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The Effects of Preovulatory Estradiol on the Uterine Environment and Conceptus Survival From Fertilization to Maternal Recognition of Pregnancy

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THE EFFECTS OF PREOVULATORY ESTRADIOL ON THE UTERINE ENVIRONMENT AND CONCEPTUS SURVIVAL FROM FERTILIZATION TO MATERNAL RECOGNITION OF PREGNANCY

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Animal Science degree and is acceptable for meeting the thesis 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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THE EFFECTS OF PREOVULATORY ESTRADIOL ON THE UTERINE ENVIRONMENT AND CONCEPTUS SURVIVAL FROM FERTILIZATION TO MATERNAL RECOGNITION OF PREGNANCY

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Preovulatory estradiol has been reported to play a critical role in pregnancy establishment and embryonic survival, but the mechanism by which estradiol exerts its effects has not been well characterized. The objective of this thesis is to determine the effects of preovulatory estradiol on the uterine environment and conceptus survival from fertilization to maternal recognition of pregnancy. Beef cows/heifers were synchronized with the CO-Synch protocol and AIed (d 0). Cows were classified by estrus expression (estrus and no estrus), and uteri were flushed to collect d16 conceptuses nonsurgically (Rep 1; n = 29), or following slaughter (Rep 2; n = 37). Uterine luminal fluid (ULF) was analyzed for protein, glucose, and interferon tau (IFNT) concentrations. For replicate 1, total cellular RNA was extracted from blood leukocytes (d 16) to determine expression of interferon-stimulated genes (ISG): ISG-15, OAS-1, and MX2. For replicate 2, total cellular RNA was extracted from caruncular (CAR) and intercaruncular (INCAR) endometrial tissue to determine relative abundance of select glucose transporters (SLC2A1, SLC2A4, SLC2A5, and SLC5A1). There was no difference in conceptus recovery rate between estrus and no estrus cows ($P = 0.20$; 48% vs 29%) or between replicates ($P = 0.46$; 44% vs 33%). There were no differences between estrus and no estrus cows for ULF protein concentration ($P = 0.36$; 2222 ± 513 vs 1547 ± 525 mg/mL).

There was no difference ($P > 0.20$) in d 16 expression of ISG-15, OAS-1, or MX2 between estrus and no estrus cows, nor a difference between cows in which a conceptus was or was not recovered. In addition, there were no differences in IFNT concentrations in the ULF among estrus and no estrus cows ($P = 0.42$), nor a difference among cows that did and did not have a conceptus recovered from them ($P = 0.71$). Cows that exhibited estrus had greater glucose concentrations in ULF ($P = 0.05$; 51 ± 1.86 vs 45 ± 1.92 mg/dL) compared to no estrus cows, but there was no difference in protein concentration in the ULF ($P = 0.36$; 2222 ± 513 vs 1547 ± 525 mg/mL). Cows in which a conceptus was recovered had greater concentrations of protein in the ULF ($P = 0.05$; 2643 ± 585 mg/mL) compared to cows in which a conceptus was not recovered (1126 ± 463 mg/mL), glucose concentration was similar between groups ($P = 0.29$; 47 ± 2.12 vs 50 ± 1.70 mg/dL). For replicate 2, in both CAR and INCAR endometria, cows that exhibited estrus had greater abundance of SLC2A1 ($P = 0.05$) and SLC5A1 ($P < 0.04$) mRNA. Presence of a conceptus tended to increase ($P = 0.10$) abundance of SLC5A1 mRNA in INCAR tissue, but had no effect ($P > 0.13$) on abundance of SLC2A1 mRNA in either tissues or SLC5A1 mRNA in CAR tissue. In CAR tissue, cows from which a conceptus was recovered had decreased SLC2A4 mRNA abundance ($P = 0.04$), but there was no effect of estrus ($P = 0.14$) and no effect of estrus or conceptus in INCAR tissue. There was no difference in SLC2A5 mRNA abundance between estrus and no estrus cows ($P > 0.20$), nor between conceptus and no conceptus cows ($P > 0.58$) in CAR or INCAR tissue. In summary, conceptus recovery rates, IFNT, and protein concentration in ULF did not differ between cows that did or did not exhibit estrus, but ULF glucose content was greater in cows that exhibited estrus. There was no difference in ULF glucose

concentration or IFNT between cows that did and did not have a conceptus, but ULF protein concentration was greater in cows from which a conceptus was recovered. Thus, there was no indication of increased conceptus survival to d 16 of pregnancy based on estrus expression, but ULF glucose and protein concentration changed based on estrus expression and conceptus presence.

CHAPTER I

REVIEW OF LITERATURE

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. Among beef cattle, fertilization rates are estimated to be 90% with calving rates of 55%. This suggests around 35% embryonic mortality, with ~70-80% of this embryonic loss occurring between day 8 and 16 post AI (Disken et al., 2006). In the United States, early embryonic loss costs the beef industry approximately 1.4 billion dollars annually (Bellows et al., 2002). With a continuously growing world population, the demand for beef will increase, and optimal reproductive efficiency in beef cattle will become a more important issue.

Possible factors that lead to early embryonic loss include: genetic defects, reproductive diseases, heat stress, and nutrition (Bridges et al., 2012). Previous research has indicated that estradiol leading up to breeding may be a critical factor for establishment of a successful pregnancy (Perry et al., 2005). Preovulatory estradiol has several roles within the female reproductive tract. It impacts follicular growth, oocyte maturation, sperm transport, uterine environment, and embryonic survival (Pohler et al., 2012). This review will start with an overview of the estrous cycle, and then go into further details about the importance of estradiol in the establishment and maintenance of pregnancy from fertilization to maternal recognition of pregnancy in cattle.

BOVINE ESTROUS CYCLE

Heifers reach puberty when gondatropin-releasing hormone (GnRH) is secreted from the hypothalamus at a sufficient amplitude and frequency to stimulate a surge of luteinizing hormone (LH) to be released from the anterior pituitary (Karsch et al., 1997). 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 due to differences in length of both the luteal phase and the follicular phase; however, the average length of the estrous cycle in cattle is 21 days. The estrous cycle usually consists of two or three waves of follicular growth in cattle (Forde et al., 2011). An estrous cycle can be divided into the follicular phase (proestrus and estrus) and the luteal phase (metestrus and diestrus). During the follicular phase, preovulatory follicles grow and develop and begin to secrete estradiol (Echternkamp and Hansel, 1973). During the luteal phase, the dominant ovarian structure is the corpus luteum (CL), which produces progesterone (Smith et al., 1994). 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 sexual receptivity. Ovulation of the preovulatory follicle occurs during this stage. In cattle, estrus usually lasts between 12-16 hours (Allrich, 1994), and estradiol concentrations peak approximately 36 hours prior to ovulation (Chenault et al, 1975). Metestrus (day 1-5) is the stage in the estrous cycle when follicular cells (granulosa and thecal cells) transform into the luteal cells that make up the CL (Smith et al., 1994). 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).

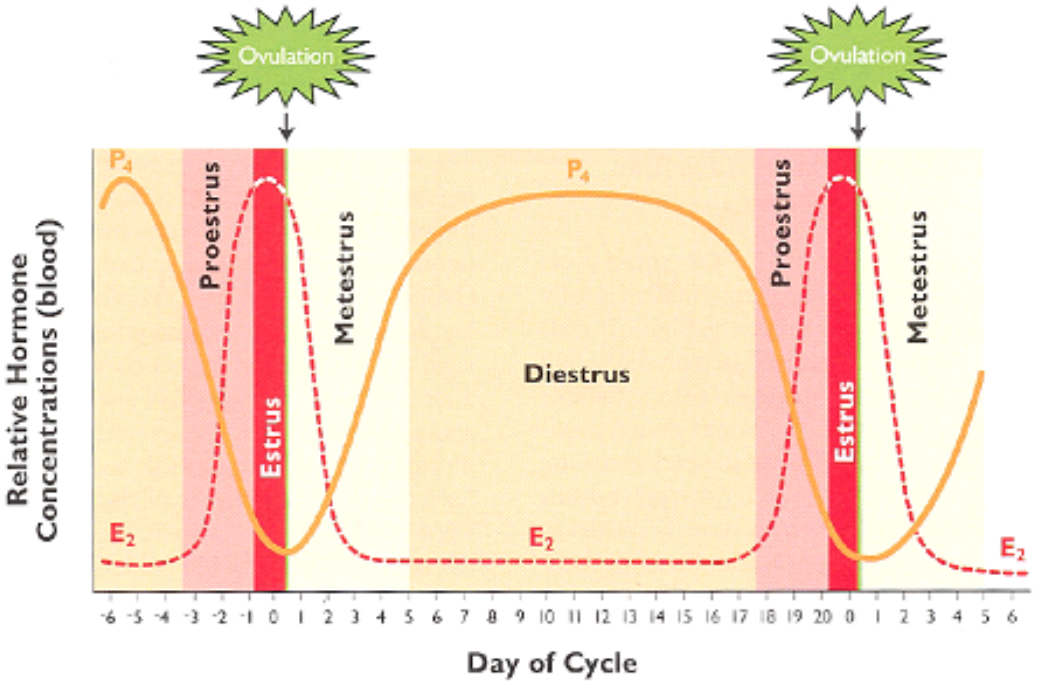

Figure 1. Hormonal profiles associated with the different stages of the bovine estrous cycle (Senger, 2003).

ESTRADIOL

Increased fertilization success, embryo quality and survival (Atkins et al., 2013), and pregnancy maintenance (Perry et al., 2005) has been associated with elevated preovulatory estradiol concentrations. 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 by the mechanism known as the two cell-two gonadotropin theory (Fortune and Quirk, 1988; Figure 2). In this 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 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).

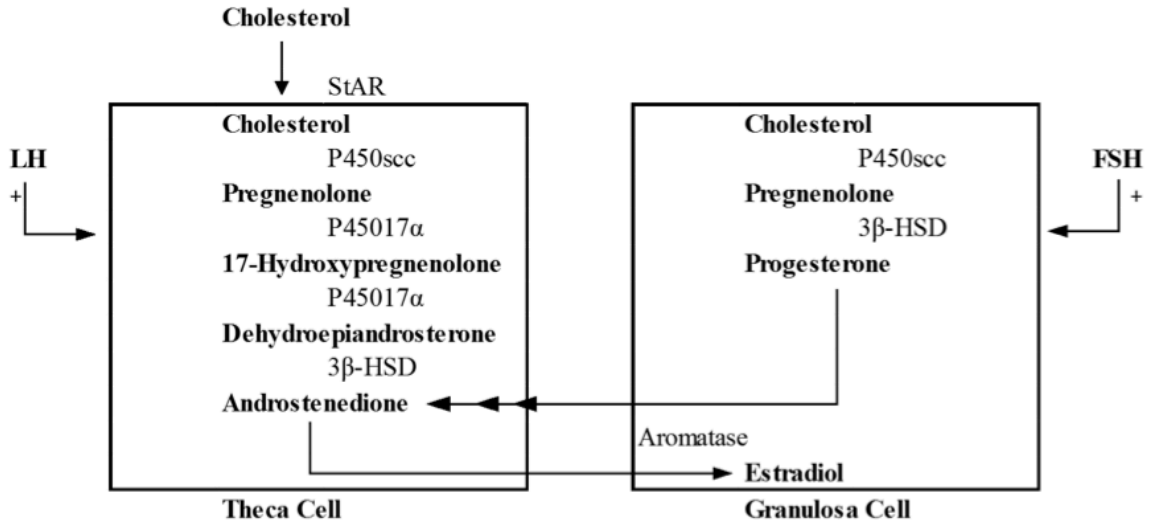

Figure 2. Two-cell, two-gonodotropin model for hormone production.

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 among 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), along with increasing luteinizing hormone receptors (Kessel et al., 1985).

Perry et al. (2014) determined that there was a positive relationship between cows that exhibited estrus between follicle size and peak estradiol concentration, but no linear relationship was seen among cows that did not exhibit estrus. Previous research has 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 time of artificial insemination and inferior progesterone production. There has also been a positive correlation between length of proestrus/estradiol exposure and pregnancy success. Bridges et al. (2010) reported that cattle that experienced a shorter proestrus period had decreased pregnancy success compared to cows that had a longer exposure to preovulatory estradiol.

Oocyte Development:

Bovine follicles containing oocytes with less progesterone and three-eight fold greater cytochrome P450 aromatase (CYP19A1) activity were more capable of being fertilized and developing into a blastocyst (Hazeleger et al., 1995; Driancourt et al., 1998). Similar findings have also been reported in in vitro fertilization studies; bovine oocytes cultured in media with increased concentrations of estradiol were more likely to develop to the blastocyst stage (Mermilloid 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., 1998).

Sperm transport:

At the initiation of estrus, uterine pH decreases from a pH of 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 may increase sperm longevity by decreasing motility, thus optimizing fertilization efficiency (Jones and Bavister, 2000). Decreased 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). Previous research has reported that ovariectomized ewes require exogenous estradiol for effective sperm transport (Allison and Robinson, 1972). Thus, sperm transport through the female

reproductive tract is optimized during estrus when estradiol concentrations are elevated (Hawk, 1983).

Uterine Environment:

Increased circulating concentrations of preovulatory estradiol have also been reported to have a beneficial impact on the uterine environment and embryo survival, however the mechanism has not been well established (Atkins et al., 2013; Jinks et al., 2013). Estradiol induced expression of endometrial receptors, production of uterine proteins (Bartol et al., 1981), and increased expression of many genes involved in uterine extracellular matrix remodeling that are necessary for embryo growth and a successful pregnancy (Bauersachs et al., 2005). Miller et al. (1977) conducted a study that determined the impact of giving large or small doses of exogenous estradiol to ovariectomized sheep. Following embryo transfer on day 4, animals that were given a small dose of estradiol had decreased uterine weight, total protein content, progesterone and estrogen receptor within the uterus, and pregnancy success compared to ewes given a larger dose. Whether or not an animal was exposed to elevated concentrations of estradiol also impacted gene expression within the endometrium (Bridges et al., 2012). Nuclear progesterone receptors in the deep glandular epithelium and mRNA abundance for estradiol receptor alpha in the uterine epithelium was decreased among animals that had decreased preovulatory concentrations of estradiol compared to animals that were exposed to elevated concentrations during the preovulatory period (Bridges et al., 2012), and these differences in mRNA expression and concentrations of receptor proteins may contribute to embryonic losses after day 15.5 of pregnancy.

