THESIS

PROFILING EQUINE ENDOMETRIAL GENE EXPRESSION DURING MATERNAL RECOGNITION OF PREGNANCY

Submitted by

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ABSTRACT

PROFILING EQUINE ENDOMETRIAL GENE EXPRESSION DURING MATERNAL RECOGNITION OF PREGNANCY

In order to maintain a pregnancy in the mare the presence of a conceptus in the uterus must be recognized by the endometrium. This is known as maternal recognition of pregnancy (MRP) and is required to prevent the secretion of prostaglandin $F_{2\alpha}$ (PGF), starting on day 14 post-ovulation, from the endometrium into circulation. The secretion of PGF initiates luteolysis of the corpus luteum, which is secreting progesterone, the hormone needed to maintain a pregnancy. However, little is known about maternal recognition of pregnancy in the mare. It is critical that the embryo is mobile throughout the entire uterine lumen to signal maternal recognition of pregnancy between days 12-14. The embryo ceases mobility on day 16 by fixing at the base of one of the uterine horns, independent of the side of ovulation. Previously, an equine specific microarray analysis was performed on days 14, 16, and 18 post-ovulation comparing endometrial gene expression between pregnant and non-pregnant mares. From this analysis, ten genes: juxtaposed with another zinc finger protein 1-like (*JAZF1*), secretory phospholipase A2 (*sPLA2*), S100 calcium binding protein G (*S100G*), estrogen receptor 1 (*ESR1*), solute carrier family 36 (proton/amino acid symporter), member 2 (*SLC36A2*), methyltransferase-like protein 7A-like (*METTL7A*), retinaldehyde dehydrogenase 1-like (*RALDH1*), eukaryotic translation initiation factor 2-alpha kinase 3 (*EIF2AK3*), dickkopf 1 homolog (*DKK1*), and adrenomedullin (*ADM*), were identified as having consistently higher or lower expression levels in the endometrium of pregnant mares at all three time points. The goal

of this study was to confirm and expand upon the results of the microarray on days 14, 16, and 18, by real time PCR (RT-PCR), and to evaluate differential gene expression on day 12. We hypothesized that the expression of the aforementioned ten genes will be the same on day 12 endometrium from pregnant mares as days 14, 16, and 18 endometrium from pregnant mares because day 12 is the start of maternal recognition of pregnancy.

To test this hypothesis, 12 normally cycling mares were utilized in a crossover design. Each mare was assigned to a random collection day (day 12, 14, 16, or 18 post-ovulation) and provided endometrial samples from a pregnant cycle and then a non-pregnant (non-mated) cycle (n=3 per day). Endometrial biopsy samples were snap frozen and stored until total RNA was isolated for RT-PCR. This analysis was consistent with the microarray results for days 14, 16, and 18. On day 12, 6 of the 10 differentially expressed genes had the same pattern of expression as day 14, but 4 of the genes had opposite expression levels on day 12.

Endometrial samples were then collected on day 13 post-ovulation (n=3) and processed for protein isolation and immunohistochemical analysis. The specificity of rabbit polyclonal antibodies for sPLA2 and DKK1 for equine endometrium were confirmed by Western Blot analysis. Upon conformation of antibody specificity, immunohistochemistry was used to determine the localization of sPLA2, DKK1, and ESR1. sPLA2 was localized to the endometrial epithelium and glandular epithelium in the endometrium from both pregnant and non-pregnant mares. DKK1 showed a localization difference between endometrial samples from pregnant and non-pregnant mares. In the endometrium from the pregnant mare, DKK1 was localized to the endometrial epithelium and the glandular cells, and in the endometrium from the non-pregnant mare DKK1 was localized throughout the glandular region, but not in the endometrial epithelium. ESR1 also showed differential localization based upon pregnancy status. In the endometrium

from the pregnant mare, ESR1 was located in the basal glandular region and not close to the lumen or in the endometrial epithelium. In endometrium from non-pregnant mares it was located throughout the entire glandular region and in the endometrial epithelium.

This experiment identified the expression patterns of ten genes, previously identified from a microarray analysis, on days 12, 14, 16, and 18 post-ovulation in the endometrium from pregnant and non-pregnant mares. The expression patterns on days 14, 16, and 18 were consistent across each day. Day 12 revealed mixed results for the expression patterns of these genes, indicating that they were undergoing transcriptional regulation based upon the presence or absence of a mobile conceptus. By determining what signal causes these genes to be higher or lower expressed in the endometrium of pregnant mares, it may lead to the identity of the signal for maternal recognition of pregnancy in the mare.

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CHAPTER I: REVIEW OF LITERATURE

Introduction

Maternal recognition of pregnancy (MRP) in all species is a sensitive process that involves communication between a conceptus and the maternal endometrium. In the horse, this communication is even more critical because there is no attachment of the conceptus to the endometrium prior to day 35 post-ovulation. Maternal recognition of pregnancy is critical so that the endometrium does not secrete prostaglandin $F_{2\alpha}$ (PGF), lysing the corpus luteum (CL) and destroying the source of progesterone, the hormone necessary to sustain a pregnancy. Pregnancy and the estrous cycle is well understood, but there is a lack of knowledge and understanding of how MRP occurs in the mare. In most other species, the signal for recognition of pregnancy has been identified. Understanding this signaling is essential for the equine industry to decrease the incidence of early embryonic loss, which is the loss of a pregnancy between days 0-35 post-ovulation. Understanding the genes that are effected by MRP is critical to determine the signaling that is occurring during maternal recognition of pregnancy in the mare and may lead to discovery of the source of the signal and the mechanisms that allow a pregnancy to be sustained.

Equine Estrous Cycle

The estrous cycle in the mare is on average 21 days long, with day 0 being the day of ovulation. It is comprised of a luteal phase and a follicular phase, which is further divided into metestrus, diestrus, proestrus, and estrus. The luteal phase is progesterone dominated and the follicular phase is estrogen dominated. In both pregnant and non-pregnant mares the hormonal profiles are the same until day 14, which is when the endometrium has recognized the presence or absence of a conceptus (Sharp et al. 1997). Prostaglandin $F_{2\alpha}$, from the endometrium, is the

hormone responsible for the demise of the CL in the absence of a conceptus, known as luteolysis (Sharp et al. 1997). Oxytocin is the stimulator for the release of PGF from the endometrium (Burns et al. 1997). Towards the end of the luteal phase (day 14), endometrial progesterone receptors are down regulated. This results in an up regulation of estrogen receptors and subsequently an up regulation of oxytocin receptors (McCracken et al. 1999). Estrogen causes hypothalamic oxytocin pulses to occur. This oxytocin then binds to the endometrium causing the endometrium to start secreting more oxytocin (McCracken et al. 1999). Circulating levels of oxytocin are highest during the mid and late luteal phase and lowest during ovulation and early diestrus (Tetzke et al. 1987). The combination of the hypothalamic pulses of oxytocin and secretion of endometrial oxytocin causes the production and secretion of endometrial PGF (McCracken et al. 1999). Administration of oxytocin during late diestrus also induces an immediate rise in the major PGF metabolite, PGFM (Goff et al. 1987).

PGF is metabolized from arachadonic acid. Upon oxytocin stimulation, arachadonic acid is secreted from the phospholipid bilayer. It is then converted to prostaglandin H2 (PGH2) by one of two enzymes (Needleman et al. 1986). These two enzymes are prostaglandin-endoperoxide synthase 1 or 2 (PTGS1 or PTGS2), previously known as cyclooxygenase 1 or 2 (COX1 or COX2) respectively (Needleman et al. 1986). PTGS1 is the constitutive isoform of the enzyme and its levels remain unaltered throughout the estrous cycle, independent of pregnancy status (Boerboom et al. 2004). PTGS2 is the inducible form that increases in mRNA and protein levels corresponding with luteolysis (Needleman et al. 1986; Boerboom et al. 2004). The increase in PTGS2 protein is not observed in pregnant mares (Boerboom et al. 2004). Prostaglandin $F_{2\alpha}$ synthase converts PGH2 into PGF (Needleman et al. 1986). PGF then enters peripheral circulation and eventually reaches the ovaries (Allen and Stewart 2001). This results

in the lysis of the CL and the continuation of the estrous cycle (McCracken et al. 1999). This process is also observed *in vitro* when endometrial cells are stimulated with oxytocin causing the release of PGF (Ealy et al. 2010). Eventually there is reduced responsiveness of the endometrium to oxytocin, which also coincides with a decreased binding capacity of the endometrium for oxytocin (Starbuck et al. 1998). During pregnancy, oxytocin responsiveness is altered indicating that adjustment of the PGF release mechanism contributes to the maintenance of the CL. Day 14 pregnant mares respond with a diminished release of PGFM following oxytocin administration, indicating that the PGF release mechanism has been altered (Goff et al. 1987). PGF release occurs throughout the entire uterine body, therefore it is necessary that the entire endometrial surface is exposed to the conceptus derived pregnancy recognition signal in order to prevent oxytocin and PGF release (Ginther 1974).

