicle reaches 8.5mm (Ginther et al., 1997). Circulating FSH concentrations decline, the smaller follicles are unable to grow (Ginther et al., 1996). The decline in FSH is driven by the increasing estradiol concentrations produced by the cohort. The production of estradiol and inhibin by the growing follicle feeds back to the hypothalamus and pituitary causing a decrease in FSH secretions (Martin et al., 1988), which leads to the atresia of all follicles except the dominant follicle. Subordinate follicles undergo atresia five days after follicular wave emergence (Adams et al., 1993).

*Dominance:*

As the dominant follicle grows, it becomes less dependent on FSH, and more dependent on LH. Luteinizing hormone is the prominent protein hormone involved in the final stages of growth of the dominant follicle (Savio et al., 1993). During this phase, a single large follicle develops to 12 to 17 mm in diameter, while the subordinate follicles undergo atresia. One distinguishing characteristic of the dominant follicle is that it acquires LH receptors on the granulosa cells, which most likely plays a role in maintaining its dominance (Bao and Gaverick, 1998). Previous research also reported that the chosen dominant follicle has increased insulin like growth factor 1 availability, which has been associated with an increased responsiveness to gonadotropins (Fortune et al., 2004). Dominant follicles typically survive for 5 to 7 days (Sirois and Fortune, 1988). During this time, a dominant follicle secretes high concentrations of estradiol, which then works with the glycoprotein inhibin to suppress FSH release from the anterior pituitary (Adams et al., 1992; Good et al., 1995). Most dominant follicles are destined for regression, which leads to a decrease in aromatase activity and estradiol production

(Price et al., 1995). This is facilitated by apoptosis when progesterone concentrations are high.

*Preovulatory Surge/Ovulation:*

During the final follicular wave, LH pulse frequency increases and estradiol concentrations peak and positively feedback to the surge center in the brain. These events cause the LH surge and ovulation. A surge typically lasts approximately 2 to 6 hours (Walters et al., 1984; Chenault et al., 1975). Ovulation is defined as the release of the ovum from the follicle. Ovulation involves cytoplasmic and nuclear maturation of the oocyte, disruption of cumulus cell cohesiveness, and thinning/rupturing of the external follicular wall. For ovulation to occur there must be: 1) a shift from estradiol to progesterone causing an increase in collagenase 2) an increase in  $\text{PGF}_2\alpha$ , which causes an increase in smooth muscle contraction and lysosomal enzyme activity 3) and an increase in  $\text{PGE}_2$  causing edema and an increase in pressure. It occurs approximately 30 hours after the onset of estrus (Plasse et al., 1970), and it marks the end of the follicular phase.

*Luteal phase:*

The dominant ovarian structure during the luteal phase is the corpus luteum (CL), it produces progesterone (Smith et al., 1994). This phase is composed of metestrus (days 1- 5), which is the stage in the estrous cycle when follicular cells (granulosa and theca cells) transform into the luteal cells that make up the CL in a process called luteinization (Smith et al., 1994). The small luteal cells that make up the CL are derived from the

theca interna cells, while the large luteal cells are derived from granulosa cells. The large luteal cells produce more than eighty percent of the progesterone (Niswender et al., 1985), thus the number of granulosa cells can greatly influence circulating progesterone concentrations. Diestrus (day 6- 14) is the longest stage of the estrous cycle, and is the period during which the CL is fully functional and progesterone secretion is at its greatest (Forde et al., 2011). In non-pregnant cattle, progesterone will remain high until around day 17 of the estrous cycle when luteolysis occurs (Rahe et al., 1980). However, if maternal recognition of pregnancy occurs, the CL will not regress and progesterone concentrations will be maintained. This progesterone is critical for the establishment and maintenance of pregnancy.

## STEROIDOGENESIS

The ovarian follicle is capable of producing androgens, estrogens, and progestins. Estradiol  $17\beta$  is the most common form of estrogen found in the body, and is produced by growing follicles within the ovary (Kaneko et al., 1991). Granulosa cells within the follicle synthesize this steroid hormone. Estradiol has been reported to cause the following actions in granulosa cells: increases cell proliferation (Goldenberg et al., 1972), increases gap junction formation (Merk et al., 1972), stimulates the action of FSH on the aromatase enzyme (Adashi and Hsueh, 1982), enhances the formation of LH receptors (Kessel et al., 1985), and enhances progesterone production following stimulation of gonadotropins (Welsh et al., 1983). The mechanism by which estradiol is synthesized is known as the two cell-two gonadotropin theory (Fortune and Quirk, 1988). In this theory, the capillary network within the theca interna is responsible for supplying

cholesterol (27C). It is the necessary precursor for all steroid hormones. Cholesterol is then transferred via steroidogenic acute regulatory protein (StAR) from the outer mitochondrial membrane to the inner mitochondrial membrane. This is the rate-limiting step for steroid hormone production (Stocco, 2001). Cholesterol is then cleaved and converted to pregnenolone via the cytochrome P450 side chain cleavage enzyme. Pregnenolone can then enter different pathways eventually leading to its conversion into a 21-carbon progestin, 19 carbon androgen, or 18 carbon estrogen (Figure 1).

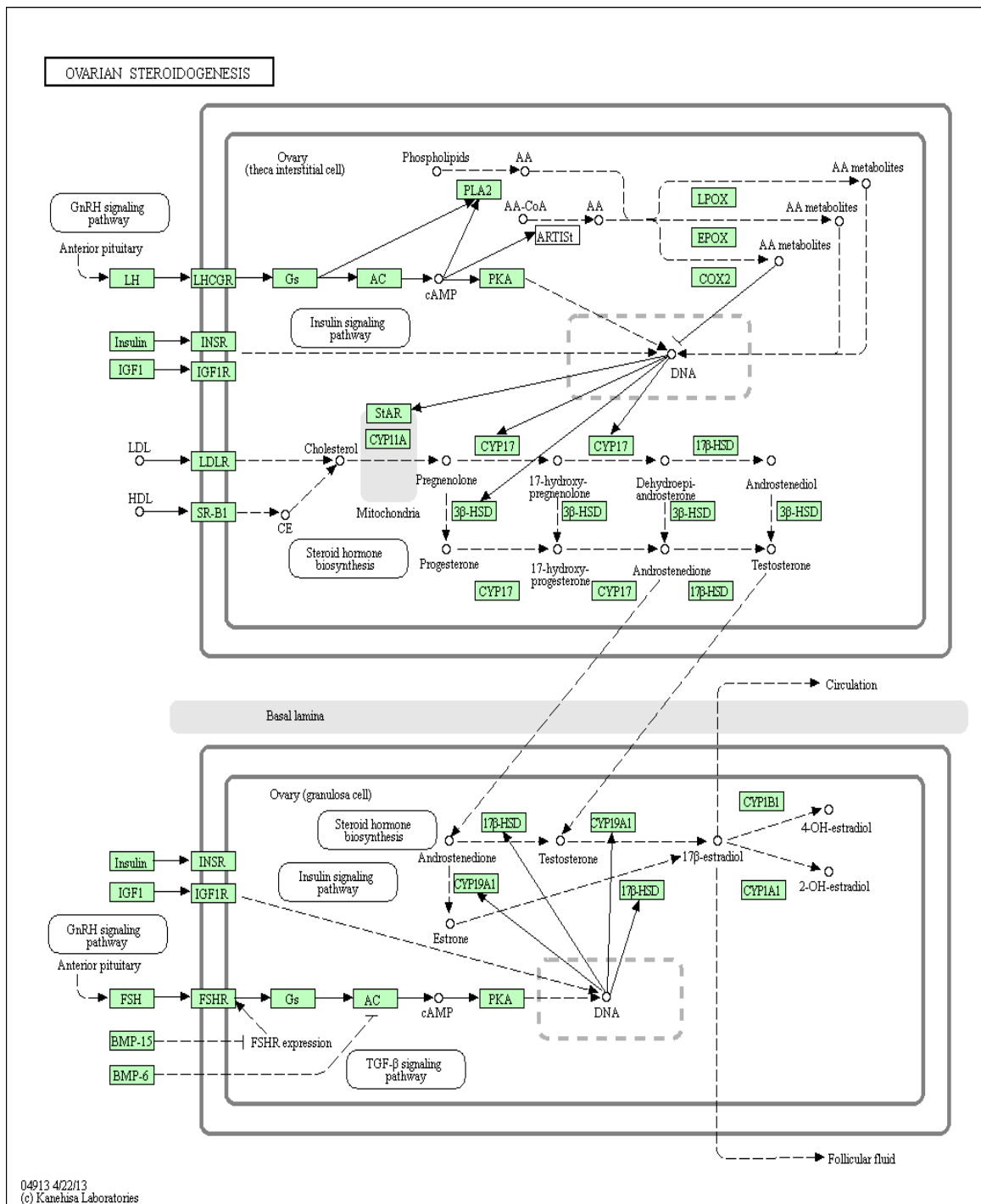


Figure 1. KEGG Ovarian steroidogenesis (Kanehisa Laboratories).

*Two cell- two gonadotropin theory:*

In the two cell- two gonadotropin theory, LH binds to its receptors on the theca interna cells, which stimulates the conversion of cholesterol into androsteindione. Androgens then diffuse across the lamina basalis into the granulosa cells (Dorrington et al., 1975). Follicle stimulating hormone (FSH) binds to its receptor on the granulosa cells to increase aromatase activity. Aromatase is the enzyme responsible for the conversion of androgens into estradiol (Bao and Garverick, 1998). The synergism between granulosa and theca interna cells is critical for maximal estrogen production (Liu and Hsueh, 1986). The estradiol that is produced during this process then enters circulation and aids in the feedback mechanism, which is responsible for causing gonadotropin secretion from the anterior pituitary and behavioral estrus.

## ESTRADIOL

Granulosa cells within the follicle produce estradiol. Estradiol is a steroid hormone, and is therefore hydrophobic. For this reason, estradiol is most often found in the bloodstream bound to the sex hormone binding globulin (Mercier-Bodard et al., 1970). Unbound estradiol is considered biologically active, and is free to interact with target tissues such as the hypothalamus, anterior pituitary, reproductive tract, and mammary glands.

*Estradiol Receptors:*

Once estradiol is free in circulation, it can bind to either plasma membrane or nuclear receptors. In the first scenario, estradiol binds to plasma membrane receptors, the

results are detectable within seconds or minutes, but it causes no genomic changes. In the second scenario, estradiol acts as a transcription factor that binds to nuclear receptors causing a conformational change, ultimately allowing the receptor to bind to chromatin and cause transcriptional changes within hours (Murdoch and Gorski, 1991; Stormshak and Bishop, 2008). These receptors are classified as estrogen receptor alpha ( $ER\alpha$ ), estrogen receptor beta ( $ER\beta$ ), and estrogen receptor gamma ( $ER\gamma$ ) (Kuiper et al., 1996). The different receptor subtypes are preferentially expressed in certain tissues, but have similar affinities. Estrogen receptor alpha is commonly expressed in the ovary, mammary glands, uterus, and brain (Kuiper et al., 1996). While  $ER\beta$  is more commonly expressed in the uterus and the mammary glands. Estrogen receptor expression changes throughout the estrous cycle, with increased expression during the follicular phase (Miller et al., 1977).

*Forms:*

The use of exogenous estradiol for synchronization of estrus in cattle is not legal in the United States. However, there are three forms of estrogens that are typically used in other countries or for research purposes. These include: estradiol-17 $\beta$ , estradiol benzoate (EB), and estradiol cypionate (ECP). There are differences in esterification, molecular weight, and polarity among the different forms of estrogens. This leads to differences in absorption, profiles, and half-lives.

Estradiol-17 $\beta$  causes the greatest increase in circulating estradiol, but decreases rapidly after peaking and reaches basal concentrations less than 24 hours after treatment. Estradiol benzoate causes a reduced peak concentration, but has a more prolonged



elevation in circulating estradiol concentration until 36 hours after treatment. Estradiol cypionate is a longer acting estrogen with sustained release at a lower concentration (Souza et al., 2005).

*Estrus:*

Initiation of estrus occurs due to increased circulating concentrations of estradiol at a time when progesterone is not high (De Silva et al., 1981). In the absence of progesterone, estradiol acts on the hypothalamus to induce behavioral estrus and an LH surge resulting in ovulation (Chenault et al., 1975). During this period of sexual receptivity, a cow will stand to be mounted by a bull or other cows (Eerdenburg et al., 1996). The effects of estradiol on the initiation of estrus appear to be an all or none effect; however, there is no absolute threshold because it differs between individual cows (Allrich, 1994). Rozell and Keisler (1990) indicated that the rate of increase in estradiol has a critical effect on estrus. Efficient estrus detection was reported to be critical for pregnancy success (Foote, 1975). It was reported that increasing heat detection from 50% to 70% results in an increase in conception rate by 14-15% (Diskin, 2008).

Cows in standing estrus within 24 hours of fixed-time AI have been reported to have greater pregnancy success (90% and 88% on days 26 and 68) than nonestrus cows (29% and 26% on days 26 and 68; Perry et al., 2005). Cows that expressed estrus have also been reported to have increased embryo survival to day 30 of gestation (Jinks et al., 2013). Madsen et al. (2015) used ovariectomized cows to demonstrate the importance of preovulatory estradiol on the survival of embryos transferred on day 7. Cows that were exposed to estradiol prior to progesterone treatment were more likely to maintain

pregnancy to day 29. However, on day 6 and 16, there were no differences in conceptus recovery rates between cows with and without elevated preovulatory estradiol concentrations (Larimore et al., 2015; Northrop et al., 2018). These results conclude that the differences in conceptus survival between animals with high and low preovulatory estradiol concentrations most likely occurs following maternal recognition of pregnancy, and before day 29 of pregnancy. We hypothesize that the differences in conceptus survival between highE2 and lowE2 animals occurs around the time of attachment (day 20).

## ROLES OF PREOVULATORY ESTRADIOL

### *Follicular cell growth:*

Estradiol concentrations within the follicle regulate steroidogenic enzyme expression. Specifically, it upregulates the action of FSH on aromatase activity (Zhuang et al., 1982), and induces the expression of FSH and LH receptors on granulosa cells (Richards et al., 1976). Estradiol also influences follicular dynamics by increasing granulosa cell mitosis and gap junction formation (Goldenberg et al., 1972; Merk et al., 1972).

Perry et al. (2014) determined that there was a positive relationship between follicle size and peak estradiol concentrations among cows that exhibited estrus, but there was no relationship among cows that did not exhibit estrus. Previous research also reported a positive relationship between follicle size, circulating concentrations of estradiol, and fertilization success among beef cattle (Perry et al., 2005). Cows that were forced to ovulate smaller follicles (<10 mm in diameter) following an injection of GnRH

experienced decreased pregnancy rates and increased late embryonic mortality (Perry et al., 2005). This decrease in fertility was associated with decreased circulating estradiol at the time of artificial insemination and inferior progesterone production.

*Oocyte Development:*

Oocytes with less progesterone and greater cytochrome P450 aromatase (CYP19A1) activity were more capable of being fertilized and developing into a blastocyst (Hazeleger et al., 1995; Driancourt et al., 1988). Similar findings have also been reported for in vitro fertilization studies; bovine oocytes cultured in media with increased concentrations of estradiol were more likely to develop to the blastocyst stage (Mermiloid et al., 1999). The positive effects of elevated concentrations of estradiol on bovine oocyte competence may be attributed to the impact of estradiol on estrogen receptors within the oocyte and surrounding cumulus cells (Driancourt et al., 1988).

*Vagina, Cervix, Mucus Production:*

The vagina and cervix function as the entry way for spermatozoa into the uterus, and the exit way for calves. The bovine cervix is composed of three cervical rings with crypts and folds. During the follicular phase, estradiol increases the amount of mucus secreted and changes the mucus properties (Mattner, 1973). This creates the proper environment for spermatozoa to move through the cervix into the uterus. Cervical mucus is composed of mucin glycoproteins that are produced by the endocervical endometrium (Pluta et al., 2012). The two types of cervical mucus that are present during estrus are sialomucin and sulfomucin. Sialomucin has a low viscosity and is produced by cells in

the basal area of the cervical crypts, while sulfomucin is very viscous and is produced by the apical areas of the cervical epithelium. Sialomucins aid in filtering out the non-motile spermatozoa. Treatment of ovariectomized ewes with an estrogenic compound resulted in an increase in the production of sialomucins on day 3 (Heydon and Adams, 1979).

*Sperm transport:*

Contractions around the time of estrus facilitates sperm transport through the reproductive tract to the site of fertilization (Hawk, 1983; Vandemark and Hays, 1952). At the initiation of estrus, uterine pH decreases from 7 to 6.5 (Perry and Perry, 2005). This change in uterine pH has been reported to increase the number of sperm that reach the site of fertilization (Larimore et al., 2015). Changes in sodium hydrogen exchangers have been reported to be responsible for these changes in uterine pH among beef cattle (Bolzenius et al., 2016) and mice (Wang et al., 2003). This rapid decrease in uterine pH at the initiation of estrus optimizes fertilization efficiency by decreasing motility and increasing sperm longevity (Jones and Bavister, 2000). A decrease in uterine pH at the time of AI has also been reported to increase pregnancy success when using a fixed- time AI protocol (Bolzenius et al., 2016). Finally, it has been reported that in ovariectomized ewes exogenous estradiol is required for effective sperm transport (Allison and Robinson, 1972).

*Oviduct/Fertilization:*

Fertilization is the process by which the female oocyte fuses with the male spermatozoa to form a single cell zygote. This process occurs within the oviduct at the ampullary isthmic junction. Oviductal fluid plays a critical role in sperm capacitation, sperm hyperactivation, fertilization, and early embryonic development. Rising estradiol has been associated with increasing bicarbonate in the oviductal mucus (Rodriguez-Martinez, 2007). Specifically, oviductal glycoprotein (OGP), is a glycoprotein that is produced during estrus. Sutton et al. (1986) reported that there was no OGP production in ovariectomized ewes; however, when given estradiol benzoate (EB), OGP production was restored. Oviductal glycoprotein has been reported to increase cleavage rates and blastocyst formation following in vitro fertilization (Hill et al., 1996). Estradiol also has a role in the regulation of another oviductal secreted glycoprotein, uterine milk protein (SERPINA14). Uterine milk protein is expressed within the endometrium of cattle and is greatest at time of estrus. The function of uterine milk protein hasn't been well-characterized, but it may be involved in embryo nutrition and growth (Ing et al., 1989). Overall, donor cows with elevated preovulatory estradiol concentrations at time of AI were more likely to have a fertilized embryo instead of an unfertilized oocyte, and they also tended to have improved embryo quality and viability (Jinks et al., 2013).

*Uterine Environment and Embryo/Conceptus:*

The uterine environment plays a critical role in embryo development, conceptus elongation, maternal recognition of pregnancy, and attachment. Increased circulating

concentrations of preovulatory estradiol has been reported to have a beneficial impact on the uterine environment and embryo survival, however the mechanism by which it improves embryo survival has not been well established (Atkins et al., 2013; Jinks et al., 2013).

Whether or not an animal was exposed to elevated concentrations of estradiol impacts gene expression within the endometrium (Bridges et al., 2012). Estradiol induces expression of endometrial receptors, production of uterine proteins (Bartol et al., 1981), and also influences the expression of many genes involved in uterine extracellular matrix remodeling (Bauersachs et al., 2005). Miller et al. (1977) conducted a study that involved giving large or small doses of exogenous estradiol to ovariectomized sheep following embryo transfer on day 4 to determine the impact on the uterine environment and endometrial gene expression. Animals that were given a small dose of estradiol had decreased uterine weight, total protein content, progesterone and estrogen receptor expression within the uterus, and pregnancy success compared to ewes given a larger dose. Bridges and others (2012) determined nuclear progesterone receptors in the deep glandular epithelium and mRNA abundance of ER $\alpha$  in the uterine epithelium was decreased among animals that had decreased preovulatory estradiol concentrations compared to animals that were exposed to elevated estradiol concentrations during the preovulatory period.

On day 6, heifers that exhibited estrus yielded embryos that were more advanced in stage and had improved quality when compared to heifers that did not exhibit estrus, however, recovery rates were not different (Larimore et al., 2015). Bridges et al. (2012) concluded that there was no difference in conceptus size and interferon tau

concentrations on day 15.5 based on preovulatory estradiol exposure. On day 16, our laboratory reported no differences in IFNT, protein, and glucose concentrations in the ULF based on preovulatory estradiol concentrations. However, there were differences in select glucose transporter expression in both intercaruncular and caruncular endometrial tissue. On day 19, Davoodi et al. (2016) reported that genes associated with maternal immune system suppression (IGLL1, CXCL10, MX2, SLPI), attachment between the endometrium and conceptus (MMP19, MYL12A), and CL maintenance (OXT, COX2, FPr, CYP11A, BAX) were favorably expressed in cows that exhibited estrus near the time of AI compared to cows that did not. Contrary to the findings by Bridges et al. (2012), Davoodi and others (2016) reported that cows that exhibited estrus yielded longer conceptuses on day 19.

## PREIMPLANTATION EMBRYO/CONCEPTUS

### *Bovine Embryo Development:*

Fertilization occurs within the oviduct adjacent to the ovary that ovulated. The embryo undergoes several cell divisions leading to a morula on day 5 (16-32 cell stage; Shea, 1981), which then enters the uterus. The first stages of embryo development depend on maternal mRNAs and proteins that are stored in the oocyte. These molecules must be degraded in order for the activation of the embryonic genome to occur around the late 4 cell or 8 cell stage (Memili et al., 1998). Between day 7 and 8, the morula develops into a blastocyst which consists of a blastocoel (fluid filled cavity). This fluid filled cavity is formed by tight junctions that have altered permeability that allows for the diffusion of water. Additionally, a blastocyst consists of an inner cell mass and a

trophectoderm layer (Flechon and Renard, 1978). The inner cell mass eventually gives rise to the fetus, while the outer trophoctoderm cells will develop into the placenta (Forde and Lonergan, 2012). On day 9, the blastocyst hatches from the acellular glycoprotein coat (zona pellucida). Hatching from the zona pellucida (ZP) is regulated by dynamic cellular components (actin) and a variety of autocrine and paracrine molecules (growth factors, cytokines, and proteases). Hatching occurs due to an initial formation of a nick in the ZP caused by hydrostatic pressure from the expanding blastocyst (Seshagiri et al., 2009). On days 11-12, the blastocyst becomes ovoid-shaped, the trophoctoderm cells begin to proliferate and the elongation process begins (Grealy et al., 1996). Blastocyst growth into an elongated bovine conceptus has not been able to be duplicated in vitro (Betteridge and Flechon, 1988). Unlike in humans and rodents, bovine blastocysts do not invasively implant in the endometrium, they are free floating in the uterus until attachment.

#### *Conceptus Elongation:*

Conceptus elongation involves exponential increases in weight and length of the trophoctoderm and the onset of extraembryonic membrane differentiation including gastrulation and the formation of the yolk sac and allantois (Guillomet, 1995; Hue et al., 2012). During elongation, the conceptus becomes more dependent on maternal uterine secretions for survival, growth, and attachment (Filant and Spencer, 2014). On day 13, the conceptus is approximately 2 mm long. By day 16 the elongated conceptus can reach 60 mm in length (Betteridge et al., 1980). The bovine conceptus is capable of doubling its length everyday between days 9 and 16 (Berg et al., 2010). The biomass of the



conceptus increases greatly during elongation. The mean weight of bovine conceptuses increased from 12.2 mg on day 16 (Lewis et al., 1982), 100 mg on day 17 (Meier et al., 2011), and 238.6 mg on day 19 (Lewis et al., 1982).

Prostaglandins (PGs) are necessary for conceptus elongation (Dorniak et al., 2011). Both the conceptus and the endometrium synthesize a variety of PGs during early pregnancy (Lewis and Waterman, 1983; Charpigny et al., 1997). There is substantially more PG production during pregnancy than during the estrous cycle, which is mostly synthesized by the conceptus not the endometrium (Lewis, 1989). The trophoderm of the elongating conceptus has a high demand for arachidonic acid and its precursors for the production of eicosanoids. Day 16 bovine conceptuses produce approximately 350ng of prostaglandins per mg of tissue during a 24-hour incubation in vitro (Lewis et al., 1982). Intrauterine infusion of PGs in ewes induced the expression of genes in the endometrium that were associated with cell proliferation and migration (Dorniak et al., 2011).

The dominant PG related enzyme that is expressed in the uterus and conceptus is cyclooxygenase, PTGS2 (Ulbrich et al., 2009). It is expressed in the conceptus trophoderm, endometrial luminal epithelium, and stromal glandular epithelium during early pregnancy. This cyclooxygenase was reported to be abundant from day 8 to day 17 in conceptuses, with maximal production occurring between days 14 and 16. It declined substantially after day 16 and was undetectable by day 25 of pregnancy (Charpigny et al., 1997). Meloxicam is a selective PTGS2 inhibitor. Intrauterine infusion of meloxicam into pregnant sheep prevented conceptus elongation (Dorniak et

al., 2011). Additionally, when meloxicam was given on day 15 after insemination, pregnancy rates were substantially reduced in heifers (Erdem and Guzeloglu, 2010).

Flunixin meglumine (FM) is a strong non-steroidal anti-inflammatory drug that non-selectively inhibits prostaglandin H Synthase activity (PGHS) (Anderson et al., 1990). Timely injections of FM on days 15 and 16 after insemination increased pregnancy rates in heifers (Guzeloglu et al., 2007). The decrease in PGHS activity most likely leads to a reduction in  $\text{PGF2}\alpha$  synthesis downstream, which may have contributed to the increased embryonic survival. The differences in results between the two prostaglandin inhibitors may be due to differences in what specific prostaglandins they inhibit. Another possible explanation for the differences is that meloxicam is highly selective and longer acting than FM (up to 72 hours). Meloxicam's actions for a longer duration of time most likely interfered with the perimplantation process causing decreased pregnancy rates (Erdem and Guzeloglu, 2010).

#### *Attachment and Placentation:*

Attachment begins around day 19, and adhesion occurs between days 21 and 22 (Peters, 1996). Uterine epithelium is primarily columnar epithelium with some multinucleated cells, while the chorion consists of cuboidal columnar cells with some binucleate cells present (Wathes and Wooding, 1980). By day 20, no interdigitation is present, however, maternal caruncles indent in trophoblastic cells leading to points of attachment occurring in the pregnant horn (King et al., 1980).

Binucleate giant cells (BNGC) are found in ruminant placentas and appear around day 20 in cattle. They originate from mononucleate trophoblast cells that

undergo acytokinetic mitoses (Wimsatt, 1951). Mononucleate trophoblast cells are located on the basal lamina and are connected by tight junctions (Dent, 1973). The mononucleate trophoblast cells consist of an irregular nucleus with a large nucleolus and finely dispersed chromatin with organelles located at the apex of the cell (Boshier and Holloway, 1977). By day 25, BNGC make up 20% of the cells in the trophoctoderm (Wathes and Wooding, 1980). Binucleate cells are not uniformly distributed, rather they appear in small clusters (Wooding, 1984). They are capable of passing through tight junctions between mononucleate cells (Morgan and Wooding, 1983). These binucleate cells migrate and fuse with maternal endometrial epithelial cells and deliver granules via trinucleate cells and syncytial plaques towards the network of capillaries in the maternal stroma (Wooding, 1984). They secrete placental lactogen, pregnancy associated glycoproteins, and synthesize estrogens and progesterone.

Placental lactogen may play a role in stimulating endometrial gland morphogenesis and differentiation, thus facilitating fetal growth and development during pregnancy in ruminants (Johnson et al., 2003). Pregnancy-associated glycoproteins are aspartic proteinases (Green et al., 2000). They have a long half-life, and have been found in maternal serum 80-100 days post partum. Pregnancy associated glycoproteins can be detected in the blood of pregnant cows during the fourth week of pregnancy, and are used in the development of bovine pregnancy tests (Haugejorden et al., 2006). The location, embryo morphology, and hormone profiles from fertilization to day 20 are depicted in figure 2.

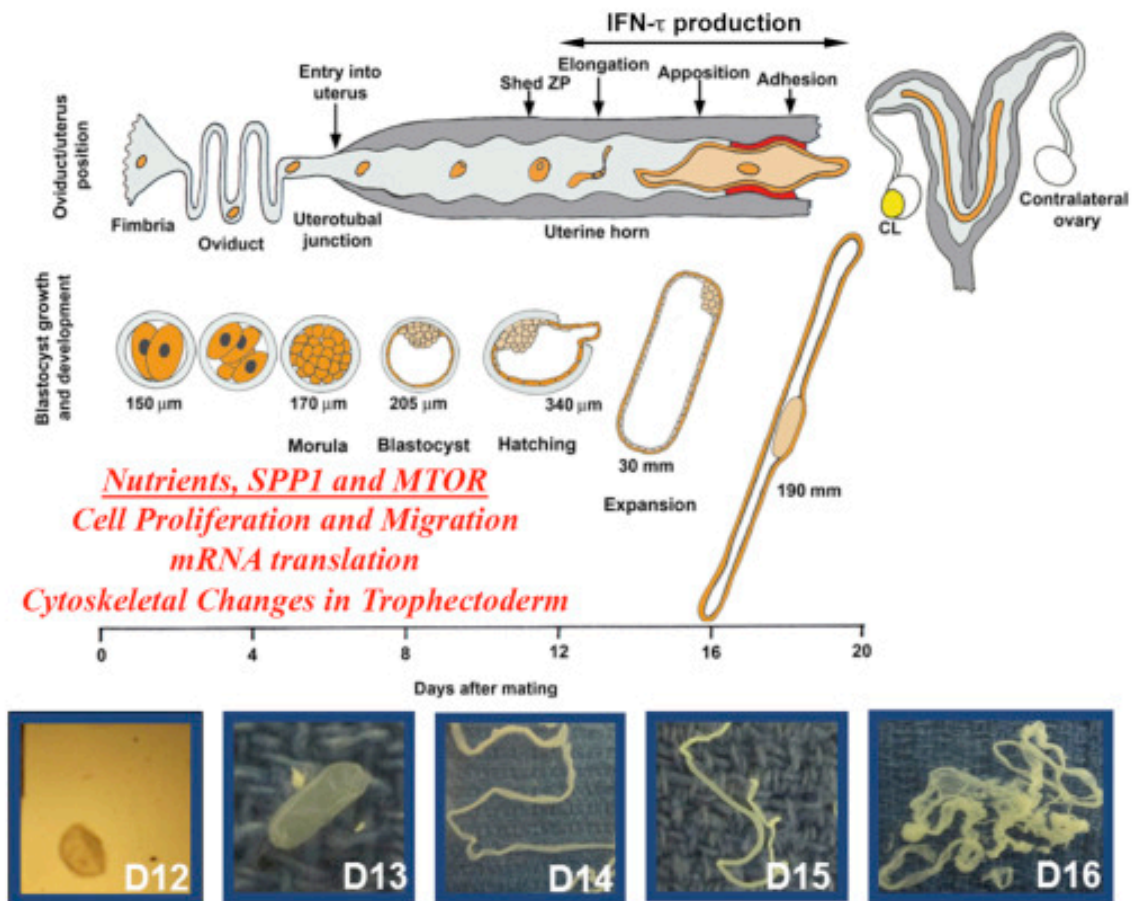


Figure 2. Conceptus location/development and hormone profiles from fertilization to attachment (Bazer, 2015).

On day 21, maternal caruncles begin to contact embryonic cells in the gravid uterine horn (Wathes and Wooding, 1980). Around day 24, interdigitation occurs (King et al., 1980), fetal membranes are present in the opposite horn, and multinucleated cells are found at the base of the horn (Wathes and Wooding, 1980). On day 26, vascularization of the trophoblast occurs (King et al., 1980). By day 28, cuboidal uninucleated cells complete interdigitation (Wathes and Wooding, 1980). Between days 20- 25 of gestation, the embryo and amnion are only ~5 grams with no cotyledons, but increases six-fold to ~30 grams in the next 5 days. At this time, the first morphologically distinguishable cotyledons appear (Neto et al., 2009). After the attachment and adhesion process, the conceptus is able to obtain nutrients and exchange gases from the mother's bloodstream.

Complete placental attachment occurs around day 42 in cattle (Betteridge and Flechon, 1988). Ruminants have epitheliochorial placentas. However, in cattle the endometrial epithelium sometimes erodes (syndesmochorial placenta). This process involves the fusion of placental cotyledons with endometrial caruncles to form convex placentomes. The number of placentomes increases between days 60 and 70 (40 to 80; Neto et al., 2010), but remains constant after day 70 of gestation (Laven and Peters, 2001). Therefore, the increase in nutrient demands is being met due to an increase in placentome length and mass rather than an increase in placentome number. Growth of placentomes coincides with vascular development, which is not completed until ~190 days (Panarace et al., 2006).

Fetal development and growth following placentation within the uterus is a complex biological event that is influenced by maternal maturity, genetic, epigenetic,

and environmental factors. These factors have an impact on size and functional capacity of the placenta, uteroplacental blood flow, transfer of nutrients and oxygen from mother to fetus, conceptus nutrient availability, endocrine environment, and metabolic pathways (Wu et al., 2014). Maternal nutrition/feed intake, maternal intestinal malabsorption, inadequate amniotic and allantoic fluid, ingestion of toxic substances, environmental temperature and stress, disturbances in maternal and fetal metabolic and homeostatic mechanisms, dysfunctional uterus, endometrium, placenta, and poor management are all factors that can negatively impact fetal growth and development (Mellor, 1983; McEnvoy et al., 2001; Redmer et al., 2004; Wu et al., 2004). A complete timeline of the major developmental events leading up to parturition are displayed in Table 1.

Table 1. Timeline for bovine embryo development.

<b>Developmental Event</b>	<b>Day</b>
Estrus	0
Ovulation	1
Fertilization	1
First cell division	2
8 cell stage	3
Migration to uterus	5-6
Maternal recognition of pregnancy	15-17
Attachment to uterus	19
Adhesion to uterus	21-22
Placentation	25
Birth	283

Adapted from: Shea, 1981; Flechon and Renard, 1978; and Peters, 1996

*Apoptosis in Preimplantation Embryos/Conceptuses:*

Apoptosis is a physiological process that involves chromatin condensation, reduction in cell volume, formation of membrane vesicles called apoptotic bodies, resulting in fragments and elimination of unnecessary, damaged and/or dangerous cells (Allen, 1987). Apoptosis is critical for normal differentiation and development of the embryo (Jacobson et al., 1997). The laboratory technique used to evaluate this process in tissue is TUNEL staining. Differential labeling of the inner cell mass cells and trophectoderm with specific fluorochromes has been used to determine the spatial occurrence of apoptosis in embryos (Handyside and Hunter, 1986). In most species, apoptosis is confined to the inner cell mass lineage. In mice, day 4 and 5 blastocysts that were flushed from the reproductive tract had 10% dead cells in the inner cell mass (ICM), while less than 3% of trophectoderm cells were dead (Hardy and Handyside, 1996). An imbalance of the trophectoderm: ICM ratio is most likely a contributing factor to enlarged offspring syndrome in domestic animals (Thompson et al., 1995). This cellular quality control within the ICM is critical since it develops into the fetus and contains the germline. It has been suggested that once programmed cell death reaches a certain threshold, it is detrimental for further development, thus eliminating non-viable offspring (Jurisicova et al., 1996). In bovine in vitro embryos, apoptosis was first observed at the 9-16 cell stage of development, decreasing at the morula stage before increasing during the blastocyst stage (Byrne et al., 1999). The effects of preovulatory estradiol on apoptosis in the trophectoderm of day 16 bovine conceptuses was previously studied in our laboratory. We concluded that there was no difference in the rate of apoptosis in



trophectoderm collected from highE2 and lowE2 animals ( $P = 0.64$ ;  $29.8 \pm 4.0\%$  vs  $27.6 \pm 4.9\%$ ; Northrop et al., 2018).

*Conceptus Gene Expression:*

*Changes Based on Day of Pregnancy:*

Brooks et al. (2016) examined the changes in gene expression throughout early pregnancy in sheep and reported that the total number of genes expressed by the conceptus increased over time (day 12- 4,198, day 14- 6,798, day 16- 8,019 genes, day 20- 8,412). Genes that were increased in the conceptus between days 12 and 14 included: PSA, IFNT, TKDP, and PPARG. Enriched pathways were associated with: cell death and survival, cellular assembly and organization, gene expression, and protein synthesis. Genes that were differentially expressed in the conceptus between days 14 and 16 included: FABP5, AFP, PTGS2, RAN, DDX54 and FADS2. Enriched pathways were associated with: cell death and survival, cellular growth and proliferation, protein synthesis, and cell cycle. Genes that were differentially expressed in the conceptus between days 16 and 20 included: ESM1, IL2RB, PAG11, CSH1, and PLET1. Enriched pathways were associated with: cellular morphology, cellular development, and molecular transport.

Mamo et al. (2011) performed RNA sequencing on bovine conceptuses on days 7, 10, 13, 16, and 19 and reported novel gene clusters associated with maternal recognition of pregnancy and implantation. They reported that a large number of transcripts were detectable at all stages of development, and a smaller number of transcripts belonged to a specific developmental stage. On day 7, top gene networks were involved in cell

signaling, cell cycle, embryonic development, and proliferation. On day 10, the top upregulated genes were: SARM1, SHBG, MYBPC3, ADAT, and LEO1. Top upregulated genes on day 13 included: HNF4A, DNMT3, NFKB, ITGB1, TP53, and SUMO3. Top gene networks were involved in cellular growth, proliferation, and DNA replication. On day 16, genes in clusters 3, 6, 8, and 9 were up-regulated, making it the largest pool of transcripts on a given day. Genes within these clusters most likely contribute to pregnancy establishment in cattle. On day 16, interferon transcripts (IFNT2, DLX3) were among the most highly expressed genes. Other upregulated genes included: AP2, PGF, FURIN, PTGS2, and pregnancy associated glycoproteins (PAGs). Transcription factor AP2 plays a key role in trophoblast cell differentiation (Blomberg et al., 2008). The PGF gene is associated with angiogenesis and regulation of VEGF (Zhao et al., 2004). The FURIN gene is associated with cleaving intracellular and extracellular precursors of various growth factors, receptors, adhesion molecules, neuropeptides, metalloproteases, viral envelope glycoproteins, and bacterial endotoxins (Mesnard and Constam, 2010). Other highly expressed genes on day 16 were associated with cell growth, proliferation, DNA replication, reproduction and development, growth factors, receptors, and integrins (APP, GPCR, IGF1R, TFAP2A, and IFNT2). Integrin transcripts were also prominent on day 19. These integrins play important roles in facilitating cell-to-cell adhesion, cell migration, and attachment of cells to the extracellular matrix around the time of attachment and implantation (Hayes, 1992). On day 19, the major gene networks were associated with metabolism, conceptus development, protein synthesis, and molecular transport.

*Changes Based on Morphology of Conceptus:*

Ribeiro et al. (2016) conducted a study that examined differences in gene expression among ovid (OV), tubular (TUB), and filamentous (FIL) embryos on day 15. There were 667 (321 upregulated, 346 downregulated) transcripts that were differentially expressed between OV and TUB embryos, 404 between TUB and FIL (250 upregulated, 154 downregulated), and 1441 between OV and FIL (725 upregulated, 718 downregulated). Differentially expressed genes among OV and TUB embryos in regard to molecular and cellular functions were associated with: cellular movement, cell to cell signaling/interaction, and cell death and survival. In regard to physiological systems, DEGs were involved in: connective tissue development, embryonic development, and organismal development. Top upregulated genes with OV as the reference included: ANKRD1, TM4SF1, PAG2, SLC27A6, DKK1, GNG2, PPARG, CYYR1, ASS1, and CHD2. Top downregulated genes with OV as the reference included: BOLA, PRSS35, ENPP3, APOA2, PDGFRA, PAM, SORL1, TBC1D4, DPP4, and CERS4.

Differentially expressed genes among TUB and FIL embryos were associated with: lipid metabolism, small molecule biochemistry, molecular transport, and cellular movement. Enriched physiological systems were associated with: embryonic development, organismal development, and reproductive system development and function. Top upregulated genes with TUB as the reference included: PTGS2, TAGLN, TP5313, CDKN1C, PAQR8, ABCG2, PPARG, SLC27A6, PLA2G7, and NOS2. Top downregulated genes with TUB as the reference included: ANF503, AQP3, STAR, PDGFRA, FUT5, GSDMC, TMEFF1, HES6, TLE1, and SLC38A11.

Differentially expressed genes among OV and FIL embryos were associated with: lipid metabolism, small molecule biochemistry, cell death and survival, cellular movement, and cellular assembly and organization. Enriched physiological systems were associated with: organismal development, connective tissue development, tissue morphology, skeletal/muscular system development, and nervous system development. Top upregulated genes with OV as a reference included: PAG2, SLC27A6, ANKRD1, PPARG, PTGS2, TM4SF1, TAGLN, C15H11, TP53I3, and SLCO2A1. Top downregulated genes with OV as a reference included: ZNF503, AQP3, PDGFRA, BOLA-N, PRSS35, CERS4, STAR, ENPP3, CGA, and TBC1D4.

There was an increase in DEGs among filamentous conceptuses relating to the ECM, cell adhesion, motility, and migration. The organization of the ECM is complex and involves interactions of ECM proteins such as fibronectin, proteoglycans, collagen V, osteonectin, laminin, nidogen, and osteopontin with various growth factors. Implantation and placentation involve the remodeling of the endometrial and chorionic ECM to aid in proliferation, differentiation, migration of binucleate cells, and attachment (Imakawa et al., 2017). Interaction between membrane proteins and the ECM creates a mechanical load on trophectoderm cells which is sensed by the cytoskeleton, leading to the generation of mechanical force using energy from hydrolyzed ATP to support cell migration (Kim et al., 2010). Differentially expressed genes relating to ECM remodeling included: actin polymerization/depolymerization proteins (AIF1, DSTN, EVL, and MTSS1), GTPases (CDC42BPG, CDC42EP2, CHN2, EFNA5, and RHOQ), actin filament cap proteins (CAPG, MTPN), actin binding proteins (ABLIM1, AFAP1, ANKRD1, CALD1, CNN3, FLNCA, MYOZ1, TAGLN,

TLN1, and SPTBN1), microtubule organizational proteins (ATXN7, BBS4, MAP7, MICAL1, NDRG1, TPPP, AND TUBB6), and myosins (MYH10, MYO1B).

Assembling and deassembling actin filaments and microtubules, along with myosin and actin crosslinker proteins allows for cell migration. Specifically, transmembrane 4 L six family member 1 (TM4SF1) is associated with cell proliferation, adhesion, motility, migration, and invasiveness in tumor lines. It interacts with integrins, actin filaments, and myosin to form the filopodia necessary for cell migration.). Additionally, there was also a reduction in the expression of some genes related to cytoskeleton organization (ARPC3, FHL3, MAPT, STMN1, TMOD1, and WIPF1). Suggesting that excessive ECM remodeling could possibly inhibit the attachment process in cattle.

Additionally, this study discovered as bovine conceptuses elongate there is an increase in the abundance of cell matrix adhesion molecules (ADAMTS1, FERMT2, F5, ITGAV, ITGA6, LAMC1, LOXL4, NID2, PLAU, TM4SF1, and THBS1). However, some adhesion molecules had decreased expression during elongation (CDH2, GJA1, JAM2, MPZL2, and SDC2; Ribeiro et al., 2016). A possible reasoning for the reduced expression of these genes may be to maintain a loose arrangement for easy migration along the cells that line the uterus.

Overall genes associated with lipid metabolism were present in all morphology comparisons. Functions such as: lipid uptake, lipid droplet formation, activation, and oxidation of fatty acids, biogenesis of peroxisomes, desaturation and elongation of fatty acids, mobilization of membrane phospholipids, biosynthesis of phospholipids, other lipid metabolites, and prostaglandin transport increased with elongation. Peroxisome proliferator activated receptor gamma (PPARG) in particular dramatically increased in

expression during elongation. It is a member of the nuclear receptor family, and functions as a ligand dependent transcription factor that controls a variety of biological processes in the placenta and other tissues (Berger and Moller, 2002). These functions include: uptake and metabolism of fatty acids, cell differentiation, cell proliferation, homeostasis, carcinogenesis, apoptosis, tissue repair, inflammation, immunity, and vascularization (Costa et al., 2010). In mice, when PPARG was knocked out it caused placental defects that lead to lethality (Barak et al., 1999). In humans, low levels of PPARG may play a role in preeclampsia in woman (McCarthy et al., 2011). In sheep, infusion of antisense oligonucleotides for PPARG into the uterus resulted in growth retarded conceptuses with reduced secretion of IFNT and prostaglandins (Brooks et al., 2015).

There was also an increase in the expression of genes related to the immune system among elongating conceptuses. The conceptus is made up of both paternal and maternal genes. These genes have the potential to be recognized as antigens that could be rejected by the maternal immune system. The expression of immune related genes changes throughout the preimplantation phase of development. The expression of the MHC-1 heavy chain (BOLA) was decreased at the onset of elongation (Ribeiro et al., 2016). This suggests that the conceptus itself can reduce antigen presentation. Cloned conceptuses have increased BOLA expression, they also are at a higher risk for pregnancy loss (Bainbridge, 2000). The elongating conceptus can also modify the expression of complement related genes. Complement 3 (C3) is a proinflammatory component of the complement system and it had decreased expression at the time of elongation (Girardi et al., 2011). While decaying accelerated factor (DAF) which

inhibits complement activation, had increased expression (Girardi et al., 2011). When DAF was knocked out in mice there was an increase in embryonic mortality due to the complement system being activated leading to the rejection of the conceptus (Girardi et al., 2011).

*Changes Based on Size of Conceptus:*

Differences in conceptus length on the same day of gestation is a result of uterine asynchrony between the conceptus and uterus. Barnwell et al. (2016) examined differences in gene expression among long (24.7 mm average) and short (4.2 mm average) conceptuses on day 15 of gestation. There were 348 differentially expressed genes among long and short bovine conceptuses. The enriched pathways associated with these genes included: metabolism and biosynthesis (lipid biosynthetic process, coenzyme and cofactor metabolism, cellular aldehyde, and cholesterol metabolism) and immune response (acute inflammatory response, regulation of phagocytosis, regulation of immune effector response, response to wounding, regulation of adaptive immune response, and antigen processing and presentation of peptide antigen via MHC class I).

Top enriched pathways in short conceptuses were involved in metabolism and ribosome/ribonucleoprotein complex biogenesis. Network analysis determined upregulated DEGs in short conceptuses were associated with: immune response, inflammation, and apoptosis. Specifically, upregulated genes in short bovine conceptuses were further related to the complement system, inflammation, and phagocytosis (Barnwell et al., 2016). This may suggest that shorter conceptuses are

being attacked by the maternal immune system, or they have delayed immune related gene expression because they are developmentally behind.