ESTRUS

Initiation of estrus occurs due to increased circulating concentrations of 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 estrus behavior 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. Initiation of estrus and the LH surge are influenced by the rate at which estradiol increases during the preovulatory period (Rozell and Keisler, 1990). Once estrus occurs, no additional amounts of estradiol can further stimulate the expression of the behavioral estrus (Allrich, 1994). Efficient estrus detection has been reported to be critical for pregnancy success (Foote, 1975).

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. (2014) 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. The critical period for pregnancy loss for control cows occurred between days 22-24 (during placental attachment). This suggests the importance of preovulatory estradiol on embryo growth and attachment.

BOVINE EMBRYO DEVELOPMENT

Fertilization occurs within the oviduct, the embryo then enters the uterus approximately around day 4 (Black and Davis, 1962). While in the uterus, the embryo undergoes several cell divisions leading to a morula (16 cell stage; Forde and Lonergan, 2012). After further differentiation, the morula develops into a blastocyst which consists of an inner cell mass and a trophectoderm layer (Forde and Lonergan, 2012). The inner cell mass eventually gives rise to the fetus, while the outer trophectoderm cells will develop into the placenta (Forde and Lonergan, 2012). During the blastocyst stage, contact with the endometrium is not necessary. Unlike in humans and rodents, bovine blastocysts do not invasively implant in the endometrium, they are free floating in the uterus until around day 19 when attachment occurs (Betteridge and Flechon, 1988). On day 9, the blastocyst hatches from the zona pellucida (Forde and Lonergan, 2012). On day 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, but by day 16 the elongated conceptus can reach 60 mm in length (Betteridge et al., 1980). The bovine blastocyst/conceptus is capable of doubling its length everyday between day 9 and 16 (Berg et al, 2010). During this time of elongation, the conceptus becomes more dependent on the maternal uterine environment and secretions from the uterus for survival, growth, and attachment (Filant and Spencer, 2014). Blastocyst growth into an elongated bovine conceptus has not been able to be duplicated in vitro (Betteridge and Flechon, 1988).

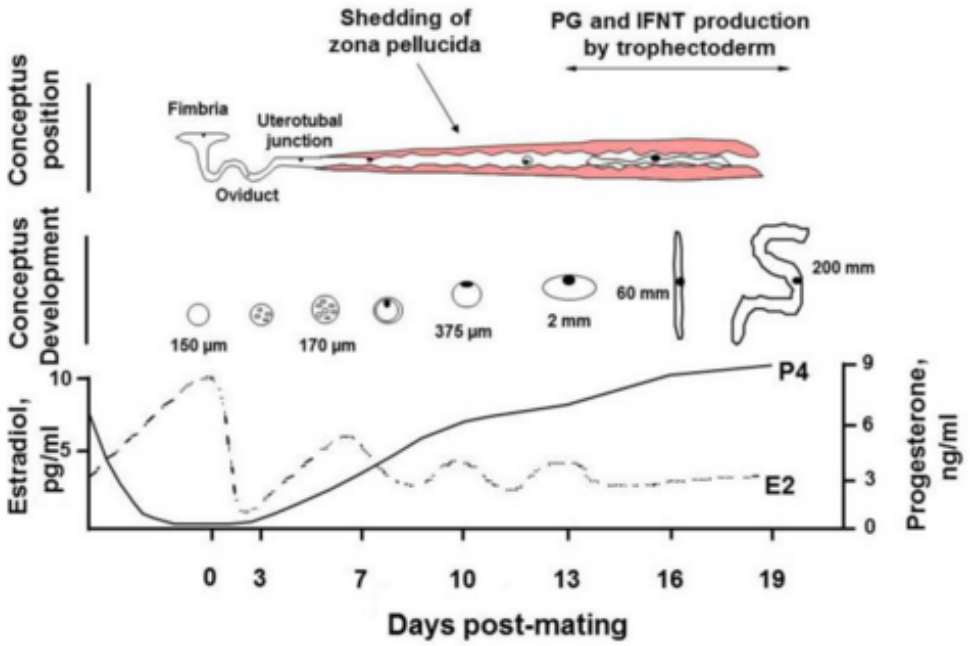

Figure 3. Conceptus position/development and hormone profiles from fertilization to attachment (Spencer, 2013).

Table 1. Timeline for bovine embryo development.

Developmental Event	**Day**
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

MATERNAL RECOGNITON 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 and quality (Ealy and Yang, 1998). The conceptus signal is critical for the prevention of the pulsatile release of $PGF_{2\alpha}$ secretion and the promotion of uterine gland secretory activity through the effects of IFNT. Previous research has reported that there is a significant amount of embryonic loss occurring around maternal recognition of pregnancy (Thatcher, et al., 2001).

Interferon Tau:

Interferon tau is a type 1 interferon with antiviral and antiproliferative properties. It is a glycoprotein with five helixes, composed of 172 amino acids (Li and Roberts, 1994). 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). 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). This causes the formation of STAT-1 transcription homodimers, which 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). Interferon stimulated genes have also been associated with uterine receptivity in cattle, and will be discussed later in further detail.

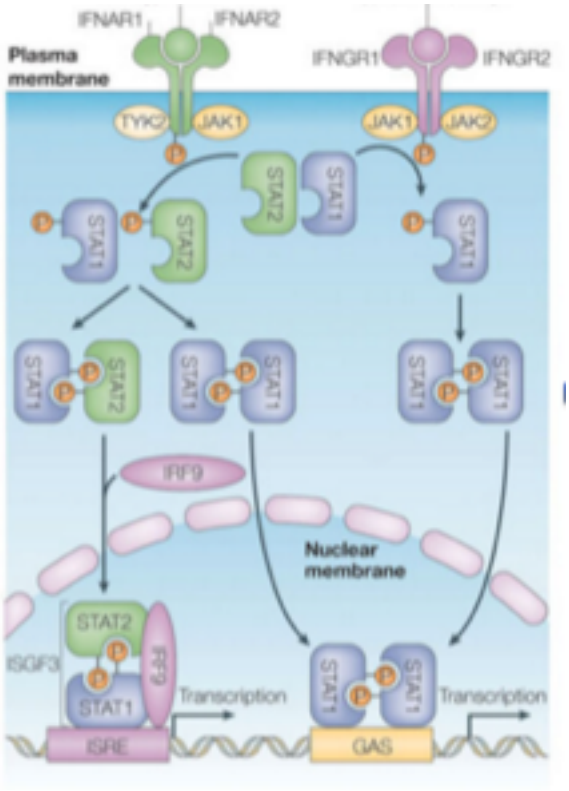

Figure 4. Signal transduction pathway for interferon tau (Decker et al., 2005).

Expression of IFNT begins in the mononuclear cells of the trophectoderm during the early morula, late blastocyst stage (day 6-7 of pregnancy; Kubisch, 1998). Interferon tau works in a paracrine manner on the endometrium, as trophectoderm cells proliferate the greater the contact with the maternal uterine lining, at the same time the IFNT signal is being amplified. Interferon tau mRNA and protein content increases dramatically between days 14-21, this coincides with elongation and trophectoderm proliferation (Ealy and Yang, 1998). Secretion of IFNT decreases rapidly at the time of uterine attachment from day 19-day 21(Ealy and Yang, 1998).

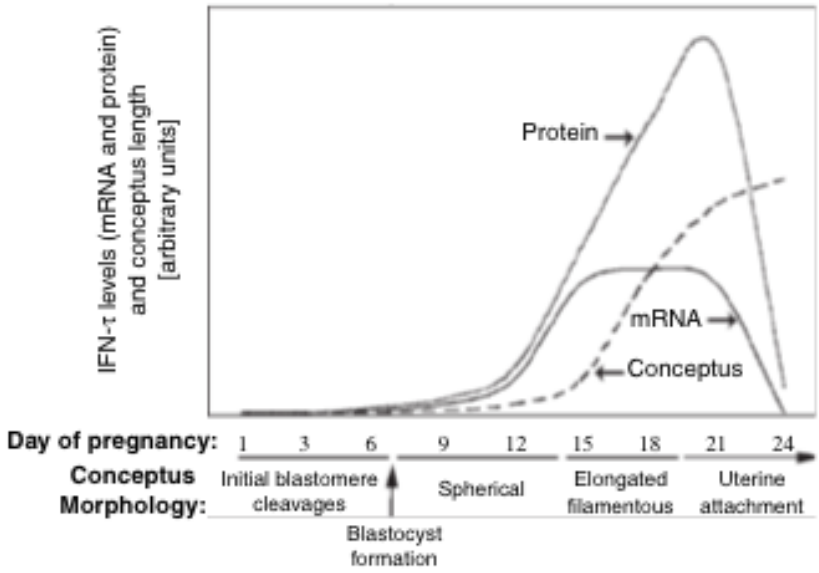

Figure 5. Bovine IFNT expression during early pregnancy (Ealy and Yang, 1998).

Impact of progesterone on IFNT and embryo viability:

An embryo must secrete sufficient amounts of IFNT by day 16 to prevent the regression of the corpus luteum. Maternal progesterone plays a key role in creating a suitable environment for this to happen. Supplementary progesterone enhances conceptus development (Garrett et al., 1988). A delayed rise in circulating concentrations of progesterone resulted in decreased embryo size and decreased quantities of IFNT being produced (Mann and Lamming, 2001). This could result in the failure of an embryo to signal its presence leading to early embryonic mortality. Furthermore, Robinson et al. (2006) also found that elongated embryos (> 10 cm) produced more IFNT compared to smaller embryos, but had similar levels of expression of IFNT mRNA.

Impact of uterine derived factors on IFNT:

Fibroblast growth factor 2 (FGF2) is a mitogen, morphogen, and angiogenic factor that plays a role in embryogenesis (Gospodarowicz, 1991). Expression of FGF2 mRNA was present in early bovine embryos starting at the blastocyst stage (Daniels et al., 2000), and supplementation of FGF2 to CT-1 bovine trophectoderm cells resulted in increased expression of IFNT after 24 hours and secretion of IFNT after 72 hours. Furthermore, supplementation of FGF2 to bovine blastocysts, stimulated IFNT production without stimulating cell numbers (Michael et al., 2006). The bovine endometrium appears to be the source of FGF2, as FGF2 was not detected in the conceptus. Thus FGF2 likely acts as regulator in IFNT production in bovine blastocysts (Michael et al., 2006). Granulocyte-macrophage colony stimulating factor (GM-CSF) is

a cytokine that is produced by the bovine endometrium (De Moraes et al., 1999) and supplementation of the bovine trophectoderm cell line (CT-1) with GM-CSF increased both IFNT secretion and increased bovine blastocyst development in culture (Imakawa et al., 1993).

Transforming growth factor beta (TGF-β) and activin B are expressed in the endometrium and have been reported to be involved in cell proliferation, differentiation, tissue remodeling, decidualisation, and establishment of pregnancy (Jones et al., 2006). In vitro, exogenous (TGF-β) in culture medium facilitated embryo development and promoted blastocyst proliferation and development (Paria & Dey, 1990). Recombinant Activin A treatment to cultured bovine embryos reduced the time taken to reach the blastocyst stage and improved hatching rates (Orimo et al., 1996).

Antiluteolytic effects:

Oxytocin is synthesized and secreted by large luteal cells, and is also secreted by the posterior pituitary (Wathes and Denning, 1992; Hooper, 1996). When oxytocin binds its receptors on the endometrium, it activates a PKC secondary messenger system leading to the pulsatile release of $PGF_{2\alpha}$ (Silvia, 1993). During a normal estrous cycle, estrogen enhances post receptor events mediated by oxytocin to ensure adequate $PGF_{2\alpha}$ pulse frequency to cause luteolysis (Bazer et al., 2012). Interferon tau is secreted by the conceptus, leading to the suppression of the estrogen receptor alpha and a decrease in oxytocin receptor mRNA (Bazer et al., 1997). Furthermore, endometrial oxytocin receptor was decreased in pregnant cows compared to cyclic cows during the luteolytic period (Thatcher et al., 1995). Prostaglandin production was further downregulated in

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). This is critical as COX2 is the rate-limiting enzyme controlling PGE2 and PGF_2 alpha synthesis; it converts arachidonic acid into PGH2 (Arosh et al., 2004). Interferon tau also alters the PGE2: PGF2 alpha ratio in favor of PGE2, which is luteotrophic (Pratt et al., 1977). Interferon tau not only has anti luteolytic effects, but also plays a role in the secretory activity of uterine glands (Godkin et al., 1984). Interferon tau binds to the apical portion of the uterine glands, and promotes protein synthesis, which is critical for preimplantation embryonic survival (Godkin et al., 1984). Ovine IFNT effects on de novo synthesis and endometrial proteins in vitro lead to an increase in secretion of 11 proteins and a decrease in six proteins, many of these have yet to be identified (Vallet and Lamming, 1991).