Early Pregnancy in the Mare

After ovulation, the oocyte travels into the oviduct where fertilization occurs. From there the conceptus stays in the oviduct until day 6 (Weber et al. 1991a). To relax the smooth muscle in the oviduct at the utero-tubal junction and enter the uterus, the conceptus secretes prostaglandin E_2 (PGE) (Allen 2001). This secretion begins at day 4.5, or when the conceptus becomes a compact morula (Weber et al. 1991a; 1991b; 1992; Allen 2001). PGE is synthesized in the same manner as PGF, except that prostaglandin E_2 synthase converts PGH2 to PGE (Needleman et al. 1986). It has a local effect and causes the relaxation of the circular smooth muscle in the oviduct (Allen 2001). Intrauterine administration of PGE also causes increased uterine tone and contractility (Gastal et al. 1998). As the conceptus enters the uterus, it is a late stage morula or an early blastocyst (Betteridge et al. 1982). Upon blastulation, the conceptus starts to develop a capsule underneath the zona pellucida (Betteridge et al. 1982). The capsule is

made up of glycoproteins resembling those of the mucin family with sialic acid making up most of the carbohydrate content (Klein and Troedsson 2011b). On day 9, there is a change in the glycosylation characteristics, ultimately resulting in the loss of the zona pellucida, and leaving the capsule surrounding the conceptus (Oriol et al. 1993). The capsule is essential for embryo survival. When it is removed from day 6-7 embryos and the embryo then placed into a recipient mare there is no establishment of pregnancy (Stout et al. 2005). The capsule will remain surrounding the embryo until day 16 when the sialic acid decreases, which also corresponds to fixation. For this reason, it is thought that the capsule acts as an anti-adhesive. The capsule starts to disappear on day 22.5 and is completely gone by day 24.5 (Oriol et al. 1993).

On day 9, there is minimal movement throughout the uterus by the conceptus, but by day 10, mobility dramatically increases (Ginther 1983b). This movement is thought to be caused by conceptus derived estrogens stimulating the release of a small amount of PGF and causing slight contractions (Stout and Allen 2001). If a cyclo-oxygenase (COX) inhibitor is administered to mares between days 10-16 it results in a significant decrease in conceptus mobility (Stout and Allen 2001). Maximum embryo mobility is between days 11-14, which corresponds to the time of maternal recognition of pregnancy (Ginther 1983b). Movement throughout the majority of the uterus is essential in order to delay PGF release from the endometrium (Ginther 1974). If the conceptus is restricted to less than two-thirds of the endometrial surface it leads to pregnancy failure, but this effect can be rescued by supplementing with exogenous progesterone (McDowell et al. 1988). During this time period the uterine tone increases, causing a change in the contractile pattern of the myometrium. It is thought the mobile conceptus is causing these changes (Gastal et al. 1996).

The conceptus ceases mobility at day 16 (Leith and Ginther 1984), and is referred to as fixation (Ginther 1983a). The conceptus fixes, but does not attach or implant, at the base of one of the uterine horns, independent of the side of ovulation (Gastal et al. 1996). There are many different factors that cause fixation. There is a sudden spasm-like increase in myometrial tone that occurs at this time (Ginther 1983b). Also, there is an increase in uterine tone and the size of the conceptus increases (Gastal et al. 1996). All of these factors result in the cessation of movement and the fixation of the conceptus.

As expected, due to the presence of a mobile conceptus, PGF concentrations at day 10 and day 14 of pregnancy are lower than the corresponding days in a cyclic mare (Douglas and Ginther 1976). Uterine flushings from mares beyond day 18 of pregnancy contain PGF concentrations similar to those seen during luteolysis, indicating that the conceptus only delays, rather than prevents the production of uterine PGF (Stout and Allen 2002). Also, when endometrium from a pregnant mare is obtained, in the absence of the conceptus, it will start to secrete the same amount of PGF as the endometrial tissue from a non-pregnant mare (Vernon et al. 1981; Watson and Sertich 1989). This suggests that the constant stimulation from the conceptus is critical for maternal recognition of pregnancy. When endometrial explants from non-pregnant mares are cultured in the same media that previously contained a conceptus there is a dramatic decrease in PTGS2, the inducible form of PTGS in PGF synthesis (Ealy et al. 2010). This indicates that not only the stimulation of the conceptus is critical, but also that it is secreting a factor that affects the endometrium. The conceptus remains fixed, but does not implant, in the uterus until day 36 of pregnancy. At day 36 the trophoblast cells become invasive and migrate into the maternal endometrium forming endometrial cups. These endometrial cups secrete equine

chorionic gonadotropin, which results in the luteinization of follicles on the ovary and an increase in progesterone secretion (Allen and Stewart 2001).

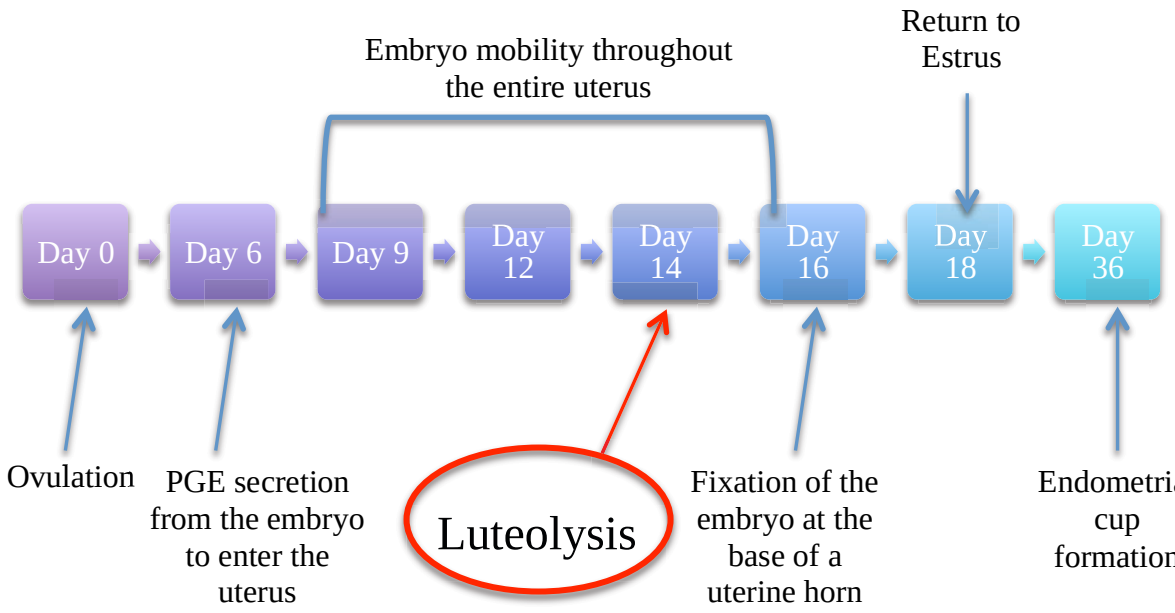

Figure 1: Key Events in Equine Pregnancy

Key events in a normal pregnancy. Day 0 is the day of ovulation. On day 6, the embryo secretes prostaglandin E_2 allowing the oviduct to relax and the embryo to enter the uterus. Embryo mobility starts on day 9 and continues until day 16 when the embryo fixes at the base of one of the uterine horns. Maternal recognition of pregnancy (MRP) is occurring between days 12-14. The embryo does not implant into the uterus until day 36 with the invasion of the endometrial cups. If MRP does not occur, luteolysis begins on day 14 with the mare returning to estrus by day 18.