Pathways enriched in long conceptuses were involved in: lipid metabolism, inflammatory response, and tissue development. Network analysis determined upregulated DEGs in long conceptuses were associated with cellular reorganization, development, proliferation, and transport. These proteins are primarily localized to the microsome, vesicular fraction, and external side of plasma membrane. Specifically, apolipoprotein A1 (APOA1), which is highly expressed in elongating conceptuses from day 7 to day 19, was upregulated in long conceptuses. Additionally, PTGS2 was also upregulated in long conceptuses. This particular gene acts as a predictor for developmental competency to term (El-Sayed et al., 2006). Interleukin 6 (IL6) was upregulated in long versus short bovine conceptuses on day 15. This cytokine is associated with rearranging actin filaments and changing cell shape (Maruo et al., 1992). Previous research has determined the important role it plays in the establishment and maintenance of pregnancy in sheep, pigs, and cows (Mathialagan et al., 1992). This suggests that long bovine conceptuses are more competent for pregnancy success (Barnwell et al., 2016).

#### *Changes Based on Fertility:*

Moraes et al. (2018) classified heifers based on previous pregnancy success (high fertile- HF, subfertile- SF, and infertile- IF). They further examined differences in conceptus gene expression among HF and SF heifers on day 17 of gestation. There were 1,287 DEGs among the HF and SF conceptuses. There were only 18 DEGs



between long and short conceptuses within the HF group. This suggests that the differences in gene expression are not due to length, but rather fertility status. There were 558 upregulated genes among HF conceptuses when compared to SF conceptuses. Gene ontology (GO) terms that were enriched among HF conceptuses included: transcription factor activity, embryo development, animal organ morphogenesis, receptor signaling, tissue morphogenesis, and growth and regulation of the actin cytoskeleton. Enriched pathways among HF conceptuses included: integrin mediated cell adhesion, FGF signaling, focal adhesion, MAPK signaling, estrogen signaling, and growth factor signaling.

There were 729 genes upregulated in SF conceptuses when compared to HF conceptuses. Gene ontology (GO) terms that were enriched among SF conceptuses included: organ development, cellular lipid metabolic process, cell adhesion, and lipid metabolic process. Enriched pathways among SF conceptuses were associated with ECM proteins. Some genes (GCM1, BMP2, FGFR2) that had decreased expression among SF conceptuses have been associated with abnormal placental development in mice. GCM1 is a transcription factor that regulates placental development and trophoblast differentiation in mice and humans (Bainbridge et al., 2012). FGFR2 functions to increase trophectoderm proliferation and IFNT production (Michael et al., 2006). BMP2 activates pathways that are necessary for endometrial function and implantation (Wetendorf and DeMayo, 2012).

## MATERNAL RECOGNITION OF PREGNANCY

In cattle, maternal recognition of pregnancy occurs around day 16 after estrus (Bazer, 1997). This physiological process is defined as the requirement for the conceptus to produce and secrete a signal that acts on the uterus and ovary to ensure the maintenance of a functional corpus luteum so progesterone production and pregnancy can be maintained (Bazer, 2013). In cattle, this signal is interferon tau (IFNT). During this critical period, it is necessary for the conceptus to secrete enough IFNT at the appropriate time to ensure that the corpus luteum does not regress. The ability of the developing embryo to secrete sufficient quantities depends on its stage of development (size) and quality (Ealy and Yang, 2009). This conceptus signal is critical for the prevention of the pulsatile release of PGF2 $\alpha$  and the promotion of uterine gland secretions.

### *Interferon Tau:*

Interferon tau is a type 1 interferon with antiviral and antiproliferative properties. It is a glycoprotein with five helices, composed of 172 amino acids with disulfide bridges between cysteine residues at 1, 29, 99, and 139 (Li and Roberts, 1994). It has a molecular weight of 19 to 24 kDa depending on glycosylation, and an isoelectric point between 5.3 and 5.8. It was first discovered in sheep when culturing conceptuses with radiolabeled amino acids and detecting a low molecular weight protein initially named protein X (Bazer, 2013). There have been 11 genomic sequences of IFNT discovered, but not all have been transcribed (Demmers et al., 2001). The signal transduction pathway that is responsible for the amplification of IFNT begins when IFNT binds to its receptor composed of IFNAR1 and IFNAR2 subunits. This activates Janus activated

kinases (JAKs) and other kinase signaling pathways (Platanias, 2005), which causes the formation of STAT-1 transcription homodimers that complex with gamma activation factor (GAF). This complex is then translocated into the nucleus where it binds to gamma activation site elements (GAS) in the promotor region of interferon-stimulated genes to amplify the effects of IFNT (Mamane et al., 1999). A different pathway with similar effects involves interferon stimulatory gene factor 3 (ISGF3G), STAT 1: STAT 2 heterodimer, and IRF9 forming a complex that moves into the nucleus to influence interferon stimulated gene expression (Stewart et al., 2002). A loss of function study was conducted to determine if inhibiting IFNT, IFNAR1, and IFNAR2 mRNA translation in ovine trophectoderm alters elongation. Morpholinos consist of a nucleic acid base and nonionic phosphorodiamidate intersubunit linkage. They are not sensitive to nucleases, and function by blocking translation of target mRNAs. Morpholino antisense oligonucleotides (MAO) were infused via osmotic pumps into ewes from days 8 to 14 postmating. Severly growth retarded and malformed conceptuses were collected from MAO-infused ewes on day 14. They had apoptotic and abnormal trophectoderm cells, and decreased IFNT concentrations in the ULF (Brooks and Spencer, 2015).

Expression of IFNT begins in the mononuclear cells of the trophectoderm during the early morula, late blastocyst stage (day 6-7 of pregnancy; Kubisch et al., 1998). Interferon tau works in a paracrine manner on the endometrium, as trophectoderm cells proliferate the greater the contact with the maternal uterine lining, resulting in signal amplification. Interferon tau mRNA and protein content increases dramatically between days 14-21, this coincides with elongation and trophectoderm proliferation (Ealy and

Yang, 2009). Secretion of IFNT decreases rapidly at the time of uterine attachment from day 19 to day 21 (Ealy and Yang, 2009).

*Factors that influence IFNT production:*

An embryo must secrete sufficient amounts of IFNT by day 16 to prevent the regression of the corpus luteum. Robinson et al. (2006) reported that elongated embryos (> 10 cm) produced more IFNT compared to smaller embryos, but both had similar levels of IFNT mRNA expression. It has also been reported that intrauterine IFNT concentrations were increased from day 14 to day 18 among pregnant animals, and was positively correlated with embryo size. Our lab reported no differences in ULF IFNT concentrations between pregnant and non-pregnant animals on day 16. There was also no difference in ULF IFNT concentration in highE2 and lowE2 animals (Northrop et al., 2018). However, Souto et al. (2011) reported on day 17.5 of gestation, conceptuses from cows with increased preovulatory estradiol concentrations tended to produce greater amounts of IFNT in the uterine lumen. Additionally, some studies have demonstrated increased IFNT production by female blastocysts (Larson and Kubisch, 1998; Kubisch et al., 2001). However, by day 14, Kimura et al. (2004) reported elongated conceptuses from super-ovulated cows had no sexual dimorphism.

*Antiluteolytic effects of IFNT:*

When an animal is not pregnant, oxytocin is synthesized and secreted by large luteal cells, and is also secreted by the posterior pituitary gland (Wathes and Denning, 1992). Oxytocin binds to its receptors on the endometrium, which activates the PKC

secondary messenger system leading to the pulsatile release of PGF<sub>2</sub> $\alpha$  from the uterine luminal epithelium and stromal glandular epithelium (Silvia, 1991). The accumulation of phospholipids in the uterine tissue is stimulated by a decrease in progesterone (day 13/14; (Bazer et al., 2011). The arachidonic acid from the phospholipids is then metabolized by prostaglandin synthase 2 and prostaglandin F synthase for the secretion of PGF<sub>2</sub> $\alpha$ . The PGF<sub>2</sub> $\alpha$  is then transported locally to the ovary by the counter-current exchange system and acts on the corpus luteum causing luteolysis (McCracken et al., 1972). Surgical removal of the uterus prolongs the lifespan of the CL in cattle, providing evidence that the luteolytic PGF<sub>2</sub> $\alpha$  is endometrial in origin (Moor et al., 1970).

When an animal is pregnant, IFNT is secreted by the conceptus and leads to the suppression of the ER $\alpha$  and a decrease in oxytocin receptor mRNA (Bazer et al., 1997). In pregnant cows, the endometrial oxytocin receptor was decreased compared to cyclic cows during the luteolytic period (Thatcher et al., 1995). Mann and Lemming (2000) reported that when high, medium, and low levels of preovulatory estradiol were given to ovariectomized cows, oxytocin binding activity in the endometrium was inhibited by the high treatment, but not by the other two treatments. Previous research has also reported that administering oxytocin to early pregnant cows negatively impacts embryo survival (Lemaster et al., 1999).

Prostaglandin production is further downregulated in pregnant cattle by an increase in the production of an endometrial prostaglandin synthesis inhibitor (EPSI) known as linoleic acid (Thatcher et al., 1994). Linoleic acid acts as a competitive inhibitor of arachidonic acid for cyclooxygenase 2 (COX2; Thatcher et al., 1995). Cyclooxygenase 2 is highly expressed in the endometrium on day 12 (Charpigny et al.,

1997). Cyclooxygenase 2 is the rate-limiting enzyme that controls PGE2 and PGF2 $\alpha$  synthesis (Arosh et al., 2004). PGE2 appears to bind to the same receptor as PGF2 $\alpha$  in bovine luteal cells (Rao, 1975). Interferon tau alters the PGE2: PGF2 $\alpha$  ratio in favor of PGE2, which is luteotrophic (Pratt et al., 1977). Intrauterine administration of PGE2 protects the CL from induced and spontaneous luteolysis in ruminants (Pratt et al., 1977), and it also stimulates luteal progesterone secretion (Marsh and LeMaire, 1974).

*Interferon tau stimulates ISG expression:*

Interferon stimulated gene (ISG) expression in the endometrium is upregulated due to IFNT secretion by the conceptus (Yankey et al., 2001). Parity status appears to influence the ISG response to IFNT (Green et al., 2010). It is speculated that this may be due to primiparous animals having larger embryos that secrete more IFNT (Green et al., 2010). Interferon stimulated genes are hypothesized to regulate uterine receptivity around the time of implantation as well as survival and growth of the conceptus (Kim et al., 2013).

There are more than 100 known ISGs, but not all are expressed equally among pregnant and cyclic animals (Samuel, 2001). Previous research has focused on increased expression of specific ISGs [Interferon stimulated protein 15 kDa (ISG15) (Austin et al., 1996), myxovirus-resistance proteins 1 and 2 (MX) (Charleston and Stewart, 1993), and 2' 5' oligoadenylate synthetase (OAS1) (Johnson et al., 2001)] in peripheral blood mononuclear cells during pregnancy. Gifford et al. (2007) reported that expression of MX2 increased as early as day 16 after insemination, and ISG15 increased around day 18 of pregnancy in cattle. Madsen et al. (2015) reported that the expression of ISG15,

MX2, and OAS1 were increased on days 17, 19, and 21 among pregnant animals compared to non-pregnant animals. Green et al. (2010) also reported that MX2 and ISG15 were increased among pregnant cows on day 18 and 20. Further research is needed to determine if measuring ISG levels prior to day 18 is accurate enough to determine early pregnancy status.

### UTERINE ENVIRONMENT

The uterine endometrium is a complex tissue comprised of luminal, superficial, and deep glandular epithelial cells as well as fibroblast-like stromal cells each having an important role in the survival of the conceptus. The luminal (caruncular) epithelium is made up of glandless dense stromal protrusions that are sites for trophoctoderm attachment. Changes in gene expression in the luminal epithelium between days 10 and 12 help to drive growth from a hatched blastocyst to a filamentous conceptus (Bazer et al., 2011), by day 20 most of the luminal epithelium disappears. Glandular (intercaruncular) epithelium contains branched glands that produce uterine histotroph to nourish the conceptus. These tissue types work together to coordinate mechanisms that stimulate: conceptus development, uterine blood flow, water and electrolyte transport, maternal recognition of pregnancy, and transport of nutrients necessary for conceptus growth and development into the uterine lumen (Bazer et al., 2012). Changes in the endometrial transcriptome are necessary for uterine receptivity and attachment to occur (Forde and Lonergan, 2012). Interferon tau, progesterone, estradiol, prostaglandins, and cortisol have been reported to regulate these changes in uterine gene expression (Brooks

et al., 2014). A suboptimal uterine environment often leads to poor development and embryonic mortality.

*Uterine Gland Development:*

Uterine gland morphogenesis is not initiated until after birth, the critical window for development occurs between days 13 and 56 postnatally (Wiley et al., 1987). During this time, exposure to steroid hormones results in permanent epigenetic ablation of uterine gland morphogenesis (Bartol et al., 1997). Chronic exposure of norgestomet (NOR- progesterone treatment) to neonatal ewes from birth permanently ablates endometrial gland morphogenesis creating the UGKO phenotype. Adult UGKO ewes have defective estrous cycles due to the inability of the endometrium to produce adequate PGF<sub>2</sub> $\alpha$  pulses (Gray et al., 2000). However, exposure of NOR to neonatal ewes does not affect development or function of the brain, hypothalamic pituitary ovarian axis, ovary, cervix, oviduct, and vagina (Gray et al., 2000; 2001). Norgestomet exposed adult ewes have lower uterocervical weights, uterine horn lengths, and uterine wet weights (Gray et al., 2001). Decreased uterine wet weights were also observed in adult cows that were exposed to progesterone and estradiol benzoate (Bartol et al., 1995). Uteri from NOR exposed ewes also lacked intercaruncular endometrial areas and had reductions in the number of endometrial folds. Endometrial width and area was also reduced in the NOR treated ewes compared to control ewes. There were no differences in the myometrium between the groups. This is most likely due to the myometrium being already differentiated into layers by birth (Taylor et al., 2000). When Gray et al. (2001) placed UGKO ewes with fertile rams; no pregnancies were ever determined on day 25



after insemination. When day 7 blastocysts were transferred into control and UGKO ewes, pregnancy was established in control ewes, but not UGKO ewes (Gray et al., 2001). This demonstrates the importance of uterine glands in conceptus development and survival.

#### *Uterine Histotroph:*

For a successful pregnancy, the conceptus and the uterine environment must be in synchrony with each other. The maternal environment needs to provide sufficient secretions for the developing embryo, these secretions are known as the uterine histotroph (Gao et al., 2009a). It is composed of a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, lipids, and glucose (Gao et al., 2009a). Conceptus cells use these molecules for nutrition, homeostasis, and cell signaling. These nutrients stimulate the nutrient sensing signaling pathway to increase messenger RNA translation (Martin and Sutherland, 2001). Cell signaling through this pathway also stimulates cell migration, invasion, cell growth and proliferation, ribosome synthesis, expression of metabolism related genes, autophagy, and cytoskeleton reorganization (Liu et al., 2008; Kim et al., 2002).

The secretions from the uterine epithelium changes throughout pregnancy. Tight junction and adheren associated proteins were moderately to abundantly present in the endometrium on day 10 of pregnancy, however, on day 12 these proteins decreased resulting in leaky channels that allow nutrient exchange between the conceptus and mother. Specifically, progesterone has been reported to alter tight and adheren junctions in the uterus (Satterfield et al., 2007). Between days 15 and 50 of pregnancy the

endometrial glands undergo significant hyperplasia, followed by hypertrophy to allow for an increase in surface area and maximal histotroph production (Moffatt et al., 1987). Specifically, during implantation the epithelial cells of the uterine lumen are highly secretory, and the trophoblast also exhibits intense pinocytotic activity (Guillomot, 1995).

*Glucose:*

Glucose appears to be detrimental to early preimplantation embryo development. Ellington et al. (1990) suggested culture media containing glucose during the first 36 hours resulted in impaired development of blastocysts; however, an increase in the lactate to pyruvate ratio stimulated embryonic development (Moore and Bondioli, 1990). The energy substrate for mammalian conceptuses switches from pyruvate and lactate to glucose at the blastocyst stage, which coincides with an increase in the expression of uterine glucose transporter proteins (Zhao et al., 2005). Later in development, glucose is known to regulate trophoblast proliferation and function (Wen et al., 2005). It is one of the main energy sources used by the conceptus for growth and development.

Glucose enhances trophoblast growth by activation of glutamine fructose-6-phosphate amidotransferase (GFAT)-mediated FKBP12-rapamycin complex-associated protein 1 (FRAP1, formerly mTOR) signaling pathway (Wen et al., 2005). Neither the conceptus nor the endometrium is capable of gluconeogenesis (Gao et al., 2009b), thus for glucose to be made available to the conceptus it must be delivered into the uterus via glucose transporters (Leese and Barton, 1984; Pantaleon and Kay, 1998). This glucose can then be used by the conceptus to make glycogen, nucleic acids, proteins, and lipids

during the peri-implantation period (Gao et al., 2009a). If glucose is not utilized for energy metabolism it is converted into fructose. The role of fructose is unclear, however; it is the most abundant hexose sugar in fetal blood and fluids in mammals (Bacon and Bell, 1948).

In sheep, total glucose content in ULF has been reported to increase six-fold between days 10 and 15 of gestation (Gao et al., 2009a; Flechon et al., 1986). During this critical period, the conceptus is transitioning from a spherical embryo to a filamentous embryo. A study in our laboratory concluded that total glucose in ULF was not significantly different among highE2 and lowE2 animals. However, animals from which a conceptus was recovered tended to have decreased glucose in ULF (Northrop et al., 2018). A possible reasoning for this is that excess glucose has been reported to negatively impact stem cell differentiation in mice (Yang et al., 2016), embryonic morphogenesis in rats, and implantation in humans (Zhou et al., 1997). The increase in glucose leads to an increased flow of the electron transport chain, increased oxygen pressure, and increased production of free radicals (Eriksson and Borg, 1991). These results indicate that an appropriate balance of glucose transport into the uterine lumen is necessary to ensure a suitable environment for a developing conceptus.

Glucose transporters are found throughout the body in various tissues. Transport of glucose is mediated by facilitative and/or sodium dependent transporters. Facilitative transporters work bidirectionally, and are energy independent (Wood and Trayhurn, 2003). Thirteen facilitative transporters have been discovered (SLC2A1-SLC2A12), many of them are present in preimplantation blastocysts (Riley and Moley, 2006). Sodium dependent transporters work against the electrochemical gradient

(SLC5A<sub>1</sub>; Zhao and Keating, 2007). Both facilitative and sodium dependent transporters work together to optimize glucose transport into the uterine lumen where it can be utilized for growth and development by the conceptus (Gao et al., 2009b). Previous literature has focused on select glucose transporters (SLC2A1, SLC2A3, SLC2A4, and SLC5A1) when examining glucose transport in the uterus. The expression of these select transporters differs among cyclic and pregnant ruminants (Gao et al., 2009).

The SLC2A1 glucose transporter is ubiquitous in humans, and is highly abundant in the bovine conceptus (Navarrete et al., 2000). Through immunohistochemistry, the SLC2A1 transporter has been localized mainly in the glandular and luminal epithelial cells (Franca et al., 2015). It was also present in the extraembryonic endoderm and trophoderm of the conceptus between days 14 and 20 of pregnancy (Gao et al., 2009b). Gao et al. (2009b) discovered that SLC2A1 mRNA was increased in pregnant ewes starting at day 10 compared to cyclic ewes. Treating ovariectomized ewes with progesterone from days 5 to 16 increased SLC2A1 mRNA 4.2 fold. Intrauterine infusion of IFNT from day 11 to 16 in ewes increased SLC2A1 mRNA 2.1 fold. Thus, expression of SLC2A1 appears to be regulated by both progesterone and IFNT in the glandular and superficial glandular epithelium.

The glucose transporter SLC2A3 has a low  $K_m$  and plays a critical role in embryonic development that cannot be compensated for by the overexpression of SLC2A1 (Ganguly et al., 2007). Mice lacking the SLC2A3 gene have restricted fetal growth and failed pregnancies (Ganguly et al., 2007). Expression of SLC2A3 mRNA

has also been detected in the extraembryonic endoderm and trophoctoderm of sheep conceptuses between days 12 and 20 of pregnancy (Gao et al., 2009b).

The glucose transporter SLC2A4 has been widely studied for its role in diabetes. It has been reported in the trophoctoderm of cows, rabbits, rats, and mice (Navarrete et al., 2000). In humans, insulin and glucose in the maternal system can regulate the expression of SLC2A4 in syncytiotrophoblasts (Ericsson et al., 2005). Gao et al. (2009b) reported that SLC2A4 expression in the extraembryonic endoderm and trophoctoderm increased between days 10 and 18 of pregnancy. Treatment of ovariectomized ewes with progesterone tended to increase SLC2A4 mRNA in the endometrium, while the combined effects of progesterone and IFNT increased SLC2A4 mRNA levels 1.9-fold (Gao et al., 2009b).

The glucose transporter SLC5A1 may function as a uniporter that transports sodium, urea, and water (Wright and Turk, 2004). Among cyclic ewes, expression of SLC5A1 mRNA increased between days 10 and 14, but decreased by day 16 (Gao et al., 2009b). Pregnant ewes had an increase in endometrial expression of SLC5A1 mRNA between days 10 and 12, and expression remained elevated through day 16 (Gao et al., 2009b). In ewes treated with progesterone, SLC5A1 mRNA abundance was greater regardless of IFNT treatment (Gao et al., 2009b).

Our laboratory examined the impact of preovulatory estradiol and conceptus presence on glucose transporter expression in caruncular and intercaruncular endometrial tissue collected on day 16. In caruncular endometria, highE2 animals had greater abundance of SLC2A1 and SLC5A1 mRNA. There was no difference in SLC2A4 and SLC2A5 mRNA abundance between highE2 and lowE2 animals. In intercaruncular

tissue, there was also no difference in SLC2A4 and SLC2A5 mRNA abundance between highE2 and lowE2 animals, while highE2 animals had increased SLC2A1 and SLC5A1 mRNA abundance.

In caruncular endometria, there was no difference in SLC2A1, SLC2A5, and SLC5A1 mRNA abundance among conceptus and no conceptus animals. However, animals from which a conceptus was recovered had decreased SLC2A4 mRNA abundance. In intercaruncular tissue, there was no difference in SLC2A1, SLC2A4, and SLC2A5 mRNA abundance, but presence of a conceptus tended to increase the abundance of SLC5A1 mRNA. These changes in glucose transporter expression may serve as a potential mechanism to regulate glucose concentration in the uterine lumen where it can be utilized for growth by the developing conceptus.

*Amino Acids:*

Amino acids originate from the blood, and are transported through the vascular wall and endometrial tissue into the uterine lumen. Amino acids in the ULF are influenced by the day of the estrous cycle and pregnancy (Groebner et al., 2011). Shortly after the embryo enters the uterus, histidine, glycine, taurine, threonine, phenylalanine, glutamate, serine, glutamine, arginine, alanine, tryptophan, valine, and leucine tended to increase in ULF (Hugentobler et al., 2007). However, the most significant increase in these nutrients occurs between days 12 and 16 of pregnancy, which coincides with rapid conceptus growth and elongation. It is assumed that the conceptus induces an increase in amino acid transport via signaling molecules which respond to progesterone and IFNT (Farin et al., 1990).

Each amino acid within the uterus has a functional role in embryo development by regulating intracellular pH (Bavister et al., 1983), osmolarity (Bavister et al., 1983), preventing ammonia toxicity (Gardner and Lane, 1993), protecting membranes (Menezo and Guerin, 1997), activating cellular functions (Kim et al., 2008; Wu et al., 2013), and promoting blastocyst growth, hatching, and protein synthesis (Frei et al., 1989; Moore and Bondioli, 1993; Grealy et al., 1996; Morris et al., 2000). Specifically, the amino acid glutamine is a major energy source used by the conceptus. It is suggested that glutamine is the preferred energy source over glucose during early embryo development (Rieger et al., 1991). Gao et al. (2009a) reported that arginine, glutamine, and leucine were 7-, 6-, and 5- fold greater in ovine uterine flush on day 15 of pregnancy compared to cyclic ewes. These amino acids are present in many cells and tissues, and they stimulate FRAP1 resulting in the activation of the target protein kinase, p70S6, which induces hyperplasia and hypertrophy during conceptus elongation in ruminants (Wu et al., 2004). Leucine and arginine also stimulate motility and growth of the trophectoderm around the time of implantation using the same signaling pathway (J. Kim and Bazer, unpublished). Additionally, arginine is utilized during protein synthesis. Within the uterus it acts as a precursor for nitric oxide and polyamines (Wang et al., 2014). Nitric oxide and polyamines are crucial for angiogenesis, embryogenesis, placental growth, utero-placental blood flow, nutrient transfer, and fetal/placental growth and development (Wu et al., 2006; Wu and Meininger, 2009). Knockdown of SLC7A1 (Arginine transporter) and NOS3 (NO transporter) resulted in retarded conceptus elongation in sheep (Wang et al., 2014).

Glycine, glutamate, lysine, serine, and glutamic acid are the most abundant amino acids in the uterine flush of pregnant and cyclic ewes (Gao et al., 2009). Glycine and serine are interconvertible, which contributes to one carbon unit metabolism that is necessary for DNA synthesis and cell proliferation. Glutamate increased 10-fold between days 10 and 14. It most likely acts as an osmolyte and energy source for the conceptus (Steeves and Gardner, 1999). Glutamate, glycine, and cysteine are involved in the synthesis of glutathione. Glutathione is the most abundant antioxidant in cells, and serves as a regulator in DNA synthesis, gene expression, and signal transduction (Wu et al., 2004; Hobbs and Kay, 1986). Glutathione increased in the uterine lumen of pregnant ewes between days 10 and 16 when the conceptus undergoes rapid growth (Gao et al., 2009a).

*Ions:*

Ionic composition in ULF is important for both sperm transport and embryo development. Calcium, sodium, potassium, and chloride function as osmolytes in uterine fluid. They participate in amino acid transport, glucose transport, enzyme-catalyzed metabolic pathways, and cell signaling events during pregnancy (Hostetler et al., 2003). They are critical for blastocyst expansion (Hobbs et al., 1986) and implantation (Wray et al., 2003). Sodium and chloride are the most prominent ions in ULF and serum. They account for 47% and 33% of the total osmolarity of extracellular fluid (Hugentobler et al., 2007). Sodium in the uterine fluid increases on day 6 and 8, which is concurrent with blastocele formation (Hugentobler et al., 2007). Extracellular chloride functions to regulate fluid secretion into the lumen (Reischl et al., 2000). In sheep, potassium in the



uterine fluid increases on day 14 compared to day 6 or 8 (Jordan et al., 1983). This coincides with implantation and acts to decrease uterine epithelium and embryo membrane potential (Casslen and Nilsson, 1984). Calcium ions play a role in uterine smooth muscle contractions and fetal implantation (Sanborn, 2000). Calcium in uterine flush appeared to vary by both day of the cycle and pregnancy. Fluctuations in calcium levels are most likely due to different calcium transporters in the uterus responding differently to progesterone and estradiol exposure (Kim et al., 2006).

#### *Essential Trace Elements:*

The fetus is dependent on the mother for supplying essential trace elements. Trace minerals are necessary for protein synthesis, activation of enzymes, and immune system functions. These trace elements are present in small quantities in the body, but can greatly influence reproduction. Inadequate transfer of these elements can result in fetal mineral deficiency. This can lead to impaired fetal growth, and central nervous system, skeleton, and metabolism abnormalities (Widdowson et al., 1974).

Copper, iodine, iron, manganese, selenium, and zinc are all trace minerals that are known to influence embryonic and fetal survival, reproductive performance, and growth (Davis and Mertz, 1987; Hidioglou, 1979). In particular, copper activates enzymes associated with iron metabolism and is needed for hematopoiesis. Most of the iodine found in the body is located in the thyroid gland because it is a necessary precursor for thyroid hormones. Specifically, the fetus requires maternal T<sub>4</sub> to support early brain development (Burrow et al., 1990). An iodine deficiency leads to reproductive problems such as: fetal resorption, abortion, and stillbirths (Hostetler et al., 2003). Manganese

plays a key role in fetal bone formation (Gamble et al., 1971). It also helps to initiate estradiol secretion by the conceptus as the signal for pregnancy recognition in pigs (Hidioglou and Shearer, 1976). In ruminants, when diets were supplemented with manganese pregnancy rates were improved (Egan, 1972). Zinc plays a role in the formation of zinc fingers, which are necessary for the binding of steroid-receptor complexes to DNA (Freedman, 1992). It acts as a cofactor in DNA synthesis and gene transcription (Chesters, 1991), and is involved in prostaglandin synthesis (Chanmugam et al., 1999). Zinc deficiencies often lead to prolonged gestations, difficult labor, low birth weights, and weak offspring (Bedwal and Bahuguna, 1974; Favier, 1992).

#### *Lipids:*

Lipids are necessary for structural/bioactive properties, providing energy to support proliferating tissue, cell signaling (Fernandis and Wenk, 2007), subcompartmentalization of cell membranes (Lingwood and Simons, 2010), and oxidation and generation of ATP (Ribeiro et al., 2016). Lipids are especially important during elongation in ruminants. During this time, there is an increase in the expression of genes associated with lipid derivatives, uptake of lipids from the extracellular space (fatty acid transporters), scavenger receptors, peroxisome formation and biosynthesis machinery, fatty acid modifications, and oxidation of fatty acids. The oxidation of fatty acids generates ATP, while lipid derivatives play a key role in cell signaling (Ribeiro et al., 2016).

The endometrium serves as the main source of lipids for conceptuses. In the uterine lumen, they are made available through exporting exosomes, microvesicles,

carrier proteins, and lipoproteins (Ribeiro et al., 2016; Figure 3). They accumulate primarily in the luminal epithelium and superficial glandular epithelium in cows (Wordinger et al., 1977). The main hormone influencing the accumulation of lipids in the uterus is progesterone (Brinsfield and Hawk, 1973). During diestrus, lipids accumulate in endometrial cells and act as a critical source of fatty acids for utilization by the conceptus.

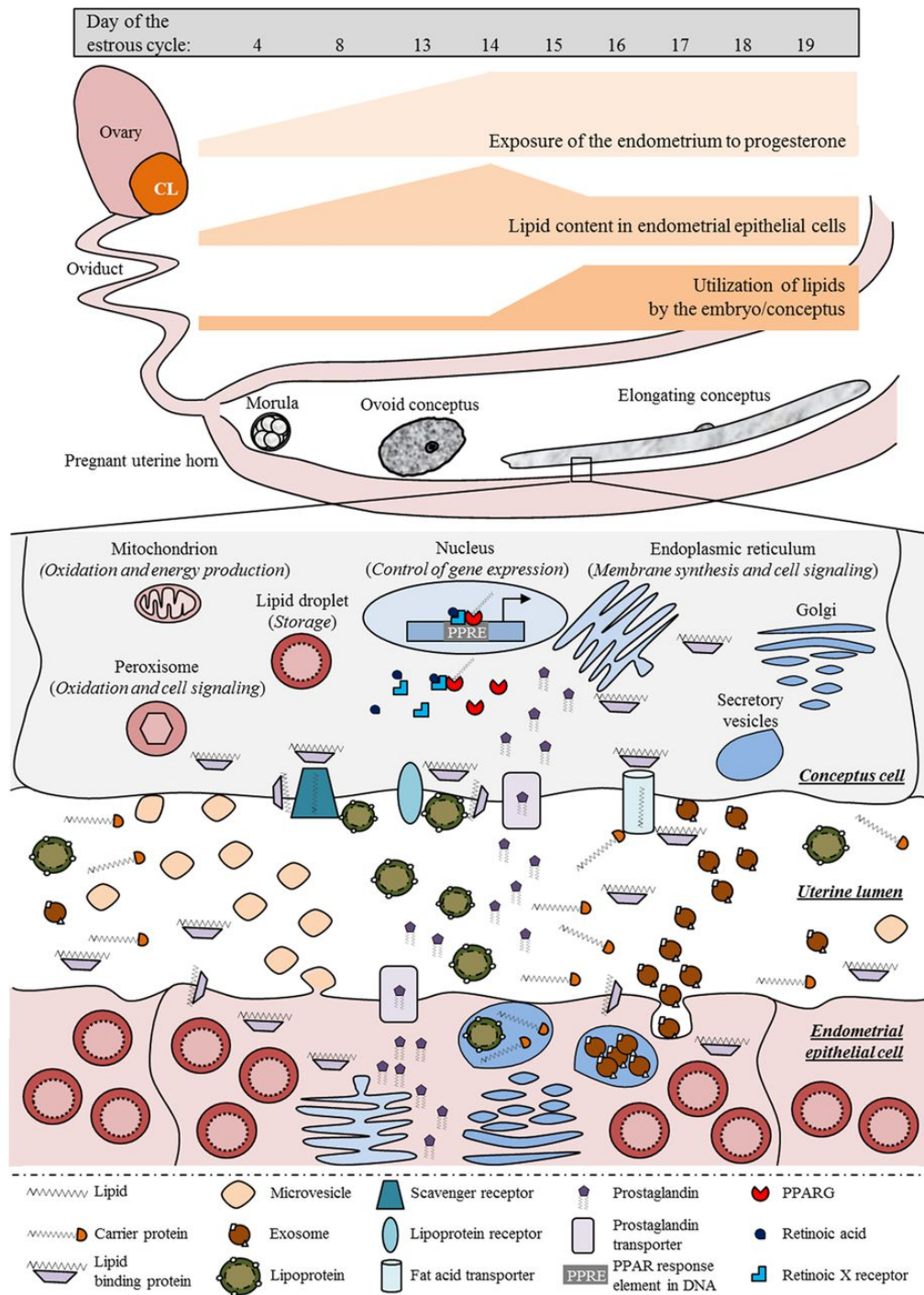


Figure 3. Lipids in the uterus during conceptus development (Ribeiro et al., 2016).

*Embryokines:*

Embryokines are regulatory molecules produced by the reproductive tract that modulate embryonic development (Hansen et al., 2014). Some embryokines that have been identified to influence preimplantation development include: CSF2 (Hansen et al., 2014), DKK1 (Hansen et al., 2014), FGF2 (Fields et al., 2011), IGF1 (Block and Hansen, 2007), and TGF $\beta$  (Neira et al., 2010). These molecules regulate key developmental events by: enhancing proliferation during the blastocyst stage (IGF1), regulating inner cell mass development (CSF2), controlling differentiation (DKK1), and inhibiting apoptosis and stress mediated developmental arrest (IGF1 and CSF2; Hansen et al., 2014). Additionally, TGF $\beta$ , FGF, and LIF used on their own accelerate embryonic development and improve the yield of hatched blastocysts (Neira et al., 2010).

Granulocyte-macrophage colony stimulating factor (CSF2) is important in the inhibition of apoptosis and intracellular regulation of endometrial, oviductal, and embryonic function during early pregnancy in cattle. It is a cytokine that is produced in the luminal epithelium (De Moraes et al., 1999). Supplementation of CSF2 in the bovine trophectoderm cell line (CT-1) increased both IFNT secretion and bovine blastocyst development in culture (Imakawa et al., 1993). Additionally, treatment with CSF2 increased IFNT production by elongated sheep conceptuses (Imakawa et al., 1993). Previous research has reported a sexual dimorphism response when embryos were exposed to CSF2. In vitro produced embryos with CSF2 from days 5-7 resulted in decreased embryo length and intrauterine accumulation of IFNT in females, but increased length and IFNT accumulation in males (Hansen et al., 2016). Treatment of bovine embryos with CSF2 from days 5-7 also increased the proportion of established

pregnancies at day 30-35 of gestation (Loureiro et al., 2009). Exposure to CSF2 from days 5-7 following insemination also changed the embryo development trajectory, gene expression, and DNA methylation as early as day 15 of pregnancy. Additionally, calves produced with CSF2 in vitro were reported to have greater increases in body weight through 13 months of age (Kannampuzha-Francis et al., 2015).

The WNT (Wingless-related mouse mammary tumor viruses) family has critical roles in development, oncogenesis, angiogenesis, inflammation, and wound repair (Van Amerongen and Nusse, 2009). This signaling pathway is regulated by the conceptus and sex steroids in the endometrium (Satterfield et al., 2008). It regulates pluripotency and helps to facilitate differentiation of the embryo (Sato et al., 2004). Dickkopf 1 (DKK1), a WNT antagonist, is an endometrial secretory protein that is potentially involved in maternal-embryo communication. When DKK1 was cultured with bovine embryos from days 5-7 of development, it resulted in embryos that were longer than control embryos on day 34 of gestation (Denicol et al., 2014).

Secretion of IGF is stimulated by GH, the bovine uterus and conceptus have receptors for GH and IGF-1 (Kolle et al., 1997). Supplementation of GH during the preimplantation period resulted in an increased ISG response in peripheral immune cells and reduced pregnancy losses (Ribeiro et al., 2014). Insulin-like growth factors 1 and 2 have been reported to increase the proportion of blastocysts that advance in development by increasing DNA and RNA synthesis at the time of compaction. They also work by limiting degradation and stimulating the incorporation of proteins via endocytosis (Brison and Schultz, 1997). Treatment of embryos with IGF-1 improved pregnancy and calving rates under heat stressed conditions (Block and Hansen, 2007). These results

may be due to IGF-1 treatment causing a more viable embryonic disc that can withstand high temperatures.

Transforming growth factor beta (TGF- $\beta$ ) and activin A are expressed in the endometrium and have been reported to be involved in cell proliferation, differentiation, tissue remodeling, decidualization, and the establishment of pregnancy (Jones et al., 2006). In vitro, exogenous TGF- $\beta$  in culture media facilitated embryo development and promoted blastocyst proliferation and development (Paria and Dey, 1990). Treatment of cultured bovine embryos with recombinant Activin A reduced the time needed to reach the blastocyst stage and improved hatching rates (Orimo et al., 1996). Production and secretion of TGF- $\beta$  and activins by epithelial glands during the secretory phase also prepares the endometrium for implantation by inducing uterine epithelial cell apoptosis and facilitating development/differentiation of the preimplantation embryo (Kamijo et al., 1998).

#### *Proteins:*

Proteins within the ULF are involved in elongation, recognition of pregnancy, implantation, and placentation. Fully elongated conceptuses when cultured in vitro produce a significant number of proteins (Bartol et al. 1985). A study from our laboratory reported that there were no differences in total protein concentrations in the ULF on day 16 between highE2 and lowE2 animals. However, animals from which a conceptus was recovered had greater protein in the ULF compared to no conceptus animals (Northrop et al., 2018). Groebner et al. (2011) examined the differences in total protein concentrations in the ULF of pregnant and non-pregnant heifers on days 12, 15,

and 18 post estrus. Total protein concentration decreased from days 15 to 18 in both pregnant and non-pregnant heifers.

*ULF Proteomic Analysis During Pregnancy in Ruminants:*

Several studies have examined protein differences in ULF between pregnant and cyclic ruminants. Koch and coworkers (2010) identified 100 of the most abundant proteins in the ULF of ewes on day 16 of pregnancy, which is when the attachment process begins. Thirty eight percent were associated with growth and remodeling, 30% nutrition, 22% immune system, 5% oxidative stress, and 5% miscellaneous. Fifteen of these proteins were significantly altered in pregnant sheep, IGHM, CC5, CC4a were significantly elevated in the pregnant group. These proteins are associated with the immunity defense system, which plays an important role in embryo and host protection (Girardi et al., 2006). Nutrition related proteins that had increased protein abundance in pregnant ewes included: AHCY, GPI, and APOA1. The AHCY protein regulates the production of homocysteinase, which is a critical amino acid associated with miscarriages, intrauterine growth restriction, and preeclampsia (De la Calle et al., 2003). There must be an optimal homocysteinase level within the ULF, otherwise increased levels can lead to oxidative stress which can negatively impact pregnancy establishment and maintenance (Micle et al., 2012). Previous research identified an increase in AHCY protein abundance in the non-gravid horn compared to the gravid horn on days 16 and 20 in sheep (Arianmanesh et al., 2016). The GPI protein is an enzyme involved in glucose metabolism and is linked to implantation (Schulz et al., 2003). The APOA1 protein is synthesized by the liver and intestine, and is a component of HDL (Zannis et al., 1985). It



is a source of nutrients for conceptuses shortly after implantation (Assemat et al., 2005). In the gravid horn, APOA1 protein abundance increased on day 20 compared to day 16 of pregnancy in sheep (Arianmanesh et al., 2016). Therefore, the conceptus can potentially exert local effects on carunucular tissue to improve the chance of survival. The proteins related to growth and remodeling that were increased in pregnant ewes included: A1BG, ACTN4, CA2, transgelin, PP9, and A2M. The A1BG protein is commonly expressed in bovine conceptus fluids (Riding et al., 2008). Carbonic anhydrase II catalyzes the hydration of carbon dioxide to bicarbonate and is critical for maintaining acid-base homeostasis (Kalifah, 1971). It is expressed in the luminal epithelium and is suggested to play an important role in endometrial gland development, morphogenesis, and remodeling (Hu and Spencer, 2005). Ledgard and others (2009) identified greater CA2 protein expression in pregnant ULF on days 16 and 18 compared to nonpregnant ULF. Arianmanesh and others (2009) reported an increase in CA2 protein in the gravid horn compared to the nongravid horn on days 16 and 20 of pregnancy in sheep. Transgelin is reported to be a biomarker for arterial vessel remodeling in the uterine tissue (Camoretti-Mercado et al., 1998). The PP9 protein catalyzes the conversion of glucose to fructose (Lee et al., 1998). Fructose is the primary sugar in fetal fluids. The protease inhibitor, A2M is produced by endometrial stroma. It plays a critical role in regulating preimplantation embryonic development and endometrial remodeling (Sayegh et al., 1995).

Previous research has characterized protein changes in the ULF on days 10,12,14,16, and 20 of pregnancy in ewes (Brooks et al., 2016). Approximately, 1,400 proteins were detected in the ULF. On day 10, the ULF contained 79 proteins, increased

to 102 proteins on day 12, 167 proteins on day 14, and 201 proteins on day 16. There was an increase in proteins in the ULF from days 10 to 16 that were associated with: cellular reorganization, proteases, and chaperoning proteins (Brooks et al., 2016). Specifically, on day 12 versus day 10, there was an increase in CP and CST6 proteins. There was a decrease in LRP2, C3, and WFDC2 proteins on day 12 versus day 10. On day 12 versus day 14, there was an increase in CST3, CTSL1, PSAP, HEXB, IGFBP1,3, and MET proteins. There was a decrease in LRP2 on day 12 versus day 14. There was an increase in AZGP1, EPRS, and RBP4 on day 14 versus day 16. While SOD3, PGK1, GC, IL1R2, HSPA8, LGALS3BP, ENO1, and CST3 proteins were decreased on day 14 versus day 16.

Forde et al. (2014) analyzed proteins in ULF on day 16 in cattle, and also quantified proteomic changes in select proteins in ULF on days 10, 13, 16, and 19. There were 1652 peptides identified in the ULF. The two largest overrepresented biological processes associated with the ULF proteins included: generation of precursor metabolites and energy, homeostatic process, monosaccharide metabolic process, proteolysis, and hexose metabolic process. The top molecular functions associated with the ULF proteins were enzyme inhibitor activity, cytoskeletal protein binding, endopeptidase inhibitor activity, actin binding, and peptidase activity. Specifically, several enzymes involved in glycolysis/gluconeogenesis were increased on day 16, which further supports the idea of the Warburg Effect in the bovine conceptus (Krisher and Prather, 2012; Vander Heiden et al., 2009). Despite more ATP being produced via the citric acid cycle, the rapidly elongating conceptuses are potentially using aerobic glycolysis to generate other types of cellular components necessary for proliferating tissues. Specifically, IDH1, is an enzyme

involved in the citric acid cycle, it had higher expression in the endometrium on days 13 and 16 compared to the conceptus, but declined at the start of implantation. Potential explanations for this decline may be that the protein is either rapidly being used up or it is only necessary for a short window of time. This study also determined that enzyme inhibitor activity was one of the top molecular functions. This is critical because it is necessary that IGFBP1, GRP, LGALS15, and CSF2 exist in their full length functional form so they can work by enhancing proliferation, migration, and attachment.

Forde et al. (2015) later analyzed protein content in ULF from cyclic and pregnant heifers on day 16, and short term in vitro cultured day 16 conceptuses. There were 334 proteins identified in cyclic heifers, 299 proteins identified in pregnant heifers, 1005 proteins identified in the 6 hour invitro culture medium, and 1012 proteins in the 24 hour invitro culture medium. More proteins present in the cultured conceptuses is most likely due to the smaller volume of fluid used (500ul versus 20ml). There were only 85 proteins identified in pregnant heifers ULF, and only 120 proteins that were present in cyclic heifers ULF. There were 54 proteins that were identified in pregnant heifers, but not in the invitro culture media which suggests that these proteins were of endometrial origin. There were 30 proteins that were unique to both pregnant heifers and produced by short term invitro cultured day 16 conceptuses (HSPE1, YWHAG, PSMC4, ACO2, ARPC5L, ACTN1, GLB1, PRKAR2A, CKMT1, EEF2, LGALS3, HNRNPA1, HNRNPF, HNRNPA2B1, IDH2, IFNT, KRT75, CAPG, MSN, NUTF2, PGM2, GPLD1, PSMA4, PSMB5, P4HB, GDI1, CSTB, HSPA8, SERPINA3, TXN, TKT). They concluded that these proteins may facilitate endometrial and conceptus interactions through microvesicular transport.

Ledgard and others (2009) examined the differences in selected proteins from pregnant and nonpregnant cows ULF on days 16 and 18. During pregnancy, there is an increase in metabolic activity in the endometrium. This particular study reported an increase in five enzymes involved in biosynthetic pathways (CA2, IDH1, NDPK, PNP, and TPI). They also reported an increase in antioxidant enzymes (PRX1 and TXN) which may play a role in protecting the trophoblast cells from oxidative stress during rapid elongation (Watson et al., 1998). Other abundant proteins in pregnant animals included: EZR (membrane-cytoskeletal linking protein; Martin et al., 2000) and HSP70 (upregulated by estradiol; Wu et al., 1996). The TIMP-2 protein was reported to be less abundant in pregnant ULF. This protein acts as a proteinase inhibitor that controls the activity of metalloproteinases and regulates ECM integrity. They also examined differences in protein abundance between the gravid and non-gravid horns. Retinol binding protein (RBP) was less abundant in the gravid horn. It appears to be controlled by progesterone, estrogen, and IFNT. Cyclic animals on day 17 have been reported to have greater RBP mRNA levels in the endometrium compared to pregnant animals (Harney et al., 1993). Another protein that was less abundant in the gravid horn was the protease, legumain (LGMN). Previous research has reported a decline in LGMN mRNA levels between days 23 and 26 in the pregnant endometrium.