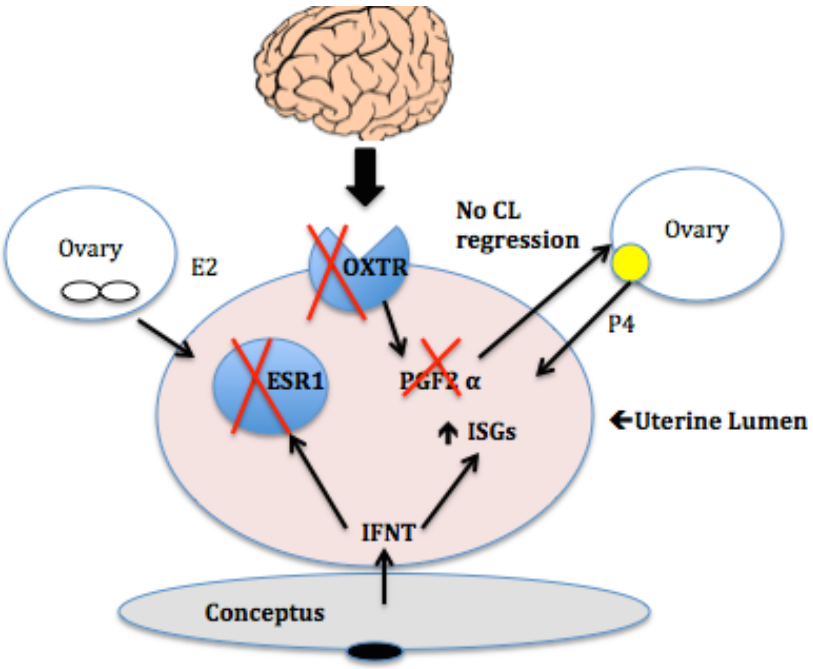

Figure 6. The actions of interferon tau on the signaling and maintenance of pregnancy (maternal recognition of pregnancy). Adapted from: Spencer et al., 2004

UTERINE ENVIRONMENT

Uterine Histotroph:

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 elongation process and endometrial secretions. Alterations in the endometrial transcriptome for uterine receptivity and attachment must occur (Forde and Lonergan, 2012). Interferon tau, progesterone, prostaglandins, and cortisol have been reported to regulate these changes in uterine endometrial gene expression (Brooks et al., 2014). 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, this is known as the uterine histotroph (Gao et al., 2009a). The uterine histotroph contains uterine epithelium secretions and molecules that are transported into the uterine lumen to provide nutrients for the developing conceptus, and it is composed of a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, and glucose (Gao et al., 2009a). A suboptimal uterine environment often leads to poor development and embryonic mortality.

Between days 15 and 50 the endometrial glands undergo significant hyperplasia, followed by hypertrophy to allow for an increase in surface area and maximal production of uterine histotroph (Moffatt et al., 1987). Nutritional deficiencies during early pregnancy have been reported to have significant effects on pregnancy outcome and postnatal health and growth (Dunn and Moss, 2014). Grey et al. (2001) placed uterine

gland knockout sheep phenotype (UGKO) with fertile rams; however, no pregnancies were ever determined on day 25 after insemination. When day 7 blastocysts were transferred into synchronized control and UGKO ewes, pregnancy was established in control ewes, but not UGKO ewes (Grey et al., 2001). This shows that endometrial glands and their secretions are necessary for conceptus development and pregnancy establishment. Progesterone has been reported to alter tight and adheren junctions in the uterus which impact uterine histotroph transport and sequestration into the uterine lumen during early pregnancy (Satterfield et al., 2007). Tight junction and adheren associated proteins were moderately to abundantly present in the endometrium on day 10 of pregnancy, however at day 12 these proteins decreased resulting in leaky channels to allow nutrient exchange between the conceptus and mother (Satterfield et al., 2007). While we do recognize its importance in embryo survival, little is known about compositional differences among cyclic and pregnant animals. Glucose, amino acids, glutathione, calcium, and sodium have been reported to be increased in uterine luminal fluid of pregnant ewes compared to cyclic ewes between day 10 and 16 of the estrous cycle (Gao et al., 2009a). The increase in these nutrients among pregnant animals indicates that conceptus regulatory molecules can stimulate nutrient transport into the uterine lumen.

Glucose:

Glucose and glutamine are known to regulate trophoblast proliferation and function. They are the main energy sources used by a conceptus for 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 to the uterus via glucose transporters (Leese and Barton, 1984; Pantaleon and Kay, 1998). Glucose can be used by the conceptus to make glycogen, nucleic acids, proteins, and lipids during the peri-implantation period (Gao et al., 2009a). In sheep, total glucose content in uterine luminal fluid 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. In mice, glucose at a blastocyst level have been reported to influence apoptotic REDOX regulating pathways, thus, mouse embryos that lacked certain glucose transporters failed to develop due to increased apoptosis (Frolova and Moley, 2011).

Transport of glucose across the plasma membrane is mediated by facilitative and/or sodium dependent transporters. Facilitative transporters work bidirectionally, and are energy independent (Wood and Trayhum, 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; Zhao and Keating, 2007). Facilitative and sodium dependent transporters work together to optimize glucose transport into the uterine lumen where it can be utilized for the growth and development of a conceptus (Gao et al., 2009b). Circulating progesterone concentrations during diestrus has been reported to influence glucose transporter expression (Shimizu et al., 2010).

The SLC2A1 glucose transporter is ubiquitous in humans, and is found in high abundance in the bovine conceptus (Navarrete et al., 2000). Through immunohistochemistry the SLC2A1 transporter has been localized mainly to glandular and luminal epithelial cells (Franca et al., 2015). It has also been found in the extraembryonic endoderm and trophectoderm of the conceptus between days 14 and 20 of pregnancy (Gao et al., 2009b). A study done by Gao et al. (2009b) found that SLC2A1 mRNA was increased in pregnant ewes starting at day 10 compared to cyclic ewes. In ovariectomized ewes that were treated 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 Km and plays a critical role in embryonic development that cannot be compensated for by the overexpression of SLC2A1 (Gangly et al., 2007). Mice lacking the SLC2A3 gene had restricted fetal growth and failed pregnancies (Gangly et al., 2007). Expression of SLC2A3 mRNA has also been detected in the extraembryonic endoderm and trophectoderm 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. Additionally, it has been found in the trophectoderm of the cow, rabbit, rat, and mouse blastocyst stage embryos (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) found that SLC2A4 expression in the

extraembryonic endoderm and trophectoderm of a conceptus remained constant in cyclic ewes, but increased in pregnant ewes between day 10 and 18 of pregnancy. Treatment of ovariectomized ewes with progesterone tended to increase mRNA for SLC2A4 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 to transport sodium, urea, and water (Wright and Turk, 2004). Among cyclic ewes, expression of SLC5A1 mRNA increased between days 10 and 14 of pregnancy, but decreased by day 16 (Gao et al., 2009b). Similarly, pregnant ewes had an increase in endometrial expression of SLC5A1 mRNA between days 10 and 12 of the cycle, but 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).

Glucose transporters are found throughout the body in various tissues (see table 2), 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 between cyclic and pregnant ruminants (Gao et al., 2009). 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.

Table 2. Summary of glucose transporter properties.

Protein	Major Sites of Expression	Functions
Facilitative Glucose Transporters		
SLC2A1	Ubiquitous distribution in tissue	Basal glucose uptake, transport across blood tissue barriers
SLC2A2	Liver, islets, kidney, small intestine	High capacity, low affinity transport
SLC2A3	Brain and nerve cells	Neuronal transport
SLC2A4	Muscle, fat, and heart, uterus	Insulin regulated transport in muscle and fat
SLC2A5	Intestine, kidney, testis, uterus	Transport of fructose
SLC2A6	Spleen, leukocytes, brain	
SLC2A7	Small intestine, colon, testis	Transport of fructose
SLC2A8	Testis, blastocyst, brain, muscle, adipocytes	Fuel supply of mature spermatozoa, insulin responsive transport in blastocyst
SLC2A9	Liver, kidney	
SLC2A10	Liver, pancreas	
SLC2A11	Heart, muscle	Muscle specific, fructose transport
SLC2A12	Heart, prostate, mammary gland	
Sodium/Glucose Cotransporters		
SLC5A1	Kidney, intestine, uterus	Glucose reabsorption in intestine and kidney
SLC5A2	Kidney	Low affinity and high selectivity for glucose
SLC5A3	Small intestine, skeletal muscle	Glucose activated sodium channel

Adapted from: Zhao and Keating, 2007

Changes in uterine gene expression:

In response to pregnancy, genes involved in cell adhesion, endometrial remodeling, and modulation change within the uterus. Spencer et al. (2008) found that more genes were altered in the intercaruncular region of the endometrium compared to the caruncular region, which is most likely due to the spread of glandular epithelial cells in this area. In particular, interferon stimulated gene (ISG) expression in the uterine endometrium is upregulated as a result of IFNT secretion by the conceptus (Yankey et al., 2001). Interferon stimulated genes are hypothesized to regulate uterine receptivity to implantation as well as survival and growth of the conceptus (Kim et al., 2012). There are more than 100 known ISGs, but not all are expressed equally among pregnant and cyclic animals (Samuel, 1991). 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 synthestase (OAS1) (Johnson et al., 2001)] in peripheral blood mononuclear cells during pregnancy.

Madsen et al. (2013) found that the expression of ISG15, MX2, and OAS1 was increased on day 17, 19, and 21 among pregnant animals compared to nonpregnant animals. 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. While, Green et al. (2010) reported that MX2 and ISG15 were greater among pregnant cows on day 18 and 20. Parity status also 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).

Early pregnancy detection is key for efficient reproductive management. Enzyme-linked immunosorbent assays (ELISAs) for pregnancy specific protein B and pregnancy-associated glycoproteins (PAGs) are used to detect pregnancies > 28 days after AI (Green et al., 2005), transrectal ultrasonography is utilized to detect pregnancy beginning around day 28, and rectal palpation can be performed beginning around day 35 (Kastelic et al., 1988); however, these options do not give us the opportunity to identify which cows are pregnant before the next expected estrus (day 21). Further research is needed to determine if measuring ISGs in maternal blood before day 18 is accurate enough to determine pregnancy status at this early stage, if this was made possible open cattle could be resynchronized for AI on day 21 after the first insemination (Lucy et al., 2004).

PROGESTERONE

The role of progesterone in the maintenance of pregnancy:

The corpus luteum is the main source of progesterone, and it is essential for the maintenance of pregnancy (McDonald et al., 1952). Reduced luteal function is often associated with infertility in ruminants (Gaverick and Smith, 1986). Adequate progesterone secretion is necessary for stimulating endometrial secretions, embryo growth and development, and maintenance of pregnancy by altering endometrial gene expression (Garrett et al., 1998). The postovulatory rise of progesterone is associated with an increase in pregnancy success (Forde et al., 2009). Pregnant cows have been reported to have greater progesterone concentrations as early as day 6 after artificial

insemination compared to open cows (Mann et al., 1999), while Funston et al. (2005) reported that augmenting progesterone had no effect on pregnancy rates in cattle.

It has been demonstrated that the magnitude of progesterone concentrations increasing following ovulation contributes to conceptus development and survival; however, the mechanism is not well understood (Shelton et al., 1990). It is suspected that progesterone induces changes is endometrial gene expression that leads to changes in uterine histotroph composition (Spencer et al., 2008). Bartol et al. (1981) determined that protein accumulation within the uterine lumen is related to length of progesterone stimulation. Supplementing pregnant cows with progesterone for four days has been reported to enhance embryo development by increasing protein secretion by the uterus on day 5 and 14 post ovulation (Garrett et al., 1988). It has also been documented that insertion of intravaginal progesterone device between day 5 and 9 of the cycle increases embryo length 16 days post artificial insemination, while supplementation between day 12 and 16 did not increase embryo length (Mann et al., 2006).

EMBRYO LOSS

In cattle, fertility is dependent on the following two criteria: animals need to be cycling naturally or by synchronization protocol and 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). Balancing the embryo-maternal environment is critical for avoiding reproductive failure (Spencer et al., 1996). Inadequate changes in endometrial gene expression can be detrimental to the survival of an embryo (Lonergan et al., 2006).

Some causes for uterine deficiencies include: failure of ovarian steroids to regulate factors in the endometrium, dysregulation of genes and receptors responsible for nutrient delivery for the development and attachment of conceptus, and the inability of the uterus to respond to the pregnancy signal from the conceptus (Bridges et al., 2014). Miller and Moore (1976) conducted an experiment using ovariectomized sheep and found that the sequence of steroid exposure is critical for embryo survival (progesterone priming, estradiol, and then progesterone after fertilization), and elevated concentrations of estradiol during the preovulatory period were critical for the maintenance of pregnancy (Madsen et al., 2015). Inadequate estradiol production before ovulation results in alterations in the expression and localization of uterine genes and proteins that are necessary for uterine function and embryo development (Bridges et al., 2014).

Progesterone following ovulation also plays a critical role in the synchrony between the uterine environment and the embryo (Rowsen et al., 1972). Decreased concentrations of preovulatory estradiol have been associated with premature luteolysis (Mann and Lamming, 2000), and an inadequate uterine environment (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). Furthermore, during the time period between maternal recognition of pregnancy and attachment, cows that received estradiol only lost 35% of their existing pregnancies, while control animals lost 75% of their existing pregnancies (Madsen et al., 2015).

Environmental factors such as ambient temperature and humidity have been correlated with seasonal decreases in pregnancy success; in particular heat stress around

the time of insemination can decrease pregnancy rates (Gwazdauskas et al., 1973; Ingraham et al., 1974; Ealy et al., 1993). Heat stress affects the degree of dominance of the selected follicle by a reduction in the steroidogenic capacity of theca and granulosa cells leading to a decrease in estradiol production (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). Due to high ambient temperatures, an increase in uterine temperature may result in an increased conceptus metabolic rate leading to changes in nutrient uptake (Biggers et al., 1987). This along with decreased nutrient secretion by the uterus may result in abnormal conceptus development or death (Biggers et al., 1987). Exposure to a period of hyperthermia for a 10 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 (Puteney et al., 1989). A possible explanation for this is thermal induction of chromosomal abnormalities while the oocyte is resuming meiosis (Putney et al., 1989).