Maternal Recognition of Pregnancy in Other Species

In many species, MRP is well defined. Maternal recognition of pregnancy is essential to extend the lifespan of the functional CL and to continue its synthesis and secretion of progesterone. Progesterone is required for secretory functions of the endometrium, embryonic development, implantation, and placentation (Bazer et al. 2008). There are two categories of mechanism of maternal recognition, luteotrophic or anti-luteolytic. Luteotrophic directly

promotes luteal function, supporting the CL, and anti-luteolytic prevents the uterine release of luteolytic PGF, which causes the lysis of the CL. Cattle, sheep, pigs, mice, and humans/non-human primates are among the most well studied species regarding maternal recognition of pregnancy.

Humans/non-human primates and rodents maintain luteal function through luteotrophic mechanisms. In primates the maternal recognition signal is chorionic gonadotropin (CG) (Ross 1978). The syncytiotrophoblast cells secrete CG from days 8-10 post-ovulation for pregnancy recognition. At the same time, days 7-9, implantation is occurring. Chorionic gonadotropin stimulates the CL to produce progesterone until there is a shift between luteal and placental progesterone secretion. Once the shift occurs, CG secretion decreases (Bazer et al. 2010). Administration of exogenous CG can also extend the luteal function of the CL and increase progesterone secretion in humans and non-human primates (Bazer et al. 2008). Rodents also maintain luteal function through luteotrophic mechanisms, with prolactin being the maternal recognition of pregnancy signal (Frasor and Gibori 2003). Prolactin, from the anterior pituitary gland, is induced by mating (Soares 2004). It is the initial maternal recognition signal until day 12 of pregnancy. After day 12 the conceptus and uterine decidua take over stimulating progesterone secretion by secreting lactogenic hormones (prolactins) (Soares 2004). These hormones act on the luteal cells through the prolactin receptors to maintain luteal cell function and the secretion of progesterone (Soares 2004).

The other mechanism for maternal recognition of pregnancy is an anti-luteolytic mechanism. The MRP signal for cattle and sheep is interferon-tau (IFNt) (Bazer et al. 1997). In sheep, IFNt is secreted between days 10-21 by the mononuclear trophoblast cells (Bazer et al. 1997). On days 11-16, the PGF concentrations are the same in pregnant and non-pregnant

animals, but pregnant animals administered PGF on day 19 or 20 do not return to estrus (Pratt et al. 1977; Bazer et al. 2008). Interferon-tau has a local effect and blocks the expression of estrogen and oxytocin receptors (Bazer et al. 1997). With the lack of estrogen and oxytocin receptors, the PGF synthesis pathway is not stimulated, which results in little PGF synthesis. There is also an increase in intrauterine PGE during pregnancy (Pratt et al. 1977). Interferon-tau is also the MRP signal in cattle (Bazer et al. 1997). It is secreted between days 12-38 of pregnancy (Bazer et al. 1997). Interferon-tau acts in the same manner in cattle as it does in sheep, by suppressing the transcription of estrogen receptor, therefore decreasing estrogen induced oxytocin receptor, decreasing the PGF cascade (Bazer et al. 1997). Ovarian follicular development is suppressed in the ovary with the CL, but not the contralateral CL. This also results in decreased levels of estrogen (Bazer et al. 1997).

Pigs also have an anti-luteolytic mechanism of maternal recognition of pregnancy and this signal is estrogen (Bazer and Thatcher 1977). Estrogen is produced by the conceptus on days 11 and 12 (Ziecik 2002). Estrogen does not cause a decrease in PGF secretion from the endometrium, but instead redirects PGF secretion from the uterine vasculature to the uterine lumen. The PGF is sequestered and metabolized in the uterine lumen to prevent it from reaching the CL and initiating luteolysis (Bazer and Thatcher 1977). There is also a shift in the ratio of PGF secretion to PGE secretion. Intrauterine application of PGE delays the lysis of the CL (Akinlosotu et al. 1986). PGF and PGE exert opposing actions on the CL, and each play a critical role in luteolysis or the maintenance of a pregnancy. Estrogen secretion from the conceptus stimulates the secretion of PGE, a luteoprotectant, from the endometrium. Prostaglandin E_2 acts in a positive feedback loop, which results in more PGE production. This keeps prostaglandin synthesis towards PGE and not PGF (Waclawik 2011).

Table 1: Maternal Recognition of Pregnancy in Various Species

Species	Maternal Recognition Signal	Type of Mechanism	Source
Bovine	Interferon Tau	Anti-luteolytic	(Bazer et al. 1997)
Ovine	Interferon Tau	Anti-luteolytic	(Bazer et al. 1997)
Porcine	Estrogen	Anti-luteolytic	(Bazer and Thatcher 1977)
Murine	Prolactin	Luteotrophic	(Frasor and Gibori 2003)
Human/Non-human Primate	Chorionic Gonadotropin	Luteotrophic	(Ross 1978)

Maternal Recognition of Pregnancy in the Mare

While MRP is well understood in many species, it remains a mystery in the horse. It is thought that during the pre-implantation phase the conceptus interacts with the uterine environment with paracrine signals in order to eventually coordinate attachment and implantation. Because implantation does not occur until day 35 in horses, the conceptus must signal its presence to the endometrium in order to inhibit luteolysis and allow progesterone secretion to continue (Bauersachs and Wolf 2012). Without this MRP, luteolysis will occur and pregnancy will be terminated due to the lack of progesterone. Different time frames have been investigated to determine when maternal recognition of pregnancy occurs. In a previous experiment, oxytocin was administered on day 8 post-ovulation to attempt to trigger the PGF synthesis pathway and initiate luteolysis. It was concluded that the oxytocin luteolytic mechanism was not in place by day 8 because administration of oxytocin failed to elicit a response and induce luteolysis (Neely et al. 1979). This means that MRP is occurring after day 8. There is also no differential gene expression in the endometrium between day 8 pregnant and non-pregnant mares (Merkl et al. 2010). It was also noted that frequent or continuous administration of oxytocin during the luteal phase blocked luteolysis, implying that MRP results

in alteration and functionality of the oxytocin receptor inhibiting its effect on luteolysis (Vanderwall et al. 2007). It has also been shown that continuous high systemic doses of oxytocin initiated prior to day 10 results in the prolongation of the luteal phase. These results imply that MRP must be initiated by day 10 (Stout et al. 1999).

Administration of oxytocin on days 7-14 post-ovulation also resulted in prolonged diestrus (Gee et al. 2012). This is further confirmed with the endometrium developing responsiveness to oxytocin administration on day 11 (Goff et al. 1987). By day 12 there is differential gene expression in the endometrium between pregnant and non-pregnant mares (Merkl et al. 2010). These genes are involved in angiogenesis and vascular remodeling, which is necessary for the endometrium to prepare for pregnancy and support conceptus growth (Klein et al. 2010). By day 13, repression of PTGS2 is noted, indicating the MRP signal is on or before day 13 (Boerboom et al. 2004). Day 13.5 endometrium also has differential gene expression between pregnant and non-pregnant mares. Some of the genes identified have also been identified to be involved in maternal recognition of pregnancy in other species. Most of the up regulated transcripts were secreted proteins and genes involved in transport and cell-signaling (Klein et al. 2010). Finally, an increase in PGF secretion is observed 14 days after ovulation, ultimately resulting in lysis of the corpus luteum (Sharp et al. 1997). All of these data indicate that MRP is occurring after day 10 but before day 14 in order to prevent luteolysis.

Since the conceptus secretes PGE to relax the smooth muscle in the oviduct allowing it to enter the uterus (the first signal of MRP in the horse), it was thought that PGE may be the MRP signal (Allen 2001). Intrauterine infusion with PGE resulted in no change in luteal function (Vanderwall et al. 1994) and there was no increase of PGE in uterine flushings or endometrial tissue associated with pregnancy indicating that PGE is not the MRP signal (Watson and Sertich

1989). Next researchers evaluated interferons like those expressed by ruminant conceptuses. Alpha 1, omega 1, and omega 2 interferons were not expressed in equine conceptuses, therefore not following the same mechanism as ruminants (Baker et al. 1991). Estrogen was evaluated to determine its effect as the MRP signal and ultimately oxytocin receptor expression. In two separate experiments, it was shown that intrauterine estradiol injection did not delay or inhibit luteolysis in the non-pregnant mare (Goff et al. 1993; Vanderwall et al. 1994). This again showed that estrogen was not the signal for MRP. Recently, the administration of plant oils and fractionated coconut oils resulted in luteal persistence in 92% of mares treated on day 10, but not the same results were seen in mares treated on days 6, 8, 12, and 14. It was thought that these oils inhibited PTGS2 (Wilsher and Allen 2011).