*Proteomic Analysis of Caruncular and Intercaruncular Endometrium:*

Wang and others (2013) examined proteomic profiles in caruncular and intercaruncular endometrium from sheep on day 17 of pregnancy. There were 1740 proteins detected in caruncular tissue, and 1813 proteins detected in intercaruncular

tissue. There were 170 proteins that were differentially expressed in the tissue types. Sixty of these proteins were upregulated in caruncular regions, while 110 were upregulated in intercaruncular regions. The protein that had the highest expression in caruncular tissue was N- acetylgalactosamine-6 sulfatase precursor (GLANS). The proteins with the highest expression in the intercaruncular tissue were: PIP4K2C, PLIN4, EML4, and ITGA1. Enriched GO terms for biological processes included: primary, cellular, and protein metabolic processes, cell adhesion, and multicellular organismal development. Enriched KEGG pathways associated with the DEPs included: focal adhesion, regulation of actin cytoskeleton, and ECM-receptor interaction. Overall bioinformatics revealed that these DEPs were primarily associated with growth and remodeling, cell adhesion, and protein transport.

Differentially expressed proteins involved in uterine tissue remodeling during pregnancy were present in both tissue types. In caruncular tissue, the GO category, proteolysis was enriched (PSMB3, NEDD8, UCHL3, USP34, TAGLN). These proteins are involved in proteasome function, ubiquitination, and vascularization. An enriched GO category in intercaruncular tissue was cell proliferation (CAMK2G, CDK5, CMPK2, and HARS). In intercaruncular tissue, the endometrial gland morphogenesis KEGG pathway was enriched (ACTA2, ACTG1, ACTN1, MYL9, and MYLK). These proteins belong to the actin and myosin family. Actin proteins are involved in cell motility, structure, and integrity. While myosin proteins are responsible for muscle movements.

Differentially expressed proteins involved in cell adhesion during pregnancy (day 17) were also present in both tissue types. In caruncular tissue, two cell adhesion proteins were increased (TJP1, POSTN). The tight junction protein, TJP1, is located on the

plasma membrane and functions to maintain cell polarity and cell contact. While POSTN is an ECM protein that aids in cell adhesion and migration. In intercaruncular tissue, the cell junction GO category was enriched. Proteins in this category included three cytoskeleton proteins (TLN1, TNS1, and VCL). These activate integrin signaling pathways which lead to cell adhesion, migration, and proliferation (Roberts et al., 2009; Humphries et al., 2007). KEGG pathways involved in focal adhesion and ECM receptor interaction were also overrepresented in intercaruncular tissue (COL1A2, COL6A2, LAMA4, LAMAC1, and ITGA1).

*ULF Proteomic Differences Based on Embryonic Sex:*

The bovine endometrium is capable of recognizing the presence of an embryo, and responding based on its potential to survive. However, little is known about whether the uterine response is different based on the sex of the embryo (Gomez et al., 2013). It has been established that male and female embryos differ based on chromosome complement, epigenetic status, and transcriptional activity (Bermejo-Alvarez et al., 2010). Previous research has determined that differing concentrations of hexoses in culture can favor one sex over another. Uterine luminal fluid collected on day 8 reported 23 DEPs between male and female embryos.

Glycolysis/gluconeogenesis enzymes and antioxidative/antistress responses were upregulated in female ULF. Uterine luminal fluid glucose concentrations were similar between the sexes; however, fructose was increased in female ULF. Other networks associated with the DEPs were NFkB complex and proteasome/ immunoproteasome

subunits. This suggests that recognition and antigen processing is different among male and female embryos (Gomez et al., 2013).

## CHANGES IN UTERINE GENE EXPRESSION DURING PREGNANCY

### *Bovine Genome:*

Cattle have a total of 60 chromosomes (29 pairs of autosomes, 1 pair of sex chromosomes). The size of the bovine genome is approximately three billion base pairs. It contains approximately 22,000 genes, 14,345 of which are common to all mammalian species (Elsik et al., 2009). The recently developed technology, RNA sequencing allows researchers to investigate specific transcriptomes. This approach to transcriptome profiling allows precise measurement of levels of transcripts and their isoforms at a given moment in time (Wang et al., 2009). There has been extensive research examining gene expression in the endometrium and conceptus during the preimplantation stage of ruminant development. This technology allows researchers to identify important information on discovering new regulatory pathways involved in uterine receptivity, conceptus elongation, trophoctoderm differentiation, conceptus-endometrial interactions, and pregnancy establishment in cattle (Brooks et al., 2016). In particular, genes involved in cell adhesion, growth, ECM remodeling, immune response, and CL maintenance have been reported to change in expression during pregnancy.

*Changes Based on Day of Pregnancy:*

Brooks et al. (2016) examined the changes in uterine gene expression (luminal and glandular) throughout early pregnancy in sheep (day 10- day 20). The luminal epithelium (LE) regulates attachment of mononuclear trophoblast cells in the uterus, by day 20 the LE disappears. They reported that in the LE, the total number of genes increased between days 10 and 20 (3,108 to 4,172). Nine genes increased (AGR2, SLC5A1, HSD11B1, RBP4, CST6) and 12 genes decreased between days 10 and 12. The DEGs were associated with organismal development, inflammatory response, and molecular transport. The protooncogene (AGR2) is involved in cell survival, migration, differentiation, and growth (Satterfield et al., 2009). There were 522 genes that increased (GRP, PFN1, S100A2, FURIN, PAPP, NUPR1, RSAD2) and 171 genes that decreased between days 12 and 14. The DEGs were associated with: lipid metabolism, cellular movement, cellular death and survival, and cellular development. The secreted metalloproteinase (PAPP) cleaves IGFBP, which then promotes cell proliferation (Laursen et al., 2001). From days 14 to 16, 138 genes increased (LY6G6C, PIP, MUC20) and 202 genes decreased (RSAD2, OST4, IFI6). The DEGs were associated with: cell signaling, cellular assembly and organization, post translational modifications, and protein synthesis. The PIP protein binds to immunoglobins and T cell receptors suggesting a possible role in immunological functions (Caputo et al., 2003). The MUC20 protein inhibits trophoblast invasion during implantation in mice and humans (Johnson et al., 2001). From days 16 to 20 there was little change in gene expression (CYP26A1, KIAA1551, SLC13A5).



Glandular epithelium (GE) has greater surface area than the LE. This suggests that GE is the main cell type influencing conceptus growth and development (Guillomet et al., 1981). The number of genes in GE increased from day 10 to day 14 (4,409 to 5,194), and then decreased on day 20 (4,316). Between days 10 and 12, 18 genes increased (SCGN, IFI6, KLF11, MEP1B) and 43 genes decreased (ODC1, S100G). The DEGs were associated with: cell death and survival, cell morphology, cell-cell signaling and interactions, and cellular function and maintenance. There were 833 genes that increased (ISG17, RSAD2, CLEC4F, OAS2, NUPR1, LGALS3BP) and 231 genes that decreased between days 12 and 14. The DEGs were associated with: cellular movement, cell death and survival, cellular growth and proliferation, and protein synthesis. Specifically, the CLEC4F protein plays a role in cell adhesion, cell signaling, glycoprotein turnover, and inflammation/immune response (Zelensky and Gready, 2005). Additionally, the LGALS3BP protein functions by modulating cell-cell and cell-matrix adhesions. From days 14 to 16, there were 246 genes that increased (S100G) and 643 genes that decreased (RHOBTB3, PABPC1, MT-ND5, KLF11). The DEGs were associated with: molecular transport, protein synthesis, cell function and maintenance, post translational modifications, and RNA trafficking. Similar to the LE, there was little change in gene expression between days 16 and 20 (SPP1, GRP, SERPINA14, STC1, SEC11C). Previous research has reported that adhesion molecules (SPP1 and GLYCAM1) are upregulated during the implantation phase in ruminants (Forde et al., 2013). The SERPINA14 protein has immunomodulatory roles, which involve blocking T cell proliferative responses, impairing natural killer cell activity, and decreasing antibody protection (Skopets et al., 1995).

*Changes Around the Time of Implantation:*

Bauersachs and others (2006) examined the differences in transcriptome profiles from endometrium collected from pregnant and non-pregnant heifers on day 18. The top biological processes that were upregulated in pregnant animals were associated with: immune response (31%), signal transduction (14%), transport (10%), and proteolysis (9%). The top molecular functions that were upregulated in pregnant animals were associated with: hydrolase activity (17%), nucleotide binding (16%), transferase activity (12%), and peptidase activity (10%). Enriched GO terms in pregnant animals were: response to stimulus, immune response, antigen presentation, complement activation, and proteolysis. Overall enriched pathways on day 18 of pregnancy included: protein modification, regulation of transcription, cell adhesion, endometrium remodeling, maternal immune modulation, and signal transduction.

Genes involved in cell adhesion that were upregulated in pregnant animals included: AGRN, CD81, CLDD4, TGM2, CLDN4, LGALS9, and LGALS3BP. Agrin (AGRN) is a proteoglycan that is involved in the formation and signaling of close cell contacts (Bauersachs et al., 2006). CD81 is a member of the tetraspanin family, it has a role in cell adhesion and other biological processes (Levy et al., 1998). CLDD4 is involved in tight junctions and likely plays a role in permeability and cell adhesion between epithelial cells in the endometrium (Tsukita and Furuse, 2002). Additionally, TGM2 is a multifunctional protein that is involved in cell adhesion and works by regulating fibronectin and integrin interaction, apoptosis, and ECM remodeling (Fesus and Piacentini, 2002). Claudins, a class of cell adhesion molecules were present in pregnant heifers, and they function within tight junctions to seal off simple and

stratified epithelia (Tsukita and Furuse, 2002). Specifically, CLDN4 was reported to cause a decrease in sodium permeability among tight junctions. Claudin 4 mRNA was also elevated around the time of implantation in humans (Carson et al., 2002).

Galectins are another class of cell adhesion molecules. They are beta-galactoside-binding lectins that act as modulators for both cell growth and adhesion. They are expressed in the endometrium of mammals, and appear to be necessary for conceptus-endometrial interactions. Specifically, LGALS9 has been reported to impact cell aggregation and adhesion (Hirashima et al., 2004). The LGALS3BP protein codes for a cell-adhesive protein in the extracellular matrix that assembles into ring like structures and binds integrins, collagens, and fibronectin (Sasaki et al., 1998).

*Changes Based on Type of Endometrium at Implantation:*

Mansouri-Attia and others (2009) examined gene expression in caruncular and intercaruncular endometrium from pregnant and cyclic cattle on day 20. There were 446 DEGs in the caruncular tissue among cyclic and pregnant animals. There were 202 DEGs that were upregulated in cyclic cows, and 244 that were upregulated in pregnant cows. Enriched pathways associated with the DEGs in the caruncular tissue were: cellular growth and proliferation, gene expression, DNA replication, recombination, repair, cellular movement, and cell death. Enriched pathways associated with the DEGs in the intercaruncular tissue were: cellular growth and proliferation, posttranslational modifications, and cell death.

The genes that had increased expression in the caruncular tissue of pregnant animals versus cyclic animals included: RSAD2, MX1, FABP3, and ISG15.

Specifically, the expression of cell adhesion molecules (galactins, integrins, and mucins) were affected by pregnancy status. The LGALS1 gene was upregulated in pregnant caruncular endometrium during implantation compared to cyclic cows (Mansouri-Attia et al., 2009). This same gene had increased mRNA expression between days 12 and 16 in the LE of pregnant sheep (Gray et al., 2004). Integrins are glycoprotein receptors that bind to ECM ligands to modify cytoskeleton organization and assist in growth and differentiation during implantation (Mansouri-Attia et al., 2009). The following integrins had increased expression among pregnant cattle: ITGAV, ITGA7, ITGA8, ITGB4, ITGB1BP1, and ADAMDEC1. Mucins are glycans that are located on the surface of the LE. Blastocysts recognize these molecules and attach to the epithelium (Aplin, 1997). This study reported MUC13 mRNA was upregulated in pregnant caruncular tissue. The genes that had decreased expression in the caruncular tissue of pregnant animals were: NPY, COL1A2, COL3A1, RARRES, and MMP2. Matrix metalloproteinases (MMP2) function by inducing ECM degradation. Another metalloproteinase, MEP1B degrades ECM components, and it had greater expression in pregnant animals on day 19 compared to nonpregnant animals (Bauersachs et al., 2006). Metalloproteinases are controlled by tissue inhibitors (TIMP). TIMP2 was increased in both caruncular and intercaruncular tissue on day 20 (Hashizume, 2007).

There were 1,295 DEGs in the intercaruncular tissue of cyclic and pregnant animals. There were 762 DEGs that were upregulated in cyclic cows, and 533 that were upregulated in pregnant cows. The genes that had increased expression in pregnant animals included: RSAD2, IF16, ISG15, MX1. The most downregulated genes

associated with pregnancy included: COL1A2, OXTR, SNAI2, and NPY. Overall, genes related to immune response were prominent in caruncular regions, while genes related to metabolic regulation were prominent in intercaruncular regions.

*Changes Based on Fertility:*

Geary et al. (2016) classified heifers based on previous pregnancy success (high fertile- HF, subfertile- SF, and infertile- IF). In vivo produced embryos were transferred into fertility classified heifers on day 7 post estrus. On day 14, endometrial biopsies were obtained for RNA sequencing. The uteri were then flushed to collect conceptuses. Recovery rate and conceptus length/area were not different among the fertility groups. However, there were 26 differentially expressed genes (Fold change > 2, FDR < 0.10) among HF and SF endometrium. There were 14 genes that were upregulated, and 12 genes that were downregulated in HF heifers. Many of the upregulated genes were associated with antimicrobial properties (TAP, MUC1) and immunoglobulins. MUC1 forms part of the glycocalyx barrier that provides innate immune protection against bacterial infections (DeSouza et al., 1999). It also regulates implantation in mice and potentially other mammals (Walker et al., 2010). There were 12 DEGs among SF and IF endometrium, with all 12 having decreased expression in SF heifers. There were only three DEGs among HF and IF endometrium, all of which were downregulated among HF heifers. The lack of variation in endometrial gene expression, along with no differences in recovery rates and conceptus development on day 14 supports the idea that differences in pregnancy loss among HF and SF heifers must occur between day 14 and day 28.

A similar study that classified heifers based on fertility (HF, SF, IF) was conducted on day 17 of gestation (Moraes et al., 2018). The heifers were synchronized, and received two in vivo embryos on day 7. The reproductive tract was flushed on day 17 to recover the conceptuses, and endometrial samples were collected. Pregnancy rate was higher in HF (71%) and SF (90%) than IF (20%) heifers. Elongating conceptuses were about two-fold longer in HF heifers compared to SF heifers. There were 168 DEGs detected in the endometrium of HF and SF pregnant heifers. Enriched GO terms included: ECM organization, cell adhesion, collagen catabolic process, locomotion, and cellular response to endogenous stimulus. Specific genes that were upregulated among HF pregnant heifers versus SF pregnant heifers included: water transporter (AQP8), lipid transporter (FABP3), and protease (MEP1B). Downregulated genes among HF pregnant heifers were associated with cell signaling (CAMK1G, IRS4) and cell adhesion. The 44 downregulated genes in SF heifers encoded for secreted factors (FABP3, MEP1B) and transporters (SLCO4C1, SLC7A1). FABP3 is involved in uptake, metabolism, and transport of long chain fatty acids (Forde et al., 2010). SLCO4C1 is involved in eicosanoid transport, while the SLC7A1 gene encodes for an arginine transporter. Arginine is critical for cell proliferation, migration, and IFNT production (Bazer et al., 2015). There were 664 genes that were commonly responsive in HF and SF heifers, these included: ISGs (MX2, ISG15, OAS2, RSAD2, BST2) and progesterone-conceptus responsive genes (DKK1, FABP3, LGALS3BP). Pathways associated with the commonly responsive upregulated genes included: IFNT signaling, cytokine signaling in the immune system, complement pathway, and toll like receptor signaling. Pathways associated with the 184 commonly responsive downregulated

genes included: ECM organization, collagen formation, integrin signaling, and focal adhesion.

*Changes Based on Ipsilateral/Contralateral Uterine Horn:*

Sanchez and others (2019) examined transcriptomic differences in the intercaruncular tissue from the ipsilateral and contralateral uterine horns on days 5, 7, 13, and 16 post estrus. Day of the cycle had the greatest impact on variation in the endometrial transcriptome. There was more variation among the ipsilateral and contralateral horn early on in the luteal phase (day 5) when compared to the late luteal phase (day 16), this may be due to an increase in blood flow towards the uterus during the periestrus period (Ford and Chenault, 1981). There were 217 DEGs between the ipsilateral and contralateral horn on day 5, 54 DEGs on day 7, 14 DEGs on day 13, and 18 DEGs on day 18. The most enriched GO analysis function among DEGs on days 5, 7, and 13 was deubiquitination. On day 16, the most enriched function was microtubule anchoring. The top canonical pathways associated with DEGs among the ipsilateral and contralateral horns were: regulation of pluripotency in stem cells, progesterone mediated oocyte maturation, endometrial cancer, ErbB signaling, and mTOR signaling. However, the differences in endometrial gene expression between the horns does not appear to impact conceptus survival to day 14 after embryo transfer on day 7.

## PROGESTERONE

### *Pregnancy establishment and maintenance:*

The corpus luteum (CL) is the main source of progesterone during pregnancy. The CL has one of the highest rates of blood flow due to each luteal cell being in direct contact with several capillaries. This extensive capillary network is what supplies nutrients, hormones, and lipoprotein-bound cholesterol to luteal cells. It is critical for efficient progesterone output (Stocco et al., 2007). The CL is essential for the establishment and maintenance of pregnancy up until at least day 200 in cattle (McDonald et al., 1952). Removal of the CL and progesterone early on in pregnancy shortened gestation, and increased dystocia and retention of fetal membranes (Estergreen et al., 1967; Chew et al., 1979).

Reduced luteal function is often associated with infertility in ruminants (Gaverick and Smith, 1986). Reasons for subluteal function include: inadequate stimulation of the preovulatory follicle by gonadotropins, decreased FSH: LH ratio, inadequate preparation of follicular cells to respond to the LH surge, exposure to progesterone, insufficient endocrine environment for the preovulatory follicle, premature stimulation of ovulation, decreased blood flow, and the premature release of PGF<sub>2</sub> $\alpha$ .

It was previously demonstrated that cows with normal developing embryos had greater progesterone concentrations on day 3 and day 6 following insemination compared to cows with degenerating embryos (Maurer and Echternkemp, 1982). A different study reported that as early as 6 days after artificial insemination, pregnant cows have greater progesterone concentrations when compared to open cows (Mann



et al., 1999). Previous research reported that cows that experienced a three-fold increase in circulating progesterone also had increased recovery rates of blastocysts and induced a 2.3-fold increase in blastocyst size on day 13 of pregnancy (Lonergan et al., 2007; Carter et al., 2008). Kerbler et al. (1997) reported that cows that experienced an earlier rise in progesterone had day 18 embryos that were more advanced developmentally, produced more IFNT, and were more capable of inhibiting the release of PGF2 $\alpha$  around the time of maternal recognition of pregnancy. Decreased pregnancy rates have also been reported in cows that experienced a delayed rise in progesterone during the early luteal phase (Shelton et al., 1990; Forde et al., 2009). The mechanism by which progesterone exerts positive effects on conceptus survival is by inducing changes in endometrial gene expression, which ultimately leads to changes in uterine histotroph composition (Spencer et al., 2008). Bartol et al. (1981) previously determined that protein accumulation within the uterine lumen is related to the duration of progesterone stimulation.

*Progesterone supplementation:*

Studies involving progesterone supplementation following artificial insemination have had mixed results. Some research supports the idea that progesterone supplementation improves reproductive performance and pregnancy rates, while others reported no differences. These differences are most likely due to differences in experimental design such as dosages, administration, and cow numbers. Mann and Lemming (1999) reported that administration of progesterone improved pregnancy rates by 5%. When cattle were treated with a CIDR on days 5 and 7 for 6 to 12 days, there

were decreased pregnancy rates among cattle that did not receive supplementary progesterone compared to those that did (13%; Macmillan et al., 1991). Other studies have focused on examining the effects of progesterone supplementation on embryo/conceptus growth and development. Supplementing pregnant cows with progesterone for four days has been reported to enhance embryo development by increasing uterine protein secretion on days 5 and 14 post ovulation (Garrett et al., 1988). Treatment of recipient cows with progesterone from day 1 to day 5 of the cycle advanced the uterine environment, and caused more pregnancies to be maintained following the transfer of embryos that were three days older (Geisert et al., 1991). It has also been documented that insertion of intravaginal progesterone devices between days 5 and 9 of the cycle resulted in longer embryos on day 16 of gestation, while supplementation between days 12 and 16 did not result in an increase in embryo length (Mann et al., 2006). Thus, revealing that to see the beneficial impact of progesterone during pregnancy it must be administered at a certain time during gestation.

Progesterone supplementation has been reported to impact endometrial gene expression. Forde et al. (2009) looked at the impact of supplemental progesterone on changes in gene expression during pregnancy. Progesterone is required for the stimulation of interferon-stimulated genes by IFNT (Bazer et al., 2008). A number of genes including interferon stimulated genes were upregulated between days 13 and 16. Specifically, genes associated with triglyceride synthesis and glucose transport were upregulated by progesterone. The conceptus uses these nutrients as fuel sources for growth and development (Cases et al., 2001). Administration of progesterone antagonists

during early conceptus development in the ewe disrupted the downregulation of the progesterone receptor in the uterine endometrium, resulting in retarded conceptus growth.

## EMBRYO LOSS

The main factor that negatively impacts profitability in beef and dairy production systems is embryonic loss. In cattle, fertility is dependent on the following two criteria: animals need to be cycling (either naturally or by synchronization protocol) and they also need to develop the appropriate endocrine conditions within the uterus to ensure a suitable environment capable of supporting a developing embryo (Hoelker et al., 2014). Pregnancy loss per day generally decreases as pregnancy progresses, and is much lower after day 60 of pregnancy. Following insemination, beef heifer embryo recovery rates decreased over time from day 4 (100%), 8 (92%), 12 (56%), 16 (66%), and 42 (58%; Diskin and Sreenan, 1980). Pregnancy loss during the later stages of gestation is often caused by specific bacterial, protozoal, or viral infections.

In cattle, embryonic loss is defined as the death of an embryo/conceptus between fertilization and day 42 of gestation. It can further be broken down into early and late embryonic loss according to when pregnancy failure occurred. Early embryonic loss is classified as occurring prior to day 27, late embryonic loss occurs from day 28 to day 42. Specifically, in cattle early embryonic loss accounts for 57% of pregnancy losses (Inskeep and Dailey, 2005). To minimize the early embryonic loss that occurs, we need to gain a better understanding of the different factors that potentially cause the problem. Possible reasons for early embryonic loss include: nutritional and environmental factors,

chromosomal abnormalities, uterine asynchrony, and inadequate hormone levels (Bridges et al., 2012).

*Nutritional factors:*

It is well known that cows that are gaining weight around breeding and throughout gestation have greater pregnancy rates than cows that are losing condition (Wiltbank et al., 1962). Malnutrition during pregnancy can severely impact embryonic and fetal development. It also impacts metabolic imprinting, thus affecting the offsprings susceptibility to chronic diseases later in life (Waterland and Jirtle, 2004). Dam nutrition can impact both the follicular and uterine environment, which ultimately has effects on conceptus growth and development. Feeding cattle diets that are high in degradable crude protein and ammonia can reduce fertility by increasing blood plasma urea leading to a decrease in uterine pH (Tamminga, 2006). This results in impaired progesterone synthesis and reduced oocyte quality (Ferreira et al., 2011). In various species, excess protein intake negatively impacts embryo hatching and survival (Chagas et al., 2007). Too little protein in cattle diets can have adverse effects on reproduction as well. When heifers were fed 85% of protein and energy maintenance requirements, they had decreased cleavage rates compared to heifers fed 100% of their maintenance requirements (Hill et al., 1970). When energy intake was 0.8 times maintenance for two weeks following AI, embryo survival was under 40% compared to heifers that received constant feed intake or changed from lower to higher feed intake (65-71%; Dunne et al., 1999).

*Environmental factors:*

Environmental factors such as ambient temperature and humidity during various time points in development have been correlated with seasonal decreases in pregnancy success. An animal undergoes heat stress when exposed to temperatures between 32 and 43 degrees Celsius with humidity around 40%. High temperatures compromise steroidogenesis, oocyte viability (Zeron et al., 2001), oocyte quality (Hansen et al., 2002), fertilization rate (Sartori et al., 2002), and pregnancy rates (Gwazdauskas et al., 1973; Ingraham et al., 1974; Ealy et al., 1993). Heat stress causes a reduction in the steroidogenic capacity of theca and granulosa cells leading to a decrease in estradiol production, which impacts the the degree of dominance of the selected follicle (Wolfenson et al., 1997). As a result, the duration and intensity of estrus is reduced (Younas et al., 1993).

Oocytes and embryos (< 3 days after conception) are the most susceptible to the adverse effects of high temperatures (Ealy et al., 1993). An increase in uterine temperature resulted in an increase in conceptus metabolic rate leading to changes in nutrient uptake (Biggers et al., 1987). This along with decreased nutrient secretion by the uterus leads to a decrease in conceptus mass (Biggers et al., 1987), developmental stage (Putney et al., 1989), and number of cells per embryo (Putney et al., 1989). Hyperthermia exposure for a ten-hour period between the onset of estrus and artificial insemination in heifers resulted in retarded embryo development and increased embryo death when examined on day 7 after insemination (Putney et al., 1989). This could possibly be due to thermal induction of chromosomal abnormalities while the oocyte is resuming meiosis. Embryos can develop the capacity to produce molecules that limit

the negative effects of heat on cellular function such as heat shock proteins (Edwards and Hansen, 1996). They work by stabilizing protein structure (Georgopoulos and Welch, 1993), inhibiting translation (Thulasiraman et al., 1998), and preventing apoptosis (Li et al., 1996).

*Chromosomal abnormalities:*

About 15% of bovine cultured oocytes possess chromosomal abnormalities (Yadav et al., 1991). The incidence of chromosomal abnormalities in bovine spermatozoa is around 2.8% (Logue and Harvey, 1978). Genetic abnormalities account for approximately 10% of embryonic losses within the first two weeks of pregnancy (King et al., 1985). Inbreeding increases the likelihood of chromosomal defects leading to embryonic mortality. Chromosomal abnormalities have been reported to cause decreased embryo growth rates (Kawarsky et al., 1996) and spontaneous abortions (Jacobs, 1990). The loss of embryos due to chromosomal abnormalities occurs early on in development usually during the first cell divisions. In sheep, prior to entry into the uteri, 6% of embryos had chromosomal abnormalities, while by day 13, 0% of the conceptuses collected had chromosomal abnormalities (Long, 1980).

*Asynchrony and Inadequate Hormone Levels:*

In cattle, synchrony between the embryo and the uterus must be within  $\pm 24$  hours of each other (Hasler, 2001). This is especially important during the period of embryonic elongation. Asynchrony between the embryo and uterus is caused by differences in the rate of embryo development or more commonly delayed or advanced progesterone

production during the luteal phase (Pope, 1988). If embryo transfer recipients and donor's uteri are not at the same stage, embryonic development is altered (Lawson et al., 1983). When embryos (day 4) were transferred into younger uteri (day 1 to 2) embryo development is retarded, if they are transferred into older uteri (day 6 to 7) embryonic development is accelerated and maintained until day 12. When asynchrony is too extreme embryos are abnormal and fail to implant (Goff, 2002). High temperatures have also been reported to increase plasma progesterins altering the uterine environment resulting in uterine asynchrony (Thatcher, 1974).

Another potential factor that can lead to embryonic loss is the failure of ovarian steroids to regulate factors in the endometrium. Miller and Moore (1976) conducted an experiment using ovariectomized sheep and reported that the sequence of steroid exposure is critical for embryo survival (progesterone priming, estradiol, and then progesterone following fertilization). Inadequate estradiol production prior to ovulation results in premature luteolysis (Mann and Lamming, 2000) and an inadequate uterine environment (Bridges et al., 2013). Estradiol and progesterone exposure modulates the expression and localization of uterine genes and proteins that are necessary for uterine function and embryo development (Bridges et al., 2013). When ovariectomized beef cows were treated with estradiol (cypionate or benzoate) embryo survival was increased to day 29 of pregnancy compared to cows that had no exposure to estradiol (Madsen et al., 2015). This same study reported that cows that received exogenous estradiol only lost 35% of their existing pregnancies, while control animals lost 75% of their existing pregnancies (Madsen et al., 2015).

## SUMMARY

Early embryonic loss is a complex issue facing the cattle industry, and gaining a better understanding of why, when, and how it occurs will aid in helping improve reproductive efficiency. Previous research indicated that estradiol leading up to breeding may be a critical factor for the establishment and maintenance of a successful pregnancy. Animals with elevated preovulatory estradiol prior to fixed time AI have improved embryo quality and pregnancy rates compared to animals with low preovulatory estradiol concentrations.

A uterine environment with appropriate endocrine conditions to support a developing conceptus is critical for a successful pregnancy. The time period that this dissertation focuses on is around maternal recognition of pregnancy (day 16). During this time, the conceptus is free floating in the uterus, and is relying on the mother's uterine histotroph for growth and development. We have previously made comparisons associated with uterine histotroph components (glucose, protein, glucose transporter expression) between highE2 and lowE2 animals on day 16 of gestation. We also reported no differences in conceptus survival based on recovery rates, apoptosis in the trophoderm, and IFNT concentrations. This dissertation further investigates the differences in uterine environment between highE2 and lowE2 animals by using mass spectrometry to examine specific protein abundance differences on day 16 in the uterine luminal fluid. Additionally, by performing RNA sequencing and real-time PCR on day 16 endometrial tissue and conceptuses we can further determine if these differences in uterine and trophoderm gene expression among highE2 and lowE2 animals possibly contribute to differences in conceptus survival.



CHAPTER II

INFLUENCE OF PREOVULATORY ESTRADIOL ON UTERINE AND  
TROPHECTODERM GENE EXPRESSION AROUND MATERNAL RECOGNITION  
OF PREGNANCY IN BEEF CATTLE

ABSTRACT

In cattle, most embryonic losses occur during the first month of pregnancy. Embryo survival and pregnancy success is increased among animals that exhibit estrus prior to fixed time AI, but there are no differences in conceptus survival to d16. The objective of this study was to examine differences in uterine and trophectoderm transcriptomes on d16 of pregnancy based on preovulatory estradiol (E2) exposure. We hypothesized that differences in uterine environment have a greater impact on pregnancy success than differences in conceptus development on d16. Beef cows/heifers were synchronized, artificially inseminated (d0), and grouped into high (highE2: n=18) or low (lowE2: n=11) preovulatory E2. Uteri were flushed to collect d16 conceptuses, and endometrial samples were collected from the ipsilateral uterine horn. Total cellular RNA was extracted from endometrium for RNA sequencing. Real-Time PCR (RT-PCR) was performed on trophectoderm (TE) RNA (n=21) to measure the relative abundance of IFNT, PTGS2, TM4SF1, C3, FGFR2, and GAPDH. Transcript abundances in the endometrium were quantified using kallisto, differentially expressed genes (DEGs) were determined using DESeq2 (FDR <0.05, FC>2), and IPA was used for pathway analysis. RT-PCR data were analyzed using the MIXED procedure in SAS. There were no differences in mRNA abundances in TE, but there were 432 DEGs among the

highE2/conceptus versus lowE2/conceptus groups, 253 were downregulated (CR2, CDH4, TROAP, COL1A2) and 179 were upregulated (PRKCG, PRND, MRAP) in the highE2/conceptus group. Top networks included: lipid metabolism, cell morphology, and embryo development. These results demonstrate greater differences in uterine function than in conceptus developmental competence between highE2 and lowE2 animals on d16.

## INTRODUCTION

The uterus is a dynamic environment whose gene expression changes drastically depending on stage of the estrous cycle and the needs of the conceptus. Advancements in microarrays and RNA sequencing technologies has allowed researchers to gain insight into changes in gene expression ultimately enhancing our understanding of the biological mechanisms associated with early pregnancy. Interferon tau, progesterone, estradiol, prostaglandins, and cortisol have been reported to regulate changes in uterine gene expression (Brooks et al., 2014). In particular, preovulatory estradiol impacts follicular growth, oocyte maturation, sperm transport, uterine environment, and embryo survival/development (Pohler et al., 2012). Specifically, estradiol acts as a transcription factor that induces endometrial receptors (Bartol et al., 1981) and the expression of many genes involved in various biological functions (Bauersachs et al., 2005).

In cattle, the embryo enters the uterus around day 4. The embryo undergoes several cell divisions leading to a morula (day 5), and then further differentiates into a blastocyst (day 7 to 8). A blastocyst consists of an inner cell mass and a trophectoderm layer (Flechon and Renard, 1978). The inner cell mass eventually gives rise to the fetus, while the outer trophectoderm cells develop into the placenta (Forde and Lonergan, 2012). On day 9, the blastocyst hatches from the acellular glycoprotein coat (zona pellucida). On days 11-12, the blastocyst becomes ovid-shaped, the trophectoderm cells begin to proliferate and the elongation process begins (Grealy et al., 1996). On day 13, the conceptus is approximately 2 mm long. By day 16 the elongated conceptus can reach 60 mm in length (Betteridge et al., 1980). In cattle, maternal recognition of pregnancy occurs around day 16 after estrus (Bazer et al., 1997), the

pregnancy recognition signal is interferon tau (IFNT). The conceptus doesn't begin to attach to the uterus until around day 19, therefore, during early pregnancy the conceptus is dependent on secretions from the uterine epithelium (uterine histotroph). The uterine histotroph is composed of a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, lipids, and glucose (Gao et al., 2009a). The conceptus utilizes these molecules for nutrition, homeostasis, cell signaling, growth, development, and survival.

Previously, our laboratory determined that there were no differences in conceptus survival to day 16 of pregnancy between highE2 and lowE2 animals based on conceptus recovery rates, apoptosis in the trophectoderm, and interferon tau concentrations in the uterine luminal fluid (ULF; Northrop et al., 2018). However, there were differences in select glucose transporter mRNA abundances in caruncular and intercaruncular endometrium (Northrop et al., 2018). The objectives of the current study were to: 1) examine the effects of preovulatory estradiol on critical genes and pathways associated with early pregnancy in cattle, and 2) examine the effects of interferon tau on gene expression during early pregnancy. We hypothesize that differences in uterine environment will have a greater impact on pregnancy success than differences in conceptus development on day 16 of pregnancy in beef cattle.

## MATERIALS AND METHODS

### *Animals:*

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee and U.S. Meat Animal Research Center (USMARC) Animal Care and Use Committees in accordance with the FASS guidelines for the care and use of agricultural animals in research.

### *Treatments:*

Angus crossed beef cows/heifers at the South Dakota State University Beef Breeding Unit (Rep 1: n=30, Rep 2: n=40) and the U.S. Meat Animal Research Center (Rep 3: n=20) were synchronized with a CO-Synch protocol [GnRH administered (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on d -9, followed by PGF2 alpha (PG; 25 mg as 5 mL of Lutalyse i.m.; Pfizer Animal Health, Madison, NJ) on d -2, and on d 0, cows were administered GnRH (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) and artificially inseminated (AIed). Estrus was monitored visually from d 0 through d 3 with the aid of EstroTect patches (Western Point, Inc., Apple Valley, MN). Animals were grouped into either highE2 (n=18) or lowE2 (n=11) based on preovulatory estradiol concentrations (replicates 1 and 2) and expression of estrus (all replicates). The threshold estradiol concentration that distinguished the two groups was 4.9 pg/mL. Previous research used a similar cutoff when evaluating changes in ovarian function associated with concentrations of estradiol before a GnRH-induced ovulation in beef cows (Larimore et al., 2016; Northrop et al., 2018).

*Ultrasonography and Detection of Estrus:*

For replicates 1 and 2, follicular dynamics were assessed by transrectal ultrasonography using an Aloka 500V ultrasound with a 7.5MHz linear probe (Aloka, Wallingford, CT) on d -9, 0, and 3 to characterize follicular development and ovulation. All follicles on each ovary > 8 mm in diameter were recorded, and only animals that ovulated following the GnRH injection at fixed-time AI were utilized in the study. Ovulation was defined as the disappearance of a previously recorded large follicle, and confirmed by changes in circulating concentrations of progesterone.

*Blood Sampling and Radioimmunoassays:*

For replicates 1 and 2, blood samples were collected by venipuncture of the jugular vein into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA). For the first replicate, blood was collected on d -2, -1, 0, then every other day through d 16. For the second replicate, blood was collected on d -2, -1, 0, then every other day through d 15. Blood was centrifuged at 1,200 x g for 30 minutes at 4°C, and plasma was collected and stored at -20°C. Radioimmunoassays (RIA) were performed on plasma samples to determine circulating progesterone concentrations (Engel et al., 2008). Intra- and interassay CVs were 4.9% and 7.5% and 6.0% and 13.2% for replicate 1 and 2, respectively, and assay sensitivity was 0.4 ng/mL. Plasma concentrations of estradiol were determined within replicate by a single assay (Perry and Perry, 2008). Intraassay CVs were 5.03% and 4.76%, for replicate 1 and 2, respectively. Assay sensitivity was 0.5 pg/mL.

*Conceptus Recovery:*

In replicate 1, uteri were flushed non-surgically using a modified Foley catheter. The catheter was inserted into the vagina through the cervix, and into the uterus. A syringe was used to inflate the balloon; animals were flushed with 100mL of flush media to maintain a constant volume. The uteri were massaged, and fluid drained through a filter above a conical tube. Flush media was assessed under a microscope at 10x to determine whether a conceptus was present or not. If no conceptus was recovered, additional flush media was added and this additional media was collected separately. The trophoctoderm (HighE2: n=6, LowE2: n=3) was separated from the embryo proper, and was then stored at -80°C.

In replicate 2, reproductive tracts were collected from the abattoir immediately following slaughter on d 16, and kept on ice. An incision was made at the anterior end of the uterine horn contralateral to the corpus luteum, a plastic tube was placed in the uterine tip and sutured to prevent any fluid loss while the other horn was clamped off. The uterine horns were flushed with 30mL of flush media, and then massaged for equal fluid distribution in the uterus. The uterine flush was then collected in a 50mL conical tube, and examined under a microscope at 10x to determine if a conceptus was present. The trophoctoderm (HighE2: n=6, LowE2: n=3) was separated from the embryo proper, and was then stored at -80°C.

In replicate 3, reproductive tracts were collected from the abattoir at USMARC immediately following slaughter on d 16. An incision was made at the anterior end of the uterine horn contralateral to the corpus luteum, a plastic tube was placed in the uterine tip and sutured to prevent any fluid loss while the other horn was clamped off.

The uterine horns were flushed with 20mL of flush media, and then massaged for equal fluid distribution in the uterus. The uterine flush was then collected in a 50mL conical tube, and examined under a microscope at 10x to determine if a conceptus was present. The trophectoderm (HighE2: n=8, LowE2: n=2) was separated from the embryo proper, and was then stored at -80°C.

*Endometrium RNA extraction and RNA sequencing:*

In replicate 1, endometrium from midway down the ipsilateral uterine horn was collected via a Jackson Uterine Biopsy instrument (Universal Surgical Instruments and Better Surgical Instrumentation; n= 23). We were unable to pass the biopsy tool on some heifers in the study. In replicate 2 (n=28) and replicate 3 (n=20), the ipsilateral uterine horn was cut anterior to the bifurcation, and endometrial tissue was collected midway down the ipsilateral horn.

For all replicates, total cellular RNA was extracted using the Qiagen RNeasy Plus Mini Kit (Austin, TX) following the manufacturer's instructions. Pure RNA was dissolved in nuclease free water, and a spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine RNA concentration for each sample. RNA integrity was then determined using an Agilent RNA Screen Tape System. Only RNA samples (Rep 1: n= 4, Rep 2: n= 5, Rep 3: n=20) with a RIN > 7 were sent to the University of Minnesota Genomic Center for RNA sequencing. They created 29 dual indexed TruSeq stranded mRNA libraries. All libraries were combined into a single pool and were sequenced across two lanes of a NovaSeq S2, 2x50-bp run. There were approximately 24M reads generated for each sample. Illumina Basis QC analysis was



performed on all paired end sequences. All libraries had mean quality scores that were equal or greater than 30, and the pools were gel sized selected to have inserts that were approximately 200 base pairs long.

*Endometrium RT-PCR Validation:*

Total cellular RNA (n=55) was diluted to 70 ng/ $\mu$ l (280ng/reaction) and RT-PCR was performed in duplicate using iScript One-Step RT-PCR Kit with SYBR Green (BioRad) and Stratagene MX 3000P QPCR machine. Expression of DDX58, ISG15, OXTR, PARP12, RSAD2, XAF1, PRSS8, CXCL10, IDO1, MUC13, CLDN4, and FABP3 was measured using the primers in table 2, and GAPDH was used as a reference gene. All of the primers were diluted to a concentration of 10  $\mu$ M. Each plate contained negative controls to assure no background contamination. The PCR program was 10 min at 50°C and 1 min at 95°C for inactivation of reverse transcriptase. Transcription was then followed by 15 sec at 95°C for melting, and 30 sec at the designated annealing temperature (table 2) for 40 cycles. All CVs were less than 20%. Amplicons were electrophoresed on 2% agarose gels to determine product size and were verified for identity by sequencing (Iowa State Genomics Core).

Table 2. Endometrium primer sequences for genes amplified during RT-PCR.

Gene	Primer	Primer Sequence	T <sub>a</sub>	Product Size (bp)	Reference
DDX58	Forward Reverse	5'-GGAAGACCCTGGACCCTACCT-3' 5'-TATACTGCACCTCTTCCTCCCTAAA-3'	60	72	Song et al., 2011
ISG15	Forward Reverse	5'-GGTATCCGAGCTGAAGCAGTT-3' 5'-ACCTCCCTGCTGTCAAGGT-3'	60	293	Green et al., 2010
OXTR	Forward Reverse	5'-ACGGTGTCTTCGACTGCTG-3' 5'-GGTGGCAAGGACGATGAC-3'	60	110	Bauersachs et al., 2006
PARP12	Forward Reverse	5'-CAACGTGAGCGTGCTGAAAA-3' 5'-AAGAGCAAGGGGTCGTTCTG-3'	60	90	Primer-Blast
RSAD2	Forward Reverse	5'-GTGGTTCCAGAAGTACGGTGA-3' 5'-CTTCTTTCCTTGACCACGGC-3'	60	103	Boruszewska et al., 2017
XAF1	Forward Reverse	5'-GAGGAGGCTCTGAGCTTGC-3' 5'-GCAGAGAAAGATGTCCGTCC-3'	64	143	Groebner et al., 2010
PRSS8	Forward Reverse	5'-ATGGGATAGGAGCCGTTGTG-3' 5'-CTGATCGGACACGAGAGAGC-3'	60	161	Primer-Blast
CXCL10	Forward Reverse	5'-CACTCCTCAACTCTTCAGGC-3' 5'-CCATTCTTTTCATTGTGGC-3'	50	262	Imakawa et al., 2006
IDO1	Forward Reverse	5'-GGGCCATGACTTATGAGAA-3' 5'-GAGGCAGCTGCTATTTCCAC-3'	60	107	Groebner et al., 2011
MUC13	Forward Reverse	5'-ACGGGCTGGTGAGACCAAACC-3' 5'-GCAGTCAGCTGTCCCCTTGC-3'	60	116	Forde et al., 2013
CLDN4	Forward Reverse	5'-CTTCTTCCATCCTTCCTGCTC-3' 5'-TCTAAACCTGTCCGTCCACTC-3'	64	164	Riedmaier et al., 2014
FABP3	Forward Reverse	5'-GAGATCAGCTTCAAGCTGGGA-3' 5'-CTTGTCCATTCCACTTCTGCAC-3'	60	121	Mansouri-Attia et al., 2009
GAPDH	Forward Reverse	5'-GATTGTCAGCAATGCCTCCT-3' 5'-GGTCATAAGTCCCTCCACGA-3'	60	94	Han et al., 2006

*Trophectoderm RNA Extraction and RT-PCR:*

Total cellular RNA was extracted using the Qiagen RNeasy Mini Kit (Austin, TX), following the manufacturer's instructions with some modifications. Trophectoderm tissue (Rep 1: n=7, Rep 2: n=5, Rep 3: n=9) was lysed in RLT buffer using a 22-gauge needle and vortexed violently. After the first RW1 wash solution step, 80 $\mu$ l of Dnase solution was added directly to the membrane, and incubated for 15 minutes at room temperature. Pure RNA was dissolved in nuclease free water, and a spectrophotometer was used to determine RNA concentration for each sample. The RNA samples were stored at  $-80^{\circ}\text{C}$ . The RNA (190 ng) was reverse transcribed into cDNA via the Bio-rad iScript cDNA synthesis kit following the manufacturer's instructions. Real-Time PCR was then performed on trophectoderm cDNA (6 ng) in duplicate using BioRad iTaq Universal SYBER Green Supermix and BioRad C1000 Touch CFX96 Real Time System.

Expression of IFNT, PTGS2, TM4SF1, C3, and FGFR2 was measured using the primers in table 3, and GAPDH was used as a reference gene. All of the primers were diluted to a concentration of 10 $\mu$ M. Each plate had negative controls to assure no background contamination. The PCR program was 5 minutes at 95 $^{\circ}\text{C}$  for melting, 15 seconds at the given annealing temperature (table 3), and 15 seconds at 70 $^{\circ}\text{C}$  for extension, for 40 cycles. All CVs were less than 20%. Amplicons were electrophoresed on 2% agarose gels to determine product size and were verified for identity by sequencing (Iowa State Genomics Core).

Table 3. Trophectoderm primer sequences for genes amplified during RT-PCR

Gene	Primer	Primer Sequence	T <sub>a</sub> (°C)	Product Size (bp)	Reference
IFNT	Forward Reverse	5'- GCTATCTCTGTGCTCCATGAGATG-3' 5'-AGTGAGTTCAGATCTCCACCCATC-3'	58	359	Shorten et al., 2018
PTGS2	Forward Reverse	5'-GCATTCTTTGCCCAGCACTTCACCC-3' 5'-CTATCAGGATTAGCCTGCTTGTCTGG-3'	58	418	Lussier et al., 2017
TM4SF1	Forward Reverse	5'- TCTTCTCCGGTATCCTGGGA-3' 5'- TCCAATGAGTGCAGCCAGTA-3'	56	155	Primer3
C3	Forward Reverse	5'-AGAACATCTGGGTCAAGGGG-3' 5'-ATCATGTTCTGCTCCCCACA-3'	56	201	Primer3
FGFR2	Forward Reverse	5'-CACCCACGGACAAAGAAATTG-3' 5'-ATGCAGAGTGAAAGGATATCCC-3'	58	113	Akbarjinejad et al., 2016
GAPDH	Forward Reverse	5'-GATTGTCAGCAATGCCTCCT-3' 5'-GGTCATAAGTCCCTCCACGA-3'	60	94	Han et al., 2006

*Data Analyses:*

The quality reads were mapped to the bovine reference genome ARS-UCD1.2 using kallisto (Bray et al., 2016). Preprocessed data (preovulatory estradiol exposure and conceptus presence interactions) were used for exploratory data analysis which included K-means clustering, hierarchical clustering, and principal component analysis (PCA) using R. Differential expression analysis was conducted on preovulatory estradiol and conceptus presence interactions using the bioconductor package, DESeq2. Genes were considered differentially expressed if  $FDR < 0.05$  and fold change was  $> 2$ . Trophectoderm data were analyzed using the MIXED procedure in SAS with GAPDH being used as a reference gene. Endometrium real-time PCR data were analyzed using the MIXED procedure in SAS with conceptus presence, preovulatory estradiol exposure, and their interaction included in the model.