SUMMARY

An adequate uterine environment is necessary for maternal and conceptus communication. The uterine environment must provide appropriate endocrine conditions to support a developing conceptus and for maternal recognition of pregnancy to occur around day 16. Previous research has established that preovulatory estradiol influences follicular growth, oocyte maturation, sperm transport, uterine environment, and embryo survival. Elevated preovulatory concentrations have been reported to have greater

pregnancy rates compared to nonestrus animals. Therefore, the next chapter will focus on the role of preovulatory estradiol on the uterine environment and conceptus survival from fertilization to maternal recognition of pregnancy.

CHAPTER II

THE EFFECTS OF PREOVULATORY ESTRADIOL ON THE UTERINE ENVIRONMENT AND CONCEPTUS SURVIVAL FROM FERTILIZATION TO MATERNAL RECOGNITION OF PREGNANCY

ABSTRACT

The role of preovulatory estradiol on the uterine environment and embryo survival has not been well established in cattle. The objective of this study is to determine the effects of preovulatory estradiol on the uterine environment and conceptus survival up until maternal recognition of pregnancy. Beef cows/heifers were synchronized with the CO-Synch protocol and AIed (d 0). Cows were classified by estrus expression (estrus and no estrus). Uteri were flushed to collect d 16 conceptuses nonsurgically (Rep 1; n = 29), or following slaughter (Rep 2; n = 37). Uterine luminal fluid (ULF) was analyzed for protein, glucose, and interferon tau (IFNT) concentrations. For rep 1, total cellular RNA was extracted from blood leukocytes (d 16) to measure the expression of interferon-stimulated genes (ISG): ISG-15, OAS-1, and MX2. For rep 2, total cellular RNA was extracted from caruncular (CAR) and intercaruncular (INCAR) endometrial tissue to measure relative abundance of glucose transporters (SLC2A1, SLC2A4, SLC2A5, and SLC5A1). There was no difference in conceptus recovery rate between estrus and no estrus cows ($P = 0.20$; 48% vs 29%). There was no difference ($P > 0.20$) in d 16 expression of ISG-15, OAS-1, or MX2 between estrus and no estrus cows, nor a difference between cows with or without a conceptus. There were no differences in IFNT concentrations in the ULF among estrus and no estrus cows ($P =$

0.42), nor a difference among cows that did and did not have a conceptus recovered (P = 0.71). Cows that exhibited estrus had greater glucose concentrations in ULF (P = 0.05; 51 ± 1.86 vs 45 ± 1.92 mg/dL). Cows in which a conceptus was recovered had greater concentrations of protein in the ULF (P = 0.05; 2643 ± 585 mg/mL vs 1126 ± 463 mg/mL). In both CAR and INCAR endometria, animals that exhibited estrus had greater abundance of SLC2A1 (P = 0.05) and SLC5A1 (P = 0.04) mRNA. Presence of a conceptus tended to increase (P = 0.10) abundance of SLC5A1 mRNA in INCAR tissue, but had no effect (P > 0.13) on abundance of SLC2A1 mRNA in either tissues or SLC5A1 mRNA in CAR tissue. In CAR tissue, cows from which a conceptus was recovered had decreased SLC2A4 mRNA abundance (P = 0.05), but there was no effect of estrus (P = 0.14) and no effect of estrus or conceptus in ICAR tissue. There was no difference in SLC2A5 mRNA abundance between estrus and no estrus cows (P > 0.20), nor between conceptus and no conceptus cows (P > 0.58) in CAR or INCAR tissue. In summary, conceptus recovery rates, IFNT, and protein concentration in ULF did not differ between cows that did or did not exhibit estrus, but ULF glucose concentration was greater in cows that exhibited estrus. Protein concentration in ULF was greater in cows from which a conceptus was recovered. Thus, there was no indication of increased conceptus survival to d 16 of pregnancy based on estrus expression, but glucose and protein in the ULF did change based on estrus expression and conceptus presence, possibly due to changes in glucose transporter expression.

INTRODUCTION

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 estrus behavior and an LH surge resulting in ovulation (Chenault et al., 1975). Preovulatory estradiol impacts follicular growth, oocyte maturation, sperm transport, uterine environment, and embryo survival (Pohler et al., 2012). Cows in standing estrus within 24 hours of fixed-time AI have been reported to have greater pregnancy success than nonestrus cows (Perry et al., 2005), and cows that exhibit estrus have also been reported to have increased embryo survival to day 30 of gestation (Jinks et al., 2013).

Miller et al. (1977) determined the impact of giving large or small doses of exogenous estradiol to ovariectomized sheep. Following embryo transfer on day 4, animals that were given a small dose of estradiol had decreased uterine weight, total protein content, progesterone and estrogen receptor within the uterus, and pregnancy success compared to ewes given a larger dose. Madsen et al. (2014) 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 embryo transfer were more likely to maintain pregnancy to day 29 compared to cows not exposed to estradiol. These studies indicate the beneficial role estradiol has in providing a uterine environment suitable for a developing embryo.

The uterine histotroph is the uterine epithelium secretions and molecules that are transported into the uterine lumen to provide nutrients for the developing conceptus. It is

composed of a mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, and glucose (Gao et al., 2009a). Glucose in particular regulates trophoblast proliferation and function (Wen et al., 2005). It is a major energy source used by the conceptus for growth and development. Glucose is delivered into the uterus where it can be utilized by the conceptus via glucose transporters (Leese and Barton, 1984; Pantaleon and Kay, 1998). In sheep, total glucose content in uterine flushes has been reported to increase six fold between days 10 and 15 of gestation (Gao et al., 2009a; Flechon et al., 1986). Thus the uterine histotroph is important for conceptus growth and development, especially during the time at which the embryo is undergoing morphological changes from spherical to filamentous (Bazer, 1975). Grey et al. (2001) placed uterine gland knockout sheep (UGKO) with fertile rams, no pregnancies were ever identified on day 25 after insemination. Blastocyst growth into an elongated bovine conceptus has not been able to be duplicated in vitro (Betteridge and Flechon, 1988). Both of these studies demonstrate that endometrial glands and their secretions are required for conceptus development and pregnancy establishment.

Among beef cattle, fertilization rates are estimated to be 90% with calving rates of 55%. This suggests approximately 35% embryonic mortality, with ~70-80% of this embryonic loss occurring between day 8 and 16 post AI (Disken et al., 2006). Therefore, the objective of this current study was to determine the impact of preovulatory estradiol during a fixed time AI protocol on uterine environment and conceptus survival from fertilization to maternal recognition of pregnancy, during a time in which early embryonic loss is most prevalent.

MATERIALS AND METHODS

Animals and Treatments:

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee. Angus crossed beef cows/heifers (Rep 1: n=29, Rep 2: n=37) at the SDSU Beef Breeding Unit were synchronized with a CO-Synch protocol. GnRH was administered (100 μg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on d -7, 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.

Ultrasonography and Estrus Detection:

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 cows 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. Estrus was monitored visually on d 0 through d 3 with the aid of EstroTect patches (Western Point, Inc., Apple Valley, MN). Cows that had greater than half of the patch scratched off were characterized as exhibiting standing estrus.

Blood Sampling and Radioimmunoassay:

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, d -1, d 0, then every other day up until d 16. For the second replicate, blood was collected on d -2, d -1, d 0, then every other day up until d 15. Blood was allowed to coagulate at room temperature prior to centrifugation at 1,200 x g for 30 minutes at 4°C. Serum was collected and stored at -20°C until radioimmunoassays were performed. Radioimmunoassays (RIA) were performed on serum samples to determine circulating progesterone concentrations using the methods described by 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. Serum concentrations of estradiol were determined within replicate by a single assay using the methods described by 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 flushing (Replicate 1):

In replicate 1, uteri were flushed nonsurgically 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; cows were flushed with 100 ml 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 to determine if a conceptus was present or not. More saline was added, if no conceptus was recovered, and this additional media was collected separately.

Reproductive tract processing (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 30 ml of saline, and then massaged for equal fluid distribution in the uterus. The uterine flush was then collected in a 50ml conical tube, and was further analyzed under the microscope to determine if a conceptus was present. The ipsilateral uterine horn was cut anterior to the bifurcation, and then cut open to expose intercaruncular and caruncular tissue, which was then dissected for further analysis of glucose transporter expression.

RNA extraction of maternal leukocytes and RTPCR (Replicate 1):

For replicate 1, d 7 and d 16 plasma and blood leukocytes were collected by jugular venipuncture in a 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA). The blood was then centrifuged at 1,200 x g for 30 minutes at 4°C. Blood leukocytes were collected, Tri-Reagent was added and mixed in 1:1 volumetric ratio (Molecular Research Center, Inc., Cincinnati, OH), and stored at -80°C until RNA isolation. SV Total RNA Isolation System (Promega, Madison, WI) was used to isolate RNA 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. Isolated RNA samples were then stored at -80°C. The RNA collected on d 7 and d 16 were diluted to 40 ng/μl and RTPCR was performed in

duplicate using iScript One-Step RT-PCR Kit with SYBR Green (BioRad) and Stratagene MX 3000P QPCR machine. Expression of ISG15, MX2, and OAS1 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 μM. Each plate had negative controls to assure no background contamination. The PCR program was 30 min at 42°C and 10 min at 95°C for inactivation of reverse transcriptase. Transcription was then followed by 30 sec at 95°C for melting, 1 min at 60°C for annealing, and 1 min at 72 °C for extension for 40 cycles. Primers were previously published for GAPDH (Han et. al., 2006), ISG15, MX2, and OAS1 (Green et al., 2010).

Table 3. Genes, primer sequences, and product size for genes amplified during RTPCR.

Gene	Primer	Primer Sequence	Product size	Reference
ISG15	Forward Reverse	5'-CAGCCAACCAGTGTCTGCAGAGA-3' 5'-CCAGGATGGAGATGCAGTTCTGC-3'	293	Green et al., 2010
MX2	Forward Reverse	5'-CTTCAGAGACGCCTCAGTCG-3' 5'-TGAAGCAGCCAGGAATAGTG-3'	232	Green et al., 2010
OAS1	Forward Reverse	5'-ACCCTCTCCAGGAATCCAGT-3' 5'-GATTCTGGTCCCAGGTCTGA-3'	199	Green et al., 2010
GAPDH	Forward Reverse	5'-GATTGTCAGCAATGCCTCCT-3' 5'-GGTCATAAGTCCCTCCACGA-3'	94	Han et al., 2006

RNA extraction of endometrial tissue and RTPCR (Replicate 2):

Caruncular and intercaruncular endometrial tissue samples were homogenized prior to RNA isolation. RNA was extracted using the Qiagen RNeasy Plus Mini Kit (Austin, Texas) 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. The RNA samples were then stored at -80°C. The RNA was diluted to 70 ng/μl and RTPCR was performed in duplicate using iScript One-Step RT-PCR Kit with SYBR Green (BioRad) and Stratagene MX 3000P QPCR machine. Expression of SLC2A1, SLC2A4, SLC2A5, and SLC5A1 was measured using the primers in Table 4, and GAPDH was used as a reference gene. All of the primers were diluted to a concentration of 10 μM. 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, 30 sec at 60°C for annealing for 40 cycles. All CVs were less than 21%. Amplicons were electrophoresed on 2% agarose gels to determine product size and were verified for identity by sequencing (Iowa State Genomics Core).

Table 4. Genes, primer sequences, and product size for genes amplified during RTPCR.

Gene	Primer	Primer Sequence	Product size	Reference
SLC2A1	Forward Reverse	5'-TAACCGCAACGAGGAGAACC-3' 5'-AGAAAACAGCGTTGATGCCG-3'	227	Rozen and Skaletsky, 2000
SLC2A4	Forward Reverse	5'-AGTTCCTAAGACAAGATGCCG-3' 5'-AGAATACGCCAAGGACCAAG-3'	103	Franca et al., 2015
SLC5A1	Forward Reverse	5'-TCACCGCCCTTTACACAATC-3' 5'-CACCATACCCTCCCACTTC-3'	132	Franca et al., 2015
SLC2A5	Forward Reverse	5'-CCATTCATCCAAGTGGGCCT-3' 5'-GTCGACGGTGGAAACTCCTT-3'	203	Rozen and Skaletsky, 2000
GAPDH	Forward Reverse	5'-GATTGTCAGCAATGCCTCCT-3' 5'-GGTCATAAGTCCCTCCACGA-3'	94	Han et al., 2006

Uterine luminal fluid (ULF) analyses:

Uterine luminal fluid underwent a 1:100 volumetric dilution. Glucose Liquicolor Kit (Boerne, TX) was used to determine glucose concentrations according to the manufacturer's instructions. Total protein concentration in the ULF was determined using the Micro BCA Protein Assay Kit (Rockford, IL) according to the manufacture's directions. Interferon tau concentration was determined for all ULF samples in Thomas Spencer's laboratory at the University of Missouri using a semi-quantitative western blot method (Satterfield et al., 2006).

Statistical Analysis:

Circulating progesterone and estradiol concentrations were analyzed by the MIXED procedure of SAS using repeated measures. Conceptus recovery rate, protein, glucose, and IFNT concentrations were analyzed using the MIXED procedure in SAS. The statistical model included: treatment (estrus or no estrus) (conceptus or no conceptus), time, and their interaction. Day 16 ISG expression and glucose transporter expression were analyzed using the MIXED procedure in SAS, and both were corrected for by the expression of GAPDH.

RESULTS

Hormone Profiles.

Serum estradiol concentrations during the preovulatory period were different between estrus and no estrus cows ($P < 0.01$; Figure 7); however, serum estradiol concentrations did not differ ($P = 0.47$) between cows that did and did not have a conceptus recovered on day 16 of pregnancy (Figure 8). There was no difference in serum progesterone concentrations between cows that did and did not exhibit estrus ($P = 0.41$; Figure 9), nor was there a difference among cows that did and did not have a conceptus recovered on day 16 of pregnancy ($P = 0.93$; Figure 10).