Researchers also evaluated gene expression profiles of equine conceptuses before, during, and after MRP. There was enhanced expression of genes involved in cholesterol transport, suggesting active synthesis of steroid hormones (Klein and Troedsson 2011a). Interestingly, there was a down regulation in genes involved in the stimulation of the immune system, suggesting the need for the conceptus to be protected from the maternal immune system (Klein and Troedsson 2011a). There was also an up regulation of fibrinogen α, β, and γ. It was thought the fibrinogen chains were released from the conceptus and acted on the endometrium (Klein and Troedsson 2011a). Down regulation of plasminogen activator and antiplasmin precursor, combined with up regulation of plasminogen activator inhibitor was identified in endometrium from pregnant mares. It was thought this system was affected in order to prevent erosion of the equine endometrium during pregnancy (Klein and Troedsson 2011a).

Finally, researchers wanted to evaluate the size and type of molecule utilized for MRP. To do this day 14 conceptuses were placed in dialysis bags with permeability to different

molecular weights and then co-incubated with endometrial explants. This narrowed the size of the signal down to be greater than 1000 Daltons, but less than 10,000 Daltons (Klein and Troedsson 2011b). The factor was later further specified to a size between 1000-6000 Daltons (Sharp et al. 1989). The conceptus secretions were treated with proteinase K and dextran-coated charcoal. The proteinase K had no effect on the anti-luteolytic activity, but the dextran-coated charcoal resulted in the removal of the anti-luteolytic activity. From this experiment, it was concluded that the MRP signal in horses was a size between 3000-6000 Daltons that is proteinase K resistant, but may be absorbed by dextran-coated charcoal (Klein and Troedsson 2011b). While a lot of information is known about the timing of the MRP signal, and what it is not, it still remains a mystery as to the actual identity of this signal.

Early Embryonic Loss

Early embryonic loss has a high prevalence in the equine industry and is defined as pregnancy loss prior to day 35 post-ovulation, or implantation. Fertilization rates in the horse are very high and approach 90%, but 77.1% of all pregnancies are lost prior to day 35 (Villahoz et al. 1985; Ball et al. 1986). The time interval with the largest loss is between days 11-15 post-ovulation. This accounts for 18.29% of mares that were diagnosed as pregnant on day 11 and no embryos were present on day 15 (Ginther et al. 1985). Of those 18.29%, 29% became pseudopregnant. This was thought to occur due to particles of the embryonic vessel still present in the uterine lumen and stimulating MRP (Ginther et al. 1985). In a separate study, 13.28% of mares suffered from early embryonic loss. Of those mares, 47.05% of pregnancies were lost prior to day 19 (Papa et al. 1998). Early embryonic loss accounts for 20% of the losses in fertile mares and 70% of the losses in subfertile mares (Carnevale et al. 2000).

Many factors are thought to cause early embryonic loss. The losses that are occurring prior to day 15 are thought to be due to the failure of MRP (Ginther et al. 1985). Endometrial disease (endometritis) is considered another major cause (Carnevale et al. 2000). Endometritis could be causing secondary luteolysis, resulting in the loss of the pregnancy (Ginther et al. 1985). Primary dysfunction of the corpus luteum is not the cause of early embryonic loss because plasma progesterone levels were similar between pregnant mares and mares suffering from early embryonic loss. The progesterone concentrations did not change until after early embryonic loss meaning the progesterone drop is an effect, not a cause, of the early embryonic loss (Papa et al. 1998). Another hypothesis is as the conceptus is triggering uterine contractility by PGF it may stimulate too much secretion resulting in the PGF entering the uterine vein and initiating luteolysis of the corpus luteum (Allen and Stewart 2001). Other factors to embryonic loss include nutrition, body condition, stallion, chromosomal abnormalities, hormonal deficiencies, stress, immune factors, and failure of the MRP signal at the appropriate time (Carnevale et al. 2000).

Conclusion

Maternal recognition of pregnancy in the mare is a delicate and complex process that is still not well understood. The exact signal and mechanism for maternal recognition of pregnancy that occurs prior to day 14 post-ovulation remains unknown. It is known that the embryo must be mobile and come in contact with over two-thirds of the endometrium, the signal acts in an anti-luteolytic manner, and the signal is between 1000-6000 Daltons. The goal of this study is to evaluate the expression patterns of ten genes identified using an equine specific microarray (Bruemmer et al. 2010) during and after maternal recognition of pregnancy in pregnant and non-pregnant mares.

CHAPTER II: PROFILING GENE EXPRESSION IN EQUINE ENDOMETRIUM DURING MATERNAL RECOGNITION OF PREGNANCY (DAYS 12, 13, 14, 16, AND 18)

Summary

The mechanism for equine maternal recognition of pregnancy is unknown. To maintain a pregnancy, the presence of a mobile conceptus prior to day 14 post ovulation (PO) must be identified to prevent endometrial secretion of prostaglandin $F_{2\alpha}$ (PGF) on days 14-16 PO, initiating luteolysis. Previously, an equine specific microarray analysis was performed on days 14, 16, and 18 PO comparing endometrium from pregnant and non-pregnant mares. Ten genes were identified as being consistently higher or lower expressed in endometrium from pregnant mares at each day. For this experiment, gene expression on days 12, 14, 16, and 18 PO was analyzed with real time PCR (RT-PCR) to analyze the microarray genes. Twelve normally cycling mares were used in a crossover design and were randomly assigned to a collection day (day 12, 14, 16, or 18 PO), providing endometrial samples from a pregnant and non-pregnant (non-mated) cycle (n=3 per day). Endometrial samples were snap frozen and total RNA was isolated for RT-PCR. Day 14, 16, and 18 were consistent with the microarray analysis, but on day 12, six of the ten differentially expressed genes had the same pattern of expression as day 14, and four of the genes had opposite expression patterns. Endometrial samples were then collected on day 13 PO (n=3) and processed for protein isolation and immunohistochemical (IHC) analysis. sPLA2 and DKK1 antibody specificity were assessed by Western Blot analysis and cellular localization was examined by IHC. These are the first data to describe gene expression and cellular localization in the endometrium at the time of maternal recognition of pregnancy for these genes. The pathway and functions of these genes may be critical in identifying the signal for maternal recognition of pregnancy in the mare.

Introduction

The interaction of the embryo with the endometrium of the equine uterus during maternal recognition of pregnancy is critical to prevent luteolysis, allowing the corpus luteum (CL) to remain intact and continue progesterone secretion. The mobility of the embryo throughout the entire uterine lumen is critical to initiate this anti-luteolytic process. In a prior experiment (Bruemmer et al. 2010), endometrial gene expression during and after maternal recognition of pregnancy (days 14, 16, and 18) was analyzed using an equine specific mircoarray containing over 42,000 probes. Analysis led to identification of 10 genes that were significantly ($P<0.001$) up or down regulated (fold change ≥ 3) in pregnant mares on all days studied (days 14, 16, and 18). These genes were also consistent in their expression levels over the three time points. Using those results, in this study the ten genes were evaluated by quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) on days 12, 14, 16, 18 to confirm and expand upon the microarray analysis. We hypothesized that on day 12 the expression patterns of the identified genes would be the same as on days 14, 16, and 18.

Materials and Methods

Horse Care

The Colorado State University Institutional Care and Use Committee approved all horse use. Mares (n=15) were used in a simple crossover design where each mare had a pregnant and non-pregnant (non-mated) cycle. Mares were monitored via transrectal palpation and ultrasonography to track their follicular development every other day. Once a follicle reached the size of 35mm or greater the mare was inseminated with at least $500x10^6$ progressively motile

sperm/mL from stallions with known fertility. The mare was evaluated via transrectal ultrasonography every day and inseminated every other day until ovulation (day 0) was detected.

Experimental Design

Mares were utilized in a crossover design with each mare being sampled in a pregnant cycle then a non-pregnant (non-mated) control cycle. Mares were randomly assigned to one collection day (day 12, 13, 14, 16, or 18 post-ovulation). Days 12, 14, 16, and 18 endometrial samples were obtained in the same year while day 13 endometrial samples were obtained the following year. Table 2 describes the design of this study.