*Pathway Analysis:*

Identification of enriched pathways was conducted using Ingenuity Pathway Analysis (IPA; Qiagen) software. For each interaction, a list of DEGs was uploaded to the IPA tool. Ingenuity Pathway Analysis functional analysis tools identified the biological functions and/or pathways that were most significant to the data set ( $P < 0.05$ ) according to a righted tailed Fisher's Exact Test. Ingenuity Pathway Analysis currently supports only Human, Mouse, and Rat species with full content, so the bovine species is supported at the ortholog level, therefore some significant genes may not be included in the analysis.

## RESULTS

Genes were filtered at a cutoff of 0.5 counts per million before fitting the data into a negative binomial distribution. There were 17,765 genes identified for the highE2/conceptus vs lowE2/conceptus comparison, 17,951 genes for the lowE2/conceptus vs lowE2/noconceptus comparison, and 18,116 genes for the highE2/conceptus vs highE2/noconceptus comparison that passed this criterion.

### *Exploratory Data Analysis:*

Counts were log<sub>2</sub> transformed prior to performing hierarchical clustering (figure 4), K means clustering (figure 5), and principal component analysis (figure 6). Enriched GO terms associated with the K means clusters were determined using iDEP (Ge et al., 2018; table 4).

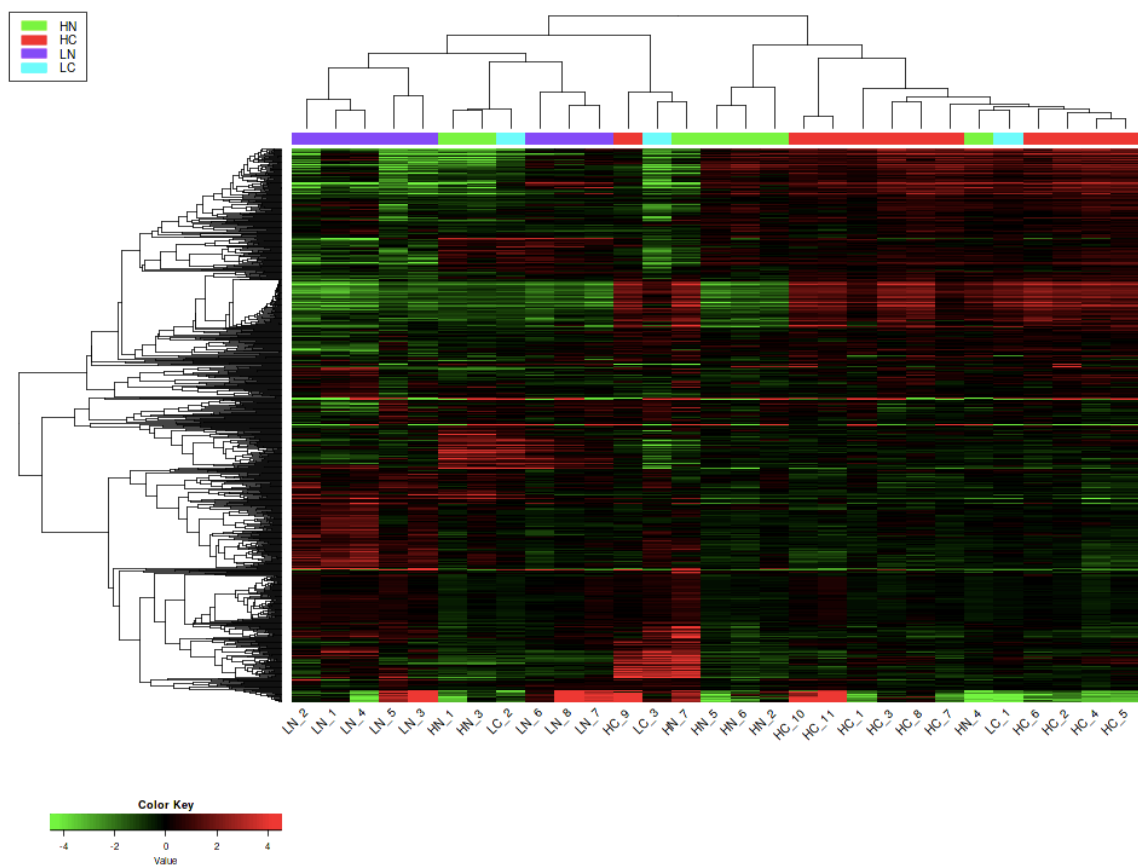


Figure 4. Hierarchical clustering heatmap for 1000 of the most variable genes among the preovulatory estradiol and conceptus presence interactions.

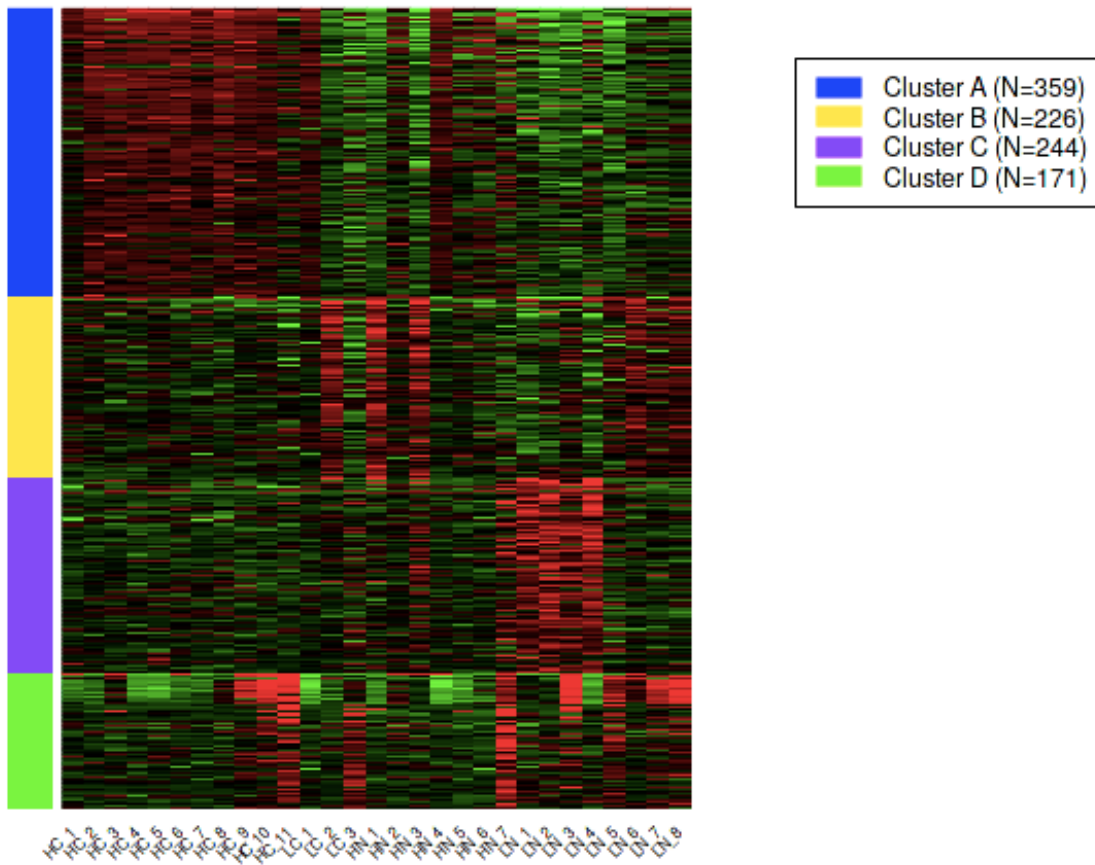


Figure 5. K Means clustering heatmap (1000 most variable genes, 4 clusters).



Table 4. Enriched pathways for each K means cluster.

Cluster	GO Biological Process	GO Cellular Component	GO Molecular Function	KEGG
A	<ul style="list-style-type: none"> <li>- Defense response</li> <li>- Response to stimulus</li> <li>- Immune system process</li> </ul>	<ul style="list-style-type: none"> <li>- Extracellular region</li> </ul>	<ul style="list-style-type: none"> <li>- Transmembrane transporter activity</li> <li>- Peptidase inhibitor activity</li> <li>- Enzyme inhibitor activity</li> </ul>	<ul style="list-style-type: none"> <li>- Influenza A</li> <li>- Hepatitis C</li> </ul>
B		<ul style="list-style-type: none"> <li>- Transmembrane transporter complex</li> </ul>	<ul style="list-style-type: none"> <li>- Gated channel activity</li> <li>- Ion channel activity</li> <li>- Inorganic transmembrane transporter activity</li> </ul>	
C	<ul style="list-style-type: none"> <li>- Multicellular organism development</li> <li>- Regulation of signaling receptor activity</li> <li>- System development</li> </ul>	<ul style="list-style-type: none"> <li>- Extracellular region</li> </ul>	<ul style="list-style-type: none"> <li>- Receptor regulator activity</li> <li>- Receptor ligand activity</li> <li>- Molecular function regulator</li> </ul>	
D	<ul style="list-style-type: none"> <li>- Immune response</li> <li>- Positive regulation of cellular process</li> <li>- Postitive regulation of metabolic process</li> </ul>	<ul style="list-style-type: none"> <li>- Extracellular space</li> </ul>	<ul style="list-style-type: none"> <li>- Chemokine activity</li> <li>- Antigen binding</li> <li>- Receptor ligand activity</li> </ul>	<ul style="list-style-type: none"> <li>- IL-17 signaling</li> <li>- TNF signaling</li> <li>- Cytokine-cytokine receptor interaction</li> </ul>

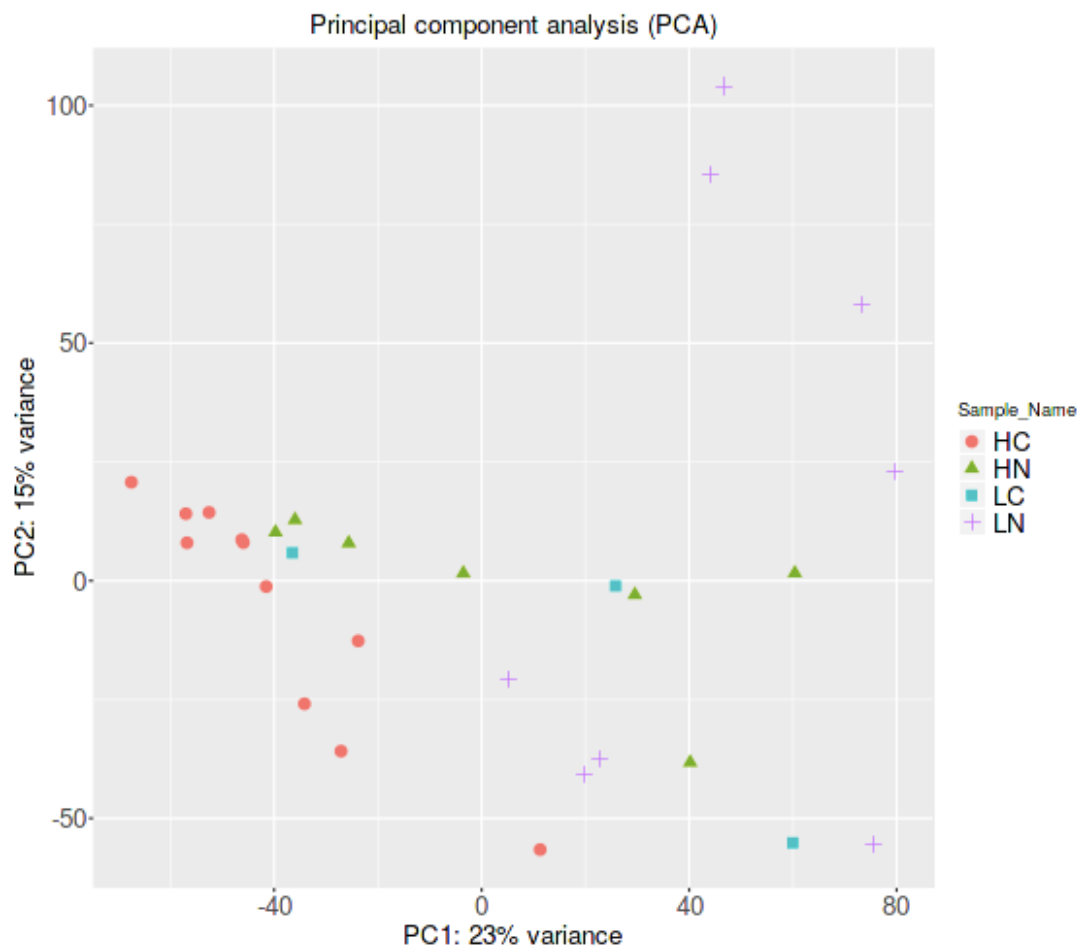


Figure 6. Principal component analysis for the different preovulatory estradiol exposure and conceptus presence interactions.

*Endometrium Gene Expression:*

The results and discussion focus on differentially expressed genes (DEGs) among the following groupings: highE2/conceptus vs lowE2/conceptus, highE2/conceptus vs highE2/noconceptus, and lowE2/conceptus vs lowE2/noconceptus. The number of DEGs within each of these comparisons is depicted in figure 7. Emphasis was given to the top canonical pathways, molecular and cellular functions, and networks.

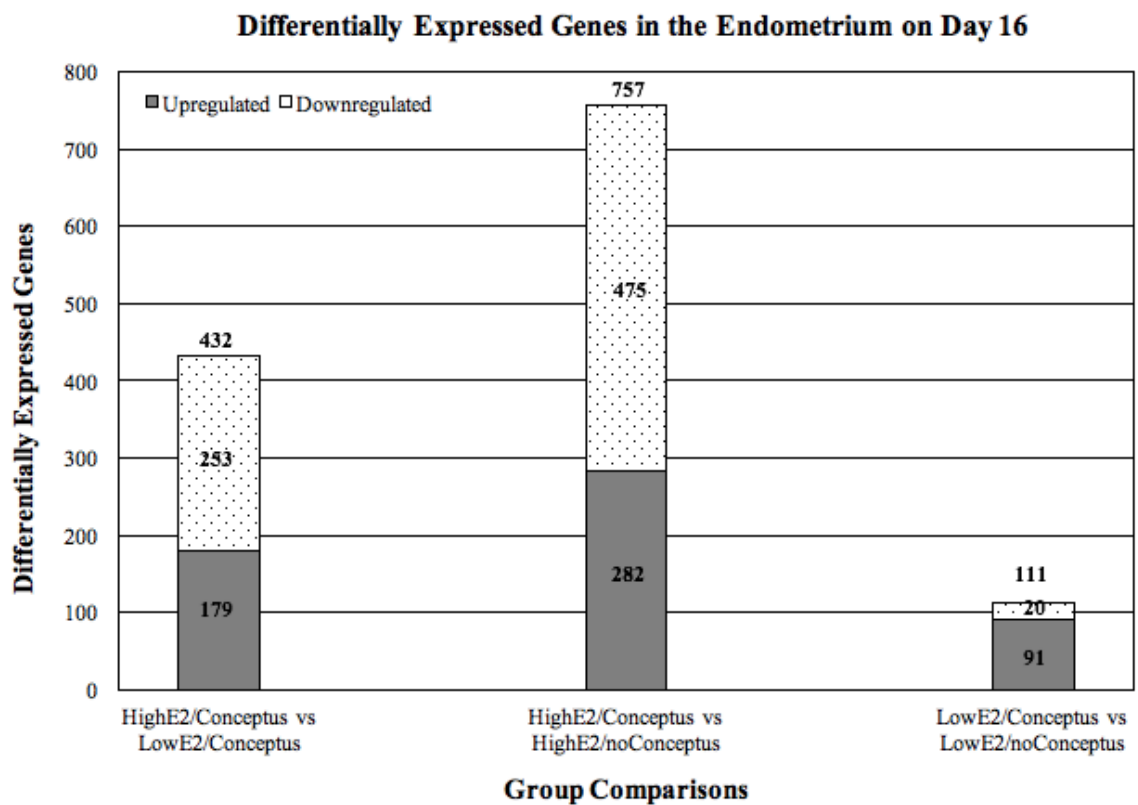


Figure 7. Upregulated and downregulated DEGs in the endometrium on day 16 in beef cattle.

Study 1- Effect of preovulatory estradiol exposure on endometrium and trophoctoderm gene expression on day 16 of pregnancy:

*HighE2/conceptus versus lowE2/conceptus:*

There were 432 DEGs between the highE2/conceptus group and the lowE2/conceptus group. Specifically, there were 253 genes that were downregulated (PRKCG, PRND, MRAP2, TDGF1, and LSMEM1) in the highE2/conceptus group (figure 8; table 5). There were 179 genes that were upregulated (PRSS2, CRYGS, C1QL2, PTPRN, FBP1) in the highE2/conceptus group compared to the lowE2/conceptus group (figure 8; table 5). The top canonical pathways associated with these DEGs included: calcium signaling (7.73E-05), caveolar mediated endocytosis signaling (1.00E-04), agranulocyte adhesion and diapedesis (1.77E-04), and axonal guidance signaling (2.37E-04; figure 9). The main molecular and cellular functions associated with the DEGs were cellular movement (97 molecules; 2.21E-03 – 9.63E-08), molecular transport (81 molecules; 1.87E-03 – 2.68E-07), cellular growth and proliferation (79 molecules; 2.02E-03 – 4.80E-07), cellular assembly and organization (74 molecules; 1.45E-03 – 1.24E-05), and cell death and survival (124 molecules; 2.22E-03 – 2.18E-05). Top network functions associated with these DEGs include: lipid metabolism, small molecule biochemistry, cell morphology, and embryonic development.

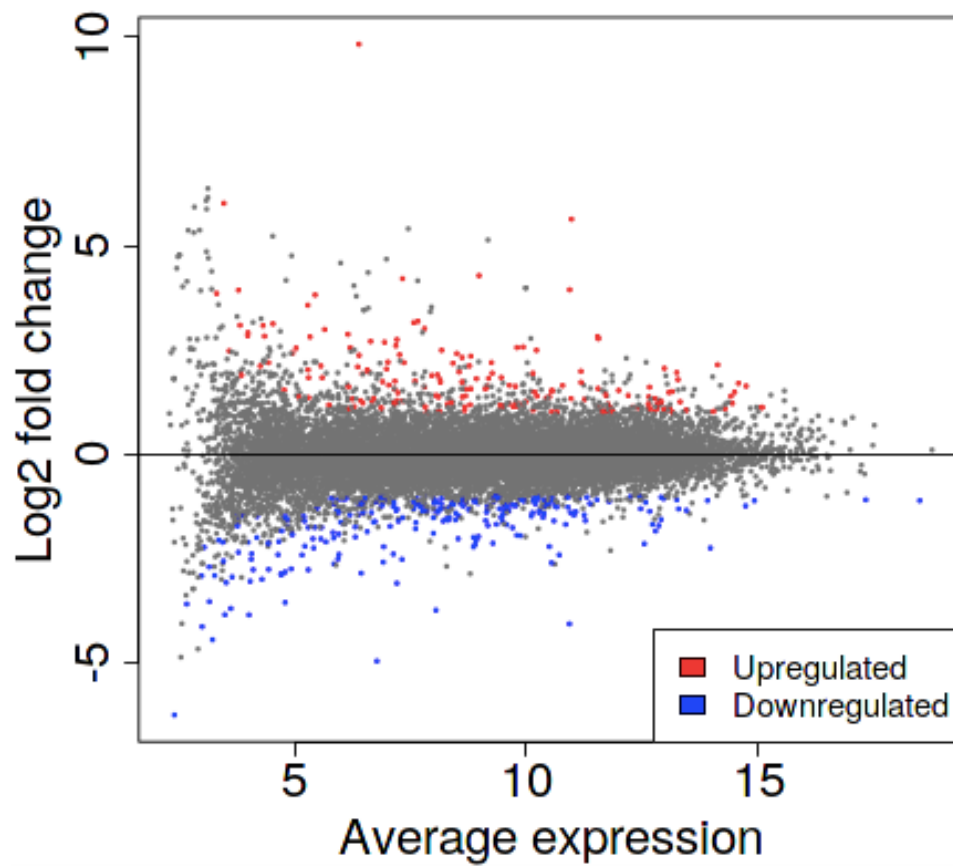


Figure 8. MA plot depicting DEGs among the highE2/conceptus vs lowE2/conceptus comparison.

Table 5. The top downregulated/upregulated characterized DEGs in the endometrium among highE2/conceptus and lowE2/conceptus animals.

Abbreviation	Gene Name	Log2Fold Change	FDR
PRKCG	Protein kinase C gamma	-4.94	0.0000014
PRND	Prion protein 2	-4.43	0.000603
MRAP2	Melanocortin 2 receptor accessory protein 2	-4.11	0.0355
TDGF1	Teratocarcinoma-derived growth factor 1	-4.05	0.0269
LSMEM1	Leucine rich single-pass membrane protein 1	-3.83	0.00767
OR9Q2	Olfactory receptor, family 9, subfamily Q, member 2	-3.83	0.0187
CD24	CD24 Molecule	-3.72	0.00384
LOC783399	Major allergen Equ c 1-like	-3.54	0.000617
CCKBR	Cholecystokinin B receptor	-3.52	0.00735
GPR183	G protein-coupled receptor 183	-3.08	0.000624
OR9G1	Olfactory receptor, family 9, subfamily I, member 1-like	-3.06	0.022
FMO1	Flavin containing monooxygenase 1	-3.03	0.0254
FAM64A	Family with sequence similarity 64 member A	-2.98	0.00181
FAAH	Fatty acid amide hydrolase	-2.93	0.0233
GLYATL2	Glycine-N-acyltransferase-like	-2.90	0.0356
ADGRE3	Adhesion G protein-coupled receptor E3	-2.89	0.0275
XIRP1	Xin actin binding repeat containing 1	-2.87	0.0407
SLC30A10	Solute carrier family 30 member 10	-2.86	0.02
TRIM9	Tripartite motif containing 9	-2.84	0.000251
IQGAP3	IQ motif containing GTPase activating protein 3	-2.81	0.00321
GRIA3	Glutamate ionotropic receptor AMPA type subunit 3	-2.76	0.0137
INA	Internexin neuronal intermediate filament protein alpha	-2.73	0.0481
HPSE	Heparanase	-2.62	0.02
CCNB1	Cyclin B1	-2.59	0.00857
LPL	Lipoprotein lipase	-2.57	0.00509
PLK5	Polo like kinase 5	2.39	0.0303
EPS8	Epidermal growth factor receptor	2.42	0.00183

kinase substrate 8-like protein			
MAP7D2	MAP7 domain-containing protein 2-like	2.43	0.0365
AGR3	Anterior gradient 3, protein disulphide isomerase family member	2.48	0.0338
IDO1	Indoleamine 2,3-dioxygenase 1	2.50	0.0182
MZB1	Marginal zone B and B1 cell specific protein	2.50	0.0101
KRT17	Keratin 17	2.57	0.00994
FGFBP1	Fibroblast growth factor binding protein 1	2.69	0.0173
ADGRF4	Adhesion G protein-coupled receptor F4	2.71	0.0123
CXCL10	C-X-C motif chemokine ligand 10	2.78	0.00133
RAB3C	RAB3C, member RAS oncogene family	2.82	0.0191
FABP3	Fatty acid binding protein 3	2.82	0.00604
CALB2	Calbindin 2	2.83	0.0438
NPC1L1	NPC1 like intracellular cholesterol transporter 1	2.85	0.0366
UPB1	Beta-ureidopropionase 1	2.88	0.000849
LBP	Lipopolysaccharide binding protein	2.92	0.0178
AHSG	Alpha 2-HS glycoprotein	2.99	0.00606
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	3.02	0.0496
BTG4	BTG anti-proliferation factor 4	3.09	0.0169
SLC5A5	Solute carrier family 5 member 5	3.13	0.00606
FBP1	Fructose-bisphosphatase 1	3.19	0.0312
PTPRN	Protein tyrosine phosphatase, receptor type N	3.82	0.00405
C1QL2	Complement C1q like 2	4.21	0.0000545
CRYGS	Crystallin gamma S	4.28	0.00000023
PRSS2	Protease, serine 2	9.82	0.00133



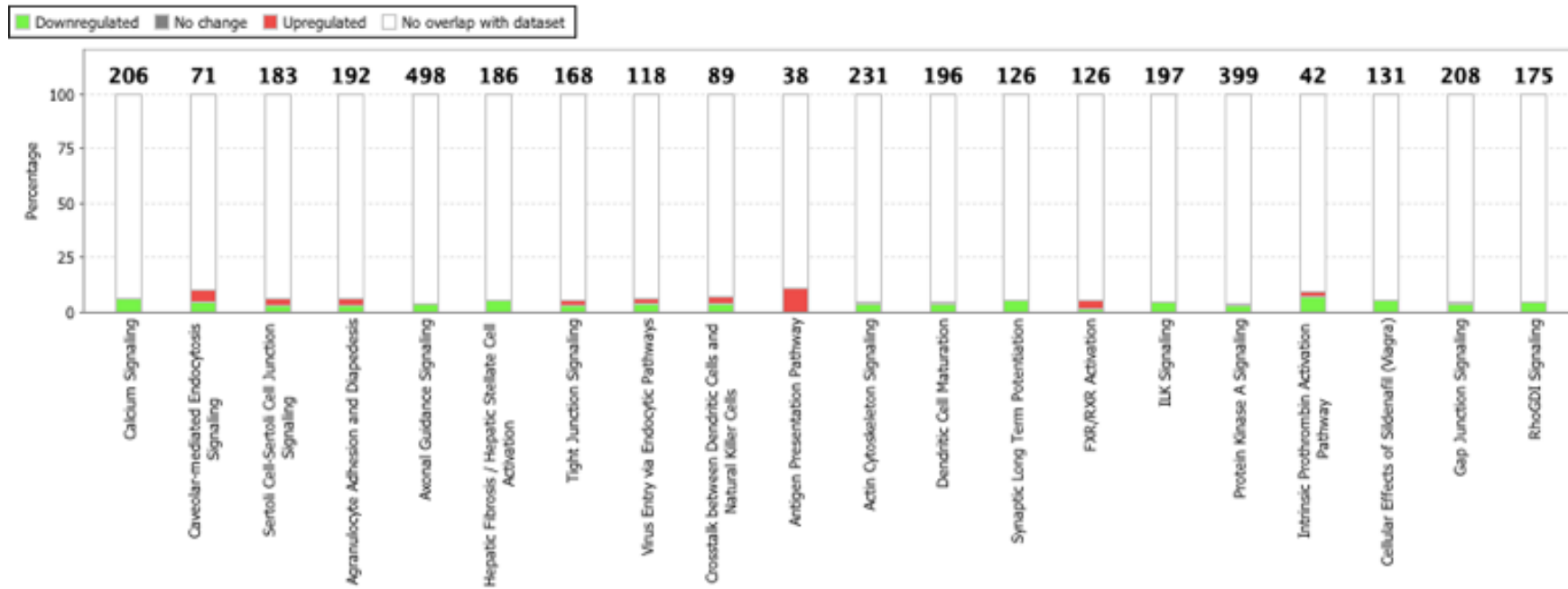


Figure 9. Top 20 canonical pathways associated with the DEGs among the highE2/conceptus vs lowE2/conceptus groups.

*RT-PCR Endometrium Validation:*

In endometrium, highE2 animals had increased mRNA abundances of PRSS8 ( $P = 0.005$ ; figure 10), CXCL10 ( $P = 0.003$ ; figure 10), IDO1 ( $P = 0.02$ ; figure 11), FABP3 ( $P = 0.04$ ; figure 11), XAF1 ( $P = 0.05$ ; figure 13), and DDX58 ( $P = 0.007$ ; figure 13) on day 16. HighE2 animals had decreased OXTR mRNA abundance ( $P = 0.02$ ; figure 12) in the endometrium compared to lowE2 animals. HighE2 animals also had a tendency to have increased mRNA abundance of MUC13 ( $P = 0.07$ ; figure 11) compared to animals with low preovulatory estradiol concentrations. There was no difference in CLDN4 ( $P = 0.26$ ; figure 10), RSAD2 ( $P = 0.17$ ; figure 12), ISG15 ( $P = 0.85$ ; figure 12), PARP12 ( $P = 0.23$ ; figure 13) mRNA abundances in the endometrium among highE2 and lowE2 animals.

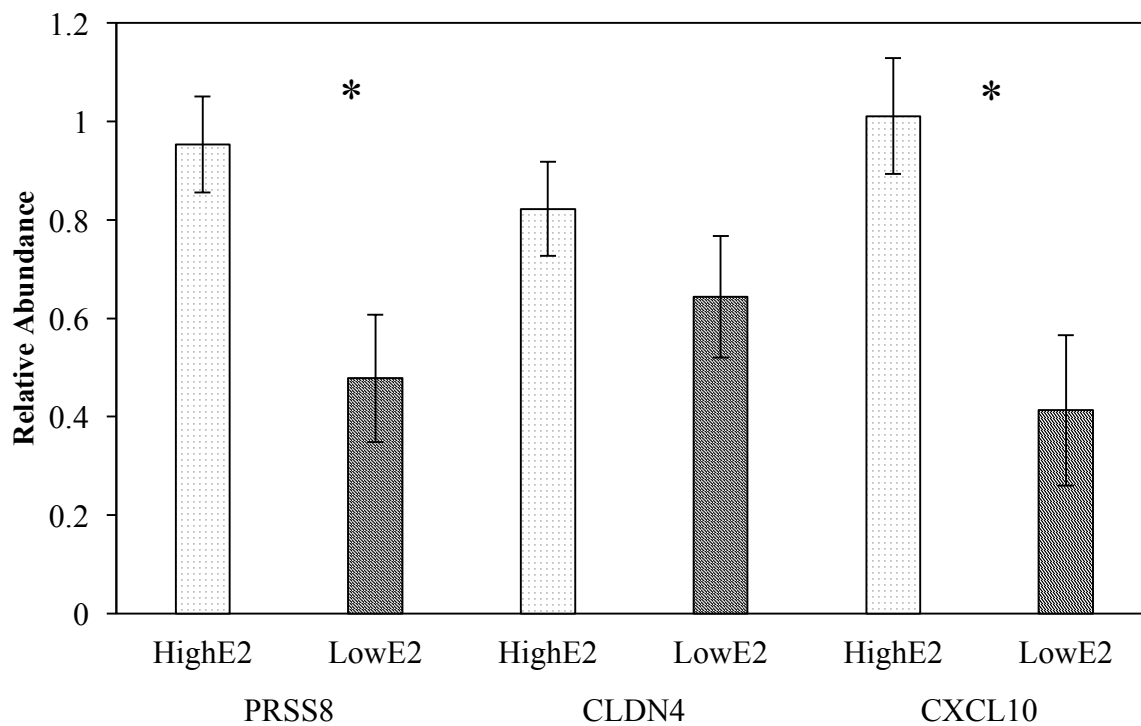


Figure 10. Endometrium mRNA abundances for PRSS8, CLDN4, and CXCL10 on day 16 among highE2 and lowE2 animals. HighE2 animals had increased PRSS8 ( $P = 0.005$ ) and CXCL10 ( $P = 0.003$ ) transcript abundances compared to lowE2 animals, CLDN4 expression was not different. ( $*P < 0.05$ )

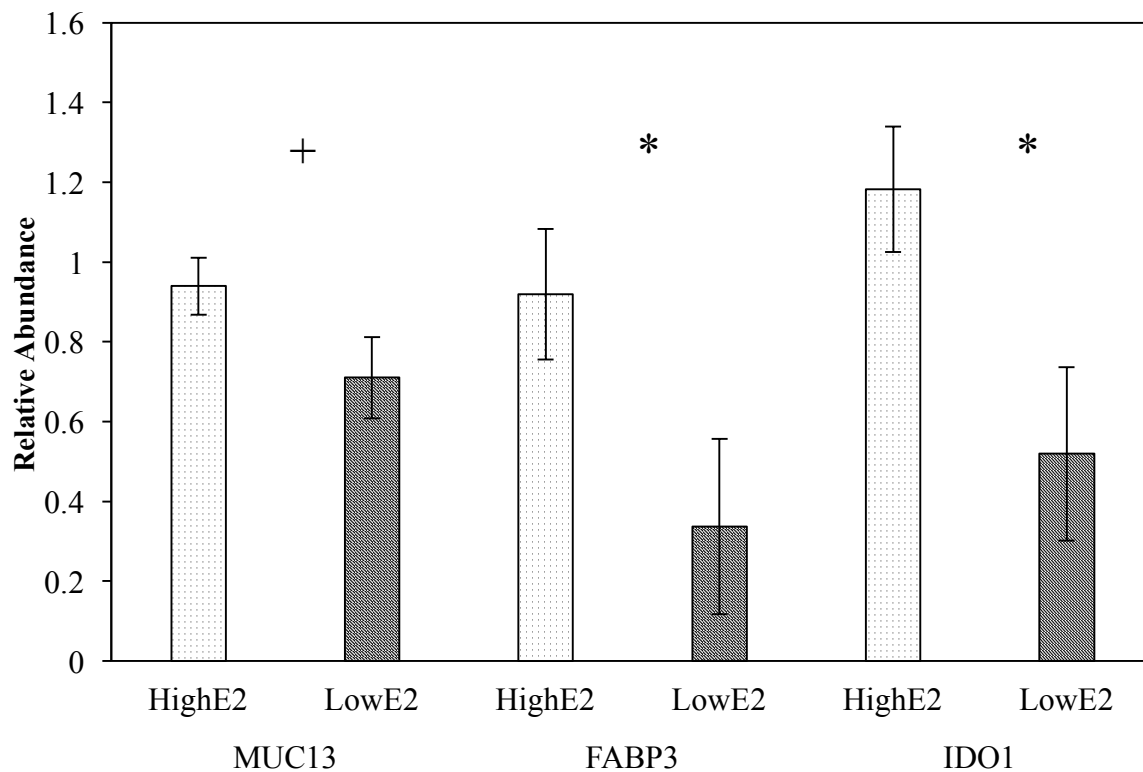


Figure 11. Endometrium mRNA abundances for MUC13, FABP3, and IDO1 on day 16 among highE2 and lowE2 animals. HighE2 animals had increased FABP3 ( $P = 0.04$ ) and IDO1 ( $P = 0.02$ ) transcript abundances compared to lowE2 animals. There was a tendency for highE2 animals to have increased MUC13 ( $P = 0.07$ ) transcript abundance in the endometrium on day 16 compared to lowE2 animals. (\* $P > 0.05$ , + $P < 0.10$ )

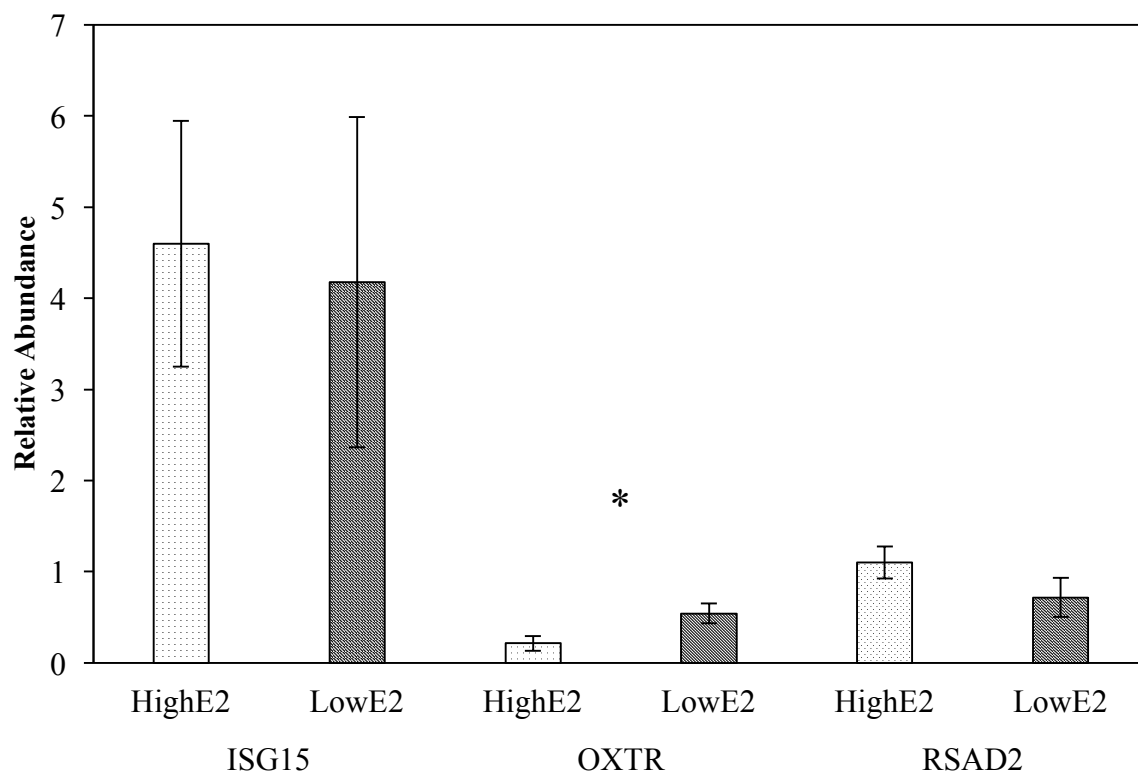


Figure 12. Endometrium mRNA abundances for ISG15, OXTR, and RSAD2 on day 16 among highE2 and lowE2 animals. HighE2 animals had decreased OXTR ( $P = 0.02$ ) transcript abundance compared to lowE2 animals. There was no difference in ISG15 and RSAD2 transcript abundances in the endometrium on day 16. ( $*P < 0.05$ )

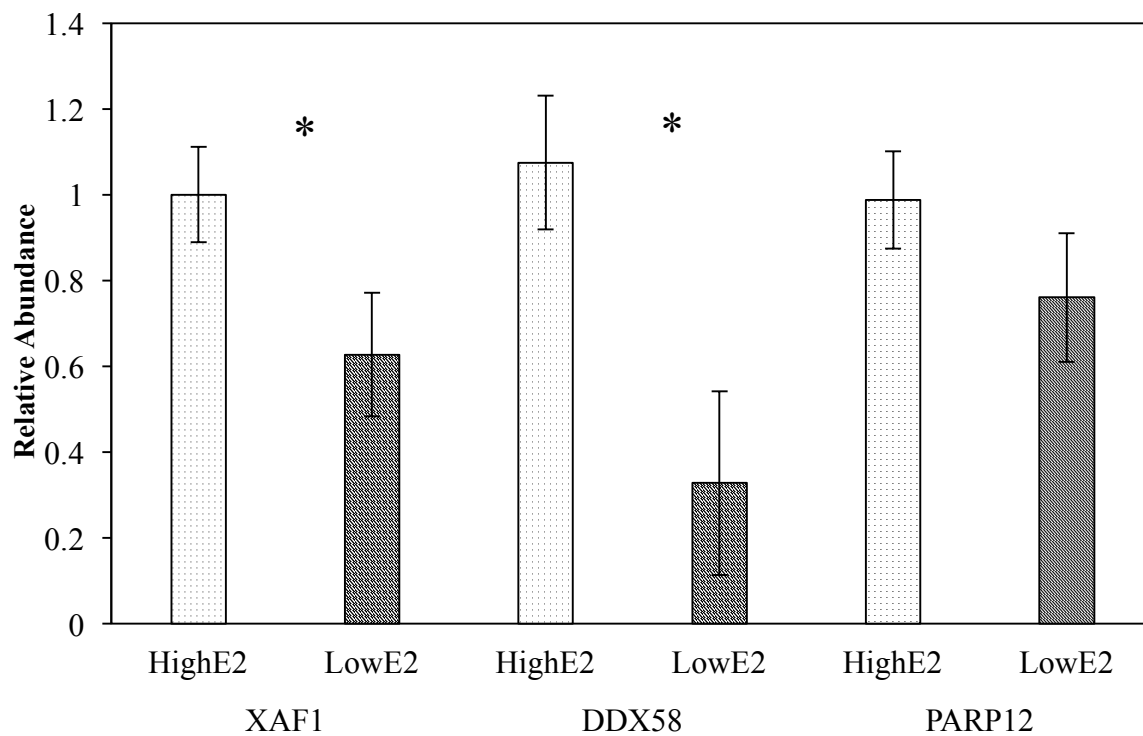


Figure 13. Endometrium mRNA abundances for XAF1, DDX58, and PARP12 on day 16 among highE2 and lowE2 animals. HighE2 animals had increased XAF1 ( $P = 0.02$ ) and DDX58 ( $P = 0.0007$ ) transcript abundances compared to lowE2 animals. There was no difference in PARP12 transcript abundances in the endometrium on day 16. ( $*P < 0.05$ )

*Trophectoderm Gene Expression:*

There were no differences in mRNA abundances for IFNT, PTGS2, TM4SF1, C3, and FGFR2 in the trophoctoderm collected from highE2 and lowE2 animals on day 16 of pregnancy ( $P > 0.22$ ; figures 14-18).

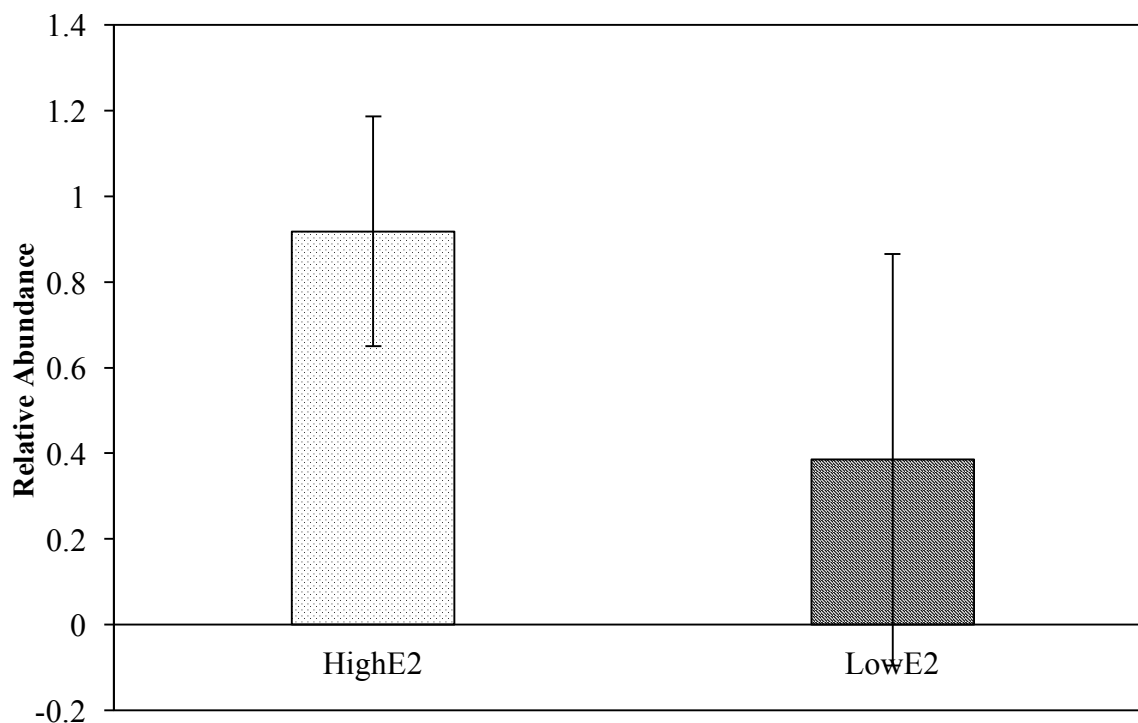


Figure 14. IFNT mRNA abundances in the trophoctoderm collected from highE2 and lowE2 animals on day 16 of pregnancy. There was no difference in IFNT mRNA abundance based on preovulatory estradiol concentrations ( $P = 0.34$ ).



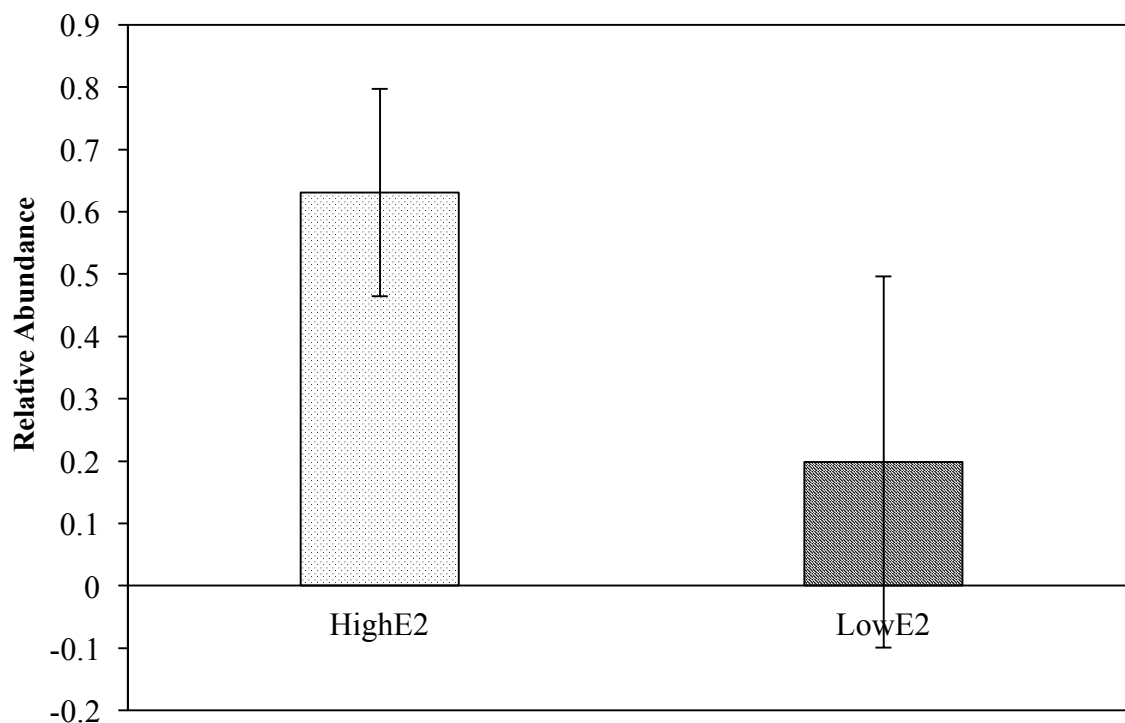


Figure 15. PTGS2 mRNA abundances in the trophoctoderm collected from highE2 and lowE2 animals on day 16 of pregnancy. There was no difference in PTGS2 mRNA abundance based on preovulatory estradiol concentrations ( $P = 0.22$ ).