Conceptus survival to d 16:

In replicate 1, day 16 ISG expression of ISG15, MX2, and OAS1 were not different ($P > 0.20$) between estrus and no estrus cows (Figure 11), nor was there a difference ($P > 0.20$) between cows that did and did not have a conceptus recovered from them (Figure 12). Interferon tau concentrations in the ULF did not differ ($P = 0.42$) between estrus and no estrus cows, nor was there a difference between cows that did and did not have a conceptus recovered from them ($P = 0.71$; Figure 13). There was no difference in conceptus recovery rates between estrus and no estrus cows ($P = 0.20$; 48% vs 29%), nor was there a difference in recovery rates between replicates ($P = 0.46$; 44% vs 33%; Figure 14).

Uterine Flush Media Analysis:

Total protein concentration in ULF was greater ($P < 0.05$) among cows that a conceptus was recovered from (Figure 15). However, there was no difference ($P = 0.36$) in total protein concentration among cows that did and did not exhibit estrus (Figure 15). Glucose concentration in ULF did not differ among cows that did and did not have a conceptus recovered ($P = 0.29$), however cows that exhibited estrus had greater glucose concentrations in their ULF compared to cows that did not exhibit estrus ($P = 0.05$; Figure 16).

Glucose Transporter Expression:

In caruncular endometria, cows that exhibited estrus had greater abundance of SLC2A1 ($P = 0.05$) and SLC5A1 ($P = 0.04$) mRNA (Figure 17), but there was no difference in SLC2A1 and SLC5A1 mRNA abundance between conceptus and no conceptus animals ($P > 0.15$; Figure 18). Cows from which a conceptus was recovered had decreased SLC2A4 mRNA abundance ($P = 0.04$; Figure 18), while there was no difference in SLC2A4 abundance between cows that did and did not exhibit estrus ($P = 0.15$; Figure 17). There was no difference in SLC2A5 mRNA abundance between estrus and no estrus cows ($P = 0.91$; Figure 17), nor between conceptus and no conceptus cows ($P = 0.58$; Figure 18) in caruncular tissue.

In intercaruncular tissue, there was no difference in SLC2A4 and SLC2A5 mRNA abundance between estrus and no estrus cows, nor between conceptus and no conceptus cows ($P > 0.20$; Figure 19, 20). Presence of a conceptus tended to increase ($P = 0.10$) abundance of SLC5A1 mRNA in intercaruncular tissue (Figure 20), while cows that

exhibited estrus had increased SLC5A1 mRNA abundance ($P < 0.01$; Figure 19). There was no difference in SLC2A1 abundance between conceptus and no conceptus cows ($P = 0.17$; Figure 20), while cows that exhibited estrus had greater SLC2A1 abundance in intercaruncular tissue ($P < 0.05$; Figure 19).

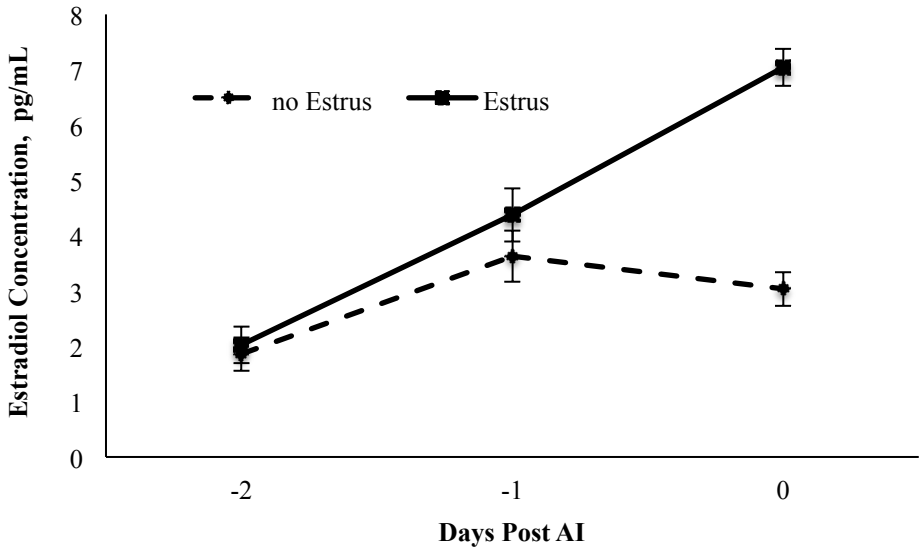

Figure 7. Circulating estradiol concentrations (pg/mL) in estrus and no estrus cows on d-2, d-1, and d0. There was an effect of estrus, time, and estrus by time ($P < 0.01$) on serum estradiol concentrations between cows that did and did not exhibit estrus.

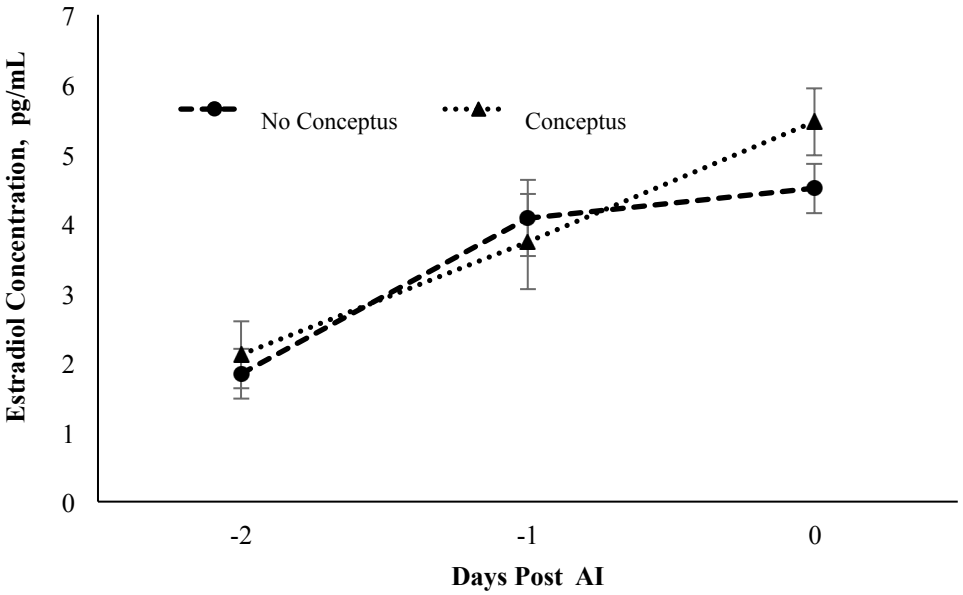

Figure 8. Circulating estradiol concentrations (pg/mL) on d-2, d-1, and d0 for cows that did and did not have a d16 conceptus recovered from them. There was no difference in estradiol concentrations between the conceptus and no conceptus cows ($P = 0.47$), while there was an effect of time ($P < 0.01$).

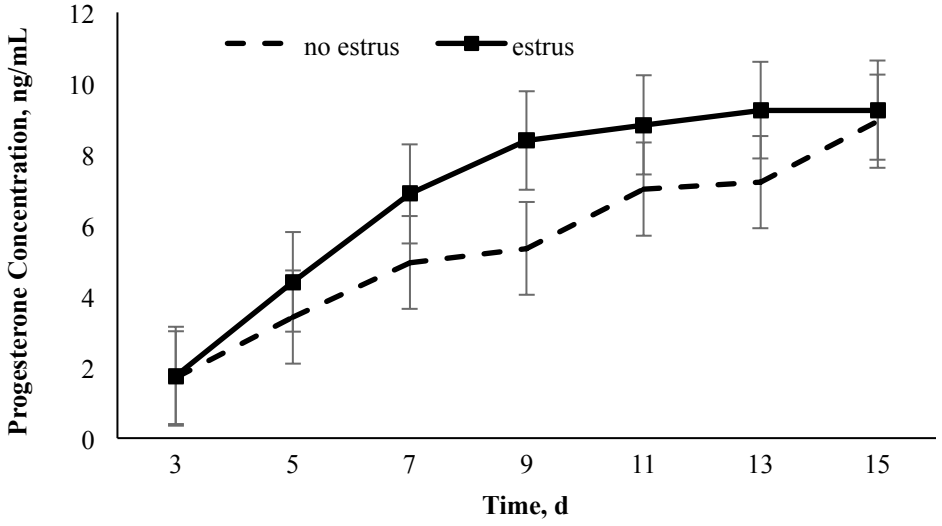

Figure 9. Circulating concentrations of progesterone (ng/mL) among estrus and no estrus cows every other day from d 3 to d 15. There was no difference in circulating progesterone concentrations among estrus and no estrus cows ($P = 0.41$), while there was an effect of time ($P < 0.01$).

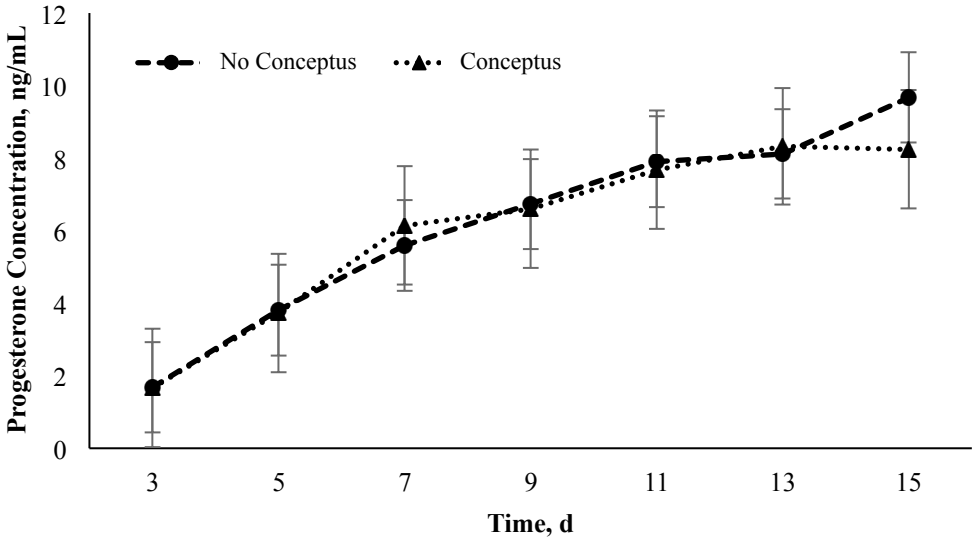

Figure 10. Circulating progesterone concentrations (ng/mL) among animals that did and did not have a d16 conceptus recovered from them. There was no difference in progesterone concentrations between the conceptus and no conceptus cows ($P = 0.93$), while there was an effect on time ($P < 0.01$).

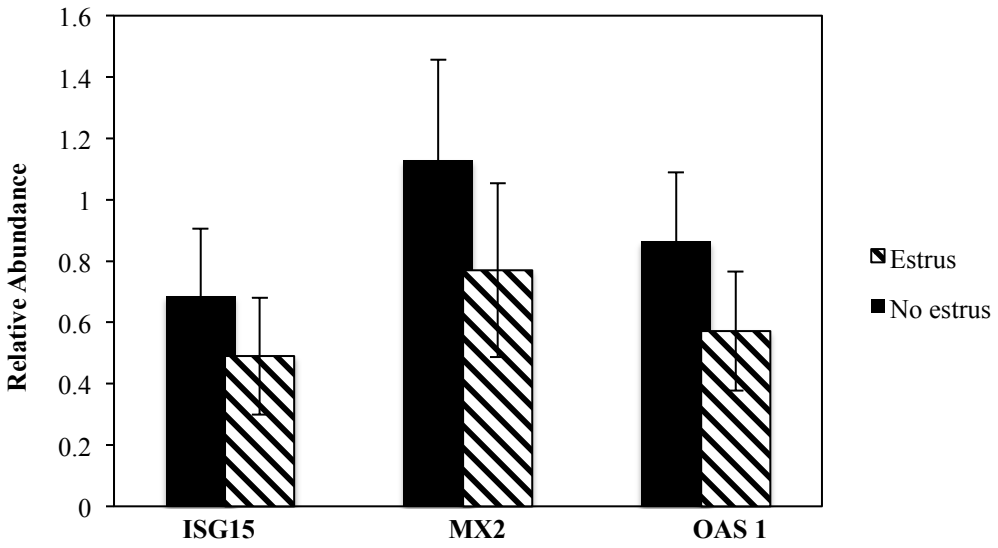

Figure 11. Day 16 interferon stimulated gene expression (ISG15, MX2, OAS1) among cows that did and did not exhibit estrus. There were no differences in ISG15, MX2, and OAS1 gene expression between estrus and no estrus cows ($P > 0.20$).

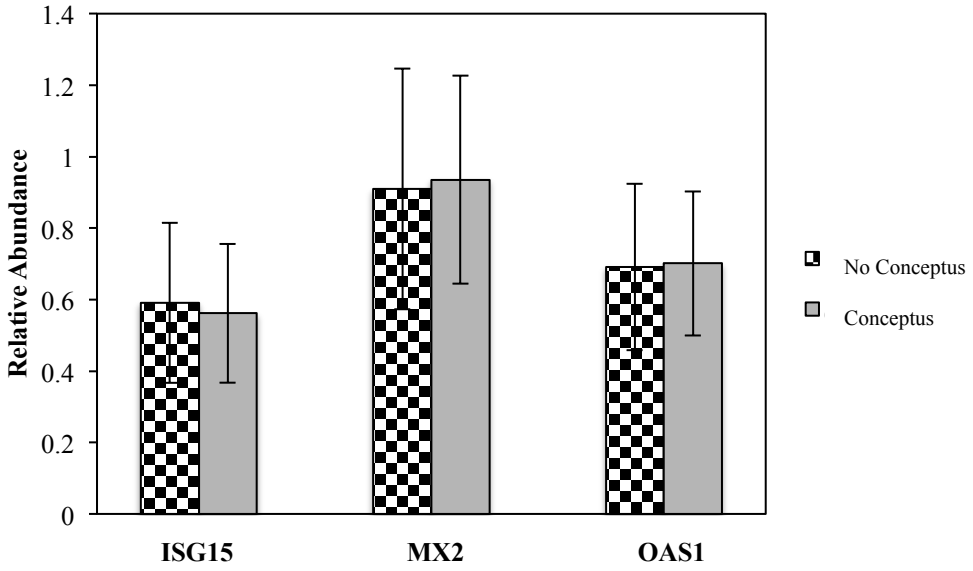

Figure 12. Day 16 interferon stimulated gene expression (ISG15, MX2, OAS1) among cows that did and did not have a conceptus recovered from them. There were no differences in ISG15, MX2, and OAS1 gene expression between conceptus and no conceptus cows ($P > 0.20$).