Table 2: Endometrial Sampling Design Layout

	Day 12		Day 13		Day 14		Day 16		Day 18	
Mare	P+	NP	P+	NP	P+	NP	P+	NP	P+	NP
1	X	X								
2	X	X								
3	X	X								
4			X	X						
5			X	X						
6			X	X						
7					X	X				
8					X	X				
9					X	X				
10							X	X		
11							X	X		
12							X	X		
13									X	X
14									X	X
15									X	X

Endometrial Biopsy Collection for Days 12, 14, 16, and 18

Mares were randomly assigned (n=3 per day) to a collection day (day 12, 14, 16, or 18 post-ovulation). On the specified collection day the mare was evaluated via transrectal

ultrasonography to confirm pregnancy status by the visualization of an embryonic vesicle. Endometrial samples were obtained non-surgically via trans-cervical biopsy punch. Each sample was rinsed in DPBS/Modified 1X (HyClone Laboratories, Inc., Logan UT) and split in half. Half of the sample was placed in TRI Reagent (Molecular Research Center, Cincinnati, OH) and frozen at -80°C until RNA isolation. The other half of the sample was snap frozen in liquid nitrogen and transferred to the -80°C until RNA isolation. This process was repeated for each mare through a non-bred cycle.

Endometrial Biopsy Collection for Day 13

Mares were randomly assigned (n=3) to collection day 13 post-ovulation. On the specified collection day the mare was evaluated via transrecetal ultrasonography to confirm pregnancy status by the visualization of an embryonic vesicle. Endometrial samples were obtained via trans-cervical biopsy punch. Each sample was rinsed in DPBS/Modified 1X (HyClone Laboratories, Inc., Logan, UT) and split into thirds. A third of the sample was placed in TRI Reagent (Molecular Research Center, Cincinnati, OH) and frozen at -80°C until RNA isolation, a third of the sample was snap frozen in liquid nitrogen and stored at -80°C until RNA isolation, and the last third of the sample was prepared to be embedded in paraffin for histological analysis by light microscopy. For light microscopy, the sample was cut in-to sections no larger than 1.5 cm. The sections were then placed in 4% paraformaldehyde (Affymetrix, Inc., Cleveland, OH) in a ratio of one part tissue to ten parts fixative and left at room temperature for one hour. They were then left at 4°C overnight. The next day, the fixative was replaced with 70% ethanol for storage. When all samples were collected and fixed they were sent to the Colorado State Veterinary Diagnostics Laboratory in Fort Collins, CO, where to be embedded in paraffin blocks.

RNA Isolation and Quantification

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) for lysis and the RNeasy Mini Kit for RNA isolation (Qiagen, Valencia, CA, Catalog #74104). Frozen tissue samples (about 30mg) were homogenized in Tri Reagent and left at room temperature for 10 minutes. Chloroform was then added to the homogenate, vortexed and left a room temperature for 8 minutes. The sample was then centrifuged at 13,200 revolutions per minute for 15 minutes, separating the sample in to three distinct layers (RNA phase, DNA phase, protein phase). The top RNA aqueous phase was transferred to a new 1.7mL tube and the DNA and protein phase was stored at -80°C for future protein isolations. The remaining RNA cleanup protocol followed the manufacturer's recommendation for the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, Catalog #74104) with the use of an RNeasy Mini spin column. All samples were treated with RNase-Free DNase set (Qiagen, Valencia, CA, Catalog #79254) to remove any DNA contamination. RNA purity and quantification were assessed using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Samples were accepted if they had 260/280 and 260/230 values above 1.7 for PCR, and samples were used for subsequent analysis.

Protein Isolation and Quantification

Protein isolation was performed utilizing RIPA lysis buffer (Appendix I). Samples (about 30mg) were homogenized in RIPA lysis buffer. The sample was placed at 4°C until bubbles broke apart. Samples were then sonicated on ice to break apart remaining cells. The remaining particles were pelleted at 10,000 revolutions per minute at 4°C for 10 minutes and the supernatant containing the protein was placed in a separate 1.7mL eppendorf tube. Proteinase

inhibitor cocktail (PIC) and phenylmethanesulfonyl fluoride (PMSF) were added to each sample (Appendix I). Samples were quantified following manufacturer's protocol for the Pierce BCA Protein Assay Kit (Thermo Scientific, Wilmington, DE, catalog #23227). Standards were prepared using the manufacturer provided bovine serum albumin at 2.0 mg/mL. Working reagent utilizing manufacturer's reagents A and B was prepared. A microplate was loaded with 25 µL of standard or unknown and 200 µL of working reagent. The microplate was then incubated for 30 minutes at 37°C. Samples were quantified using spectrophotometry with the Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT). Sample concentrations were determined based upon the standard curve prepared with the standards. If concentrations were below 1.0 µg/µL the sample was reisolated. The samples were stored at -80°C until further analysis.

Reverse Transcription

cDNA was created using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, Catalog #170-8890) using the manufacturer's protocol. A 20µL reaction was made up with 4µL of 5x iScript reaction mix, 1µL of iScript reverse transcriptase, 1µg of RNA template, and nuclease-free water. It was incubated for 5 minutes at 25°C, 30 minutes at 42°C, and then 5 minutes at 85°C. The cDNA was then immediately added to the qRT-PCR reaction mix for PCR analysis.

Sequencing

Equine specific PCR forward and reverse primers were designed using Primer3 (http://primer3.wi.mit.edu/) with a product size between 115-135 base pairs, a primer length between 19-27 base pairs, a primer Tm between 60-65°C, and a GC% content between 40-60. Designed primers for each of the genes can be found in Appendix II. Each product was sequenced to verify the specificity of the primer. cDNA was generated from the RNA template

from endometrial samples using the same protocol as the cDNA for qRT-PCR. A 10µL PCR reaction mix was generated using 5.7µL of nuclease free water, 2.0µL of 5x GoTaq buffer (Promega, Madison, WI, Catalog #M791A), 0.1µL GoTaq DNA polymerase (Promega, Madison, WI, Catalog #M300A), 0.2µL of 10mM dNTP mix (Quanta Biosciences, Gaithersberg, MD, Catalog #84032), 1.0µL of cDNA, and 1µL of 5µM primer in 10mM Tris-HCl. The reaction was incubated at 94°C for 5 minutes, then 40 cycles consisting of 15 seconds at 94°C, 30 seconds at 60°C and 15 seconds at 72°C, and then a final incubation for 3 minutes at 72°C. PCR products were run through a 2% agarose gel to identify the PCR products. The PCR products were isolated from the gel following manufacturer's recommendation for the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA, Catalog #28704). The PCR product was excised from the gel and dissolved in buffer QG. Isopropanol was added and the sample was then placed in a Qiaquick spin column. The sample was washed with buffer PE to rinse away remaining gel and eluted in nuclease-free water. Samples were then prepared to be sent to sequencing at the Colorado State University Proteomics and Metabolomics Facility, Fort Collins, CO.

qRT-PCR

The validated primers were used to analyze the mRNA expression levels of the samples with qRT-PCR. Two positive controls were used, *GAPDH* and *TUBA1B*, and the primer sequences are listed in Appendix II. Each reaction was 6µL total with 3µL of SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA, Catalog #1725260), 0.9µL of nuclease-free water, 1µL of cDNA at a concentration of 50ng/µL, and 1µL of primer at a concentration of 5µM. qRT-PCR was performed using a LightCycler480 PCR system (Roche, Indianapolis, IN). The PCR conditions consisted of 30 seconds at 95°C for enzyme activation, 40 cycles consisting of denaturation at 95°C for 5 seconds and annealing and extension at 60°C for 30 seconds, and

ending in a melt curve analysis of the PCR product at 65-95°C in 0.5°C increments with 2 seconds at each step. The gene of interest was considered to be present at a Cp value of less than 37 and each amplicon was evaluated based upon a correct amplification curve and a singular melt peak.

Statistical Analysis

Raw Cp values from endometrial samples from pregnant and non-pregnant mares were normalized by using the geometric mean of *GAPDH* and *TUBA1B* because they have the same expression level the same across all time points independent of pregnancy status. The samples were then compared using a paired, two-tailed student's t-test for statistical difference within each day. Samples were also analyzed across days 12, 14, 16, and 18 by a one-way ANOVA with a Tukey's Honestly Significant Difference Test (HSD). Samples were considered statistically different at $P<0.05$ for both analyses, within and across days.