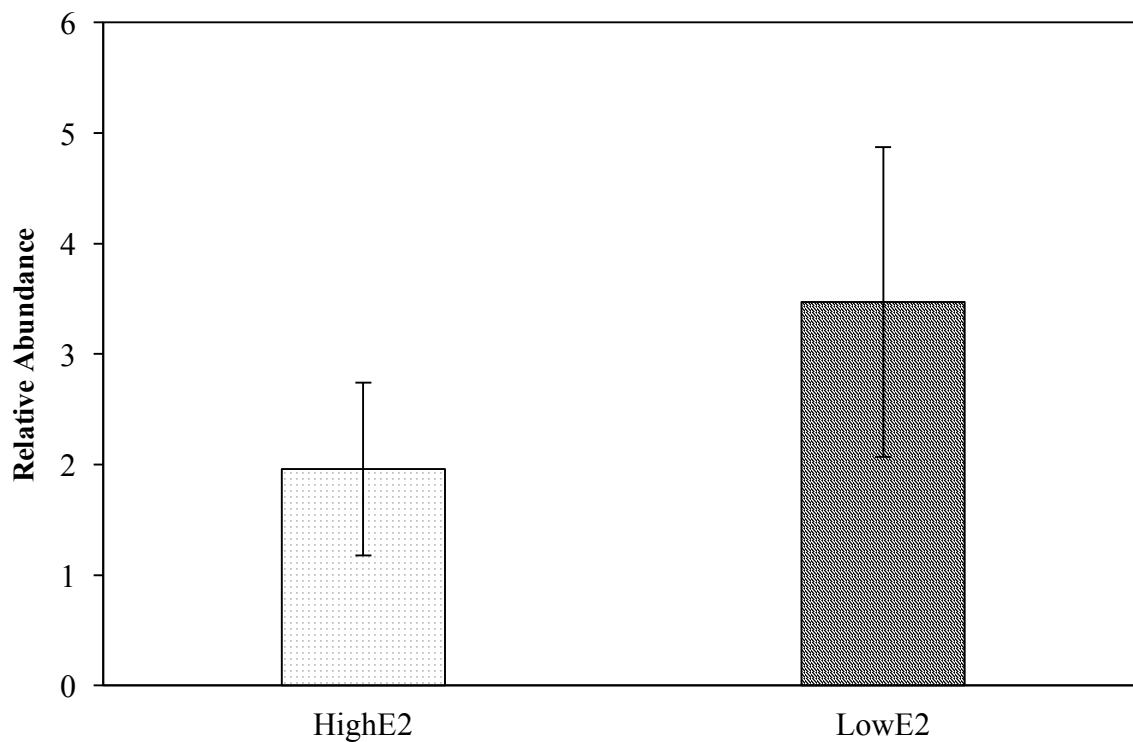


Figure 16. TM4SF1 mRNA abundances in the trophectoderm collected from highE2 and lowE2 animals on day 16 of pregnancy. There was no difference in TM4SF1 mRNA abundance based on preovulatory estradiol concentrations ( $P = 0.36$ ).

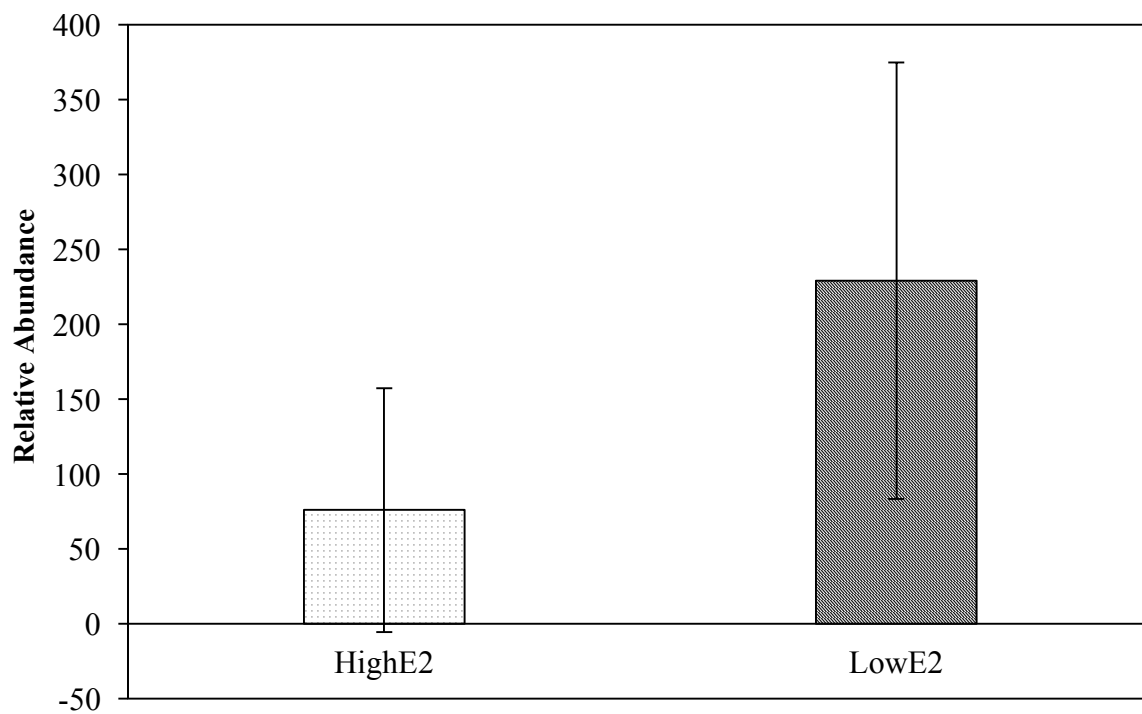


Figure 17. C3 mRNA abundances in the trophoctoderm collected from highE2 and lowE2 animals on day 16 of pregnancy. There was no difference in C3 mRNA abundance based on preovulatory estradiol concentrations ( $P = 0.37$ ).

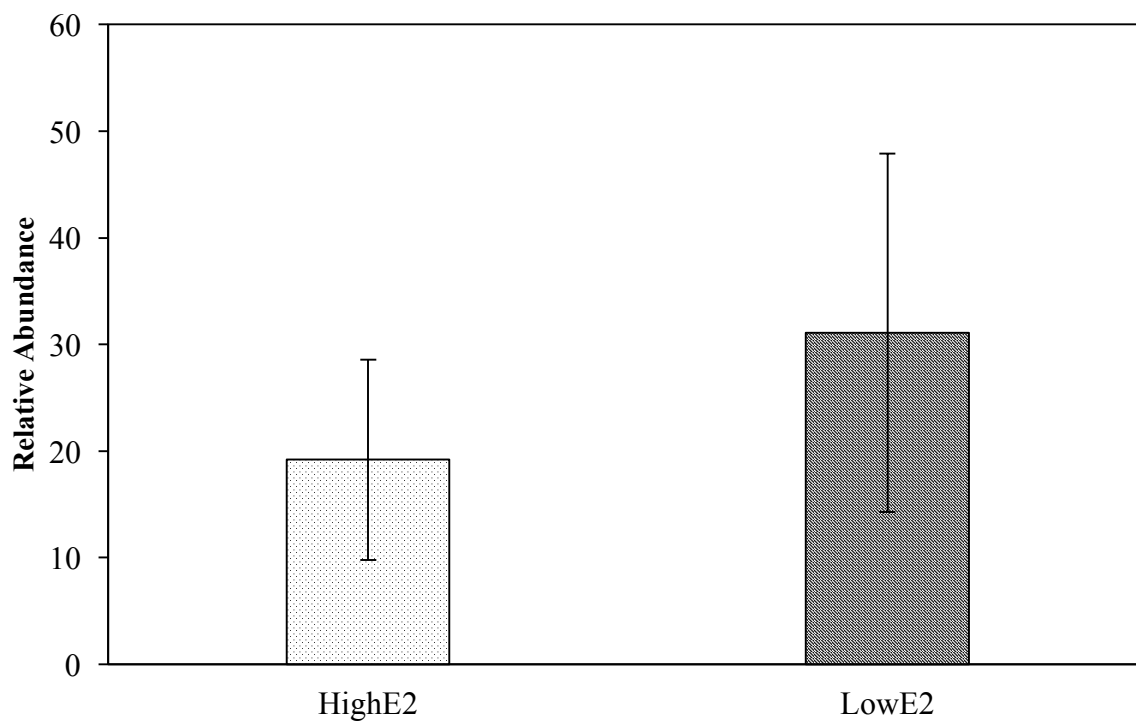


Figure 18. FGFR2 mRNA abundances in the trophoctoderm collected from highE2 and lowE2 animals on day 16 of pregnancy. There was no difference in FGFR2 mRNA abundance based on preovulatory estradiol concentrations ( $P = 0.54$ ).

Study 2- Effect of conceptus presence (interferon tau) on endometrium gene expression on day 16 of pregnancy/estrous cycle:

*HighE2/conceptus versus highE2/noconceptus:*

There were 757 DEGs between the highE2/conceptus group and the highE2/noconceptus group. Specifically, there were 282 genes that were upregulated (RRAGA, APOBEC321, PRM3, JCHAIN, RESP18) in the highE2/conceptus group compared to the highE2/noconceptus group (figure 19; table 6). There were 475 genes that were downregulated (SLC9A4, BSP3, CA1, CDK5R2, RBBP8NL) in the highE2/conceptus group (figure 19; table 6). The top canonical pathways associated with these DEGs included: interferon signaling ( $1.91E-06$ ), activation of IRF by cytosolic pattern recognition receptors ( $2.03E-05$ ), hepatic stellate cell activation ( $1.90E-04$ ), axonal guidance signaling ( $2.35E-04$ ), and the complement system ( $2.44E-04$ ; figure 20). The main molecular and cellular functions associated with the DEGs were: cell-cell signaling (113 molecules;  $6.54E-04 - 1.43E-09$ ), molecular transport (129 molecules;  $1.39E-04 - 6.58E-08$ ), cell signaling (40 molecules;  $4.48E-04 - 2.18E-07$ ), cellular movement (141 molecules;  $5.48E-04 - 2.45E-07$ ), and carbohydrate metabolism (74 molecules;  $3.49E-04 - 5.38E-07$ ). Top network functions associated with these DEGs include: antimicrobial response, inflammatory response, lipid metabolism, small molecule biochemistry, and carbohydrate metabolism.

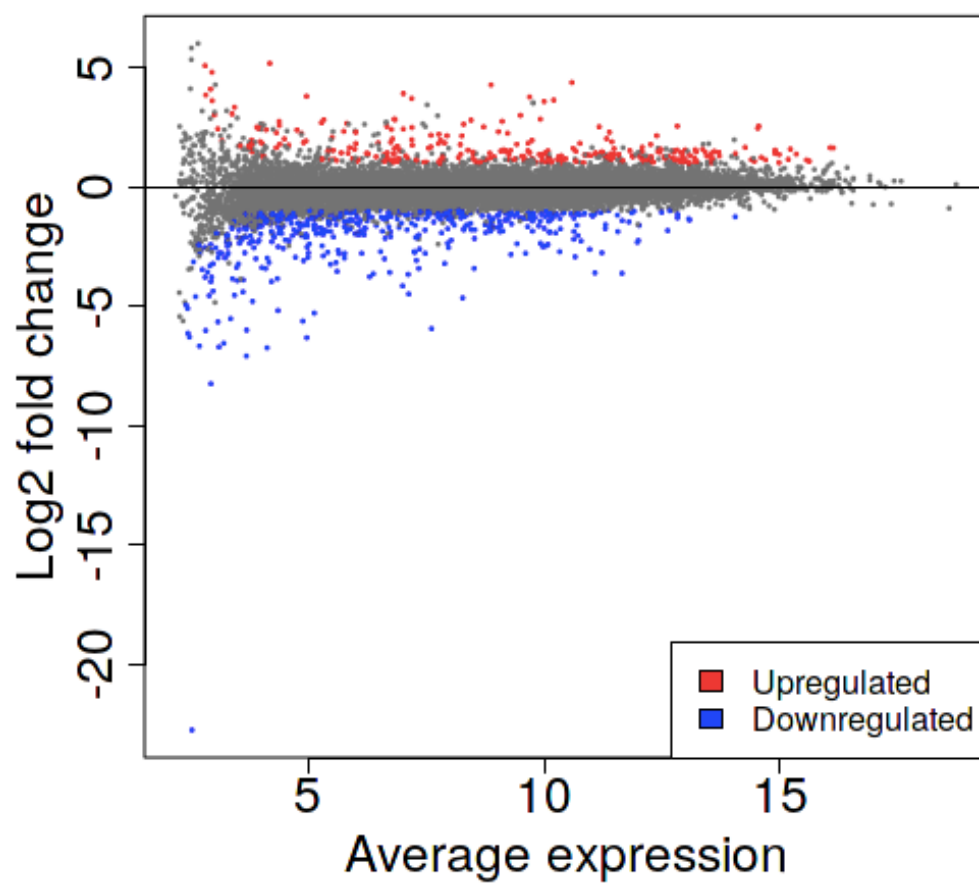


Figure 19. MA plot depicting DEGs among the highE2/conceptus vs highE2/noconceptus comparison.

Table 6. The top downregulated/upregulated characterized DEGs in the endometrium among highE2/conceptus and highE2/noconceptus animals.

Abbreviation	Gene Name	Log2Fold Change	FDR
SLC9A4	Solute carrier family 9 member A4	-22.78	0.000000000891
BSP3	Binder of sperm 3	-8.26	0.000858554
CA1	Carbonic anhydrase 1	-6.75	0.0000457
CDK5R2	Cyclin dependent kinase 5 regulatory subunit 2	-6.72	0.000325192
RBBP8NL	RBBP8 N-terminal like protein	-6.56	0.000120094
BSP1	Binder of sperm 1	-6.14	0.030413264
GSX1	GS homeobox 1	-6.03	0.036796029
SLC13A5	Solute carrier family 13 member 5	-5.95	0.00000482
WNT7A	Wnt family member 7A	-5.63	0.00000232
DISP3	Dispatched RND transporter family member 3	-5.53	0.0000227
AGXT	Alanine-glyoxylate aminotransferase	-5.11	0.044704538
PIWIL4	Piwi like RNA-mediated gene silencing 4	-4.96	0.021995747
DAPL1	Death associated protein like 1	-4.81	0.000119798
CD24	CD24 molecule	-4.66	0.000119798
TEX29	Testis expressed 29	-4.61	0.009006198
KRT5	Keratin 5	-4.57	0.004654873
CNGA1	Cyclic nucleotide gated channel alpha 1	-4.55	0.000545743
PRKCG	Protein kinase C gamma	-4.50	0.0000094
HTR1A	5-hydroxytryptamine receptor 1A	-4.41	0.010200026
IGF2BP1	Insulin like growth factor 2 mRNA binding protein 1	-4.38	0.001413202
SLC26A3	Solute carrier family 26 member 3	-3.98	0.000172319
TAF7L	TATA-box binding protein associated factor 7 like	-3.94	0.002319392
PRG3	Proteoglycan 3	-3.91	0.047353191
MMRN1	Multimerin 1	-3.85	0.000746992
SLC26A4	Solute carrier family 26 member 4	-3.79	0.001913373
C1QL2	Complement C1q like 2	2.42	0.0230679

MMP9	Matrix metalloproteinase 9	2.43	0.039639887
RSAD2	Radical S-adenosyl methionine domain containing 2	2.45	0.015664537
CCL2	C-C Motif Chemokine Ligand 2	2.49	0.000672892
LOC520778	Ubiquilin 3-like	2.50	0.016502573
CRYGS	Crystallin gamma S	2.51	0.003230429
CXCL10	C-X-C motif chemokine ligand 10	2.51	0.000290207
ISG15	ISG15 ubiquitin-like modifier	2.54	0.014497037
IFIT3	Interferon induced protein with tetratricopeptide repeat	2.54	0.002985721
DUOX1	Dual oxidase 1	2.57	0.043659938
ULBP11	UL16-binding protein 11	2.61	0.042818379
CCL8	C-C Motif Chemokine Ligand 8	2.62	0.000187357
UPB1	Beta-ureidopropionase 1	2.65	0.0002389
PTPRN	Protein tyrosine phosphatase, receptor type N	2.68	0.001643261
AQP8	Aquaporin 8	2.74	0.022807165
ISG20	Interferon stimulated exonuclease gene 20	2.74	0.004920751
GRIP2	Glutamate receptor interacting protein 2	2.79	0.001458079
TGM5	Transglutaminase 5	2.81	0.010349331
IDO1	Indoleamine 2,3-dioxygenase 1	2.83	0.0000466
TMEM40	Transmembrane protein 40	2.83	0.0000279
DUOXA1	Dual oxidase maturation factor 1	3.05	0.011602779
RESP18	Regulated endocrine specific protein 18	3.33	0.002268262
JCHAIN	Joining chain of multimeric IgA and IgM	3.58	0.010980856
PRM3	Protamine 3	3.61	0.002836028
RRAGA	Ras-related GTP-binding protein A	5.08	0.015861075



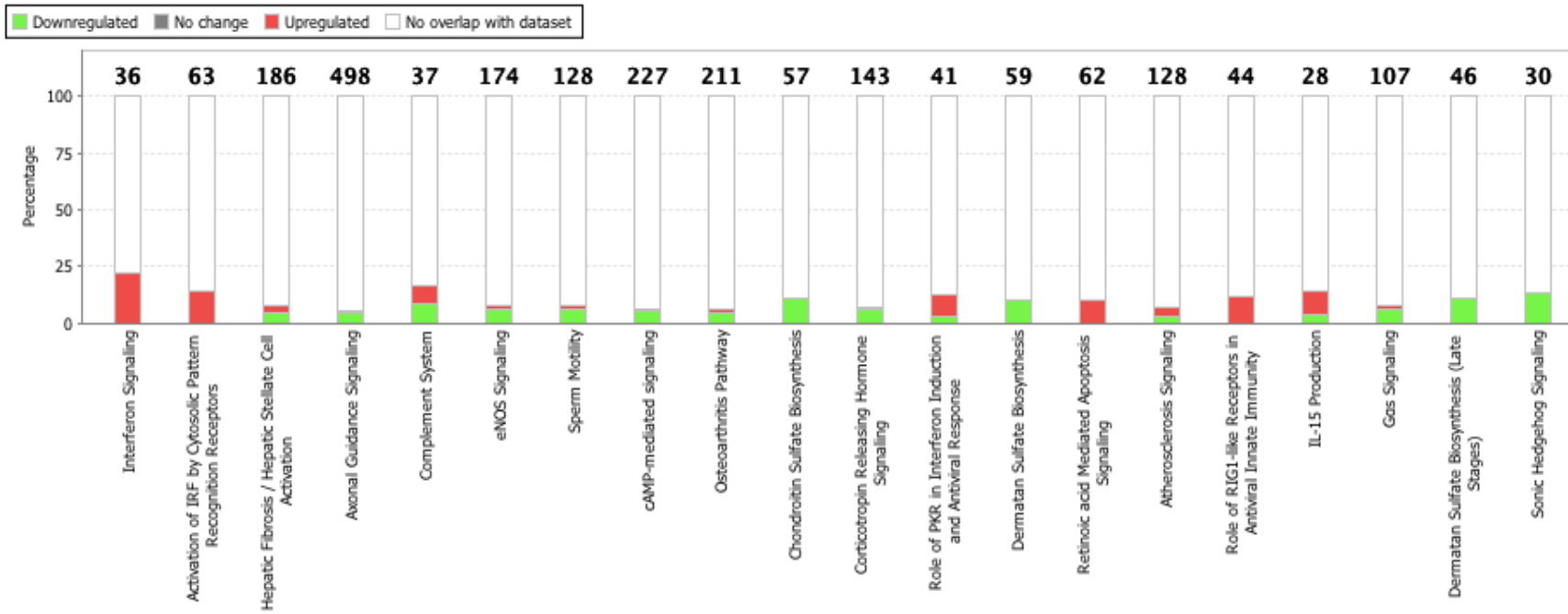


Figure 20. Top 20 canonical pathways associated with the DEGs among the highE2/conceptus vs highE2/noconceptus groups.

*LowE2/conceptus and lowE2/noconceptus:*

There were 111 DEGs between the lowE2/conceptus group and the lowE2/noconceptus group. Specifically, there were 91 genes that were upregulated (MX2, ISG15, BST2, IFIT1, RSAD2) in the lowE2/conceptus group compared to the lowE2/noconceptus group (figure 21; table 7). There were 20 genes that were downregulated (MMP1, SERPINA1, SMPX, OXTR, RSP23) in the lowE2/conceptus group (figure 21; table 7). The top canonical pathways associated with these DEGs included: activation of IRF by cytosolic pattern recognition receptors ( $1.87E-14$ ), interferon signaling ( $4.77E-13$ ), role of RIG1-like receptors in antiviral innate immunity ( $4.76E-07$ ), role of pattern recognition receptors in recognition of bacteria and viruses ( $6.22E-07$ ), and UVA-induced MAPK signaling ( $5.09E-05$ ; figure 22). The main molecular and cellular functions associated with the DEGs were: cell signaling (10 molecules;  $1.06E-02 - 4.32E-11$ ), post translational modification (16 molecules;  $1.11E-02 - 1.81E-08$ ), protein folding (4 molecules;  $1.81E-08 - 1.81E-08$ ), protein trafficking (9 molecules;  $3.54E-03 - 1.81E-08$ ), and the cell cycle (21 molecules;  $1.06E-02 - 7.93E-06$ ). Top network functions associated with these DEGs include: antimicrobial response, inflammatory response, cellular development, cellular growth and proliferation, and endocrine system development and function. Regardless of preovulatory estradiol exposure, there were 63 DEGs in common when comparing endometrium samples from animals that did and did not have a conceptus recovered (figure 23).

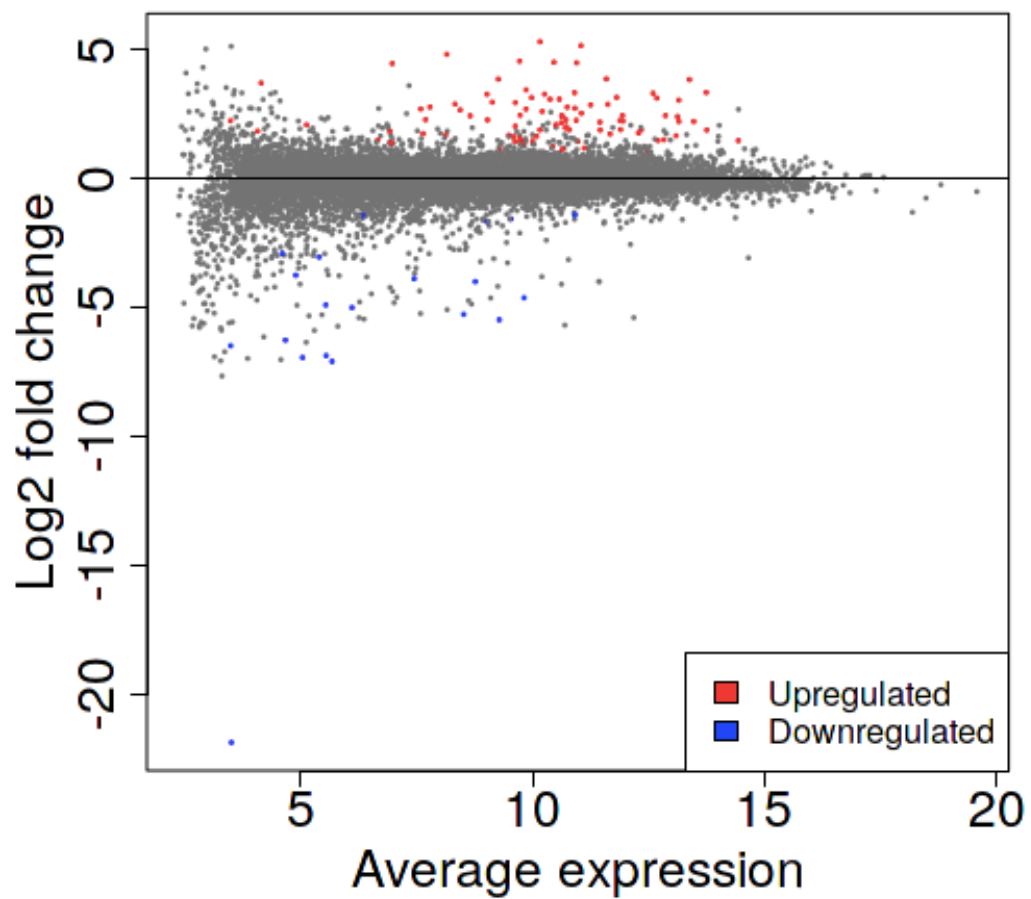


Figure 21. MA plot depicting DEGs among the lowE2/conceptus vs lowE2/noconceptus comparison.

Table 7. The top downregulated/upregulated characterized DEGs in the endometrium among lowE2/conceptus and lowE2/noconceptus animals.

Abbreviation	Gene Name	Log2Fold Change	FDR
MMP1	Matrix metalloproteinase 1	-21.89	0.000000545
SERPINA1	Serpin family A member 1	-7.11	0.013544244
SMPX	Small muscle protein, X-linked	-6.88	0.010538977
RPS23	40S ribosomal protein S23	-6.50	0.017263631
OXTR	Oxytocin receptor	-5.49	0.043428372
ADAMTS18	ADAM metalloproteinase with thrombospondin type 1 motif 18	-5.02	0.017869681
OSTN	Osteonin	-4.92	0.010427075
CLDN10	Claudin 10	-4.64	0.005636542
MGAT5B	Acetylglucosaminyltransferase B	-4.01	0.014714355
NDST4	N-deacetylase and N-sulfotransferase 4	-3.76	0.049252873
RHBDL3	Rhomboid like 3	-3.06	0.000292332
PLIN4	Perilipin-4	-2.91	0.038202134
MDF1	MyoD family inhibitor	-1.68	0.005318663
PIGR	Polymeric immunoglobulin receptor	-1.57	0.004575342
ABHD15	Abhydrolase domain containing 15	-1.44	0.010095709
EPSTI1	Epithelial stromal interaction 1	2.76	0.0000894
CYP1A1	Cytochrome P450, subfamily I	2.76	0.0000108
IRF7	Interferon regulatory factor 7	2.85	1.02E-05
HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase	2.87	0.00000168
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	2.88	0.004227525
CMPK2	Cytidine/uridine monophosphate kinase 2	2.94	0.000117797
IFI44	Interferon induced protein 44	2.95	0.0000585
MAFF	MAF bZIP transcription factor F	3.07	0.011035713
OAS1Y	2',5'-oligoadenylate synthetase 1, 40/46kDa	3.12	0.0000494
IFIT3	Interferon induced protein with tetratricopeptide repeats 3	3.13	0.010928799
UBA7	Ubiquitin like modifier activating enzyme 7	3.14	0.0000818

OAS1X	2',5'-oligoadenylate synthetase 1, 40/46kDa	3.26	0.000128784
USP18	Ubiquitin specific peptidase 18	3.26	0.00000598
DDX58	DExD/H-box helicase 58	3.32	0.000000535
IFI6	Interferon alpha inducible protein 6	3.33	0.0000208
IFI44L	Interferon induced protein 44 like	3.43	0.00000792
MX1	MX dynamin like GTPase 1	3.83	0.0000000787
ZBP1	Z-DNA binding protein 1	3.85	0.00000288
NR4A1	Nuclear receptor subfamily 4 group A member 1	3.86	0.014236076
OAS2	2'-5'-oligoadenylate synthetase 2	4.45	0.000000623
RSAD2	Radical S-adenosyl methionine domain containing 2	4.48	0.0000102
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	4.55	0.00000751
BST2	Bone marrow stromal antigen 2	4.81	0.0000000219
ISG15	ISG15 ubiquitin-like modifier	5.15	0.0000000459
MX2	MX dynamin like GTPase 2	5.30	0.0000000243

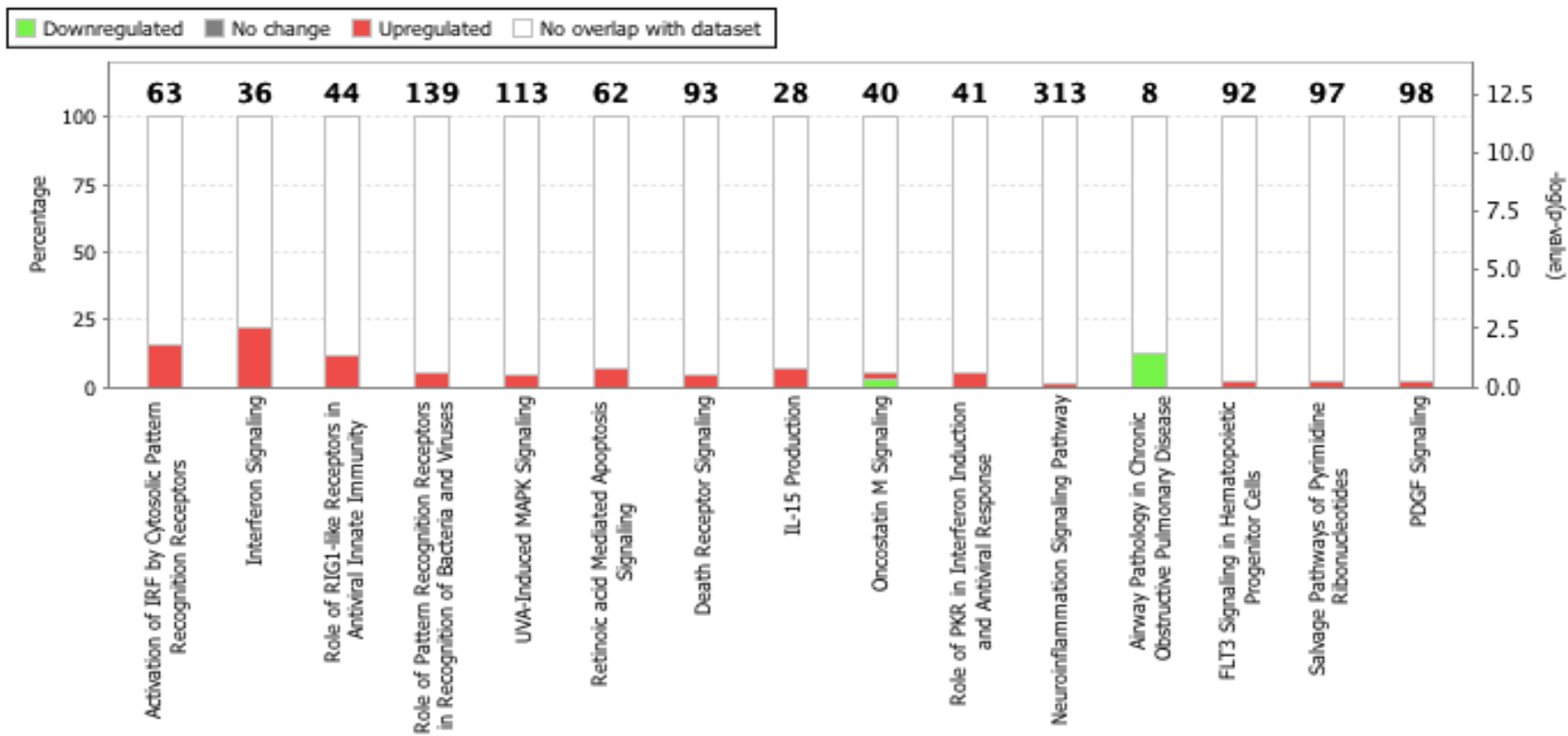


Figure 22. Top canonical pathways associated with the DEGs among the lowE2/conceptus vs lowE2/noconceptus groups.

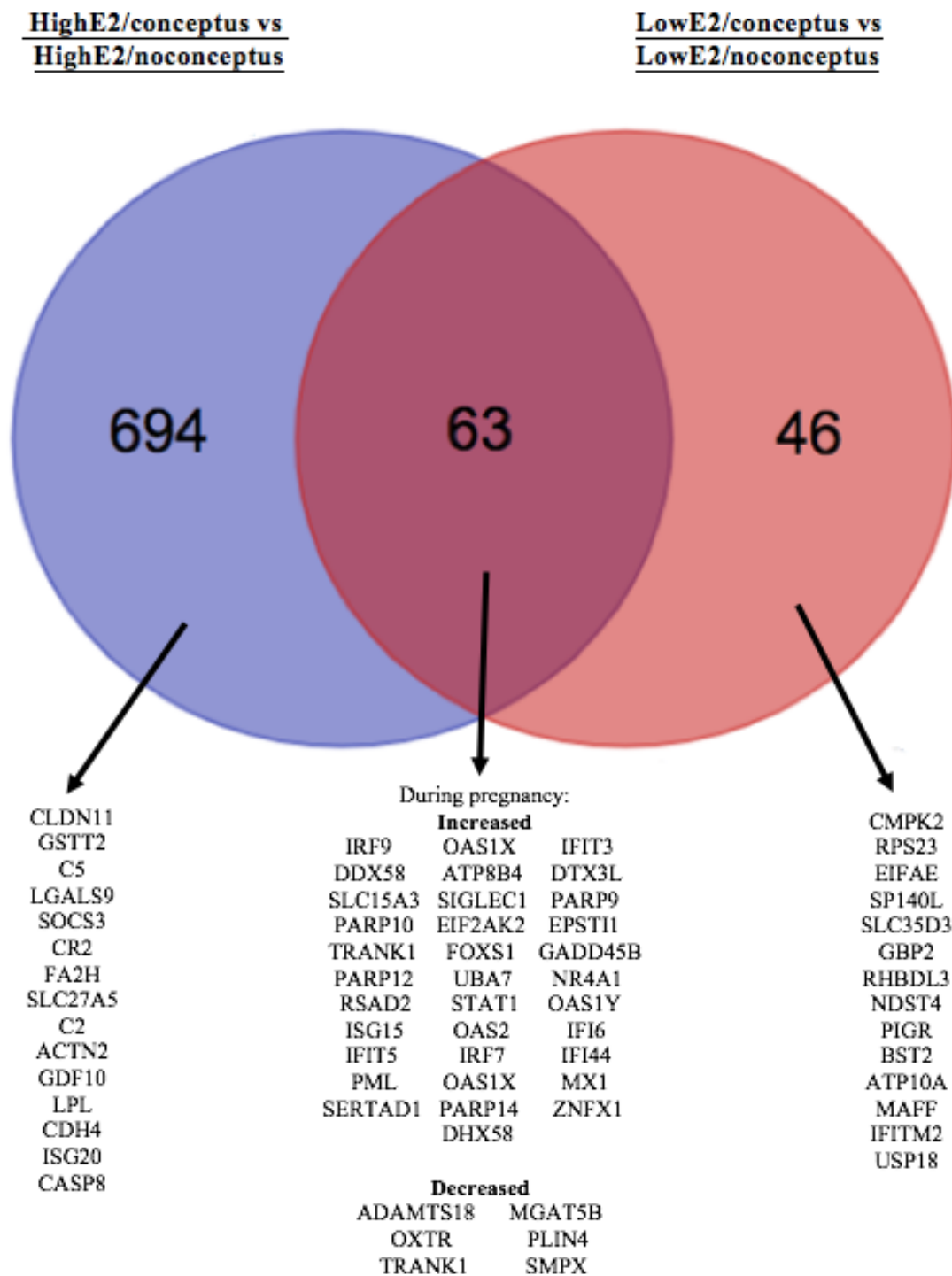


Figure 23. A comparison examining the effects of IFNT, by comparing differentially expressed genes among highE2/conceptus vs highE2/noconceptus and lowE2/conceptus vs lowE2/noconceptus groups.

*RTPCR Endometrium Validation:*

In endometrium, animals from which a conceptus was recovered had increased mRNA abundances of ISG15 ( $P = 0.006$ ; figure 24), RSAD2 ( $P < 0.0001$ ; figure 24), XAF1 ( $P < 0.0001$ ; figure 25), DDX58 ( $P = 0.002$ ; figure 25), PARP12 ( $P < 0.0001$ ; figure 25), FABP3 ( $P = 0.01$ ; figure 27), IDO1 ( $P = 0.002$ ; figure 27), CXCL10 ( $P = 0.0005$ ; figure 26), CLDN4 ( $P = 0.03$ ; figure 26), and PRSS8 ( $P = 0.01$ ; figure 26) on day 16. There was no difference in OXTR ( $P = 0.20$ ; figure 24) and MUC13 ( $P = 0.12$ ; figure 24) transcript abundances in the endometrium among animals that did and did not have a conceptus recovered. There was a conceptus and preovulatory exposure interaction for CXCL10 ( $P = 0.04$ ), and a tendency for an interaction for DDX58 ( $P = 0.06$ ) and IDO1 ( $P = 0.06$ ; appendix A).



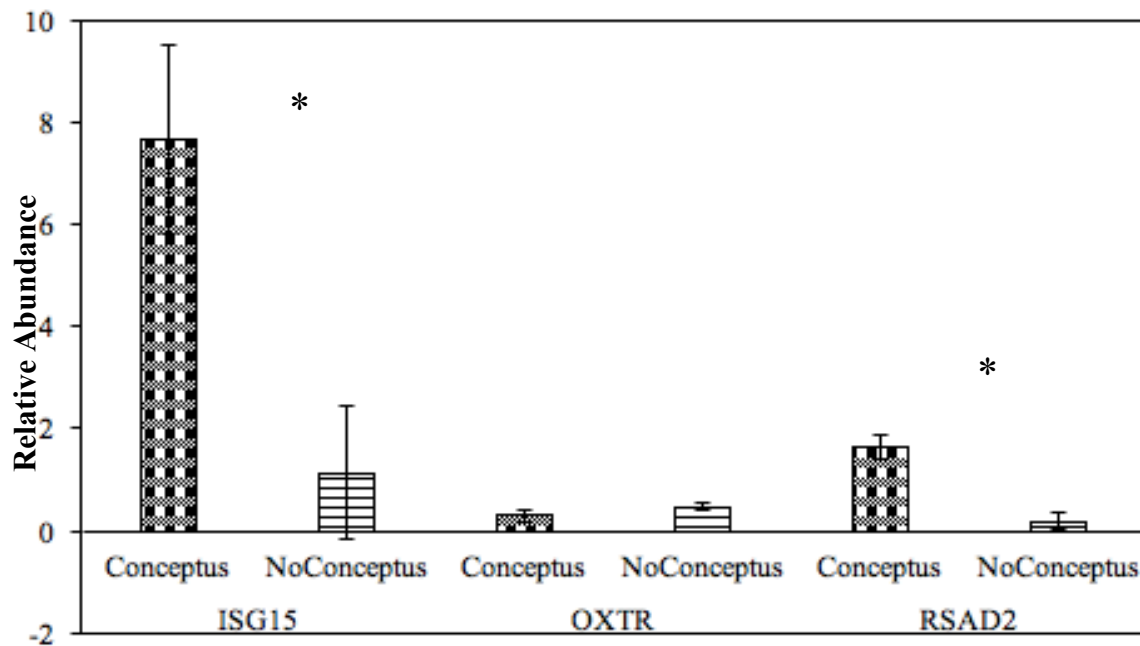


Figure 24. Endometrium mRNA abundances for ISG15, OXTR, and RSAD2 on day 16 among animals that did and did not have a conceptus recovered. Animals with a conceptus had increased ISG15 ( $P = 0.006$ ) and RSAD2 ( $P < 0.0001$ ) transcript abundance compared to animals without a conceptus. There was no difference in OXTR ( $P = 0.20$ ) transcript abundance in the endometrium on day 16. (\* $P < 0.05$ )

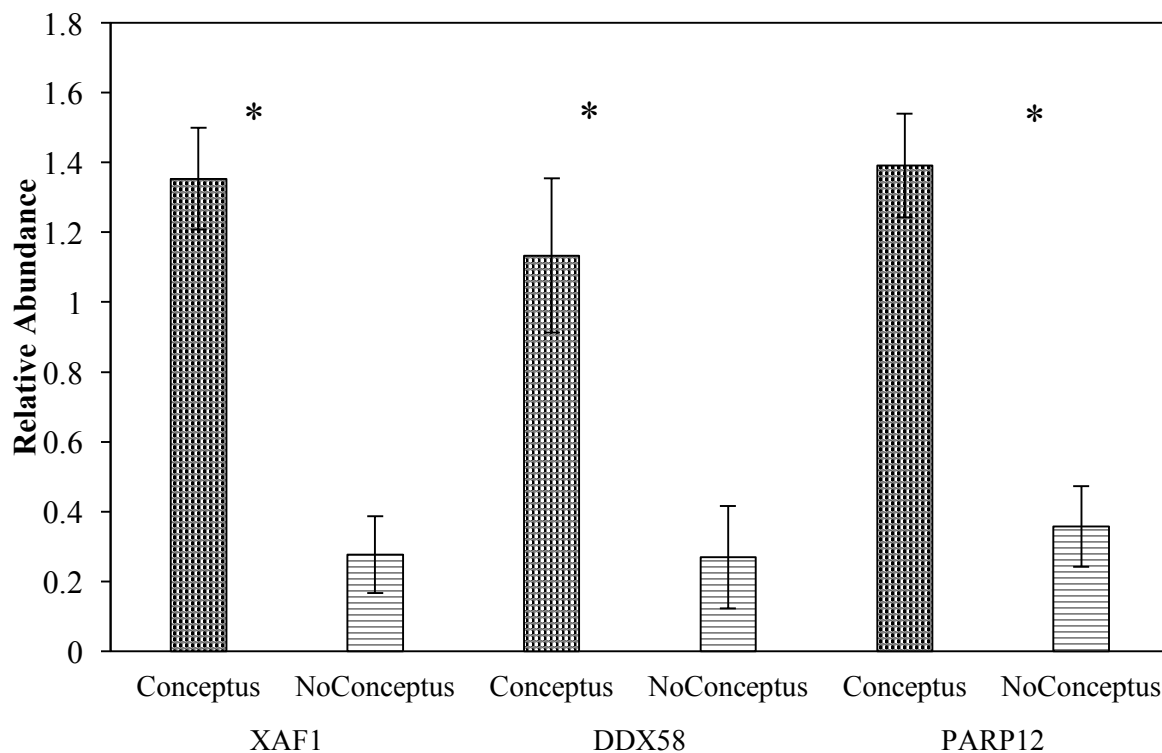


Figure 25. Endometrium mRNA abundances for XAF1, DDX58, and PARP12 on day 16 among animals that did and did not have a conceptus recovered. Animals with a conceptus had increased XAF1 ( $P < 0.0001$ ), DDX58 ( $P = 0.002$ ), and PARP12 ( $P < 0.0001$ ) transcript abundance compared to animals without a conceptus on day 16.

(\* $P < 0.05$ )

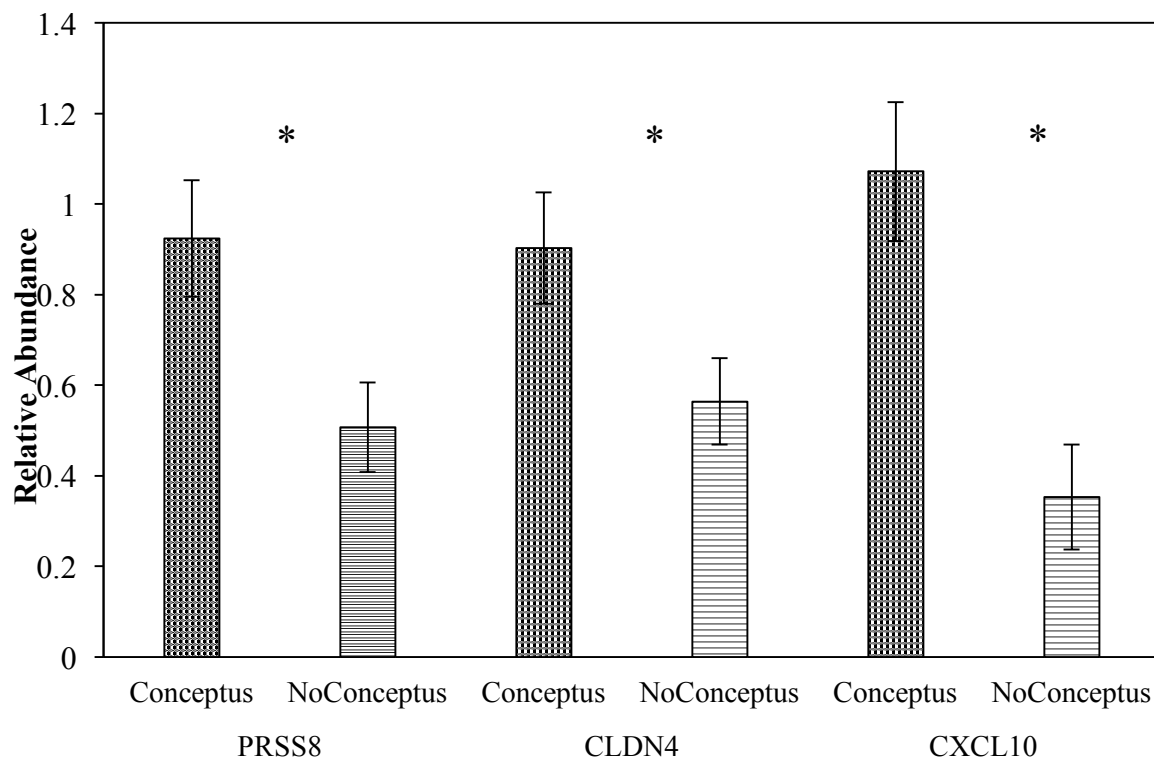


Figure 26. Endometrium mRNA abundances for PRSS8, CLDN4, and CXCL10 on day 16 among animals that did and did not have a conceptus recovered. Animals with a conceptus had increased PRSS8 ( $P = 0.01$ ), CLDN4 ( $P = 0.03$ ), and CXCL10 ( $P < 0.0005$ ) transcript abundance compared to animals without a conceptus on day 16.