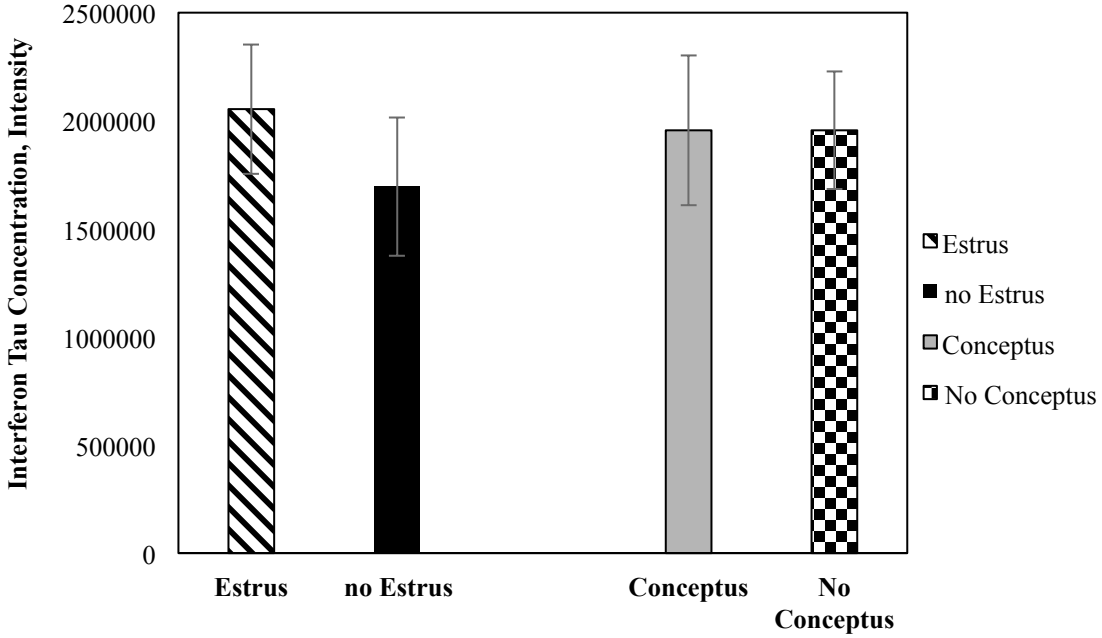

Figure 13. Interferon tau concentration (intensity) in ULF for cows that did/did not exhibit estrus and cows that did/did not have a conceptus recovered from them. There were no differences in interferon tau concentration when comparing estrus/no estrus cows ($P = 0.42$) or conceptus/no conceptus cows ($P = 0.71$).

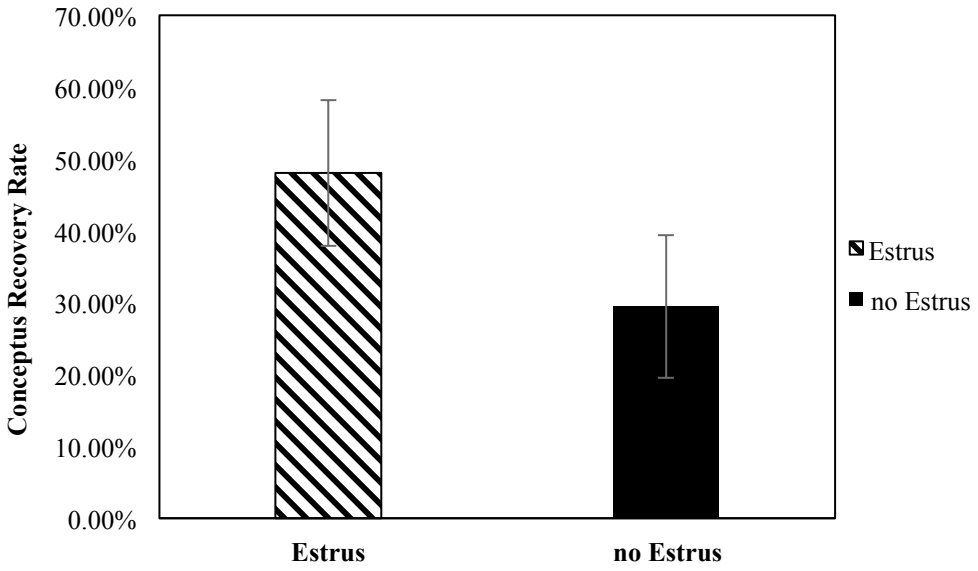

Figure 14. Conceptus recovery rates did not differ between estrus and no estrus cows (P = 0.20; 48% vs 29%). Nor was there a difference between replicate 1 and 2 (P = 0.46; 44% vs 33%).

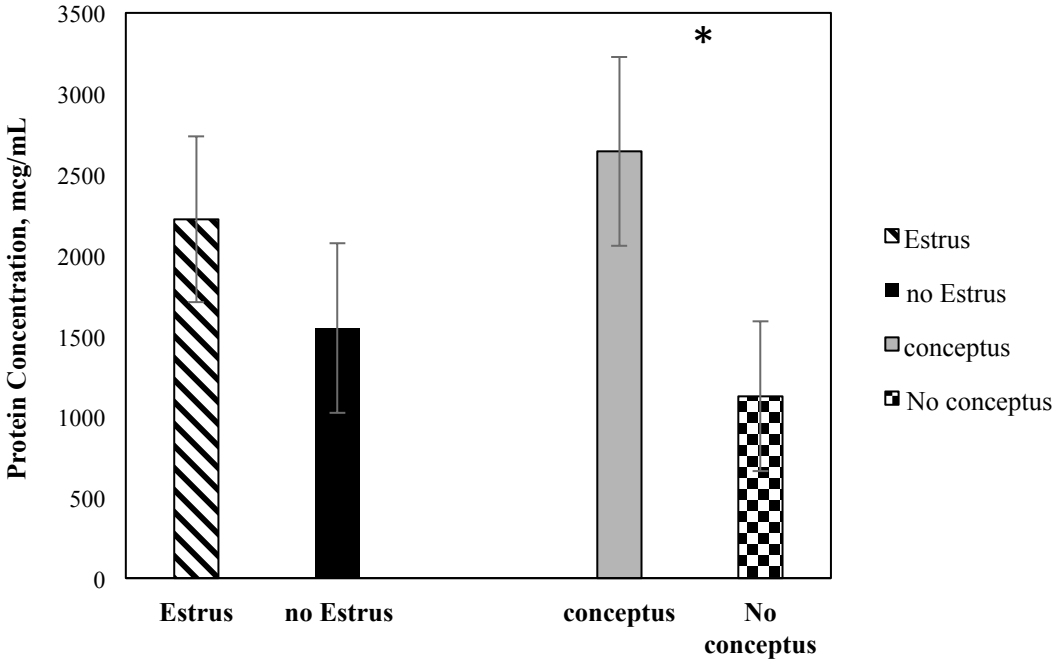

Figure 15. Total protein concentration (mcg/mL) in ULF for cows that did/did not exhibit estrus and cows that did/did not have a conceptus recovered from them. There was no difference in protein concentration among estrus and no estrus cows ($P = 0.36$), while cows that had a conceptus recovered from them had greater protein content in the ULF compared to the no conceptus cows. * $P = 0.05$

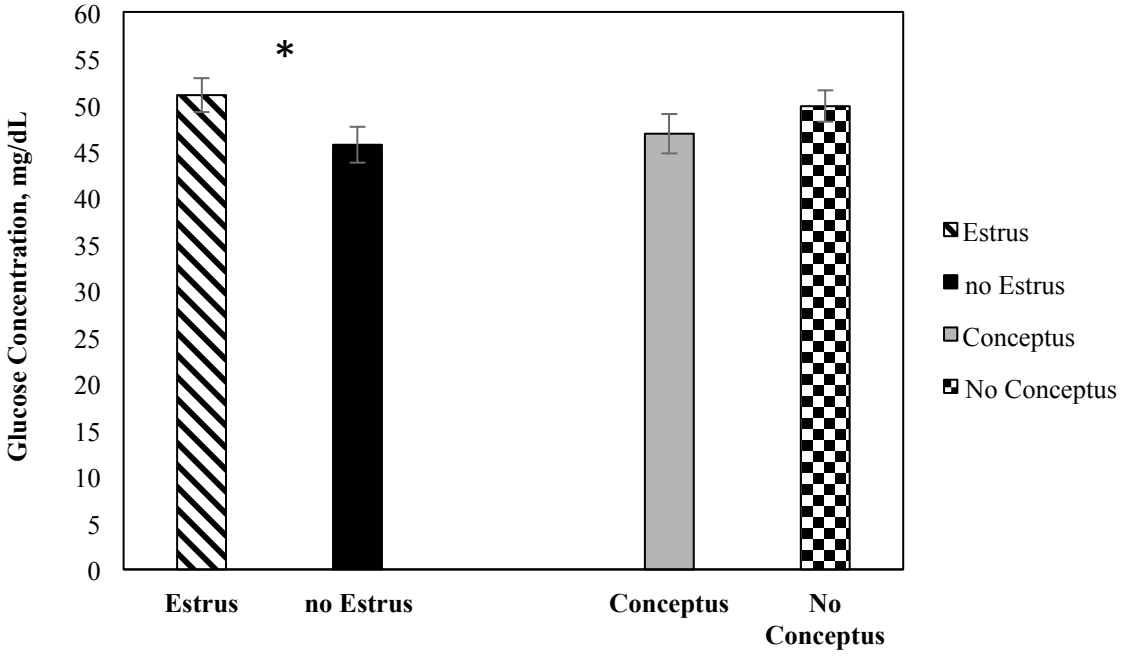

Figure 16. Glucose concentration (mg/dL) in ULF for cows that did/did not exhibit estrus and cows that did/did not have a conceptus recovered from them. Cows that exhibited estrus had a greater glucose concentration in the ULF. There was no difference in glucose concentration among the conceptus/no conceptus cows ($P = 0.29$). * $P = 0.05$

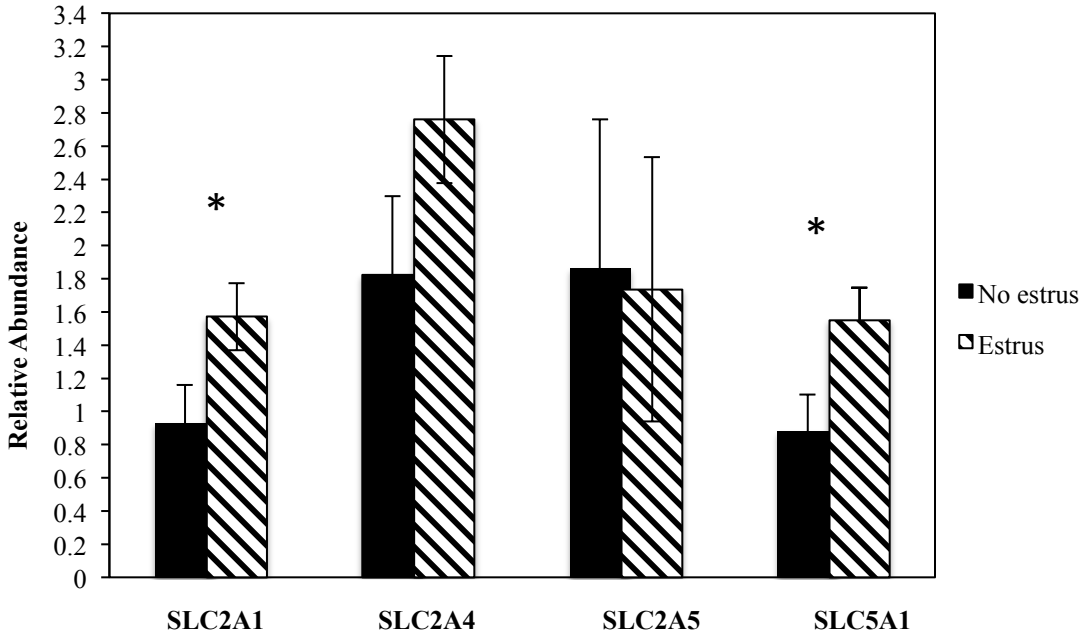

Figure 17. Glucose transporter expression (SLC2A1, SLC2A4, SLC2A5, SLC5A1) in caruncular endometrial tissue among cows that did and did not exhibit estrus. Cows that exhibited estrus had greater SLC2A1 ($P = 0.05$) and SLC5A1 ($P = 0.04$) mRNA abundance.