Gene Ontology

Gene ontology was evaluated for each gene of interest. Chromosome locations were determined for the horse, but gene ontologies were based upon human ontologies. All ontologies were determined using AgBase (http://www.agbase.msstate.edu/).

Western Blot Analysis

Western blot analysis was used to determine the specificity of DKK1 and sPLA2 antibodies that were not designed against equine peptide, but have been used in various species. For each western blot, 40µg of endometrial protein from pregnant and non-pregnant mares was loaded into each well of a 12% 1.0mm SDS-page polyacrylamide gels (Appendix III). Samples

were incubated with a 4:1 6x buffer to DTT mix for 10 minutes at 90°C. Samples were then transferred to the wells and run for 30 minutes at 30mA and transferred to Protran nitrocellulose membranes (GE Healthcare Life Sciences, Pittsburgh, PA, Catalog #10402468) for 1 hour at 100V at 4°C. Membranes were blocked in 5% blocking buffer (5% non-fat dried milk in 1X TBST) for 1 hour at room temperature and washed with 1X TBST. Membranes were then incubated with either primary anti-phospholipase A2 rabbit polyclonal antibody (1:1000 in 5% milk TBST, Abcam, San Francisco, CA, ab23705) or primary anti-DKK1 rabbit polyclonal antibody (1:1000 in 5% milk TBST, Abcam, San Francisco, CA, ab93017) overnight at 4°C. The next day membranes were washed 3 times in 1X TBST and incubated with horseradish peroxidase conjugated goat polyclonal secondary antibody to rabbit IgG (1:2000 in 5% milk TBST, Abcam, San Francisco, CA, ab6721) for 1 hour at room temperature. Membranes were again washed 3 times with 1X TBST and exposed to ECL Plus Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburgh, PA, Catalog #RPN2232) for 5 minutes. Membranes were imaged for analysis on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA).

ESR1 Immunohistochemistry

The protocol for immunohistochemistry for ESR1 was adapted from (Wilsher et al. 2011). 5μm sections of tissue were placed on a slide and incubated overnight at 37°C. Slices were then taken through a standard dewaxing and dehydration process using Citrisolve (Thermo Scientific, Wilmington, DE, Catalog# 22-143975) and an alcohol gradient. The slices were then blocked from endogenous peroxidase with 1% H_2O_2 in 70% methanol for 30 minutes and rinsed in water. Microwave assisted antigen retrieval was performed by boiling slides in 10mM sodium citrate buffer (pH 6.0) for 2 bursts of 5 minutes and then left in the hot sodium citrate for 30 minutes.

Then they were washed with running water for 5 minutes. Slides were blocked from nonspecific binding by incubation with 10% normal goat serum (NGS) in TBS containing 1% bovine serum albumin (BSA) in a humidity chamber at room temperature for 30 minutes. The slides were then incubated overnight at 4°C in a humidity chamber with primary mouse monoclonal estrogen receptor antibody (1:20 in TBS with 1% BSA and 2% NGS, Leica Microsystems, Buffalo Grove, IL, Catalog #NCL-ER-6F11). The next day the slides were washed in TBS with BSA solution and the horseradish peroxidase conjugated bioatinylated goat anti-mouse secondary antibody (1:200 in TBS with 1% BSA and 2% NGS, Vector Laboratories, Burlingame, CA, Catalog #BA-9200) as applied for 30 minutes in a humidity chamber at room temperature. The slides were washed in TBS with BSA solution and incubated with Vectastain ABC solution (Vector Laboratories, Burlingame, CA, Catalog #PK-6000) for 30 minutes per manufacturer's protocol. After the ABC solution, Immpact DAB Peroxidase Substrate Solution (Vector Laboratories, Burlingame, CA, Catalog #SK-4105) was applied for 7 minutes per manufacturer's instructions. The reaction was stopped by running tap water for 5 minutes. The slides were taken back through the alcohol gradient to dehydrate the slices. Once dry the slides were mounted with cytoseal (Thermo Scientific, Wilmington, DE, Catalog #8310-4). A negative control was created for each slice by incubating with TBS with 1% BSA and 2% NGS and no primary antibody. Slides were then imaged with light microscopy.

sPLA2 and DKK1 Immunohistochemistry

5µm sections of tissue were placed on a slide and incubated overnight at 37°C. Slices were then taken through a standard dewaxing and dehydration process using Citrisolve (Thermo Scientific, Wilmington, DE, Catalog# 22-143975) and an alcohol gradient. Antigen retrieval was performed by boiling the slides in 10mM sodium citrate (pH 6.0) for 20 minutes and leaving in

the hot sodium citrate for one hour. The slides were washed in phosphate buffered saline (PBS) for 10 minutes and blocked in 10% normal goat serum (NGS) in PBS with 1% bovine serum albumin (BSA) at room temperature for 2 hours. The slides were blotted dry and incubated in either primary anti-phospholipase A2 rabbit polyclonal antibody (1:200 in PBS with 1% BSA, Abcam, San Francisco, CA, ab23705) or primary anti-DKK1 rabbit polyclonal antibody (1:200 in PBS with 1% BSA, Abcam, San Francisco, CA, ab93017) overnight at 4°C in a humidity chamber. The next day, slides were washed in PBS for 10 minutes and incubated at room temperature in a humidity chamber with 0.3% H_2O_2 in PBS for 15 minutes to suppress endogenous peroxidase activity and reduce background staining. The slides were then incubated with horseradish peroxidase conjugated goat polyclonal secondary antibody to rabbit IgG (1:5000 in PBS with 1% BSA, Abcam, San Francisco, CA, ab6721) for 1 hour in a humidity chamber at room temperature. The slides were rinsed in PBS three times for 5 minutes and Immpact DAB Peroxidase Substrate Solution (Vector Laboratories, Burlingame, CA, Catalog #SK-4105) was applied for 10 minutes per manufacturer's instructions. To stop the color reaction the slides were rinsed in running water for 5 minutes. The slides were then taken back through the alcohol gradient for dehydration. Once dry the slides were mounted with cytoseal (Thermo Scientific, Wilmington, DE, Catalog #8310-4). A negative control was created for each slice by incubating with TBS with 1% BSA and 2% NGS and no primary antibody. Slides were then imaged with light microscopy.

Results

Microarray Analysis

Previously, a microarray experiment performed on days 14, 16, and 18 was used to identify gene expression patterns in the endometrium of pregnant and non-pregnant mares (Bruemmer et al. 2010). The data were analyzed to uncover log 2-fold differences in gene expression with regard to day and pregnancy. Genes were considered significantly differential expressed with $P<0.001$ and a fold-change greater than 3. Table 3 lists the seven genes that had higher expression in endometrium from pregnant mares at each time point and Table 4 lists the three genes that had lower expression in endometrium from pregnant mares at each time points.

qRT-PCR

qRT-PCR for day 12 endometrial samples from pregnant and non-pregnant mares revealed differential patterns of expression of *JAZF1, sPLA2, S100G, ESR1, SLC36A2,* and *DKK1,* that were also consistent with the microarray results. Other genes, *METTL7A, RALDH1*, *EIF2AK3,* and *ADM*, expression levels according to qRT-PCR were not consistent with the microarray results for days 14, 16, and 18. Figure 2 shows the relative expression levels of each of the genes for day 12.