(\* $P < 0.05$ )

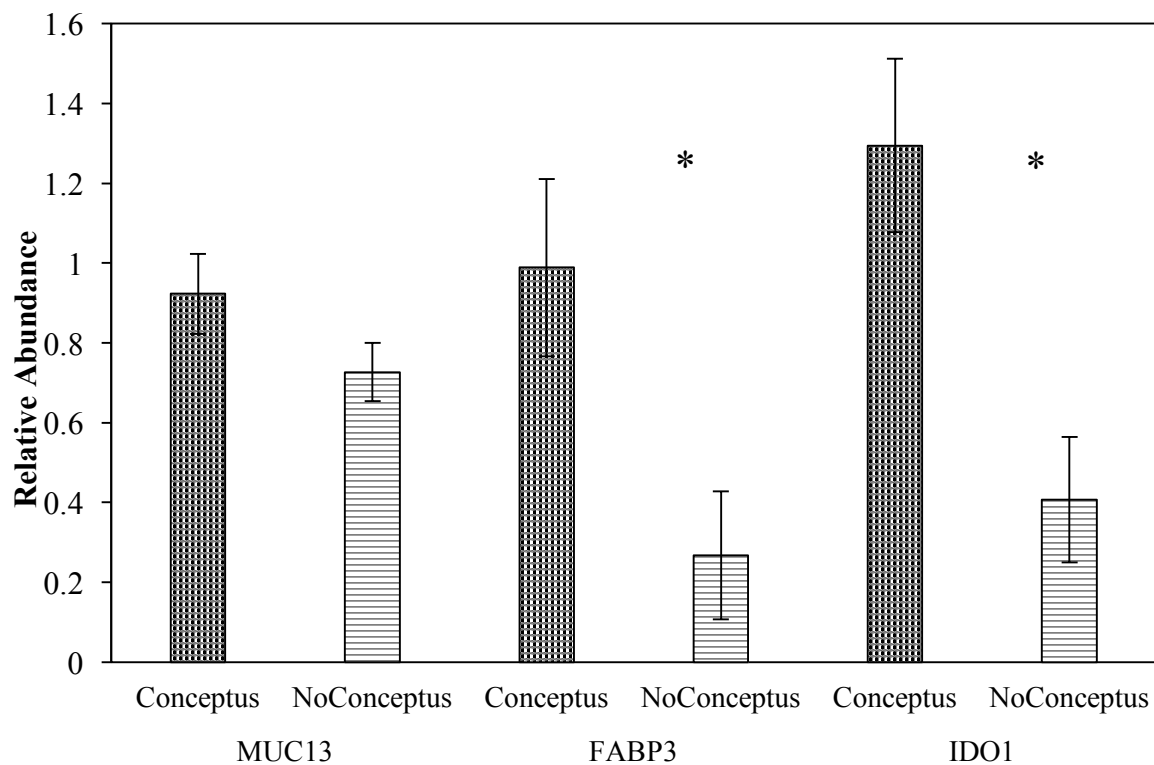


Figure 27. Endometrium mRNA abundances for MUC13, FABP3, and IDO1 on day 16 among animals that did and did not have a conceptus recovered. Animals with a conceptus had increased FABP3 ( $P = 0.01$ ) and IDO1 ( $P = 0.002$ ) transcript abundance compared to animals without a conceptus on day 16. There was no difference in MUC13 ( $P = 0.12$ ) transcript abundance. (\* $P < 0.05$ )

## DISCUSSION

### Study 1:

Gene expression in the endometrium is mainly regulated by the complex interactions of estradiol and progesterone. These hormones act as transcription factors by binding to nuclear receptors causing conformational changes, ultimately allowing the receptor to bind to chromatin and cause transcriptional changes within hours (Murdoch and Gorski, 1991; Stormshak and Bishop, 2008). Specifically, there has been extensive research regarding the impact of preovulatory estradiol on the uterine environment and embryo survival in cattle.

On day 6, heifers that exhibited estrus yielded embryos that were more advanced in stage and had improved quality when compared to heifers that did not exhibit estrus, however, recovery rates were not different (Larimore et al., 2015). Bridges et al. (2012) concluded that there was no difference in conceptus size and interferon tau concentrations on day 15.5 based on preovulatory estradiol exposure. On day 16, Northrop and others (2018) determined that there were no differences in conceptus survival based on apoptosis in the trophectoderm, IFNT, protein, and glucose concentrations in the ULF among highE2 and lowE2 animals. On day 19, Davoodi et al. (2016) reported that genes associated with maternal immune system, attachment between the endometrium and conceptus, and CL maintenance were favorably expressed in cows that exhibited estrus near the time of AI compared to cows that did not. Additionally, they reported that cows that exhibited estrus also yielded longer conceptuses. When ovariectomized beef cows were treated with estradiol (cypionate or benzoate) embryo survival was increased to day 29 of pregnancy compared to cows that had no exposure to

estradiol (Madsen et al., 2015). However, prior to the current study there was little known about the impact of preovulatory estradiol on uterine and trophectoderm transcriptomes around the critical period of maternal recognition of pregnancy. Gaining a better understanding of the differences in critical genes and enriched pathways in the uterus and trophectoderm among highE2 and lowE2 animals may help elucidate the underlying biological mechanisms that potentially lead to increased embryo survival and pregnancy rates among highE2 animals. The following discussion focuses on biological processes associated with the DEGs among highE2 and lowE2 animals on day 16 in cattle.

*Endometrial Remodeling:*

The remodeling of the endometrium and chorionic extracellular matrix is critical for successful implantation and placentation. Specifically, the remodeling of the ECM supports proliferation, differentiation, migration of binucleate cells, and attachment (Imakawa et al., 2017). Other studies in ruminants have reported genes involved in this process being expressed during gestation (Bauersachs et al., 2006; Ribeiro et al., 2016). In the current study, MYLK, ADAM12, TAGLN, ACTA2, MYL9, COL1A2, COL3A1, HSPE, and ADAMTS15 were downregulated, while PRSS8, LGMN, and GRN were upregulated when comparing the highE2/conceptus and lowE2/conceptus groups. Ribeiro and others (2016) also reported both an upregulation and downregulation of genes involved in ECM organization. This suggests that excessive endometrial remodeling may negatively impact conceptus attachment. Proteases play an important role in ECM degradation, and they can be divided into three categories: serine, cysteine,

and metalloproteinases (Barrett, 1994). Specifically, PRSS8 is a serine protease that is suggested to play a role in endometrial epithelial morphology establishment, tissue remodeling, and trophoblast invasion during early pregnancy in the rhesus monkey (Lin et al., 2006). Additionally, PRSS8 knockout mice exhibited lethality due to placental insufficiency (Hummler et al., 2013). Real time PCR results with additional animals added determined that there was an effect of preovulatory estradiol and conceptus presence on PRSS8 transcript abundance in the endometrium on day 16. Legumain (LGMN) is a cysteine protease that was also reported to be upregulated in the endometrium of day 18 pregnant heifers (Bauersachs et al., 2006). Ledgard and others (2009) reported LGMN being elevated in the gravid uterine horn compared to the nongravid horn on day 18 in cows. Both PRSS8 and LGMN had increased expression in the highE2/conceptus group suggesting that the uterus is undergoing changes in the organization of the ECM in preparation for pregnancy.

*Adhesion:*

Adhesion molecules play a critical role in the attachment process between the fetal chorionic binucleate cells and the luminal epithelium. In the current study, CLDN4, F5, MUC13, ITGA3, CLEC4F, ITGB5 were upregulated, while CDH4 and TROAP were downregulated when comparing the highE2/conceptus and lowE2/conceptus groups. Integrins are a large family of cell surface receptors that mediate cell-cell adhesion, cell migration, growth/differentiation, and attachment of cells to the ECM around the time of implantation (Hayes, 1992; Mansouri-Attia et al., 2009). Specifically, it has been established that ITGB5 and a series of other integrins

are expressed on the apical surface of the trophectoderm and uterine luminal epithelium in preparation for implantation in sheep (Johnson et al., 2000). Claudins are another class of cell adhesion molecules that function within tight junctions to seal off simple and stratified epithelia (Tsukita and Furuse, 2002). In humans, claudin 4 (CLDN4) had increased mRNA expression around implantation (Carson et al., 2002). Bauersachs and others (2006) reported that CLDN4 mRNA was upregulated in pregnant heifers on day 18 compared to cyclic heifers. They proposed that CLDN4 may act as a marker for endometrial receptivity. Mucins are a family of heavily glycosylated proteins, these proteins contain glycans that are recognized by the blastocyst (Alpin, 1997). Specifically, MUC13 mRNA was upregulated in pregnant carunucular tissue on day 20 of pregnancy in cattle (Mansouri-Attia et al., 2009). Additionally, MUC13 mRNA was increased in the endometrium of highly fertile and subfertile heifers compared to infertile heifers on day 17 of gestation (Moares et al., 2018). Forde and others (2013) also reported increased expression in the endometrium as pregnancy progressed (day 13 to day 19), however, expression was not significant based on pregnancy status. The increased expression of select cell adhesion molecules (ITGB5, CLDN4, MUC13) among pregnant animals implies that the endometrium is undergoing changes in order to prepare for attachment.

#### *Immune:*

The developing conceptus is made up of both maternal and paternal genes. Therefore, strict regulation of the maternal immune system is critical for the maintenance of pregnancy. The following genes associated with immune response



were increased when comparing the highE2/conceptus and lowE2/conceptus groups: IDO1, BPI, BOLA-NC1, C2, CFB, C1QL2, OAS2, S100A12, CD48, MIC1, CXCL10, and CXCL11. Indoleamine 2,3-dioxygenase (IDO1) is the enzyme that catalyzes the rate limiting step in tryptophan catabolism. Previous research determined that placental cells express IDO, which causes tryptophan depletion leading to the suppression of T cell proliferation at maternal-fetal interface (Munn et al., 1998). In pregnant mice, treatment with an IDO inhibitor resulted in the inhibition of tryptophan catabolism, which initiated maternal lymphocytes to facilitate fetal rejection (Munn et al., 1998). Real time PCR results with additional animals added further confirmed sequencing data, that there was an effect of preovulatory estradiol, conceptus presence, and an interaction on IDO1 transcript abundance in the endometrium on day 16. Chemokines are multifunctional molecules that recruit immune cells to inflammatory regions (Red-Horse et al., 2001). Specifically, there is abundant chemokine expression at the maternal-fetal interface. In humans, leukocytes are infiltrated to the implantation site, and they are responsible for maintaining an appropriate balance between embryo protection and accepting hemiallogenic tissues (Red-Horse et al., 2004). In mice and goats, chemokine receptors are present in the endometrium and trophoblast cells which implies that they may also play a role in adhesion between the maternal endometrium and conceptus (Dominguez et al., 2003; Nagaoka et al., 2003). Imakawa and others (2006) reported CXCL11 mRNA expression was highest in the endometrium on day 17 of pregnancy, and CXCL10 was highest on day 20 of pregnancy in sheep. In ruminants, CXCL10 appears to attract trophoblasts to the endometrium and promote adhesive activity (Imakawa et al., 2006). On day 17, there was more than an 11-fold

increase in CXCL10 expression in the uterus of pregnant cows (Cerri et al., 2012). CXCL10 mRNA expression was also downregulated in subfertile dairy cows compared to fertile cows on day 17 of pregnancy (Walker et al., 2012). Additionally, CXCL10 mRNA expression in the uterus was favorably expressed on day 19 of gestation among cows that exhibited estrus around the time of AI compared to animals that did not (Davoodi et al., 2016). In the current study, real time PCR results with additional animals added further determined that there was an effect of preovulatory estradiol, conceptus presence, and an interaction on CXCL10 transcript abundance in the endometrium on day 16.

*Metabolic:*

An increase in metabolites during pregnancy is necessary to ensure adequate nutrients for the growth, development, and survival of the conceptus. The following genes associated with metabolic function were upregulated: FABP3, SLC2A1, SLC5A5, SLC27A2, SLC27A5, AMPD3, FBP1, and ACO2, while LPL, SLC7A2, SLC38A4, and FAAH was downregulated when comparing the highE2/conceptus and lowE2/conceptus groups.

Lipids are essential for structural properties, providing energy for proliferating tissue, cell signaling, and generation of ATP. The endometrium is the main source of lipids, which are especially important during rapid conceptus elongation in ruminants. FABP3 is involved in the uptake, metabolism, and transport of long chain fatty acids (Forde et al., 2010). Long chain fatty acids are needed for cell growth and production of eicosanoids. Forde and others (2010) reported that FABP3 mRNA was upregulated

in the luminal epithelium between days 13 and 16 of pregnancy in cattle. On day 17, FABP3 mRNA in the endometrium was increased among high fertility heifers compared to subfertile heifers (Moares et al., 2018). FABP3 also had increased expression in the caruncular tissue of pregnant animals compared to cyclic animals on day 20 of gestation (Mansouri-Attia et al., 2009). Additionally, in the current study real time PCR results with additional animals added further confirmed that there was an effect of preovulatory estradiol and conceptus presence on FABP3 transcript abundance in the endometrium on day 16. SLC27A2 is a fatty acid transporter that is also involved in lipid metabolism. Previous research has reported that SLC27A2 had increased expression on day 17 of pregnancy in intercaruncular tissue of pregnant dairy heifers (Cerri et al., 2012).

Glucose is one of the main energy sources used by the conceptus for growth and development, it is known to regulate trophoblast proliferation and function (Wen et al., 2005). Transport of glucose into the uterus is mediated by facilitative and/or sodium dependent transporters. The SLC2A1 transporter has been localized mainly in the glandular and luminal epithelial cells (Franca et al., 2015). This glucose transporter appears to be regulated by both progesterone and IFNT in the glandular epithelium (Gao et al., 2009). Previously our laboratory reported that highE2 animals had increased SLC2A1 mRNA abundance in intercaruncular and caruncular tissue compared to lowE2 animals on day 16.

*Trophectoderm Gene Expression:*

The five trophectoderm genes were selected based on the findings from previous studies that determined their importance in conceptus growth, development, and survival. Interferon tau (IFNT) is secreted by the embryo and its production is consistent with size, therefore the ability of the embryo to signal its presence and prevent luteal regression is dependent on growth rate (Robinson et al., 2006). Prostaglandin-endoperoxide synthase 2 (PTGS2) is the rate limiting enzyme involved in prostaglandin synthesis. It has been reported that PTGS2 expression increased in bovine conceptuses on day 16 and 19 of gestation (Mamo et al., 2011). When a PTGS2 inhibitor was infused into the uterus, conceptus elongation was inhibited in sheep (Dorniak et al., 2011). Additionally, when meloxicam was given to heifers on day 15 after insemination, pregnancy rates were reduced (Erdem and Guzeloglu, 2010). Barnwell and others (2016) also reported that PTGS2 was upregulated in long versus short conceptuses on day 15 of gestation. Fibroblast growth factor receptor 2 (FGFR2) is activated in the elongating conceptus through FGF2 in the endometrium. It is responsible for increasing trophectoderm proliferation and IFNT production (Michael et al., 2006). On day 17, conceptuses recovered from subfertile beef cattle had decreased FGFR2 expression compared to highly fertile animals (Moraes et al., 2018). Transmembrane 4 superfamily member 1 (TM4SF1) encodes for a protein that is involved in cell proliferation, adhesion, motility, migration, and invasiveness (Wright and Rudy, 2000; Shih et al., 2009; Zukauskas et al., 2011). The gene, TM4SF1 has previously been reported to increase in expression as a bovine conceptus elongates from ovid to tubular to filamentous (Ribeiro et al., 2016). The complement system is involved in embryo and maternal protection (Girardi et al., 2006).

Specifically, C3 has been reported to have decreased expression as the conceptus elongates (Ribeiro et al., 2016), and has increased abundance among short vs long conceptuses (Barnwell et al., 2016). Real time PCR revealed there were no differences in trophoctoderm transcript abundance for all five of these genes among highE2 and lowE2 animals. This further supports our previous findings that conceptus survival to day 16 of pregnancy is not different based on preovulatory estradiol exposure.

#### Study 2:

Interferon tau acts in a paracrine manner on the endometrium to suppress the transcription of ESR1 and OXTR genes, thus preventing the pulsatile release of PGF2 $\alpha$  (Spencer and Bazer, 1996). In addition to its antiluteolytic effects, IFNT also induces the transcription of several genes associated with conceptus elongation, uterine receptivity, and implantation (Shorten et al., 2018; Spencer and Hansen, 2015). These genes are classified as either: classical or nonclassical interferon stimulated genes (ISGs). Classical ISGs are induced by other type I interferons, while nonclassical ISGs are typically induced by progesterone before IFNT can stimulate their expression. Nonclassical ISGs are reported to be important in nutrition, proliferation, migration, and conceptus attachment (Spencer et al., 2007; 2008). Previous research has examined specific ISGs in peripheral blood leukocytes as being a potential early pregnancy detection biomarker. However, the expression of these genes is influenced by parity (Green et al., 2010).

In the current study, regardless of estradiol exposure there were 63 differentially expressed genes in common among the highE2/conceptus vs highE2/noconceptus and

the lowE2/conceptus vs lowE2/noconceptus groups. Among these DEGs were several classical ISGs (ISG15, OAS, STAT1, IRF9, RSAD2, MX1, IFIT3, IFIT5, DDX58) and nonclassical ISGs (PARP12, HERC6, ZNFX1; Sandra et al., 2015). Additionally, as expected, OXTR was downregulated in the endometrium of pregnant animals on day 16 after artificial insemination. RNA sequencing determined that lowE2 animals with a conceptus had decreased (44.6 fold) OXTR expression compared to lowE2 animals without a conceptus. While highE2 animals with a conceptus had decreased (6.6 fold) OXTR expression compared to highE2 animals without a conceptus on day 16. Contrary to sequencing results, real time PCR with additional animals added determined that there was no difference in OXTR transcript abundance among animals that did and did not have a conceptus recovered. A possible explanation may be that some animals potentially experienced embryonic loss prior to day 16, and the IFNT that was produced by that conceptus has impacts on gene expression in the uterus even after loss.

ISG15 is a ubiquitin like protein that is hypothesized to be critical for establishing the microenvironment at the uterine-placental interface during early pregnancy in ruminants (Joyce et al., 2005). Deletion of ISG15 in mice resulted in 50% pregnancy loss around the time of early placentation (Ashley et al., 2010). Madsen and others (2015) reported the expression of ISG15 was increased in blood leukocytes of pregnant animals as early as day 17 compared to non-pregnant animals. In the current study, RNA sequencing determined that lowE2 animals with a conceptus had increased (35.5 fold) ISG15 expression compared to lowE2 animals without a conceptus. While highE2 animals with a conceptus had increased (5.8 fold) ISG15 expression compared to highE2 animals without a conceptus on day 16. Real time PCR with additional animals

added further validated these findings that animals with a conceptus had increased ISG15 transcript abundance in the endometrium on day 16.

Radical S-adenosyl methionine domain containing 2 (RSAD2) is an antiviral gene that is induced by type 1 (Mansouri et al., 2009), 2 (Seo et al., 2011) and type 3 (Fitzgerald, 2011) interferons. It is hypothesized to play an important role in preventing viral infection by modulating innate immune responses in the uterus around implantation (Song et al., 2007). Walker and others (2010) reported RSAD2 was the most differentially expressed gene among pregnant and cyclic animals on day 17. The same study determined that RSAD2 expression was 3-fold greater in caruncular tissue compared to intercaruncular tissue. This may serve as a mechanism to prevent immunological attack of the foreign conceptus, because caruncles are the areas where attachment occurs. In the current study, RNA sequencing determined that lowE2 animals with a conceptus had increased (22.3 fold) RSAD2 expression compared to lowE2 animals without a conceptus. While highE2 animals with a conceptus had increased (5.5 fold) RSAD2 expression compared to highE2 animals without a conceptus on day 16. Real time PCR with additional animals added further validated these findings that animals with a conceptus had increased RSAD2 transcript abundance in the endometrium on day 16.

The poly (ADP-ribose) polymerase superfamily is not only involved in the protein modification process, ADP ribosylation, they also appear to play a role in the regulation of the cytoskeleton and membrane trafficking. The functions associated with the four members that were identified in the current study (PARP9, PARP10, PARP12, PARP14) is not well understood, but they have been mentioned previously in other

studies that examined uterine and conceptus gene expression during early pregnancy in ruminants (Klein et al., 2006; Bauersachs et al., 2008; 2009; Forde et al., 2012; Sandra et al., 2015). Specifically, PARP12 was previously reported to have increased mRNA expression in the endometrium of pregnant cattle on day 16 compared to cyclic animals, and was directly upregulated by in vivo IFNT infusion for 2 hours (Forde et al., 2012). Additionally, PARP12 had increased mRNA expression on day 18 of pregnancy when compared to cyclic cattle (Bauersachs et al., 2006), and it was also increased in the blood on day 18 in pregnant dairy cows (Forde et al., 2012). In the current study, RNA sequencing determined that lowE2 animals with a conceptus had increased (3.7- 5.4-fold) PARP expression compared to lowE2 animals without a conceptus. While highE2 animals with a conceptus had increased (2.1-2.7 fold) PARP expression compared to highE2 animals without a conceptus on day 16. Real time PCR with additional animals added further validated these findings that animals with a conceptus had increased PARP12 transcript abundance in the endometrium on day 16.

XIAP Associated Factor 1 (XAF1), along with other proapoptotic genes, help to mediate TNF-alpha induced apoptosis in preparation for implantation (Groebner et al., 2011). It has been reported that XAF1 mRNA increased 2.9-fold on day 15, and 15.1-fold on day 18 among pregnant heifers. Additionally, XAF1 mRNA expression increased in glandular epithelial and stromal cells when cultured with IFNT in vitro (Groebner et al., 2011). It is proposed that XAF1 may act on endometrial cells to control lymphocyte cell death to help protect the semi-allogenic conceptus. In the current study, RNA sequencing determined that lowE2 animals with a conceptus had increased (5.4-fold) XAF1 expression compared to lowE2 animals without a conceptus. While highE2



animals with a conceptus had increased (3-fold) XAF1 expression compared to highE2 animals without a conceptus on day 16. Real time PCR with additional animals added further validated these findings that animals with a conceptus had increased XAF1 transcript abundance in the endometrium on day 16.

It has been established that DEAD box polypeptide 58 (DDX58) is induced in endometrial cells by interferon tau via classical STAT1 mediated cell signaling independent of progesterone (Song et al., 2011). It has been hypothesized that DDX58 may increase antiviral response within the pregnant uterus in order to protect against viral infection (Song et al., 2011). Additionally, Forde and others (2012) reported increased DDX58 mRNA expression in the endometrium of pregnant animals on day 16 compared to cyclic animals. RNA sequencing determined that lowE2 animals with a conceptus had increased (10-fold) DDX58 expression compared to lowE2 animals without a conceptus. While highE2 animals with a conceptus had increased (3-fold) DDX58 expression compared to highE2 animals without a conceptus on day 16. Real time PCR with additional animals added further validated these findings that animals with a conceptus had increased DDX58 transcript abundance in the endometrium on day 16.

In the current study, there were also DEGs that function in other biological processes in addition to just maintaining appropriate antiviral responses. The lysosomal histidine and peptide transporter, SLC15A3, increases in the endometrium during pregnancy (day 12-day 18), and was also stimulated by IFNT in vitro in the glandular epithelium and stroma cells (Groebner et al., 2011). It is hypothesized that it plays a role in protein synthesis during conceptus elongation (Groebner et al., 2011). In the current study, this transporter had increased mRNA abundance in the endometrium of pregnant

animals. The disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) family is known to cleave a wide range of substrates in the ECM. Specifically, ADAMTS18 increased in the endometrium during pregnancy in pigs, and is reported to be involved in conceptus adhesion to the uterine surface (Meyer et al., 2019). This study determined that ADAMTS18 had decreased mRNA abundance in the uterus of pregnant animals on day 16. The orphan nuclear receptor, NR4A1, is reported to play a role in metabolism, homeostasis, and inflammation (Pearen and Muscat, 2010; Zhao and Bruenne, 2010). The progesterone receptor induces NR4A1 transcription, which then regulates several junctional proteins that impact vascular permeability during implantation (Goddard et al., 2014). Additionally, NR4A1 mRNA was upregulated at the implantation site compared to interimplantation tissue in the uterus of pregnant mice, it was proposed that it may be involved in apoptosis (Ma et al., 2006). This study concluded NR4A1 had increased mRNA expression in the endometrium of pregnant animals.

In conclusion, this study identified critical genes and pathways in the uterus that are impacted by preovulatory estradiol and interferon tau around maternal recognition of pregnancy in cattle. The lack of differences in trophoctoderm gene expression further supports our previous findings that conceptus survival to day 16 of pregnancy is not affected by preovulatory estradiol exposure. Overall, these results demonstrate greater differences in uterine function than in conceptus developmental competence on day 16. Therefore, the differences in pregnancy success among highE2 and lowE2 animals most likely occurs after maternal recognition of pregnancy and before day 29 in cattle.

## CHAPTER III

INFLUENCE OF PREOVULATORY ESTRADIOL ON UTERINE LUMINAL FLUID  
PROTEOMICS AROUND MATERNAL RECOGNITION OF PREGNANCY IN BEEF  
CATTLE

## ABSTRACT

Proteins within the uterine luminal fluid (ULF) are involved in elongation, recognition of pregnancy, implantation, and placentation. Previous research has established that elevated preovulatory estradiol increased embryo survival and pregnancy rates. However, on d16 of pregnancy no differences in conceptus recovery rates or interferon tau concentrations were detected between animals with elevated or low concentrations of estradiol pre-breeding. The present study evaluated the effects of preovulatory estradiol and conceptus presence on the d16 ULF proteome. Beef cows/heifers (n=28) were synchronized, artificially inseminated (d 0), and grouped into high (highE2) and low (lowE2) preovulatory estradiol. On d16, animals were slaughtered and uteri were flushed. Two independent ULF pools were created for each of the following groups: highE2/noconceptus, highE2/conceptus, lowE2/noconceptus, and lowE2/conceptus. Pools were analyzed using a 2D LC-MS/MS based 8plex iTRAQ quantitative method. Scaffold Q+ was used to quantitate peptide and protein identifications, and FDR was adjusted using the Benjamini-Hochberg procedure. Ingenuity Pathway Analysis was used to analyze upregulated and downregulated proteins. There were 48 differentially expressed proteins (DEPs) between highE2/conceptus and lowE2/conceptus groups, 19 of which were upregulated (IDH2, ACTN4, GOT1, ACAA1, GPLD1) and 29 were downregulated (UTMP, C3, ANXA8,

ANXA1, FGG) in the highE2/conceptus group. The main functions associated with these DEPs were carbohydrate and nucleic acid metabolism, small molecule biochemistry, cellular development, and cellular function and maintenance. These data provide insight into differences in specific protein abundances in the ULF that may contribute to improved conceptus survival among highE2 animals.

## INTRODUCTION

The importance of preovulatory estradiol in the establishment and maintenance of pregnancy has been well established. Cows in standing estrus prior to fixed-time AI have been reported to have greater pregnancy success than nonestrus animals (Perry et al., 2005). Specifically, when ovariectomized beef cows were treated with estradiol (cypionate or benzoate) embryo survival was increased to day 29 of pregnancy compared to cows that had no exposure to estradiol (Madsen et al., 2015). Furthermore, cows that received exogenous estradiol only lost 35% of their existing pregnancies, while control animals lost 75% of their existing pregnancies (Madsen et al., 2005). Despite there being extensive research regarding the importance of preovulatory estradiol during early pregnancy, little is known about its impact on uterine protein abundances.

An adequate uterine environment is necessary for maternal and conceptus communication. The uterine environment must provide sufficient nutrients and endocrine conditions for the establishment and maintenance of pregnancy. In cattle, the elongating conceptus is free-floating in the uterus until the time of attachment (day 20). During this time of elongation, the conceptus is relying on the maternal environment and the secretions from the uterus for growth, development, and survival. These secretions from the uterine epithelium is termed the uterine histotroph. It is composed of a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, and glucose (Gao et al., 2009). These nutrients stimulate the nutrient sensing signaling pathway to increase the translation of messenger RNA, which is critical for conceptus development (Martin and Sutherland, 2001). Cell signaling through this pathway also stimulates cell migration, invasion, and cell growth and proliferation (Liu et

al., 2008). Proteins in particular are important for the elongation of the trophoblast, recognition of pregnancy, implantation, and placentation. Estradiol has been reported to induce the expression of uterine proteins (Bartol et al., 1981).

When Gray and others (2001) placed uterine gland knockout sheep (UGKO) with fertile rams, no pregnancies were identified on day 25 after insemination. Additionally, blastocyst growth into an elongated bovine conceptus has not been able to be duplicated in vitro (Betteridge and Flechon, 1988). These studies demonstrate that endometrial glands and their secretions are necessary for pregnancy establishment and conceptus development.

In cattle, maternal recognition of pregnancy occurs around day 16 after estrus (Bazer et al., 1997). This physiological process is defined by the requirement for the conceptus to produce and secrete a signal that acts on the uterus and ovary to ensure the maintenance of a functional corpus luteum so progesterone production and pregnancy can be maintained (Bazer, 2013). This signal in cattle is interferon tau (IFNT), and the ability of the developing embryo to secrete sufficient quantities of IFNT depends on its quality and stage of development (size; Ealy and Yang, 2013). Previously our laboratory determined that there were no differences in conceptus survival to day 16 of pregnancy between highE2 and lowE2 animals based on conceptus recovery rates, apoptosis in the trophectoderm, and IFNT concentrations. However, glucose and protein concentration in the ULF was influenced by preovulatory estradiol concentrations and conceptus presence (Northrop et al., 2018). Therefore, the objectives of the current study were to: 1) examine the differences in ULF proteomics on day 16 of pregnancy based on preovulatory estradiol exposure, and 2) examine the effects of conceptus presence (interferon tau) on

day 16 of pregnancy/estrous cycle. Specifically, we hypothesized that: 1) beef cows and heifers with elevated preovulatory estradiol (highE2) concentrations prior to fixed-time AI would have a more suitable ULF protein profile for conceptus development and survival, and 2) the secretion of IFNT by the conceptus will impact the abundance of several proteins needed for uterine receptivity and pregnancy maintenance.

## MATERIALS AND METHODS

### *Animals:*

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

### *Treatments:*

Reproductive cycles of Angus crossed beef cows/heifers (n=39) at the South Dakota State University Beef Breeding Unit were synchronized with a CO-Synch protocol [GnRH administered (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on d -9, followed by PGF2 alpha (PG; 25 mg as 5 mL of Lutalyse i.m.; Pfizer Animal Health, Madison, NJ) on day -2, and on d 0, cows were administered GnRH (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) and artificially inseminated]. Estrus was monitored visually from d 0 through d 3 with the aid of EstroTect patches (Western Point, Inc., Apple Valley, MN). Animals were grouped into either highE2 (n=12) or lowE2 (n=16) based on preovulatory estradiol concentrations and expression of estrus (d -2 to d 0). The threshold estradiol concentration that distinguished the two groups was 4.9 pg/mL. Previous research used a similar cutoff when evaluating changes in ovarian function associated with concentrations of estradiol before a GnRH-induced ovulation in beef cows (Larimore et al., 2016).



*Transrectal Ultrasonography:*

Follicular dynamics were assessed by transrectal ultrasonography using an Aloka 500V ultrasound with a 7.5MHz linear probe (Aloka, Wallingford, CT) on d -9, d 0, and d 3 to characterize follicular development and ovulation. All follicles on each ovary > 8 mm in diameter were recorded, and only animals that ovulated following the GnRH injection at fixed-time AI were utilized in the study (n=28). Ovulation was defined as the disappearance of a previously recorded large follicle, and confirmed by changes in circulating concentrations of progesterone.

*Blood Sampling and Radioimmunoassay:*

Blood samples were collected by venipuncture of the jugular vein into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA) on d -2, d -1, d 0, then every other day through d 15. Blood was centrifuged at 1,200 x g for 30 minutes at 4°C, and plasma was collected and stored at -20°C. Radioimmunoassays (RIA) were performed on plasma samples to determine circulating concentrations of progesterone and estradiol (Engel et al., 2008; Perry and Perry, 2008). For estradiol, the intra-assay CV was 4.76%, respectively, and assay sensitivity was 0.5 pg/mL. For progesterone, intra- and interassay CVs were 7.5% and 13.2%, respectively, and assay sensitivity was 0.4 ng/mL.

*Uterine Luminal Fluid Collection and Conceptus Recovery:*

Reproductive tracts were collected from the abattoir immediately following slaughter on d 16, and kept on ice. An incision was made at the anterior end of the

uterine horn contralateral to the corpus luteum, a plastic tube was placed in the uterine tip and sutured to prevent any fluid loss while the other horn was clamped off. The uterine horns were flushed with 30mL of flush media, and then massaged for equal fluid distribution in the uterus. The uterine flush was then collected in a 50mL conical tube, and examined under a microscope at 10x to determine if a conceptus was present.

*Mass Spectrometry:*

There were 28 samples of uterine luminal fluid that were used to make a total of eight pools that were sent to the Mass Spectrometry facility at the University of Minnesota. There were two independent pools made for each of the following groups: lowE2/noconceptus, lowE2/conceptus, highE2/noconceptus, and highE2/conceptus. Each animal was represented equally within the appropriate pool. Protein quantification was conducted using a 2D LC-MS/MS based 8plex iTRAQ quantitative method. The samples were pooled, reduced, alkylated by MMTS, digested with Trypsin, and labeled with iTRAQ reagents. Scaffold Q+ (version Scaffold\_4.8.4, Proteome Software Inc., Portland, OR) was used to quantitate Label Based Quantitation (iTRAQ) peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Normalization was performed iteratively (across samples and

spectra) on intensities, as described in Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples Using ANOVA (Oberg et al., 2008). Medians were used for averaging. Spectra were log-transformed, pruned of those matched to multiple proteins, and weighted by adaptive intensity weighting logarithm. The FDR was adjusted using Benjamini-Hochberg procedure ( $P < 0.05$ ) to identify significance based on permutation tests.

*Pathway Analysis:*

Identification of enriched pathways associated with differentially expressed proteins (DEPs) in the ULF on day 16 of pregnancy was conducted using Ingenuity Pathway Analysis (IPA; Qiagen) software. For each group comparison, a list of DEPs was uploaded to the IPA tool. Ingenuity Pathway Analysis functional analysis tools identified the biological functions and/or pathways that were most significant to the data set ( $P < 0.05$ ) according to righted tailed Fisher's Exact Test. Ingenuity Pathway Analysis currently supports only Human, Mouse, and Rat species with full content, so the bovine species is supported at the ortholog level, therefore some significant proteins may not be included in the analysis.

## RESULTS

Proteins in the uterine luminal fluid (ULF) were identified and quantified using mass spectrometry. This approach detected a total of 6,989 peptides and 1,269 proteins in the ULF from the 28 animals. The results and discussion focus on differentially expressed proteins (DEPs) among the following groupings: highE2/conceptus vs lowE2/conceptus, highE2/conceptus vs highE2/noconceptus, and lowE2/conceptus vs lowE2/noconceptus. The number of significant DEPs within each of these comparisons is depicted in figure 28. Emphasis was given to the top canonical pathways and molecular and cellular functions.

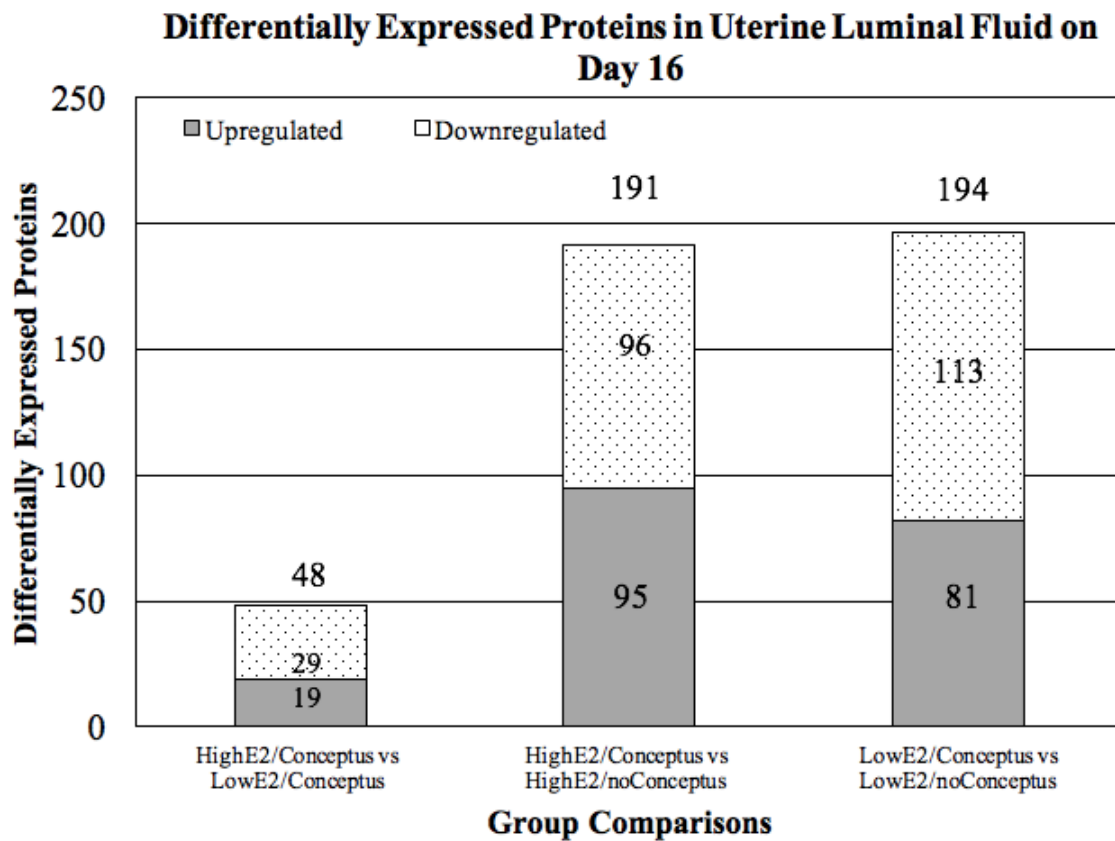


Figure 28. Upregulated and downregulated differentially expressed proteins (DEPs) in uterine luminal fluid (ULF) on day 16 in beef cattle.

Study 1- Effect of preovulatory estradiol exposure on uterine luminal fluid protein composition:

*HighE2/conceptus versus lowE2/conceptus:*

There were 48 DEPs between the highE2/conceptus group and the lowE2/conceptus group. Specifically, there were 29 proteins that were downregulated (UTMP, ORM1, ANXA8, ANXA1, and FGG) in the highE2/conceptus group (table 8). There were 19 proteins that were upregulated (GOT1, GSTM4, TST, ACAA1, and GPLD1) in the highE2/conceptus group compared to the lowE2/conceptus group (table 8). The top canonical pathways associated with these DEPs included: acute phase response signaling ( $3.17E-05$ ), aryl hydrocarbon receptor signaling ( $5.06E-04$ ), coagulation system ( $6.39E-04$ ), and PPAR signaling ( $9.87E-04$ ; figure 29). The main molecular and cellular functions associated with the DEPs were carbohydrate metabolism (4 molecules;  $3.99E-02$  -  $1.10E-06$ ), nucleic acid metabolism (8 molecules;  $4.60E-02$  -  $3.29E-06$ ), small molecule biochemistry (14 molecules;  $4.60E-02$  -  $3.29E-06$ ), cellular development ( $4.01E-02$  -  $9.92E-05$ ), and cellular function and maintenance (12 molecules;  $4.91E-02$  -  $9.92E-05$ ). Protein network interactions for the DEPs for this comparison are depicted in figure 30.

Table 8. The 48 characterized DEPs in ULF between the highE2/conceptus and lowE2/conceptus animals.

Abbreviation	Protein Name	Log2Fold Change	FDR
UTMP	Uterine milk protein	-2.02	0.00027
ORM1	Alpha-1-acid glycoprotein	-1.31	< 0.0001
ANXA8	Annexin A8	-1.23	< 0.0001
ANXA1	Annexin A1	-0.89	< 0.0001
FGG	Fibrinogen gamma-B chain	-0.88	0.00031
FGB	Fibrinogen beta chain	-0.86	< 0.0001
APLP2	Amyloid beta precursor like protein 2	-0.73	0.001
SLC25A5	ADP/ATP translocase 2	-0.71	< 0.0001
PHB2	Prohibitin	-0.7	< 0.0001
COX4I1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	-0.68	0.001
PHB	Prohibitin-2	-0.67	< 0.0001
ANXA2	Annexin A2	-0.65	< 0.0001
FGA	Fibrinogen alpha chain	-0.65	< 0.0001
HIST1H2AC	Histone H2A	-0.64	0.001
ANXA4	Annexin A4	-0.61	0.00039
ANXA3	Annexin A3	-0.59	0.00016
RPN1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	-0.59	0.00051
HIST1H4	Histone H4	-0.55	0.001
IMMT	MICOS complex subunit MIC60	-0.54	0.00015
ANXA11	Isoform 2 of Annexin A11	-0.53	0.001
H2AFY	Core histone macro-H2A	-0.53	< 0.0001
C3	Complement C3	-0.49	< 0.0001
RAP1B	Ras-related protein Rap-1b	-0.47	0.001
APOA1	Apolipoprotein A-I	-0.46	0.001
HIST1H1E	Histone cluster 1 H1 family member e	-0.42	0.0001
EZR	Ezrin	-0.42	< 0.0001
A2M	Alpha-2-macroglobulin	-0.36	0.001
ANPEP	Aminopeptidase N	-0.27	< 0.0001
PLEC	Plectin	0.2	< 0.0001
ATP5B	ATP synthase subunit beta,	0.25	0.001

mitochondrial			
HSPD1	60 kDa heat shock protein, mitochondrial	0.26	0.00068
IDH2	Isocitrate dehydrogenase [NADP], mitochondrial	0.26	0.001
CNDP2	Cytosolic non-specific dipeptidase	0.29	0.00039
ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	0.3	0.001
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	0.32	0.001
ACTN4	Alpha-actinin-4	0.32	< 0.0001
IDH1	Isocitrate dehydrogenase [NADP]	0.33	0.00061
PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	0.37	0.00042
SPTBN1	Spectrin beta chain	0.38	< 0.0001
GPD1L	Glycerol-3-phosphate dehydrogenase [NAD(+)]	0.39	0.001
ALDH2	Aldehyde dehydrogenase, mitochondrial	0.4	0.00049
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4	0.4	< 0.0001
GPLD1	Phosphatidylinositol-glycan- specific phospholipase D	0.56	< 0.0001
ACAA1	Acetyl-CoA acyltransferase 1	0.58	0.00067
TST	Thiosulfate sulfurtransferase	0.58	< 0.0001
GSTM4	Glutathione S-transferase Mu 1	0.66	0.001
GOT1	Aspartate aminotransferase, cytoplasmic	1.02	< 0.0001



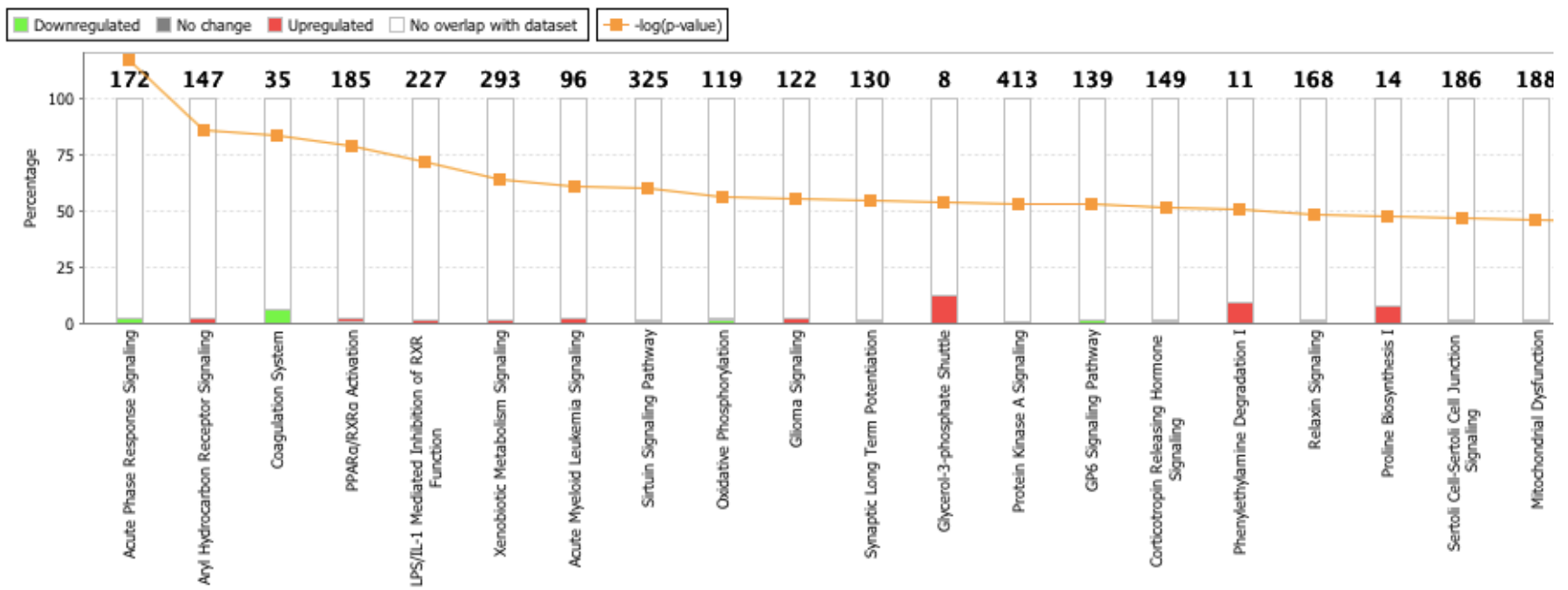


Figure 29. Top 20 canonical pathways associated with the DEPs among the highE2/conceptus vs lowE2/conceptus groups.