* Significance within a transporter ($P < 0.05$)

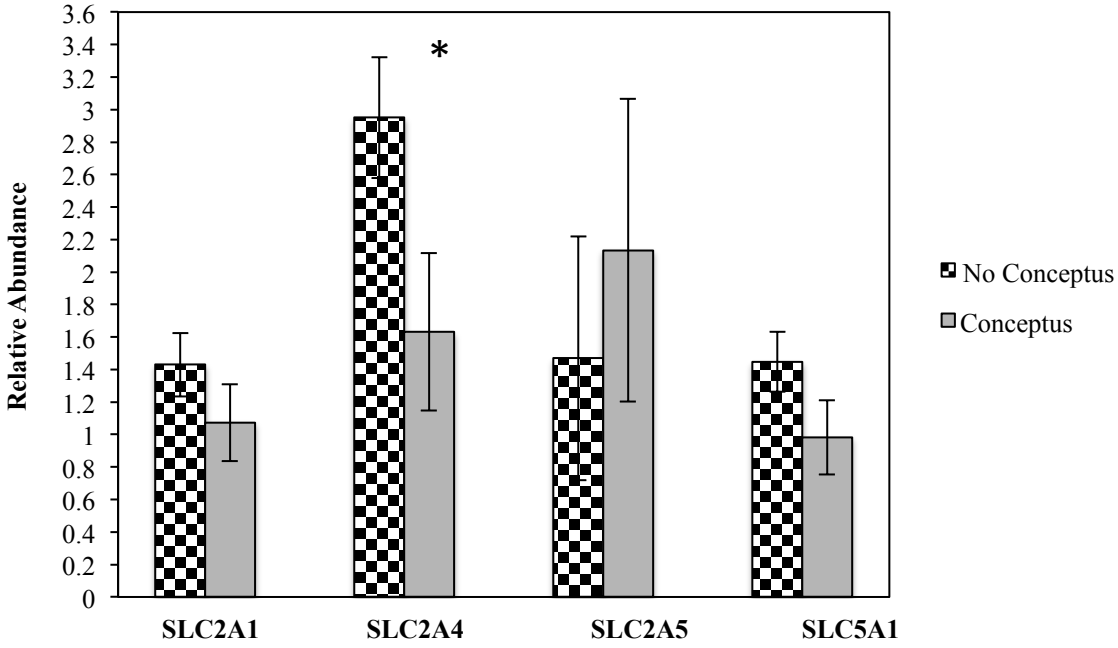

Figure 18. Glucose transporter expression (SLC2A1, SLC2A4, SLC2A5, SLC5A1) in caruncular endometrial tissue among cows that did and did not have a conceptus recovered from them. Cows from which a conceptus was recovered had decreased SLC2A4 abundance ($P = 0.05$).

* Significance within a transporter ($P < 0.05$)

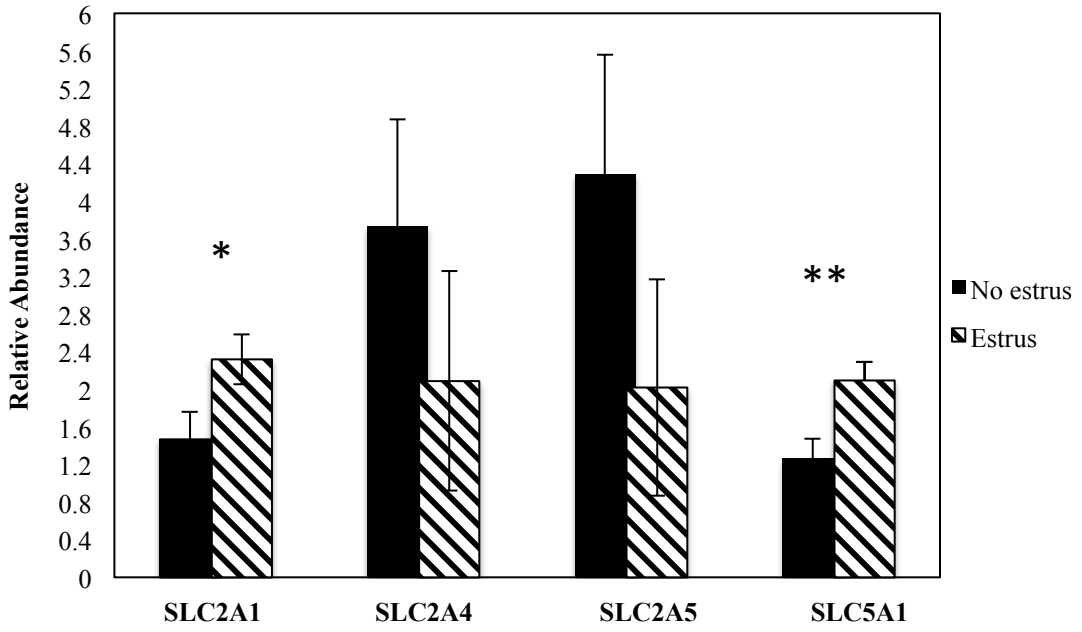

Figure 19. Glucose transporter expression (SLC2A1, SLC2A4, SLC2A5, SLC5A1) in intercaruncular endometrial tissue among cows that did and did not exhibit estrus. Cows that exhibited estrus had greater SLC2A1 ($P = 0.05$) and SLC5A1 ($P < 0.01$) mRNA abundance.

* Significance within a transporter ($P < 0.05$)

** Significance within a transporter ($P < 0.01$)

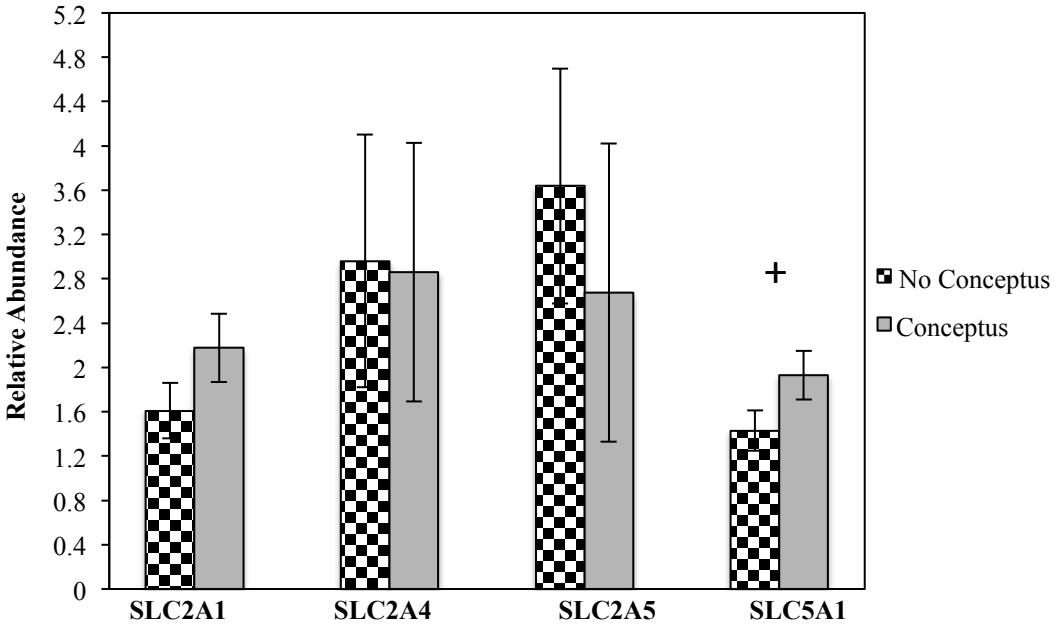

Figure 20. Glucose transporter expression (SLC2A1, SLC2A4, SLC2A5, SLC5A1) in intercaruncular endometrial tissue among cows that did and did not have a conceptus recovered from them. Presence of a conceptus tended to increase SLC5A1 mRNA abundance ($P = 0.10$).

+ Tendency within a transporter (+ $P < 0.10$)

DISCUSSION

It has been well established that the initiation of estrus occurs due to increased circulating concentrations of estradiol at a time when progesterone is not present (De Silva et al., 1981), and as previously demonstrated (Perry and Perry, 2008), cows that exhibited estrus had greater preovulatory estradiol concentrations compared to cows that did not exhibit estrus. However, in the current study, serum estradiol concentrations did not differ between cows that did and did not have a conceptus recovered on day 16 of pregnancy. The corpus luteum is the main source of progesterone, and it is essential for the maintenance of pregnancy (McDonald et al., 1952). Adequate progesterone secretion is necessary for stimulating endometrial secretions, embryo growth and development, and maintenance of pregnancy by altering endometrial gene expression (Garrett et al., 1998). The postovulatory rise of progesterone is associated with an increase in pregnancy success (Forde et al., 2009). The current study found no differences in circulating progesterone concentrations between cows that did and did exhibit estrus, and there was also no difference in cows that did and did not have a conceptus recovered from them.

Previous research found that ovariectomized cows that were exposed to estradiol prior to progesterone treatment were more likely to maintain pregnancy to day 29, but it was hypothesized that embryo survival to maternal recognition of pregnancy was similar between cows that were and were not exposed to estradiol (Madsen et al., 2015). In cattle, maternal recognition of pregnancy occurs around day 16 after estrus (Bazer, 1997). The conceptus must produce and secrete interferon tau (IFNT), which 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). Expression of IFNT begins

in the mononuclear cells of the trophectoderm during the early morula, late blastocyst stage (day 6-7 of pregnancy; Kubisch, 1998). Robinson et al. (2006) reported that intrauterine IFNT concentrations were increased from day 14 to day 18 among pregnant animals, and was positively correlated to embryo size. In the current study, there was no difference in conceptus recovery rates between cows that did and did not exhibit estrus, and IFNT concentrations on day 16 of pregnancy in the ULF did not differ between cows that did and did not exhibit estrus. The lack of differences in IFNT concentration in the ULF may be due to early embryonic loss occurring.

Interferon stimulated gene expression in the uterine endometrium is upregulated as a result of IFNT secretion by the conceptus (Yankey et al., 2001). Interferon stimulated genes are hypothesized to regulate uterine receptivity to implantation as well as survival and growth of the conceptus (Kim et al., 2012). In the present study, there were no differences in ISG15, OAS1, and MX2 expression on day 16 between cows in which a conceptus was or was not recovered. Previous work has reported that expression of MX2 increased as early as day 16 after insemination, and ISG15 increased around day 18 of pregnancy in cattle (Gifford et al., 2007). Green et al. (2010) reported MX2 and ISG15 expression was greater in pregnant compared to non-pregnant cows on day 18 and 20. This further suggests conceptus survival to maternal recognition of pregnancy is similar between cows that do and do not exhibit estrus prior to fixed-time AI.

For embryo survival to occur the maternal uterine environment needs to provide sufficient nutrients to the developing embryo, these nutrients are provided in what is known as the uterine histotroph (Gao et al., 2009a). The uterine histotroph is composed of a mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino

acids, proteins, and glucose (Gao et al., 2009a). Estradiol has been reported to induce endometrial receptors and expression of uterine proteins (Bartol et al., 1981), as well as induce the expression of many genes involved in uterine extracellular matrix remodeling that are necessary for embryo growth and a successful pregnancy (Bauersachs et al., 2005). In addition, IFNT has also been reported to influence uterine gene expression (Chen et al., 2006). When pregnant and cyclic ewes were compared, 30 genes were up regulated and nine were down regulated during pregnancy. Many of the upregulated genes were associated with antiviral responses, while the downregulated genes were associated with preventing the regression of the corpus luteum. Miller et al. (1977) conducted a study administering small and large doses of estradiol; ovariectomized ewes given a small dose of estradiol had decreased uterine weight, total protein content, and pregnancy success prior to reaching maternal recognition of pregnancy compared to ewes given a larger dose. However, preovulatory estradiol concentrations did not cause differences in ULF protein concentration in the current study on day 16 of pregnancy, while cows that had a conceptus recovered from them had a greater protein in the ULF compared to cows in which no conceptus was recovered. The difference between the current study and the study by Miller et al. (1977) is likely in the timing of uterine collection. Miller examined the uterus prior to maternal recognition of pregnancy, and in the current study uteri were flushed/collected after maternal recognition had occurred. Thus, the stimulation of uterine genes by IFNT may have masked a difference in uterine protein between estrus and no estrus cows.

Glucose is a major fuel source used by the conceptus; it is transported into the uterus via glucose transporters (Leese and Barton, 1984; Pantaleon and Kay, 1998).

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). In sheep, total glucose content in uterine flushes has been reported to increase six fold between days 10 and 15 of gestation, during this time period the embryo is undergoing morphological changes from spherical to filamentous (Gao et al., 2009; Flechon et al., 1986). In the current study, glucose concentrations in the ULF were greater in cows that exhibited estrus compared to cows that did not exhibit estrus. However, there was no difference in ULF glucose concentration between cows that did and did not have a conceptus recovered from them. Thus, this increase in glucose among cows that exhibited estrus may contribute to increased pregnancy success on day 30 among cows that exhibit estrus prior to fixed-time AI.

Transport of glucose across the plasma membrane is mediated by facilitative and/or sodium dependent transporters. Glucose transporters are found throughout the body in various tissues (Zhao et al., 2007). 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 differed between cyclic and pregnant ruminants (Gao et al., 2009b). In the present study, in caruncular endometria, cows that exhibited estrus had greater abundance of SLC2A1 and SLC5A1 mRNA. There was no difference in SLC2A4 and SLC2A5 abundance between cows that did and did not exhibit estrus. In intercaruncular tissue, there was no difference in SLC2A4 and SLC2A5 mRNA abundance between estrus and no estrus cows, while cows that exhibited estrus had increased SLC2A1 and SLC5A1 mRNA abundance.

In caruncular endometria, there was no difference in SLC2A1, SLC2A5, and SLC5A1 mRNA abundance between conceptus and no conceptus cows. However, cows 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 abundance of SLC5A1 mRNA. A study done by Gao et al. (2009b) found that SLC2A1 mRNA was increased in pregnant ewes starting at day 10 compared to cyclic ewes. They found that expression of SLC2A1 appeared to be regulated by both progesterone and IFNT. According to Gao et al. (2009b), SLC2A4 mRNA abundance also increased in pregnant ewes between day 10 and 18 of pregnancy, and treatment of ovariectomized ewes with progesterone and IFNT increased SLC2A4 mRNA levels 1.9 fold. Previous research has also reported that pregnant ewes had an increase in endometrial expression of SLC5A1 mRNA between days 10 and 12 of the cycle, and expression remained elevated through day 16 (Gao et al., 2009b). However, in in the present study there was no difference in circulating concentrations of progesterone or concentrations of IFNT in the ULF. Furthermore, the reduction in SLC2A4 may indicate the ability of the conceptus to partially regulate glucose in the uterine environment, as excess glucose has been reported to negatively impact stem cell differentiation in mice (Yang et al., 2016) and to negatively impact implantation in humans (Zhou et al., 1997). Franca et al. (2015) reported no differences in SLC2A1, SLC2A4, SLC2A5, and SLC5A1 transcript abundance or in uterine concentrations of glucose on day 10 between large follicle (LF)-large CL (LCL) cows versus small follicle (SF)-small CL (SCL) cows. However, large follicle cows had greater plasma estradiol concentrations on day -2, -1, and 0 compared to SF-SCL cows. 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.