Table 3: Microarray Results for Days 14, 16, and 18 with Higher Expression in Endometrium from Pregnant Mares

Gene Symbol	Gene Name	Chromosome Number	Gene Ontology Biological Processes	Gene Ontology Molecular Function	Gene Ontology Cellular Component
JAZF1	Juxtaposed with another zinc finger 1-like	4	Regulation of transcription, DNA dependent Negative regulation of transcription from RNA polymerase II promoter	Nucleic acid binding Transcription corepressor activity	Nucleus Transcriptional repressor complex
S100G	S100 calcium binding protein G	10	N/A	Calcium ion binding Vitamin D binding	Basolateral plasma membrane Apical plasma membrane
SLC36A2	Solute carrier family 36 (proton/amino acid symporter) member 2	14	Ion transport Amino acid transport	Hydrogen:amino acid symporter activity Glycine transmembrane transporter activity	Cytoplasm Plasma membrane
METTL7A	Methyltransferase-like protein 7A-like	6	Metabolic Process Methylation	Methyltransferase activity Transferase activity	Endoplasmic reticulum Membrane
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3	15	Regulation of translation Endoplasmic reticulum overload response	Protein kinase activity Nucleotide binding	Endoplasmic reticulum membrane Cytoplasm

Table 3 Continued

Gene Symbol	Gene Name	Chromosome Number	Gene Ontology Biological Processes	Gene Ontology Molecular Function	Gene Ontology Cellular Component
DKK1	Dickkopf 1 homolog	1	Negative regulation of canonical Wnt receptor signaling pathway Cell morphogenesis involved in differentiation	Protein binding Signal transducer activity	Extracellular region Plasma membrane
ADM (*AMPP*)	Adrenomedullin	7	Positive regulation of angiogenesis Progesterone biosynthetic process	Receptor binding Hormone activity	Extracellular region Cytoplasm

Table 4: Microarray Results for Days 14, 16, and 18 with Lower Expression in Endometrium from Pregnant Mares

Gene Symbol	Gene Name	Chromosome Number	Gene Ontology Biological Processes	Gene Ontology Molecular Function	Gene Ontology Cellular Component
sPLA2 (Pla2g5)	Secretory phospholipase A2	2	Phospholipid metabolic process Lipid catabolic process	Phospholipid binding Phospholipase A2 activity	Extracellular region Endoplasmic reticulum membrane
ESR1	Estrogen receptor 1	31	Regulation of apoptotic process Uterus development	Steroid hormone receptor activity Sequence-specific DNA binding transcription factor activity	Nucleus Cytoplasm
RALDH1 (*Aldh1a1*)	Retinaldehyde dehydrogenase 1-like	23	Cellular aldehyde metabolic process Retinol metabolic process	Retinal dehydrogenase activity Aldehyde dehydrogenase activity	Cytoplasm

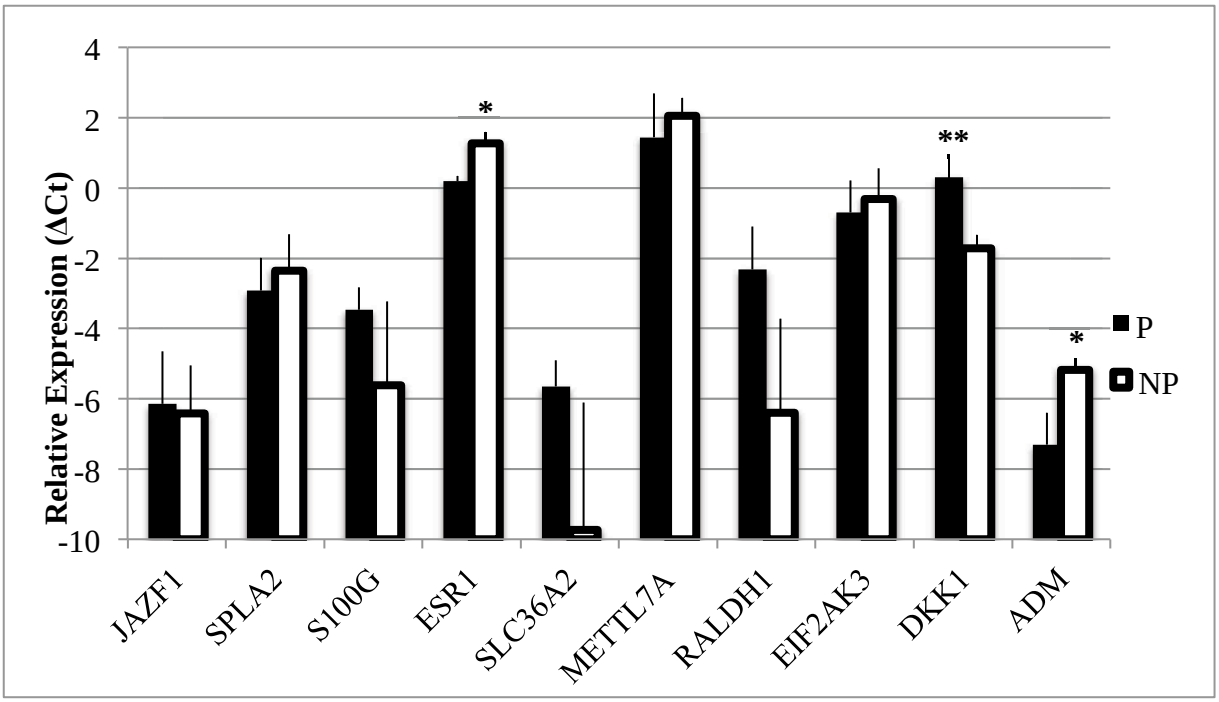

Figure 2: Day 12 Endometrial mRNA qRT-PCR Results

This figure demonstrates the higher and lower expression levels of each of the genes on day 12 endometrium from pregnant and non-pregnant mares. Expression levels are represented by the ΔCt and error bars represent the standard error of the mean. One star denotes $P<0.05$ and two stars denotes $P<0.01$.

qRT-PCR analysis on day 14 endometrium from pregnant and non-pregnant mares revealed expression patterns of each of the genes that was also consistent with the prior microarray results. *JAZF1 ,S100G, ESR1, SLC36A2, METTL7A,* and *EIF2AK3*, showed differences in expression levels between endometrium from pregnant and non-pregnant mares. *METTL7A* and *EIF2AK3* were up-regulated in the endometrium from pregnant mares on day 14. Figure 3 displays the relative expression levels for each of the genes.

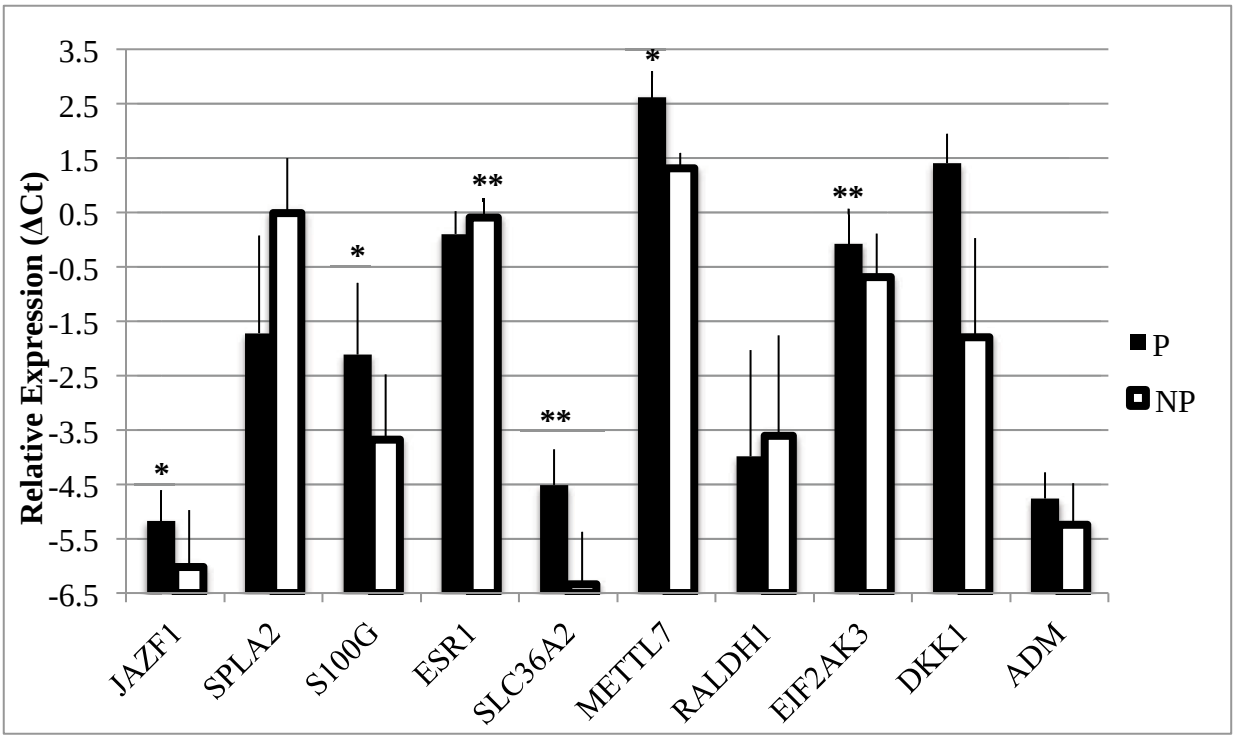

Figure 3: Day 14 Endometrial mRNA qRT-PCR Results

This figure demonstrates the higher and lower expression levels of each of the genes on day 14 endometrium from pregnant and non-pregnant mares. The regulation in the pregnant endometrium is consistent with the microarray results Expression levels are represented by the ΔCt and error bars represent the standard error of the mean. One star denotes $P<0.05$ and two stars denotes $P<0.01$.