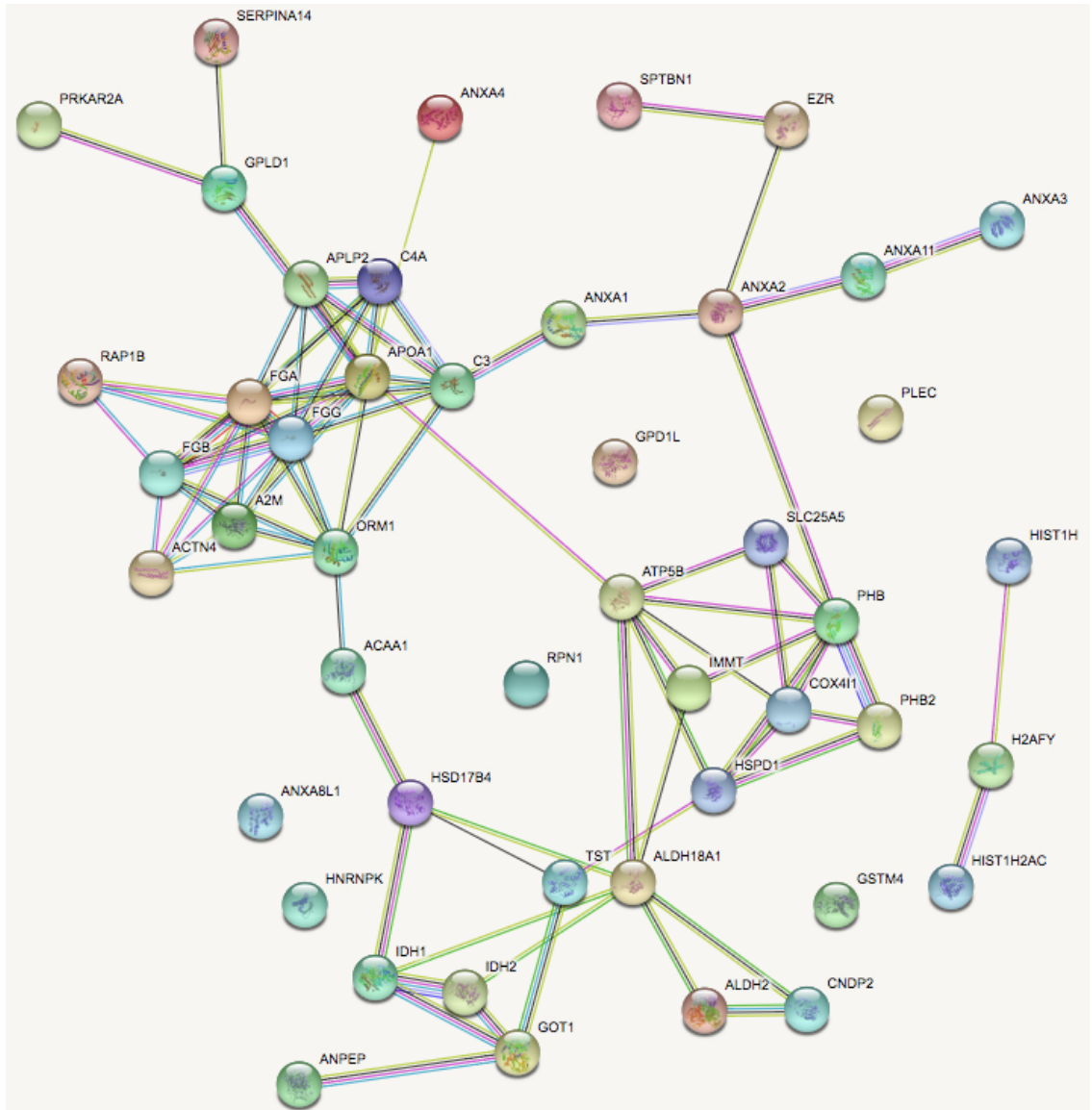


Figure 30. Protein network interactions for the DEPs among the highE2/conceptus and lowE2/conceptus groups. (STRING)

Study 2- Effect of conceptus presence (interferon tau) on uterine luminal fluid protein composition:

*HighE2/conceptus versus highE2/noconceptus:*

There were 191 DEPs between the highE2/conceptus and highE2/noconceptus groups. Specifically, there were 96 proteins that were upregulated (GOT1, ACAA1, HS, TST, CKMT1) in the highE2/conceptus group compared to the highE2/noconceptus group (table 9). There were 95 proteins that were downregulated (OVGP1, IGFBP1, LTF, GRP, SMPDL3A) in the highE2/conceptus group (table 9). The top canonical pathways associated with these DEPs included: epithelial adherens junction signaling ( $7.92E-08$ ), TCA cycle ( $2.50E-06$ ), tight junction signaling ( $2.67E-05$ ), and the protein ubiquitination pathway ( $8.13E-05$ ; figure 31). The main molecular and cellular functions associated with the DEPs were: cell death and survival (69 molecules;  $3.09E-03 - 2.29E-15$ ), cellular assembly and organization (47 molecules;  $3.04E-03 - 2.31E-09$ ), protein synthesis (37 molecules;  $3.09E-3 - 3.14E-09$ ), cell to cell signaling and interaction (36 molecules;  $3.04E-03 - 1.06E-08$ ), and cellular movement (44 molecules;  $2.96E-03 - 6.74E-07$ ). Protein network interactions for the DEPs for this comparison are depicted in figure 32.

Table 9. The top 25 downregulated/upregulated characterized DEPs in ULF among the highE2/conceptus and highE2/noconceptus animals.

Abbreviation	Protein Name	Log2Fold Change	FDR
OVGP1	Oviduct-specific glycoprotein (Fragment)	-1.8	0.007
IGFBP1	Insulin-like growth factor-binding protein 1	-1.31	0.001
LTF	Lactotransferrin	-1.18	< 0.0001
GRP	Gastrin-releasing peptide	-1.05	0.001
SMPDL3A	Acid sphingomyelinase-like phosphodiesterase 3a	-1.05	0.006
ANXA8	Annexin A8	-0.98	< 0.0001
SERPINA3-1	Serpin A3-1	-0.92	0.001
CTSF	Cathepsin F	-0.91	< 0.0001
TIMP2	Metalloproteinase inhibitor 2	-0.9	0.003
NUCB1	Nucleobindin-1	-0.88	0.004
GAS6	Growth arrest specific 6	-0.86	0.0001
UTMP	Uterine milk protein	-0.86	0.00017
ALPL	Alkaline phosphatase, tissue-nonspecific isozyme	-0.85	< 0.0001
MAN2A2	Alpha-mannosidase	-0.84	< 0.0001
CTSA	Lysosomal protective protein	-0.83	< 0.0001
APLP2	Amyloid beta precursor like protein 2	-0.81	0.001
STC1	Stanniocalcin-1	-0.81	0.004
CHGA	Chromogranin-A	-0.81	< 0.0001
DAG1	Dystroglycan	-0.81	< 0.0001
MFGE8	Lactadherin	-0.8	< 0.0001
GPC1	Glypican-1	-0.79	0.00063
VDAC1	Voltage-dependent anion-selective channel protein 1	-0.79	0.002
PSAP	Isoform 2 of Prosaposin	-0.77	0.003
AGT	Angiotensinogen	-0.75	< 0.0001
FAM234A	Protein FAM234A	-0.75	< 0.0001
RPS28	40S ribosomal protein S28	0.49	0.003
IVD	Isovaleryl-CoA dehydrogenase, mitochondrial	0.49	0.004
CTNNB1	Catenin beta-1	0.49	0.007
ATP5A1	ATP synthase subunit alpha, mitochondrial	0.49	< 0.0001

SPTBN1	Spectrin beta chain	0.49	< 0.0001
GOT2	Aspartate aminotransferase, mitochondrial	0.5	0.00022
CCAR2	Cell cycle and apoptosis regulator 2	0.5	0.0038
ATP5B	ATP synthase subunit beta, mitochondrial	0.5	< 0.0001
ACO2	Aconitate hydratase, mitochondrial	0.5	< 0.0001
KRT18	Keratin 18	0.53	0.00026
DLD	Dihydrolipoyl dehydrogenase	0.53	0.001
TALDO1	Transaldolase	0.54	0.00074
ALDH2	Aldehyde dehydrogenase, mitochondrial	0.55	< 0.0001
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4	0.56	< 0.0001
EIF3C	Eukaryotic translation initiation factor 3 subunit C	0.57	0.001
PSPH	Phosphoserine phosphatase	0.57	< 0.0001
CS	Citrate synthase, mitochondrial	0.58	0.002
AMACR	Alpha-methylacyl-CoA racemase	0.6	0.003
SUCLG2	Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial	0.64	0.006
MDH2	Malate dehydrogenase, mitochondrial	0.64	< 0.0001
CKMT1	Creatine kinase U-type, mitochondrial	0.73	< 0.0001
TST	Thiosulfate sulfurtransferase	0.75	< 0.0001
HS	10 kDa heat shock protein, mitochondrial	0.79	< 0.0001
ACAA1	Acetyl-CoA acyltransferase 1	0.8	0.00043
GOT1	Aspartate aminotransferase, cytoplasmic	0.86	< 0.0001

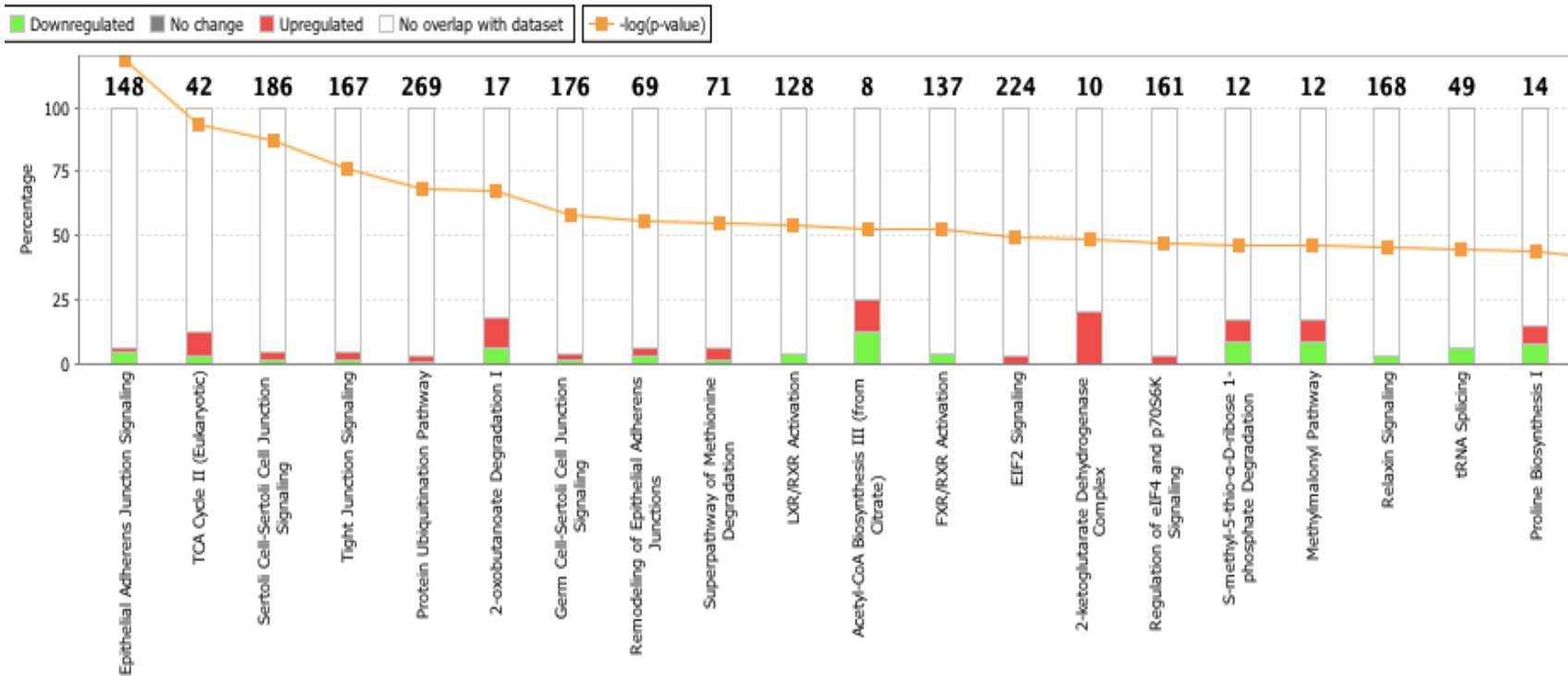


Figure 31. Top 20 canonical pathways associated with the DEPs among the highE2/conceptus vs highE2/noconceptus groups.

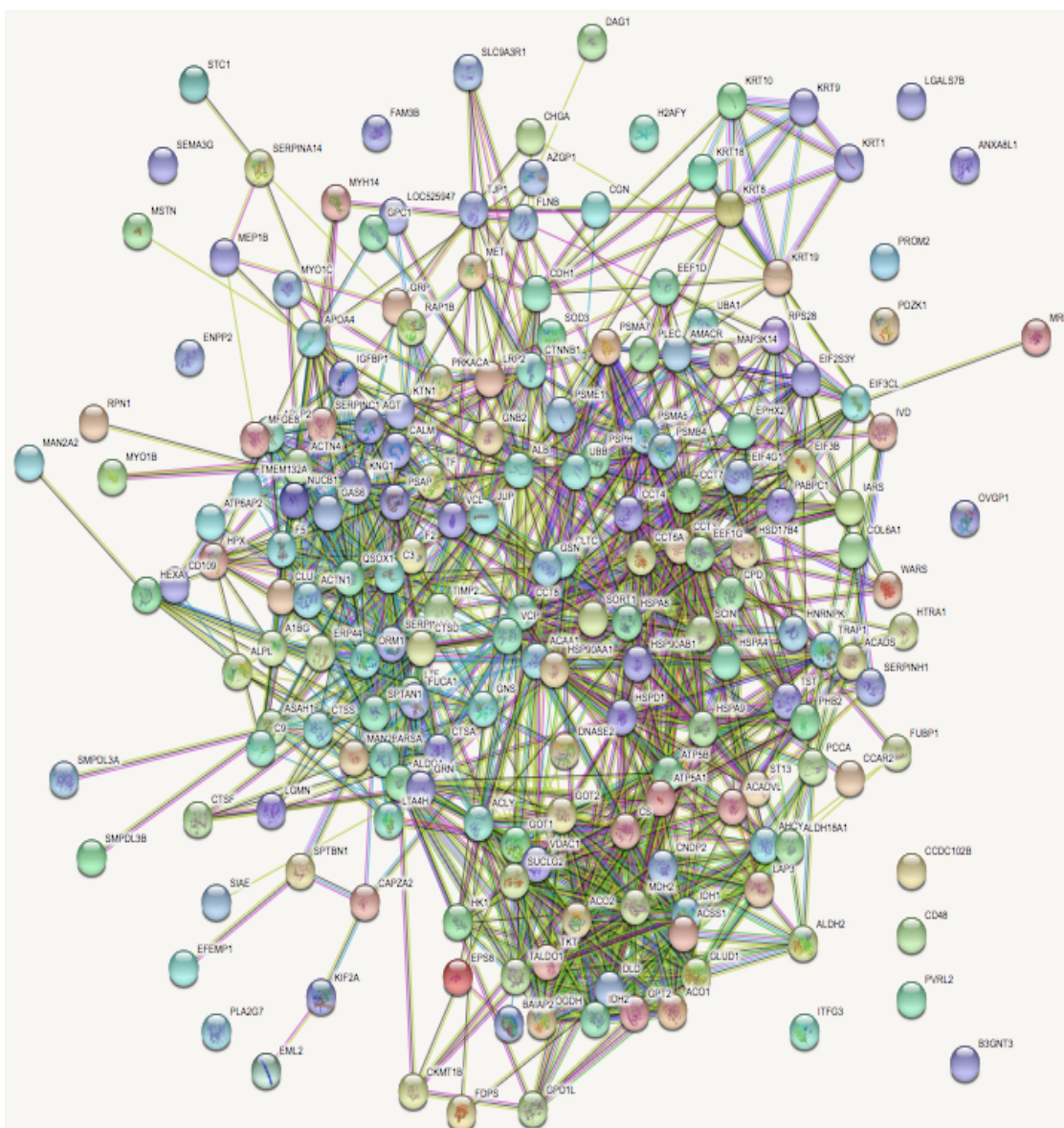


Figure 32. Protein network interactions for the DEPs among the highE2/conceptus and highE2/noconceptus groups (STRING).

*LowE2/conceptus and lowE2/noconceptus:*

There were 194 DEPs among the lowE2/conceptus and lowE2/noconceptus groups. Specifically, there were 81 proteins that were upregulated in the lowE2/conceptus group compared to the lowE2/noconceptus group (table 10). There were 113 proteins that were downregulated (ALDH18A1, MCCC2, DDX1, UBA2, NUMA1) in the lowE2/conceptus group (table 10). The top canonical pathways associated with these DEPs included: fatty acid beta oxidation ( $1.24E-06$ ), mitochondria dysfunction ( $7.65E-05$ ), 4- aminobutyrate degradation ( $1.06E-04$ ), valine degradation ( $1.59E-04$ ), and glutamate degradation ( $3.49E-04$ ; figure 33). The main molecular and cellular functions associated with the DEPs were: cell death and survival (70 molecules;  $5.83E-03 - 2.55E-12$ ), amino acid metabolism (13 molecules;  $5.66E-03 - 1.29E-10$ ), small molecule biochemistry (55 molecules;  $5.66E-03 - 1.29E-10$ ), cell-cell signaling and interaction (32 molecules;  $5.08E-03 - 1.08E-09$ ), and cellular compromise (11 molecules;  $1.88E-03 - 4.19E-08$ ). Protein network interactions for the DEPs for this comparison are depicted in figure 34. Regardless of preovulatory estradiol exposure, there were 77 DEPs in common when comparing ULF samples from animals that did and did not have a conceptus recovered (figure 35).



Table 10. The top 25 downregulated/upregulated characterized DEPs in ULF between the lowE2/conceptus and lowE2/noconceptus animals.

Abbreviation	Protein Name	Log2Fold Change	FDR
ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	-1.07	< 0.0001
MCCC2	Methylcrotonoyl-CoA carboxylase 2	-0.98	< 0.0001
DDX1	ATP-dependent RNA helicase DDX1	-0.97	0.002
UBA2	UBA2 protein	-0.91	0.001
GSTM1	Glutathione S-transferase Mu 1	-0.85	< 0.0001
PSPC1	Paraspeckle component 1	-0.83	0.001
PDZK1	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	-0.83	0.001
PSAT1	Phosphoserine aminotransferase	-0.83	< 0.0001
ANP32A	Acidic leucine-rich nuclear phosphoprotein 32 family member A	-0.81	0.001
GNMT	Glycine N-methyltransferase	-0.81	0.005
TALDO1	Transaldolase	-0.8	< 0.0001
RBMX	RNA-binding motif protein, X chromosome	-0.79	0.001
SULT1A1	Sulfotransferase 1A1	-0.79	0.005
SFPQ	Splicing factor proline and glutamine rich	-0.78	0.00019
ACAT1	Acetyl-CoA acetyltransferase, mitochondrial	-0.72	0.00011
DCPS	m7GpppX diphosphatase	-0.72	0.004
EPHX2	Epoxide hydrolase 2	-0.7	0.00053
PDHA1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	-0.69	0.001
CTNNB1	Catenin beta-1	-0.66	0.00023
MDH2	Malate dehydrogenase, mitochondrial	-0.66	< 0.0001
OGDH	2-oxoglutarate dehydrogenase, mitochondrial	-0.66	< 0.0001
TST	Thiosulfate sulfurtransferase	-0.66	< 0.0001
ACTN1	Alpha-actinin-1	-0.64	< 0.0001
CCAR2	Cell cycle and apoptosis regulator 2	-0.64	< 0.0001
KRT7	Keratin, type II cytoskeletal 7	-0.63	< 0.0001
CTSZ	Cathepsin Z	0.78	0.001

FAM213A	Redox-regulatory protein FAM213A	0.78	0.001
APLP2	Amyloid beta precursor like protein 2	0.78	0.004
CTSL	Cathepsin L1	0.79	0.001
RPN1	Dolichyl-diphosphooligosaccharide- -protein glycosyltransferase subunit 1	0.79	< 0.0001
HIST1H2AC	Histone H2A	0.8	0.001
DNASE2	Deoxyribonuclease-2-alpha	0.8	0.005
GRN	Granulin precursor	0.82	0.00013
COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	0.83	0.006
IMMT	MICOS complex subunit MIC60	0.89	< 0.0001
APMAP	Adipocyte plasma membrane- associated protein	0.93	0.00026
PHB2	Prohibitin-2	0.96	< 0.0001
ANXA8	Annexin A8	0.98	0.0038
LGMN	Legumain	0.99	< 0.0001
SLC25A5	ADP/ATP translocase 2	1.03	< 0.0001
TAPBP	TAP binding protein	1.04	0.003
PHB	Prohibitin	1.04	< 0.0001
LYZ	Lysozyme C, milk isozyme	1.05	0.002
ANXA1	Annexin A1	1.06	< 0.0001
COX4I1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	1.08	0.001
RBP4	Retinol-binding protein 4	1.2	< 0.0001
ECSOD	Superoxide dismutase [Cu-Zn]	1.26	0.00017
STC1	Stanniocalcin-1	1.29	0.002
VDAC2	Voltage-dependent anion-selective channel protein 2	1.38	< 0.0001
UTMP	Uterine milk protein	1.59	0.001

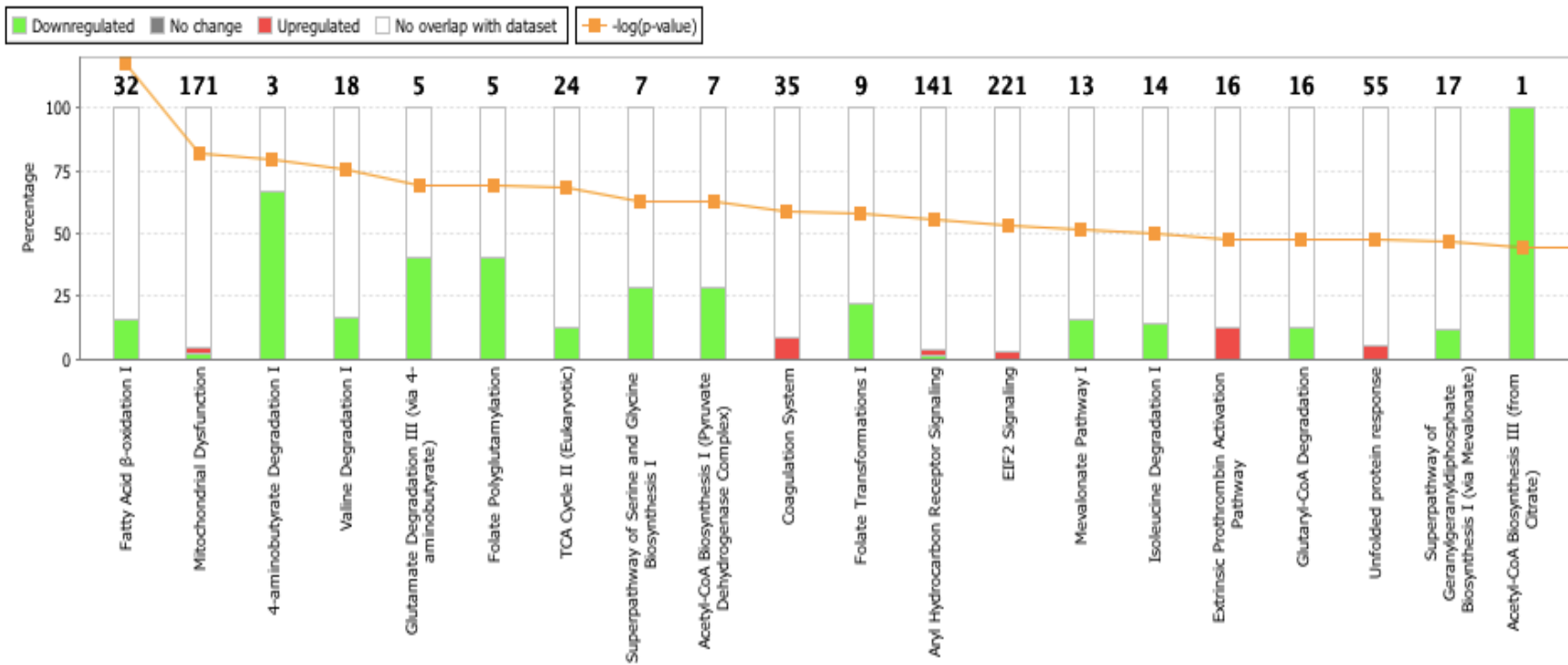


Figure 33. Top 20 canonical pathways associated with the DEPs among the lowE2/conceptus vs lowE2/noconceptus groups.

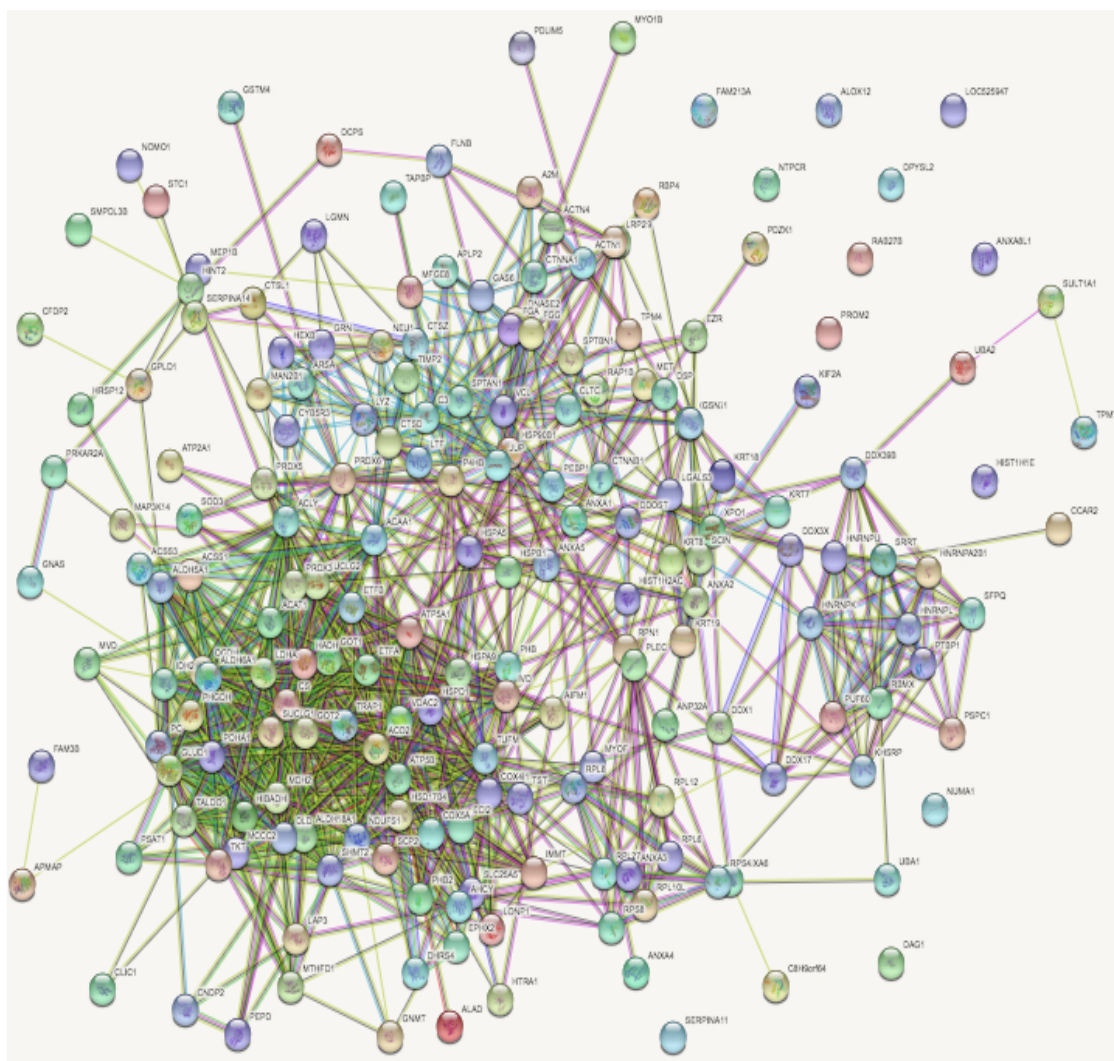


Figure 34. Protein network interactions for the DEPs among the lowE2/conceptus and lowE2/noconceptus groups (STRING).

HighE2/Conceptus vs  
HighE2/Noconceptus

LowE2/Conceptus vs  
LowE2/Noconceptus

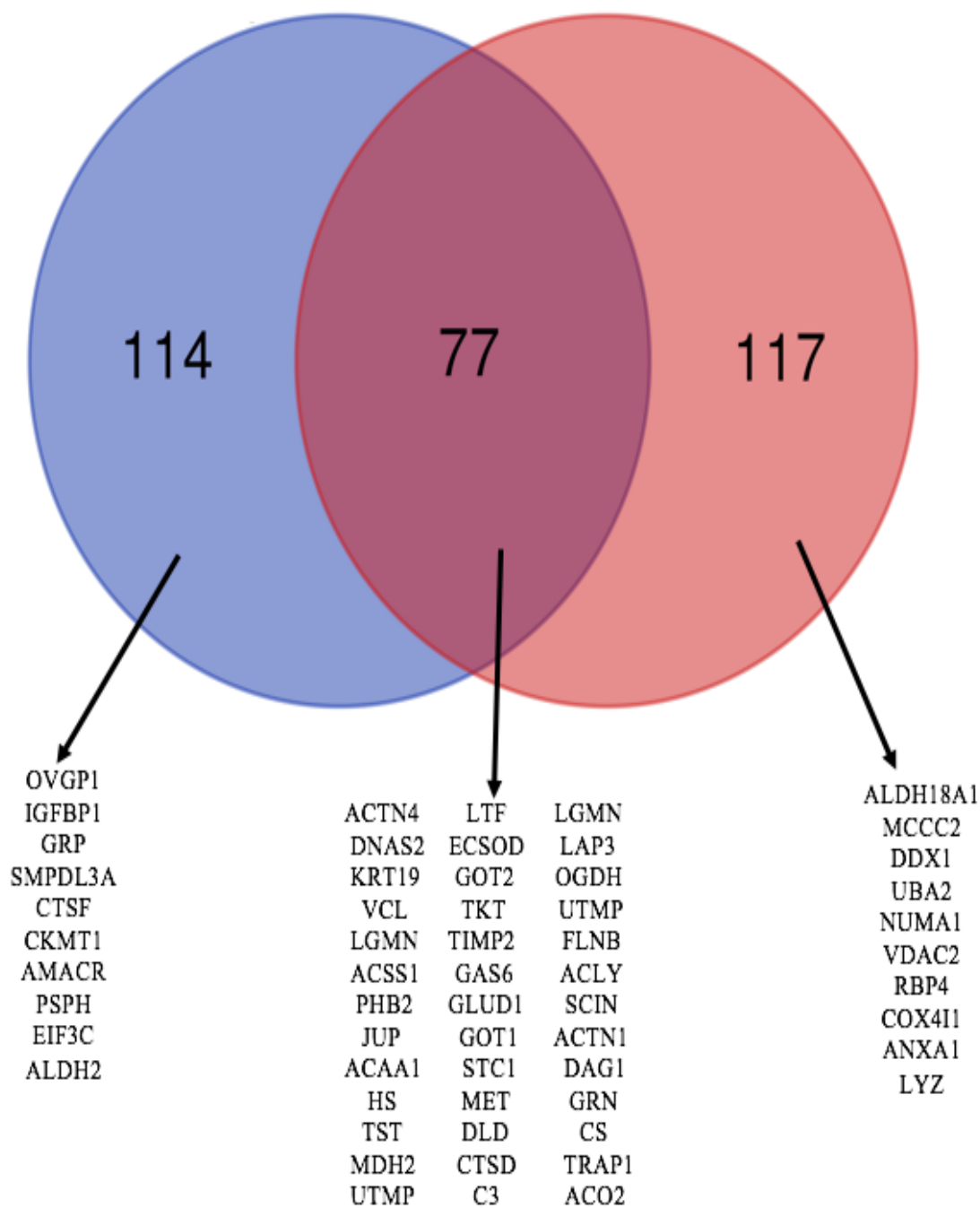


Figure 35. A comparison examining the effects of IFNT, by comparing differentially expressed proteins among highE2/conceptus vs highE2/noconceptus and lowE2/conceptus vs lowE2/noconceptus groups.

## DISCUSSION

The results from these studies indicate that preovulatory estradiol exposure and presence of a conceptus (IFNT) influences the uterine proteome on day 16 of the estrous cycle and pregnancy. The uterus is a dynamic environment whose composition changes drastically depending on stage of the estrous cycle and the needs of the conceptus. During pregnancy, proteins are necessary for conceptus elongation, pregnancy recognition, and preparation for implantation and placentation. Previous researchers have focused on differences in uterine protein profiles among pregnant/cyclic ruminants during various time points in the preimplantation phase of development (Koch et al., 2010; Forde et al., 2014; Forde et al., 2015; Brooks et al., 2016; Arianmanesh et al., 2016) however, little is known about the effect preovulatory estradiol can have on specific protein abundances in the uterine luminal fluid. The main purpose of this study was to gain a better understanding of the differentially expressed proteins (DEPs) among the different groupings and what role they may have in pregnancy maintenance and conceptus development.

### Study 1:

Specifically, for the highE2/conceptus and lowE2/conceptus comparison there were 48 differentially expressed proteins. There were 19 proteins that were upregulated (GOT1, GSTM4, TST, ACAA1, and GPLD1) and 29 proteins that were downregulated (UTMP, ORM1, ANXA8, ANXA1, and FGG) in the highE2/conceptus group compared to the lowE2/conceptus group. There were 6 DEPs (ANXA8, APLP2, PHB, ANPEP, ALDH2, GPLD1) that were also considered differentially expressed (FDR<0.10) at the

mRNA level (unpublished-chapter II). The lack of similarities in mRNA and protein expression may be attributed to: post-transcriptional (alternative splicing, transport, mRNA stability), translational (miRNAs), and post-translational (phosphorylation, ubiquitination, methylation, acetylation) regulatory factors.

During pregnancy, there is an increase in metabolic activity of the endometrium due to the exponential growth of the conceptus, this is reflected by the top molecular and cellular functions being associated with carbohydrate metabolism and nucleic acid metabolism. Protein abundance of enzymes that are critical in metabolic pathways were increased among highE2/conceptus animals versus lowE2/conceptus animals on day 16. These enzymes included: acetyl-CoA acyltransferase 1 (ACAA1), isocitrate dehydrogenase [NADP] (IDH1), isocitrate dehydrogenase, mitochondrial (IDH2), glycerol-3-phosphate dehydrogenase (GPD1L), and aspartate aminotransferase (GOT1). The IDH1 and IDH2 proteins are involved in the citric acid cycle (ATP production). Isocitrate dehydrogenase was reported to be more abundant in pregnant bovine endometrium compared to non-pregnant endometrium on day 18 (Berendt et al., 2005). A different study also reported it being more abundant in the ULF from the gravid horn on day 18 of pregnancy compared to non-gravid horns (Ledgard et al., 2009). Forde and others (2014) determined that the IDH1 protein is secreted by the endometrium at the initiation of conceptus elongation through pregnancy recognition, then it decreases by day 19 of pregnancy which coincides with implantation. It has been suggested that it may only be necessary for a short period of time by the conceptus, or it is a protein that is being turned over rapidly by the uterus (Forde et al., 2014).

On day 16 in sheep, around the time of attachment, actinin 4 (ACTN4) protein was elevated in pregnant ULF compared to non-pregnant ULF. This protein also had increased abundance in ULF among the highE2/conceptus group when compared to the lowE2/conceptus group. The alpha actinin family proteins are involved in growth and remodeling. They can bind to filamentous actin and regulate cytokinesis, cell adhesion, spreading, migration, and signaling (Kao, 2015). An increase in cell adhesion and migration activity possibly suggests that the uterus is preparing for attachment.

Heat shock proteins have many critical roles in reproduction, they are also one of the first proteins produced during embryogenesis (Neuer et al., 2000; Bensusade and Morange, 1983). They are involved in inter-organelle transport, inhibition of aggregation of denatured polypeptides, antigen presentation, pro-apoptotic activity, and assist in proper folding of polypeptides (Yu et al., 2012; Witkin et al., 1996). The HSP60 family is expressed in a highly constitutive manner, especially during the secretory phase in humans when the endometrium is receptive to embryo implantation (Neuer et al., 2000). In the current study, there was increased protein abundance of HSP60 among highE2/conceptus animals compared to lowE2/conceptus animals. However, Arianmanesh and others (2016) reported a downregulation of HSP60 protein expression in caruncular endometrium of gravid horns during the post implantation period (day 20) in sheep. It is important to keep in mind that these differences in expression may be due to the different species, time period, collection method (ULF versus endometrial tissue), or differences between gene expression and protein concentrations.

Uterine milk protein (UTMP) functions are not well understood, but may include: protease inhibition, nutrition of conceptus, growth, and maternal immune suppression



(Khatib et al., 2007). Expression has been reported to be increased following stimulation with estradiol, and detection of the protein revealed increased concentrations localized to the glandular epithelium on the day of estrus (Ulbrich et al., 2009). In the present study, the highE2/conceptus group had decreased UTMP abundance on day 16. This is supported by the report that expression, as determined by Real-time RT-PCR, decreased during the midluteal phase, but increased again on Day 18 in pregnant cows (Ulbrich et al., 2009). A possible explanation for these changes could be that an excess of this particular protein can negatively impact the survival of the conceptus at different stages of pregnancy.

The innate and adaptive immune system must be appropriately regulated to prevent the rejection of the conceptus during pregnancy, as it is considered foreign to the female body. The complement component system functions in both embryonic and host protection (Girardi et al., 2006). Additionally, in humans, an intact complement system during early pregnancy at the placental interface optimizes placental development and function (Regal et al., 2015). However, the complement 3 protein (C3), a component of the complement system, had decreased abundance in the highE2/conceptus group compared to the lowE2/conceptus group. Ribeiro and others (2016) reported decreased C3 expression as the conceptus undergoes morphological changes from spherical to filamentous. Barnwell et al. (2016) examined differences in gene expression among long and short bovine conceptuses on day 15 of gestation. They reported increased C3 mRNA abundance among short conceptuses. In humans, previous research has established that excessive or misdirected complement activation can lead to pregnancy complications such as: pregnancy loss, fetal growth restriction, and preterm birth (Regal et al., 2015).

Therefore, the uterus and/or conceptus may be downregulating the abundance of this protein to avoid pregnancy loss.

Forde and others (2015) previously analyzed protein content in ULF from cyclic and pregnant heifers on day 16. They further analyzed proteins specifically produced by day 16 conceptuses in culture media. Thirty proteins were identified to be unique to ULF from pregnant heifers and produced by short term in vitro cultured conceptuses on day 16. In the current study, 28 of these proteins were identified on day 16 in the uterine luminal fluid. There were 20 proteins that were upregulated, and 8 proteins that were downregulated in the highE2/conceptus group compared to the lowE2/conceptus group. They concluded that these proteins could possibly be involved in facilitating interactions between the conceptus and endometrium during pregnancy recognition. If this is true, these proteins may contribute to the improved pregnancy rates observed in cows that display estrus during a timed AI protocol (Madsen et al., 2015; Richardson et al., 2016).

#### Study 2:

Interferon tau has also been reported to regulate changes in uterine protein expression (Brooks et al., 2014). Expression of IFNT begins in mononuclear cells of the trophectoderm during the late morula, early blastocyst stage (Kubisch et al., 2001). Interferon tau mRNA and protein content increases dramatically between days 14-21, this coincides with elongation and trophectoderm proliferation. Interferon tau secretion decreases rapidly around the time of uterine attachment (day 19-21; Ealy and Yang, 2009). Therefore, we further examined protein abundance in ULF among highE2 and

lowE2 animals that did and did not have a conceptus recovered to determine the effects of IFNT on protein abundances on day 16 of pregnancy regardless of estradiol exposure.

There were 194 DEPs among the highE2/conceptus versus highE2/noconceptus comparison, 55 of these DEPs (LTF, PDZK1, MEP1B, IDH1, WARS, SCIN, TST, GOT1) were also considered differentially expressed (FDR<0.10) at the mRNA level (unpublished-chapter II). There were 195 DEPs among the lowE2/conceptus versus lowE2/noconceptus comparison, 1 of these DEPs (KRT7) was considered differentially expressed (FDR<0.10) at the mRNA level (unpublished-chapter II). Some of the differentially expressed proteins among cyclic and pregnant comparisons fall under the category of non-classical interferon stimulated genes/proteins, meaning their expression in the endometrium is stimulated by IFNT, but they are not involved in the typical type I interferon response. These include: ARSA (Forde et al., 2013), EZRIN (Ledgard et al., 2009), LGMN (Ledgard et al., 2009), CTSD, CTSL, CTSS, CTSZ (Song et al., 2005), IGFBP1 (Simmons et al., 2009), GRP (Song et al., 2005), and RBP4 (Dore et al., 1994).

Cathepsins (CTSD, CTSL, CTSS, CTSZ) are lysosomal proteinases that function by degrading extracellular matrix molecules such as: collagens, laminin, fibronectin, and proteoglycans (Salamonsen, 1999). There is evidence that suggests the production of cathepsins, other proteases, and their inhibitors by both the endometrium and conceptus regulate tissue remodeling and angiogenesis at implantation (Salamonsen, 1999; Salamonsen et al., 1995). In sheep, CTSD, CTSL, CTSS, and CTSZ mRNA abundance increased between day 10 and day 20 of pregnancy (Song et al., 2005). Specifically, in mice, CTSL and CTSB inhibitors were administered in high and low doses. Animals given large doses resulted in complete failure of implantation, while smaller doses caused

abnormal embryonic development and reduced decidualization (Afonso et al., 1997). In the current study, the highE2/conceptus group had a decrease in the protein abundances of CTSD and CTSS compared to the highE2/noconceptus group on day 16. For the lowE2/conceptus group, there was an increase in CTSD, CTSL, and CTSZ protein abundances compared to the lowE2/noconceptus group on day 16. These data suggest that IFNT and preovulatory estradiol exposure affects the abundances of cathepsins in order to maintain optimal protease activity in preparation for attachment.

Retinol binding proteins are necessary for the transport of retinoids. Retinoids are important for normal function, proliferation, and differentiation of cells within the uterus. It has also been suggested that they play a role in the production and transport of uterine secretions to the fetus for growth and development (Bates, 1983). Specifically, RBP4 was one of the most abundant proteins in ULF identified on day 16 of pregnancy in heifers (Forde et al., 2014). This study reports increased RBP4 abundance among lowE2 animals with a conceptus, when compared to lowE2 animals without a conceptus. Previously retinoic acid has been reported to inhibit interferons at a transcriptional level (Blalock and Gifford, 1977). Excessive retinol binding proteins and vitamin A transport into the uterine lumen may lead to decreased IFNT production among lowE2/conceptus animals, thus potentially causing conceptus loss after day 16.

In summary, both preovulatory estradiol and interferon tau impacted protein abundance within the uterine luminal fluid on day 16 in cattle. Specifically, the differences in protein abundances in the ULF among the highE2/conceptus and lowE2/conceptus groups may solely reflect differences in metabolism, growth, and

immune processes. However, these differences in protein abundance may also contribute to improved conceptus survival among highE2 animals.