In summary, there were no differences in conceptus survival based on recovery rates, IFNT concentrations, and ISG expression among cows that did and did not exhibit estrus. However, glucose transporter expression in the endometrium, and also glucose and protein concentration in the ULF was influenced by preovulatory estradiol and conceptus presence. Thus, differences in conceptus survival to pregnancy determination among cows that exhibit estrus prior to fixed-time AI is not a factor in conceptus survival to maternal recognition of pregnancy, but transport of glucose to the uterus among cows that exhibited estrus may contribute to the increased pregnancy success at these later time points.

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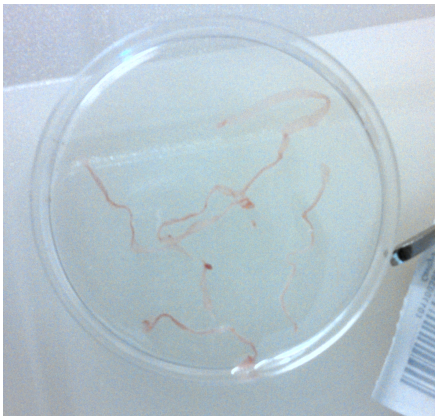
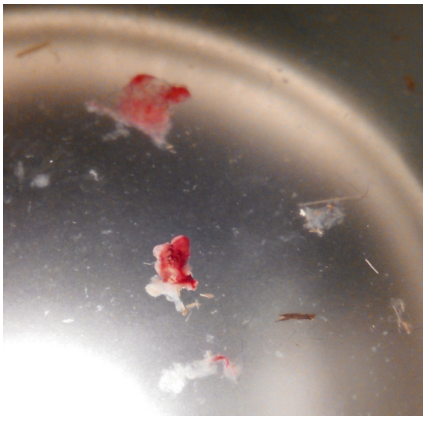
APPENDIX

REPLICATE 1 CONCEPTUS DATA			
ID	Estrus Expression	Conceptus Presence	Trophectoderm Fragment Lengths (mm)
0043	YES	NO	
69	NO	NO	
0081	YES	NO	
0099	NO	NO	
0114	YES	NO	
0154	YES	YES	55.17, 36.15, 28.32, 50.64, 56.31, 26.39
197	NO	YES	3.25
3028	YES	NO	
4209	NO	YES	27.7, 65.6
5217	NO	NO	
6072	YES	YES	27.58
8359	YES	YES	61.96
9039	YES	YES	51.44, 33.58, 58.36, 72.11, 16.49, 65.49
9043	NO	NO	
9173	YES	NO	
Y103	NO	NO	
Y110	YES	NO	
Y111	NO	NO	
Y113	NO	NO	
Y116	NO	NO	
Y142	NO	NO	
Y192	YES	NO	
Z203	NO	NO	
Z207	YES	YES	27.8, 76.58, 8
Z245	YES	NO	
Z255	NO	YES	41.1, 10.78, 13.92, 3.84, 4.55
Z301	NO	NO	
Z305	YES	NO	
Z309	YES	YES	82.82, 65.97, 85.83, 12.4, 37.43, 78.19, 28.58, 53.06, 40.91, 61.51, 18.75, 16.46, 53.69, 41.04, 26.48, 10.19

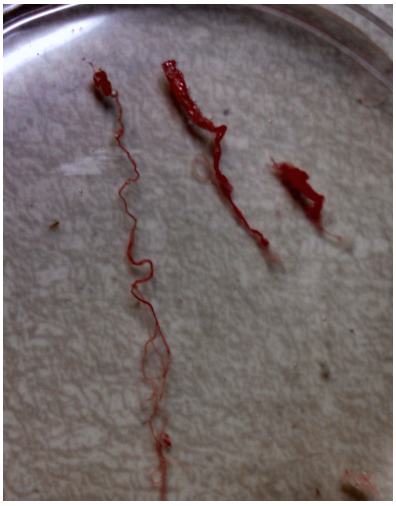
Table A1: Day 16 replicate 1 conceptus data from nonsurgical flushing.



D16 conceptus- ID 0154



D16 conceptus- ID 197



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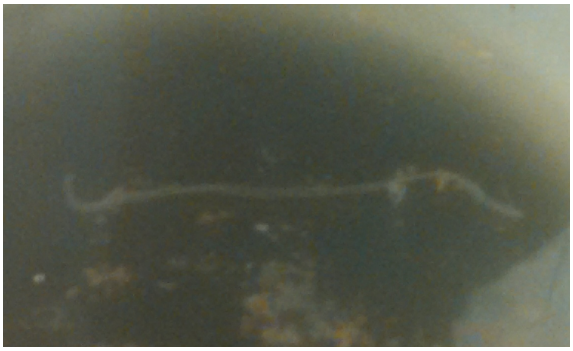
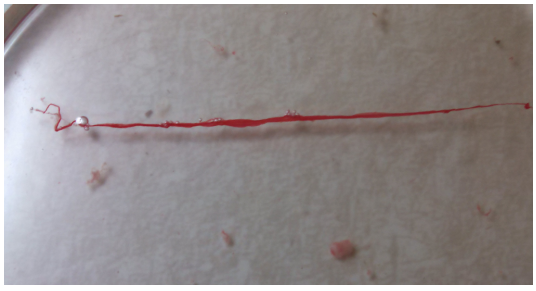


D16 conceptus- ID 6072



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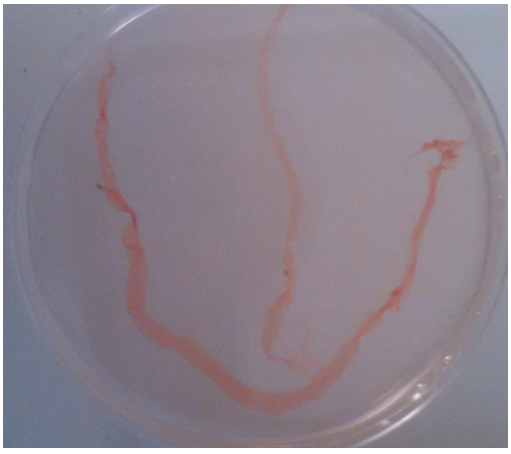
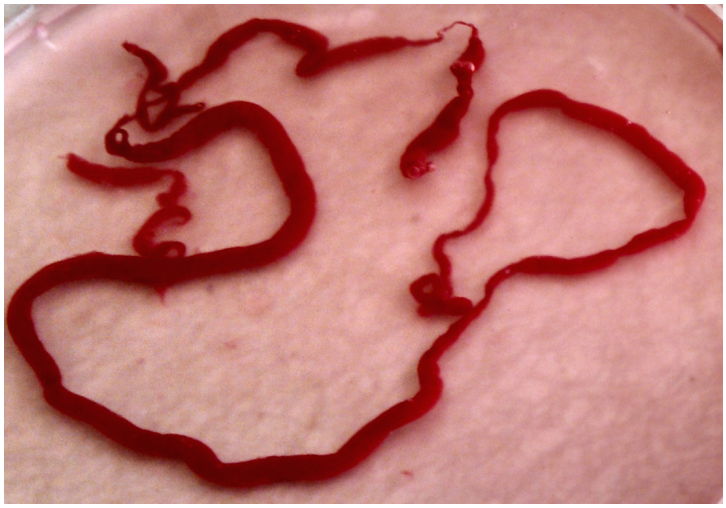


D16 conceptus- ID 9039

D16 conceptus- ID Z207

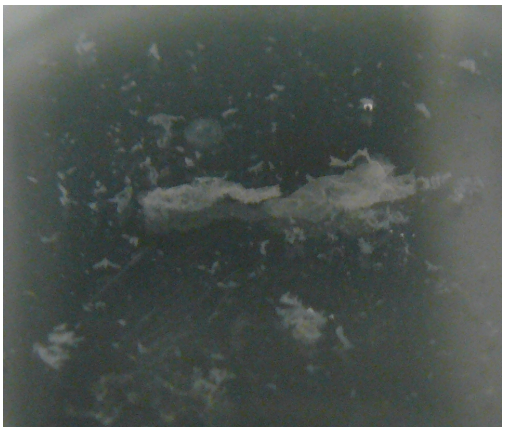


D16 conceptus- ID Z255

REPLICATE 2 CONCEPTUS DATA			
ID	Estrus Expression	Conceptus Presence	Trophectoderm Fragment Lengths (mm)
8	NO	NO	
0043	NO	YES	13.49, 1.86
067	YES	YES	2.18, 0.71
69	NO	NO	
71	YES	NO	
0081	NO	NO	
0099	NO	NO	
0114	NO	NO	
117	YES	YES	15.43, 2.66
197	NO	YES	25.97, 0.88
244	YES	YES	3.17, 1.88
254	YES	NO	
3028	NO	NO	
3327	YES	NO	
4209	NO	YES	14.12, 0.93
5217	NO	NO	
5342	NO	YES	35.24, 1.11
6072	NO	NO	
8359	NO	NO	
9039	NO	NO	
9043	NO	NO	
9173	NO	NO	
Y066	NO	NO	
Y103	NO	NO	
Y110	YES	YES	3.28, 1.03
Y111	NO	NO	
Y113	NO	NO	
Y116	NO	NO	
Y142	NO	NO	
Y192	YES	NO	
Z203	YES	NO	
Z207	NO	NO	
Z245	NO	YES	40.51, 0.96
Z255	NO	NO	
Z301	NO	NO	
Z305	NO	NO	
Z309	NO	NO	

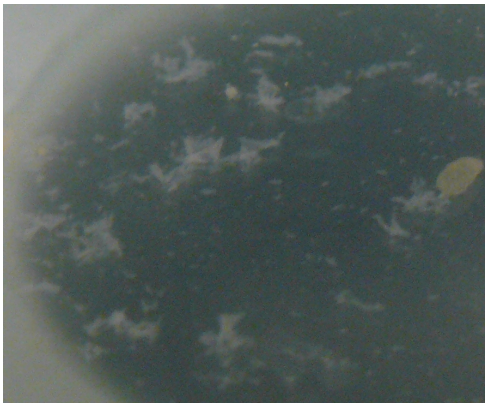
Table A2: Day 16 replicate 2 conceptus data from flushing following slaughter.

D16 conceptus- ID 0043



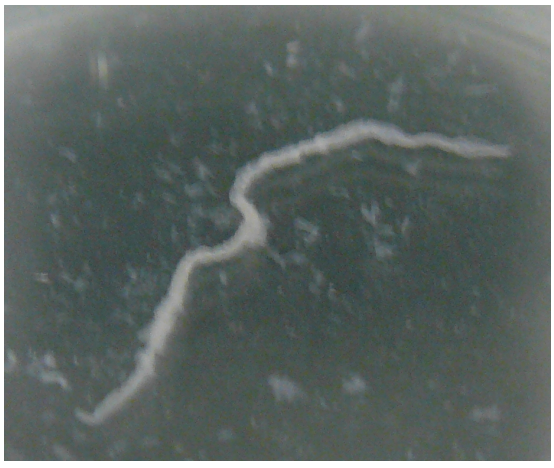
D16 conceptus- ID 117

D16 conceptus- ID 197

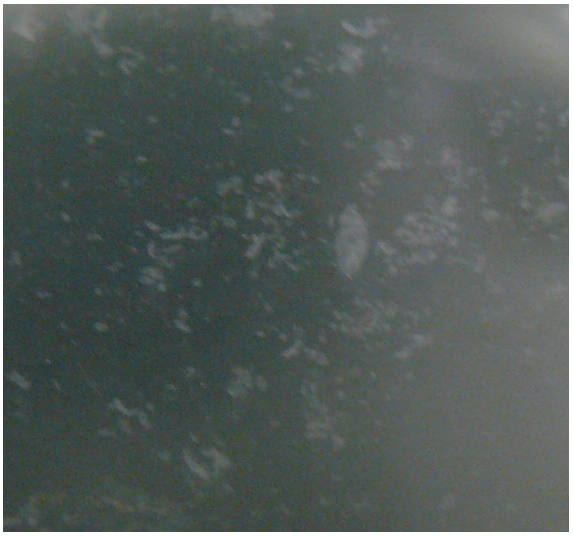


D16 conceptus- ID 244

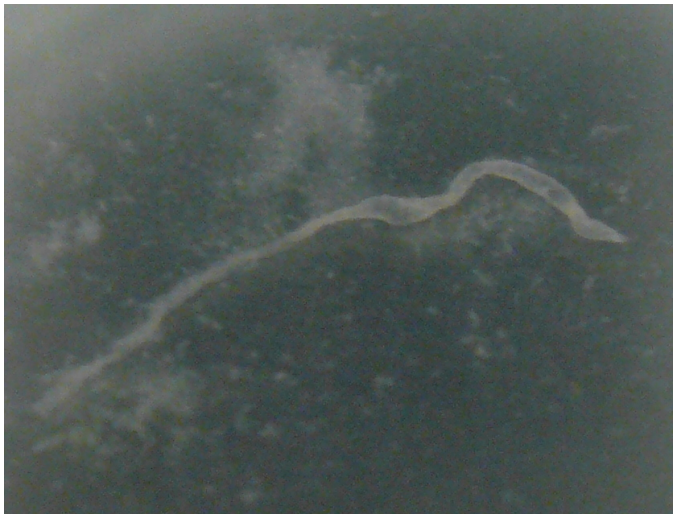
D16 conceptus- ID 4209



D16 conceptus- ID 5342



D16 conceptus- ID Y110



D16 conceptus- ID Z245