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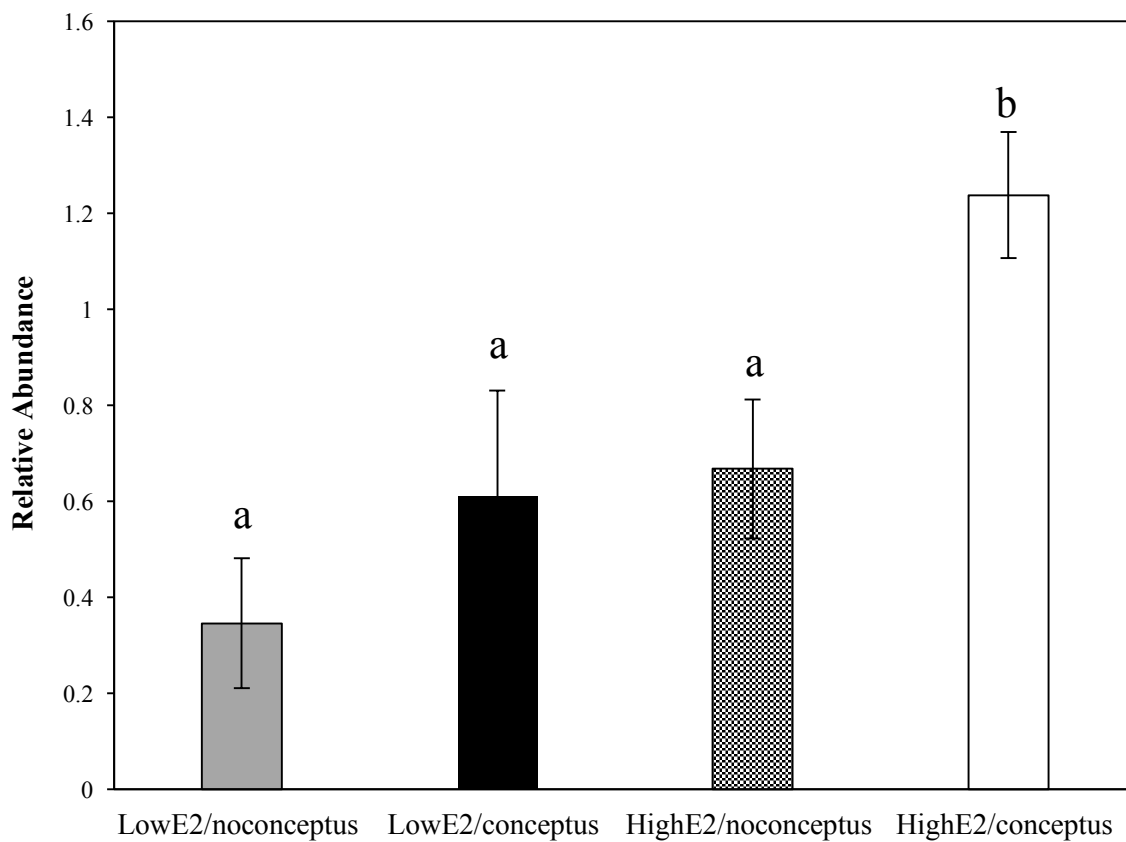
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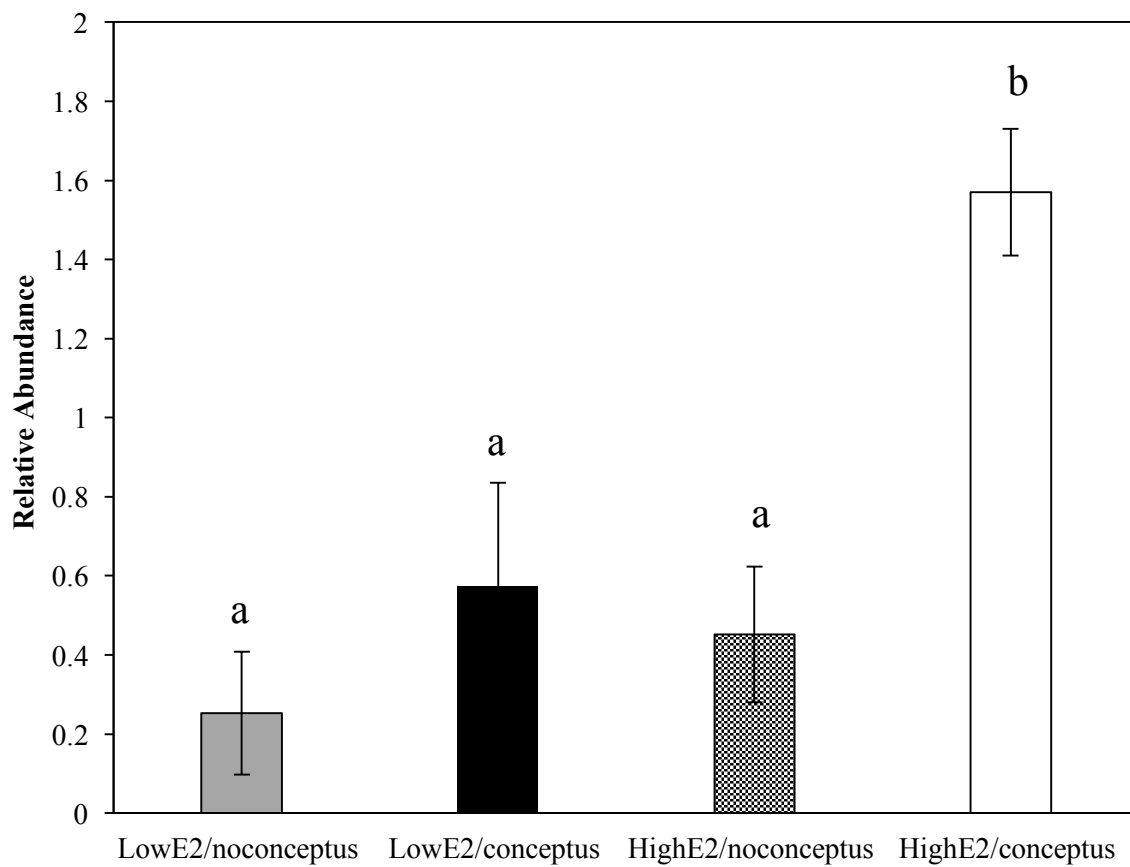
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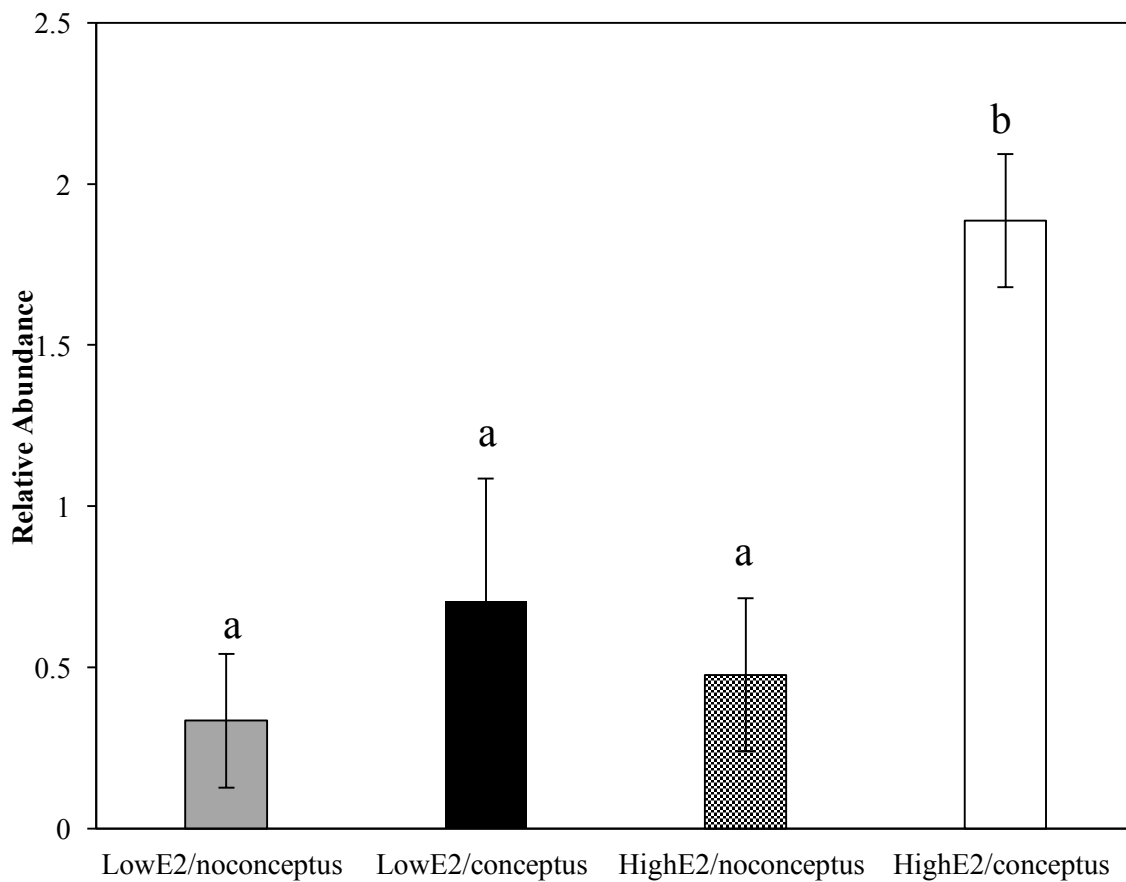
## APPENDIX A



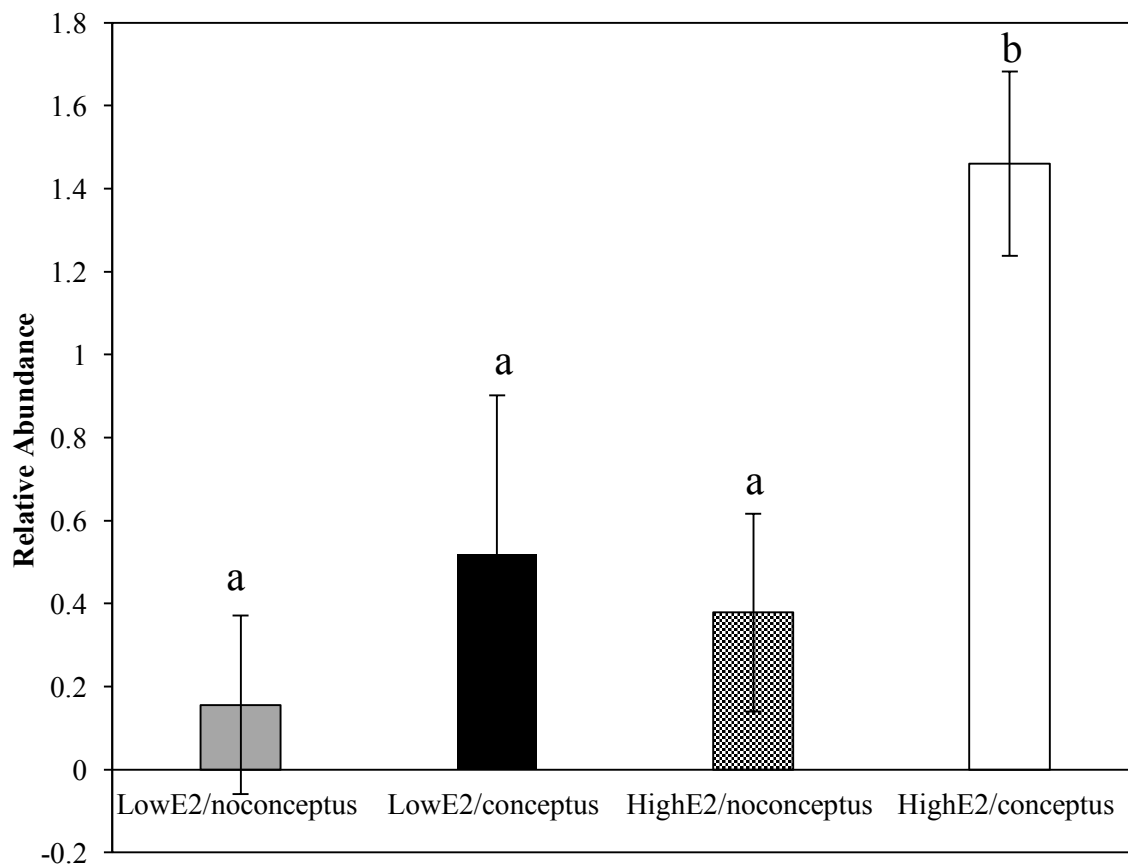
Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.35$ ) on PRSS8 mRNA abundance in endometrium on day 16.



Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.04$ ) on CXCL10 mRNA abundance in endometrium on day 16.

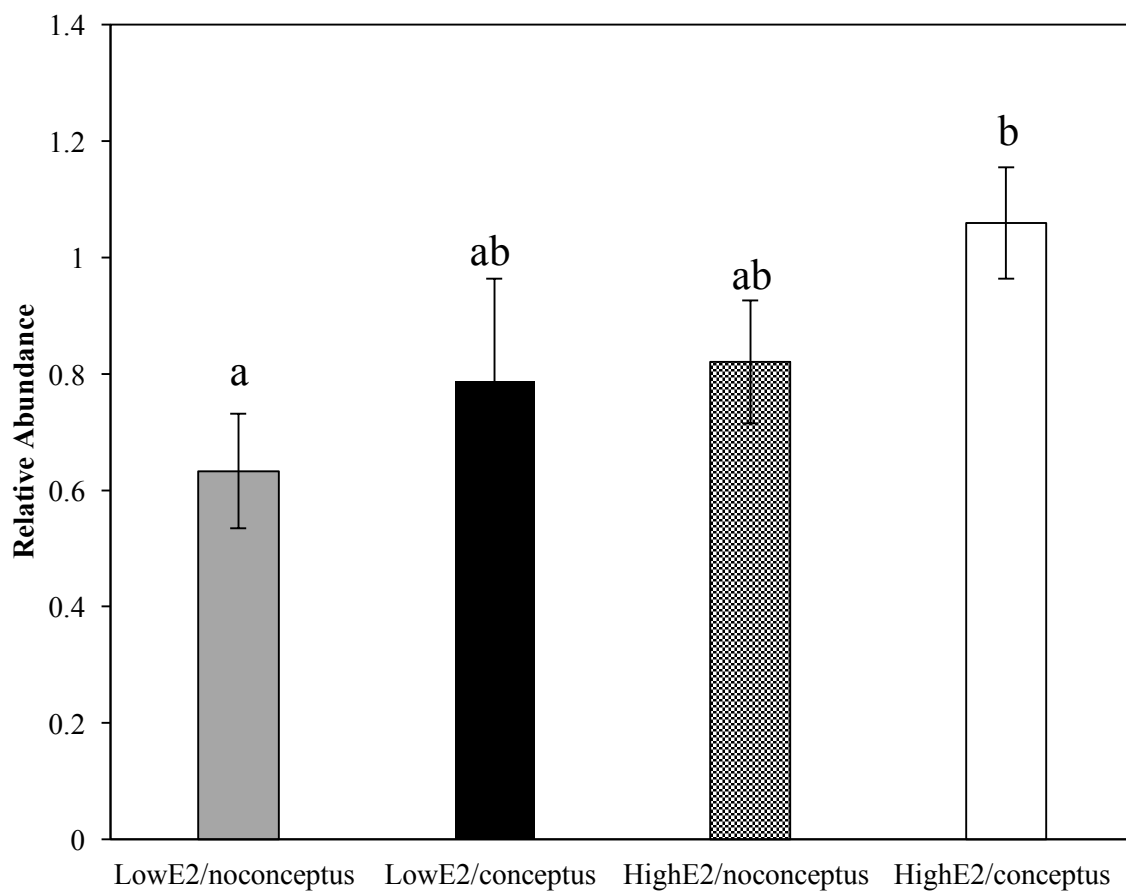


Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.06$ ) on IDO1 mRNA abundance in endometrium on day 16.

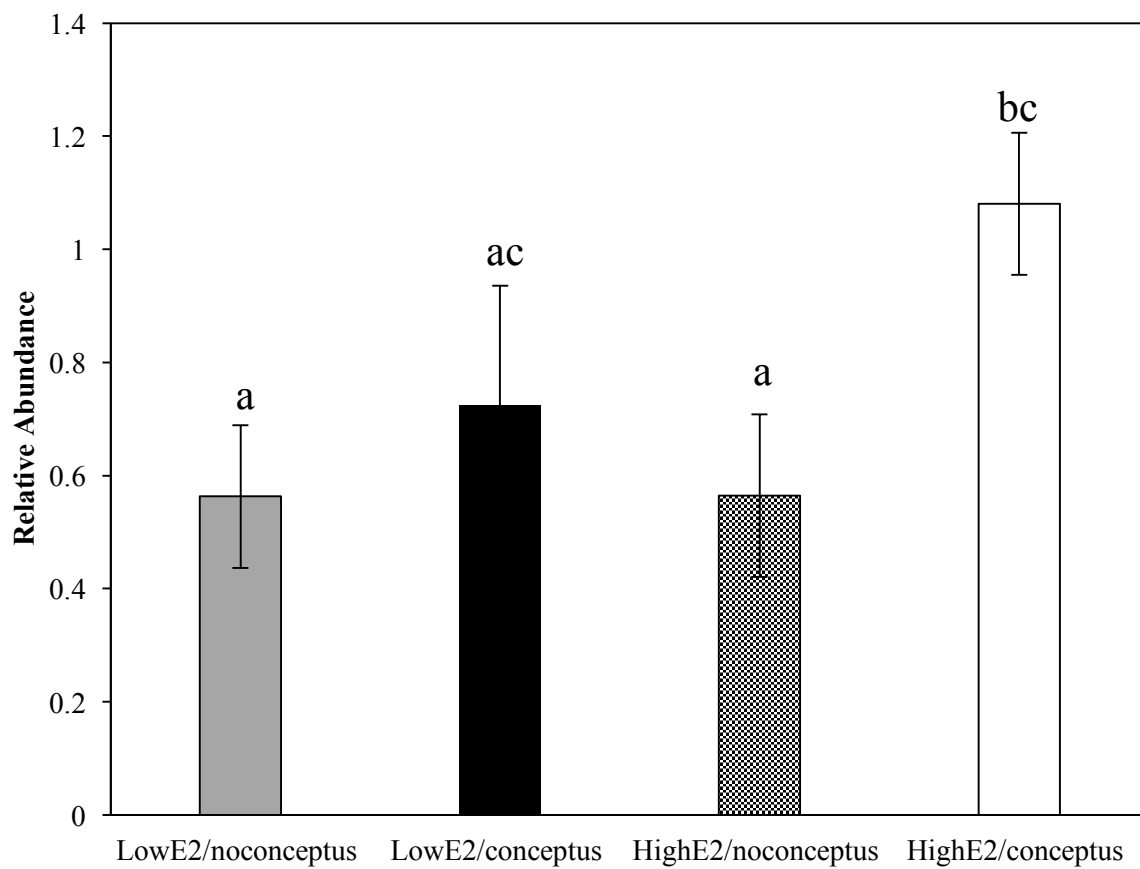


Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.20$ ) on

FABP3 mRNA abundance in endometrium on day 16.



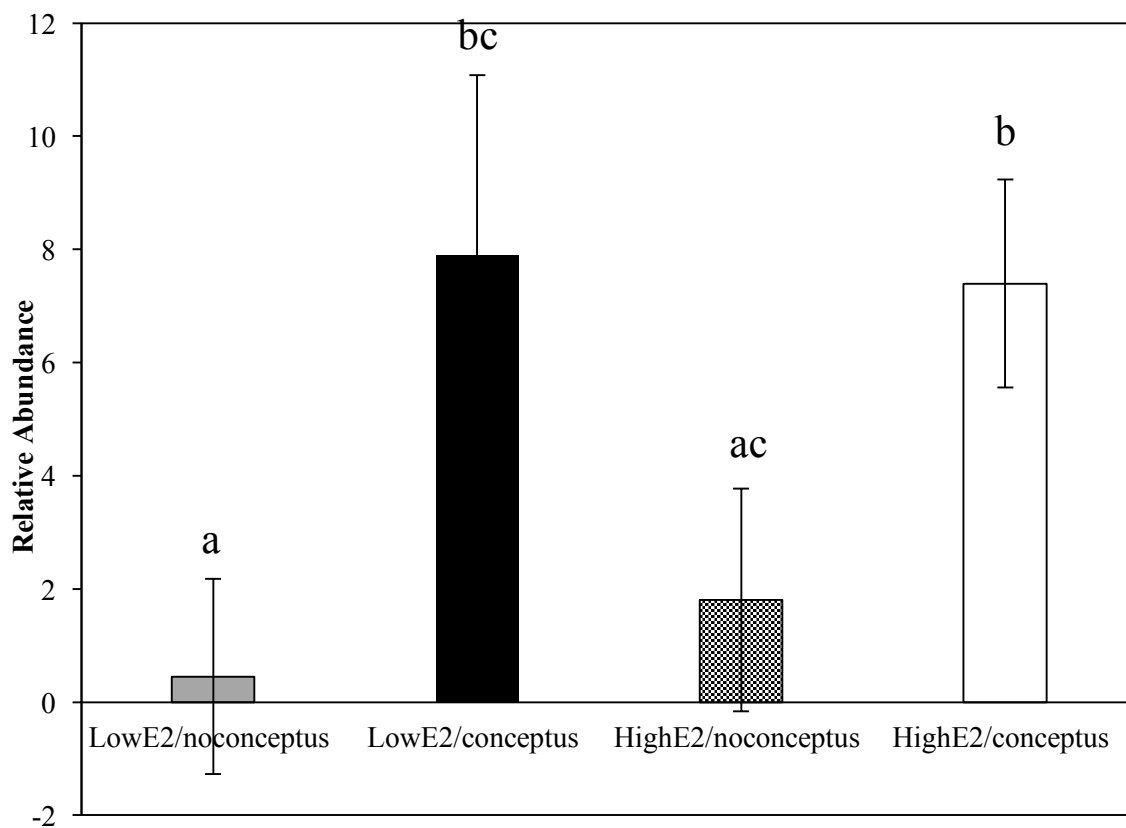
Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.73$ ) on MUC13 mRNA abundance in endometrium on day 16.



Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.26$ ) on

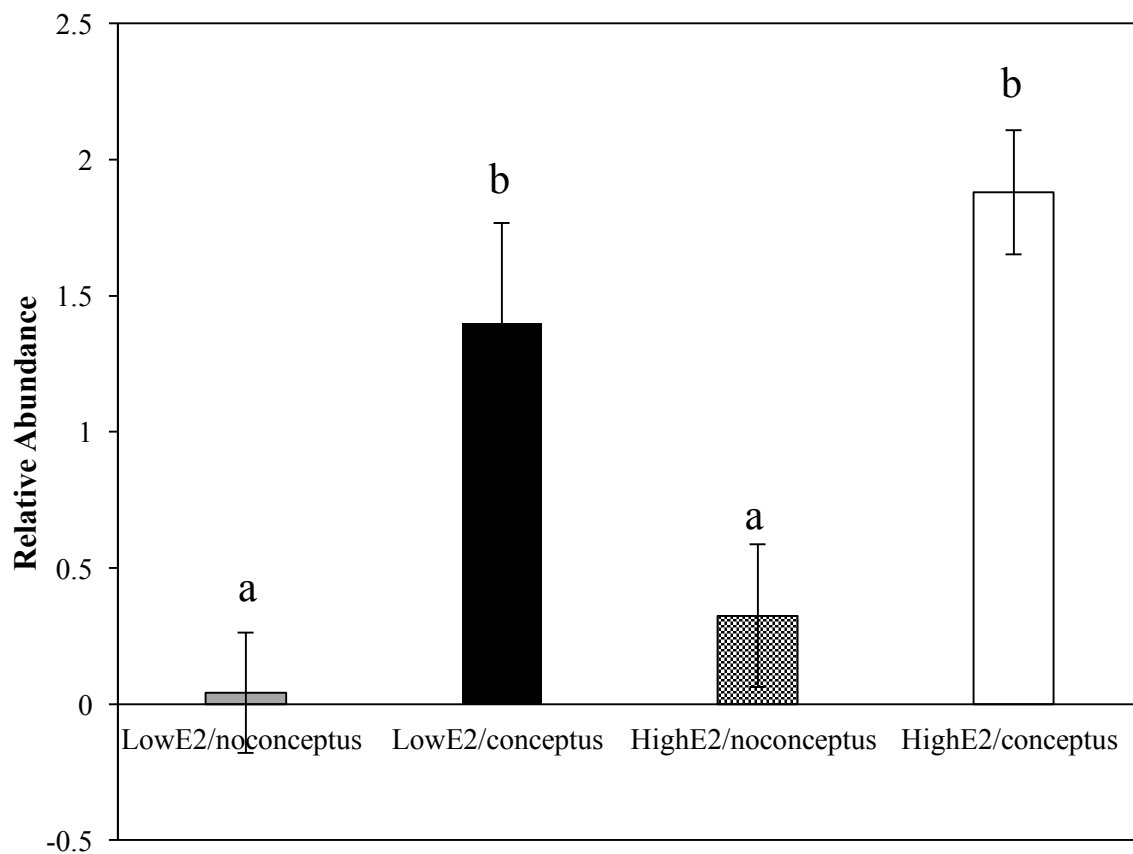
CLDN4 mRNA abundance in endometrium on day 16.





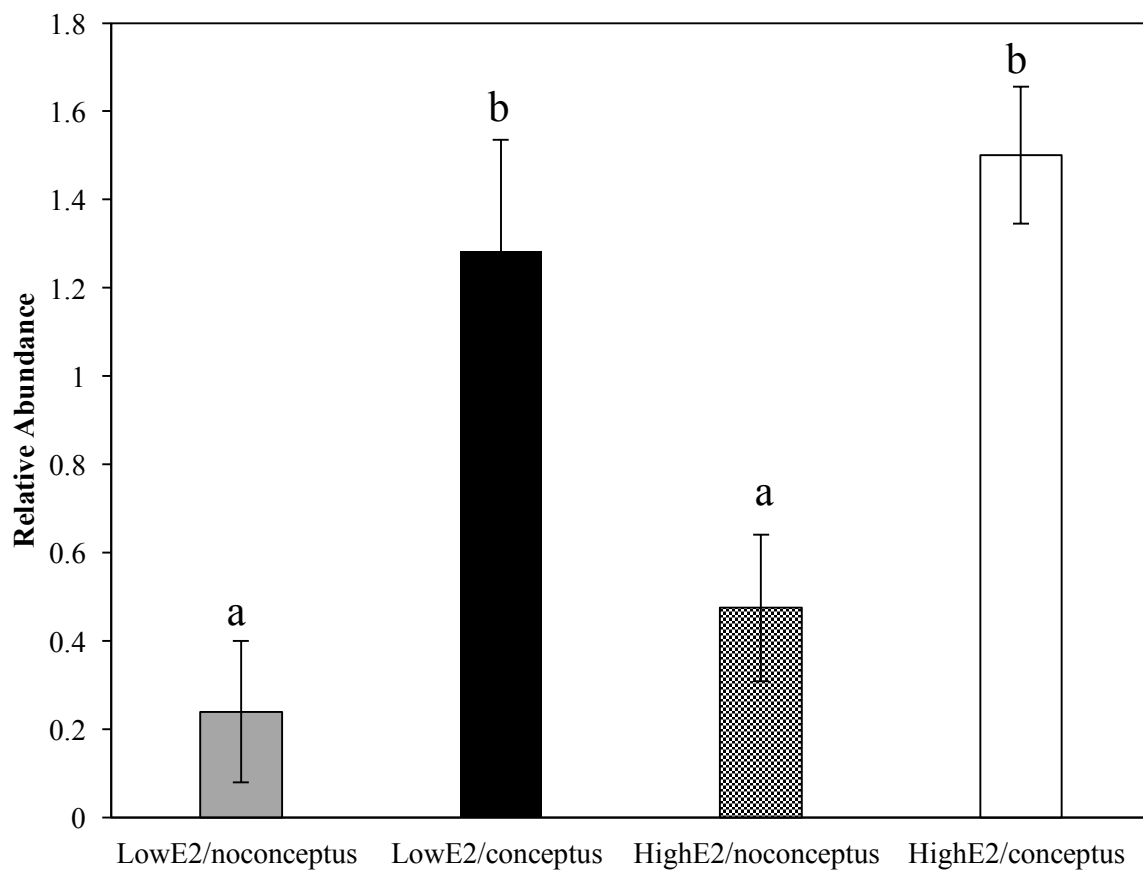
Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.68$ ) on

ISG15 mRNA abundance in endometrium on day 16.



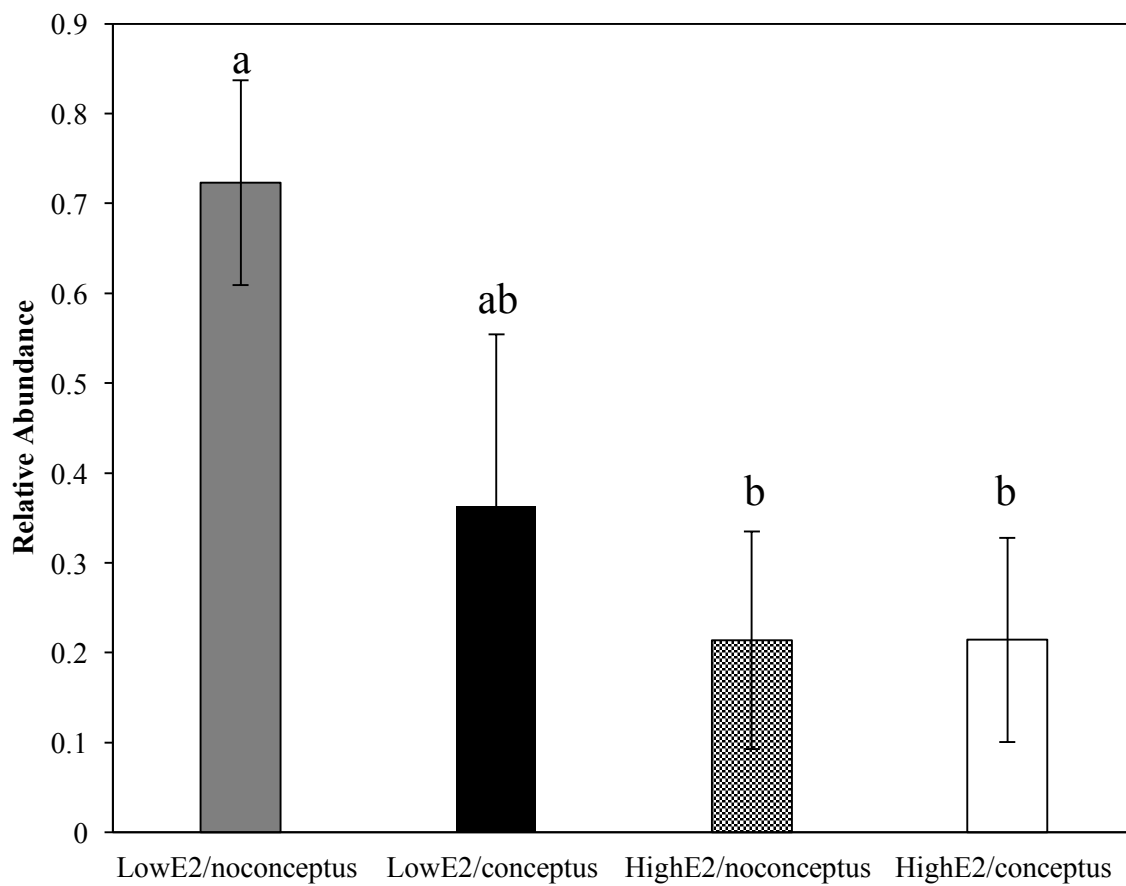
Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.72$ ) on

RSAD2 mRNA abundance in endometrium on day 16.

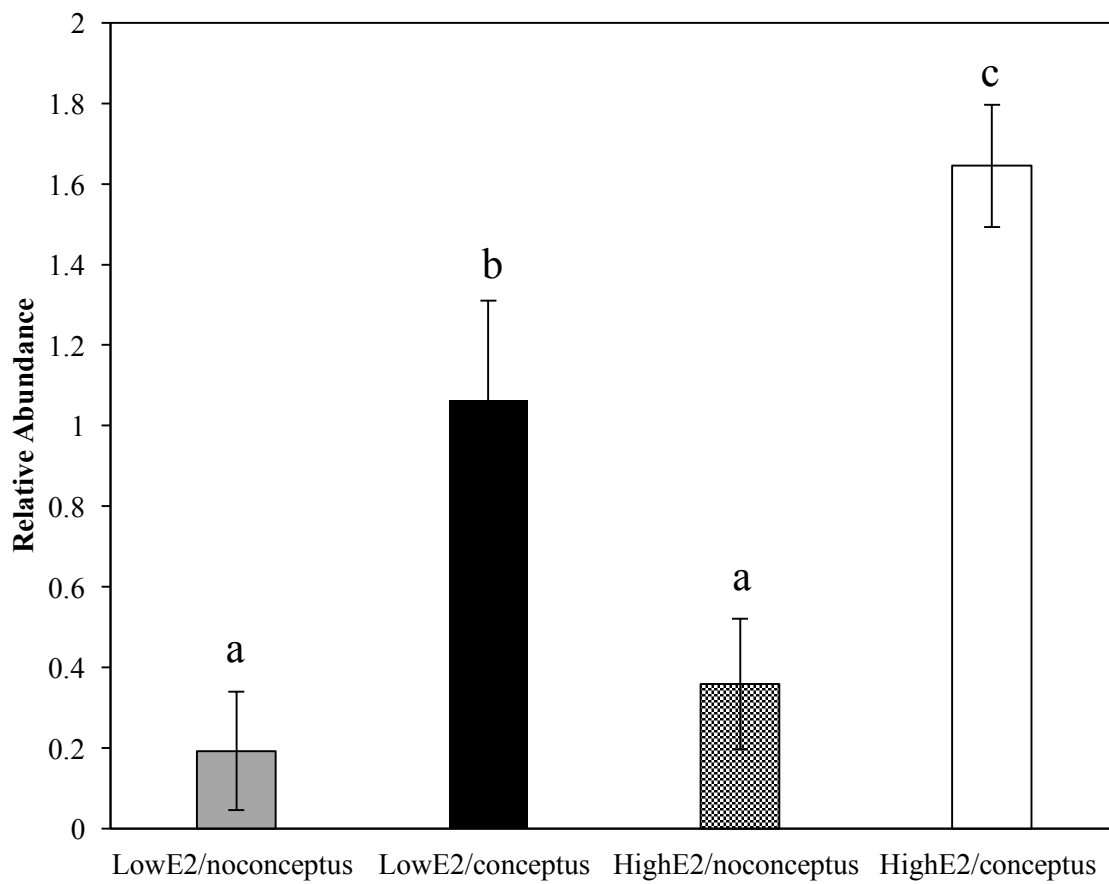


Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.96$ ) on

PARP12 mRNA abundance in endometrium on day 16.

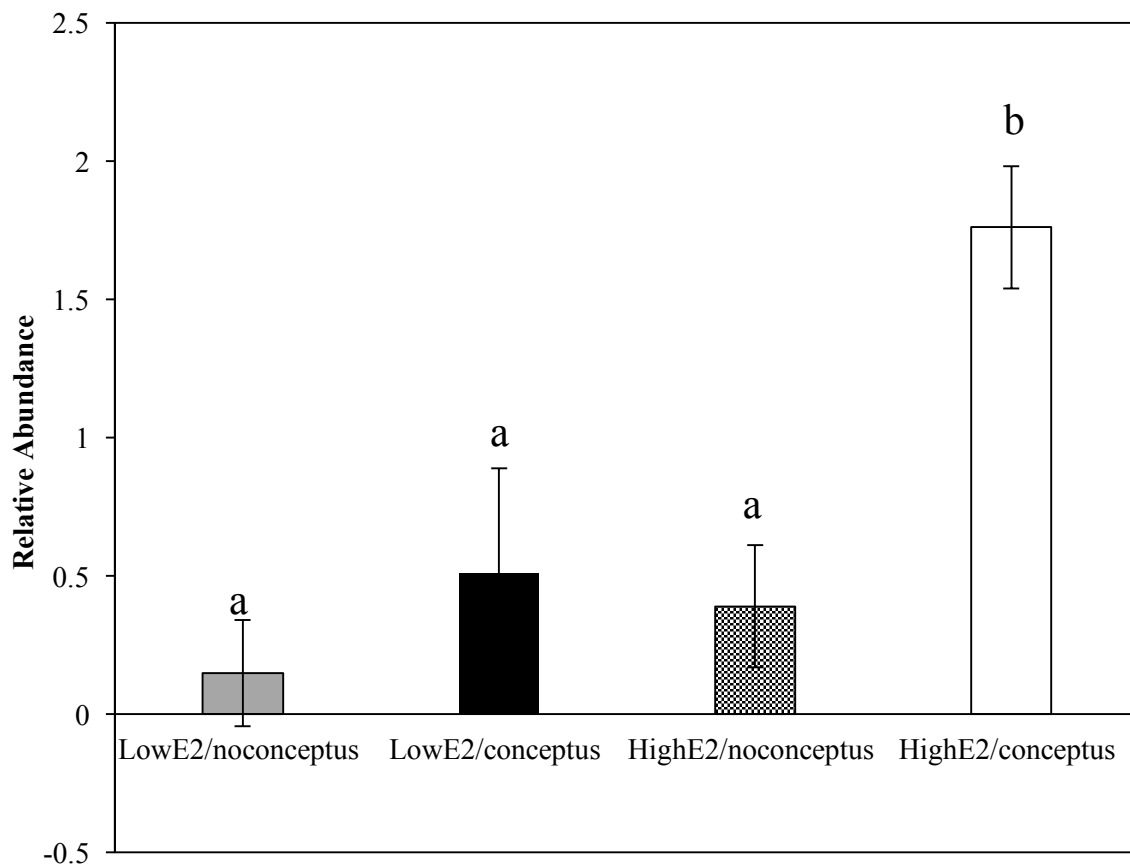


Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.20$ ) on OXTR mRNA abundance in endometrium on day 16.



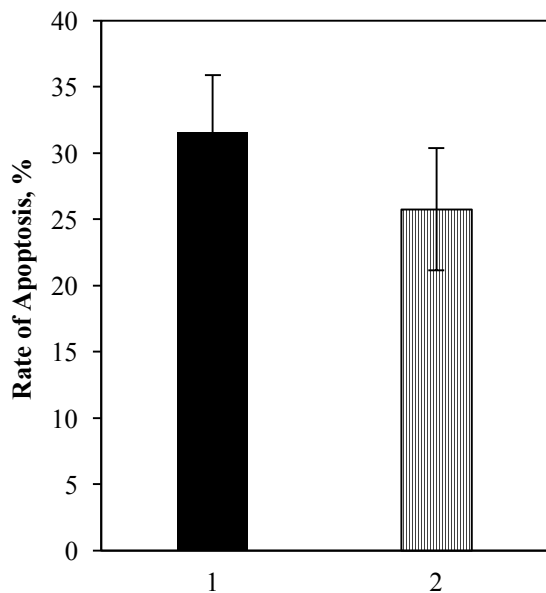
Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.26$ ) on

XAF1 mRNA abundance in endometrium on day 16.

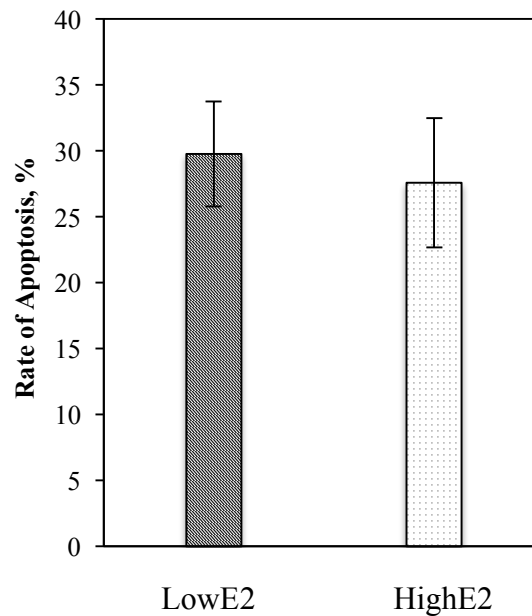


Preovulatory estradiol exposure and conceptus presence interaction ( $P = 0.06$ ) on DDX58 mRNA abundance in endometrium on day 16.

## APPENDIX B

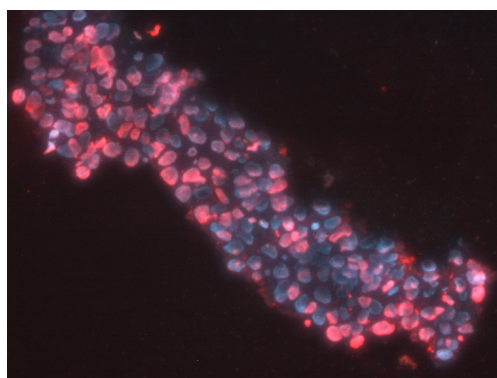
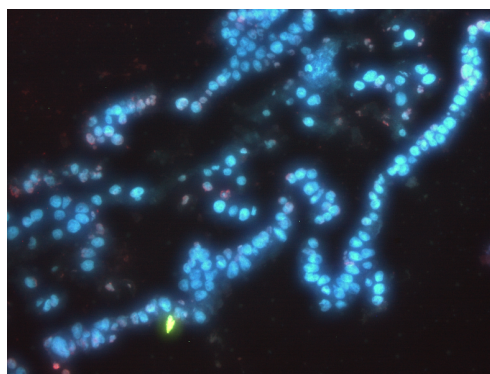


There was no difference in the rate of apoptosis in the trophoblast between replicate one and two ( $P = 0.22$ ).

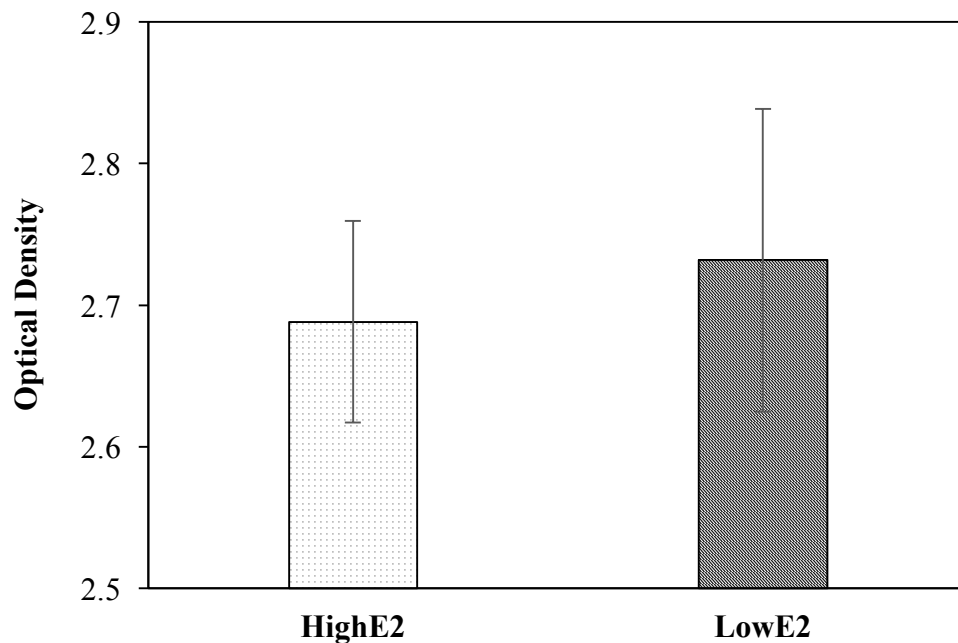


There was no difference in the rate of apoptosis in the trophoblast collected from lowE2 and highE2 animals ( $P = 0.64$ ).

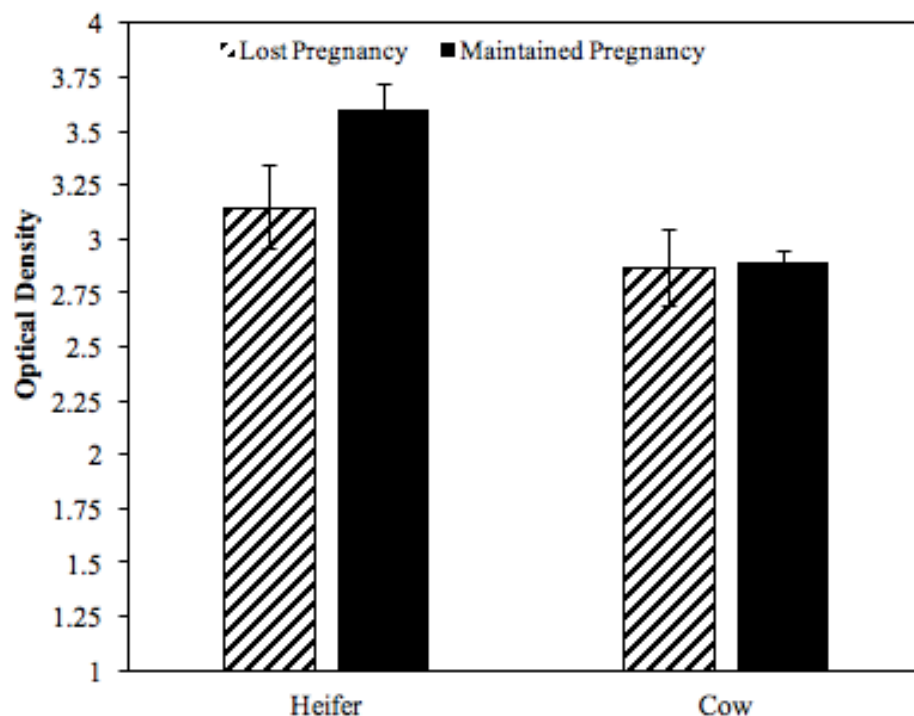
Fluorescent BrdU Staining: Red- apoptotic cells, Blue- normal cells



## APPENDIX C



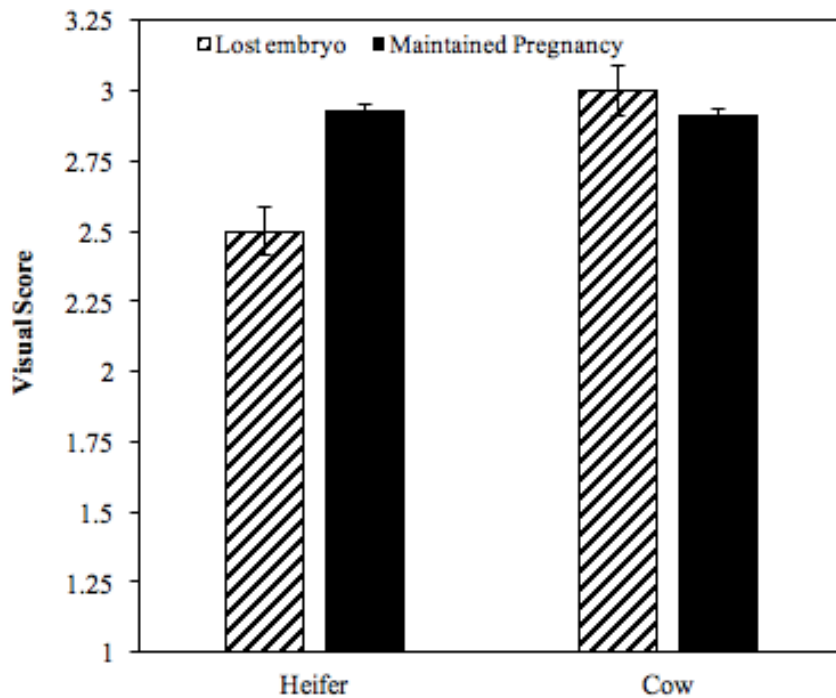
IDEXX bovine pregnancy test. There was no difference in PAG concentrations among highE2 and lowE2 animals ( $P = 0.73$ ).



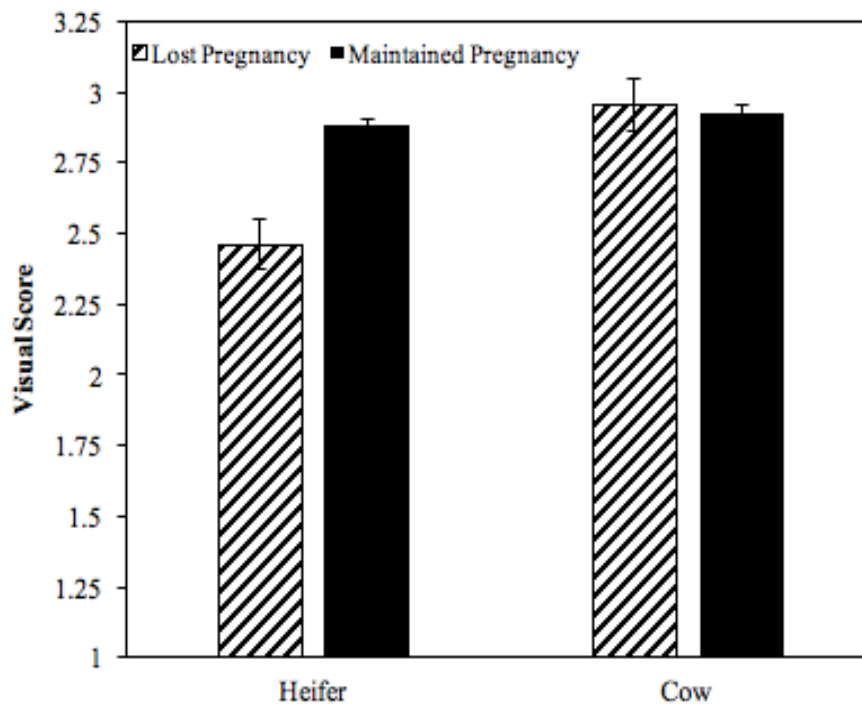
IDEXX bovine pregnancy test. Animals that experienced pregnancy loss had decreased circulating PAGs ( $P=0.04$ ). Cows had decreased circulating PAGs compared to heifers ( $P=0.0002$ ). There was a tendency for a loss and age interaction ( $P=0.06$ ).

bovine pregnancy test.





IDEXX Rapid Visual test (Technician 1). Animals that lost a pregnancy scored lower than animals that maintained their pregnancy ( $P=0.005$ ). Cows were scored higher than heifers ( $P=0.0001$ ). There was a loss and age interaction ( $P<0.0001$ ).



IDEXX Rapid Visual test (Technician 2). Animals that lost a pregnancy scored lower than animals that maintained their pregnancy ( $P=0.003$ ). Cows were scored higher than heifers ( $P<0.0001$ ). There was a loss and age interaction ( $P<0.0009$ ).