On day 16, qRT-PCR in on endometrium from pregnant and non-pregnant mares revealed expression patterns consistent with the microarray results. *ESR1, SLC36A2, EIF2AK3,* and *ADM* were up or down regulated in the endometrium from pregnant mares. Figure 4 shows the relative expression regulation status of each of the genes on day 16.

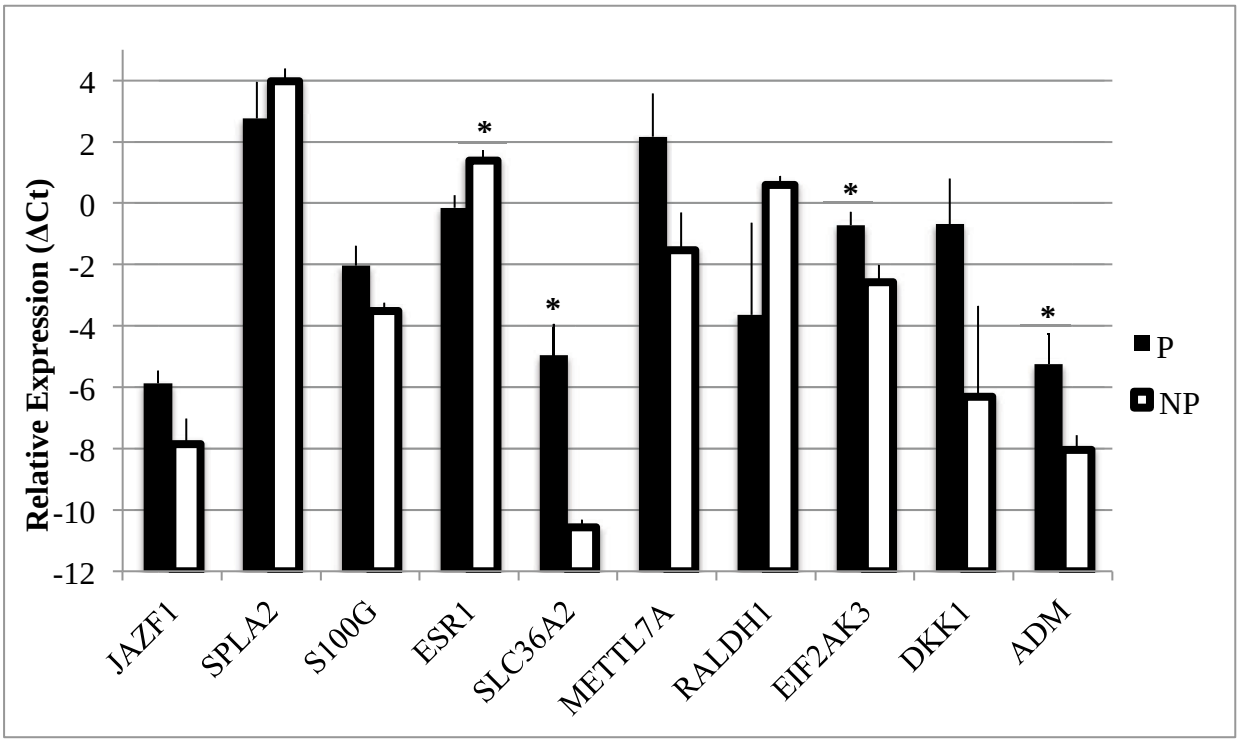

Figure 4: Day 16 Endometrial mRNA qRT-PCR Results

This figure demonstrates the higher and lower expression levels of each of the genes on day 16 endometrium from pregnant and non-pregnant mares. The regulation in the pregnant endometrium is consistent with the microarray results Expression levels are represented by the ΔCt and error bars represent the standard error of the mean. One star denotes $P<0.05$.

Day 18 qRT-PCR results were also consistent with the microarray results for all genes. *sPLA2* and *RALDH1* were among the few significantly differentially expressed genes. *ADM* and *JAZF1* were the only two genes not to show a significant higher or lower expression level in endometrium from pregnant mares. Figure 5 shows the relative expression of each of the genes on day 18.

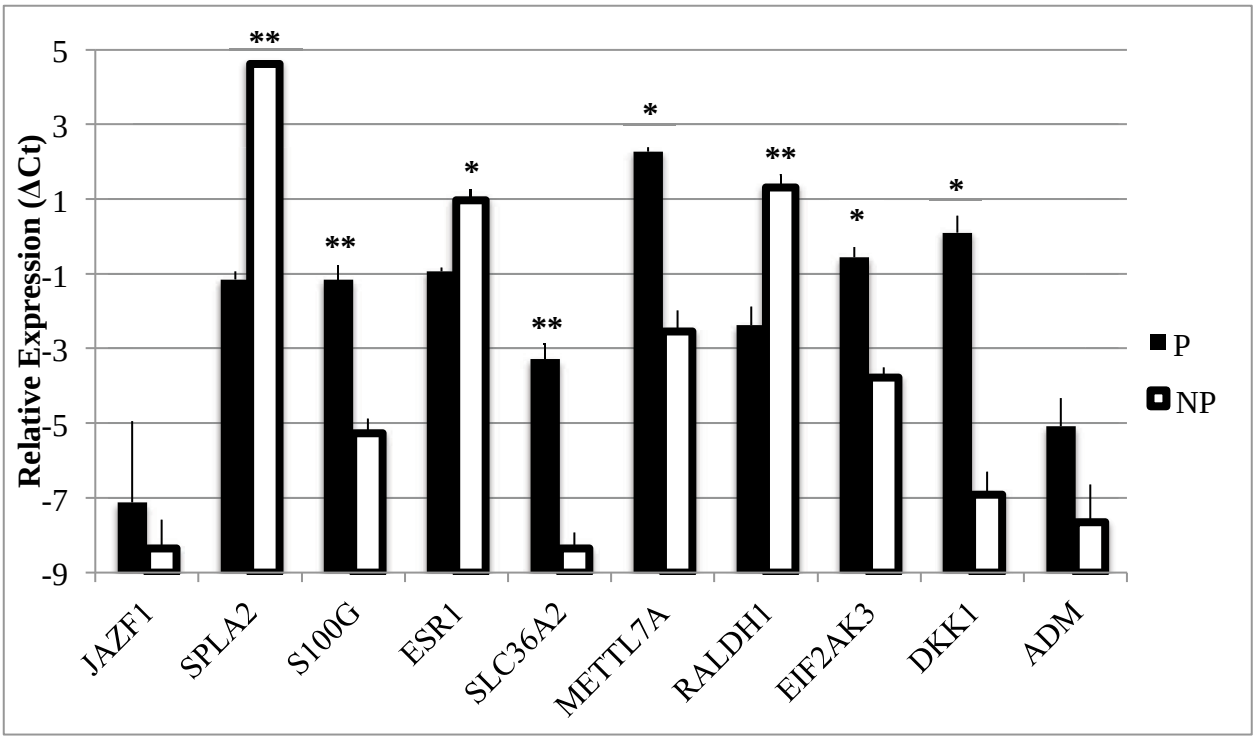

Figure 5: Day 18 Endometrial mRNA qRT-PCR Results

This figure demonstrates the higher and lower expression levels of each of the genes on day 18 endometrium from pregnant and non-pregnant mares. The regulation in the pregnant endometrium is consistent with the microarray results Expression levels are represented by the ΔCt and error bars represent the standard error of the mean. One star denotes $P<0.05$ and two stars denotes $P<0.01$.

Expression patterns were also analyzed for each gene across days 12, 14, 16, and 18 in endometrium from pregnant and non-pregnant mares. *JAZF1* was not statistically different across the days. Figure 6A shows the expression pattern of *JAZF1* across days and Figure 6B depicts the relative expression of *JAZF1* across days in endometrium from pregnant versus non-pregnant mares.

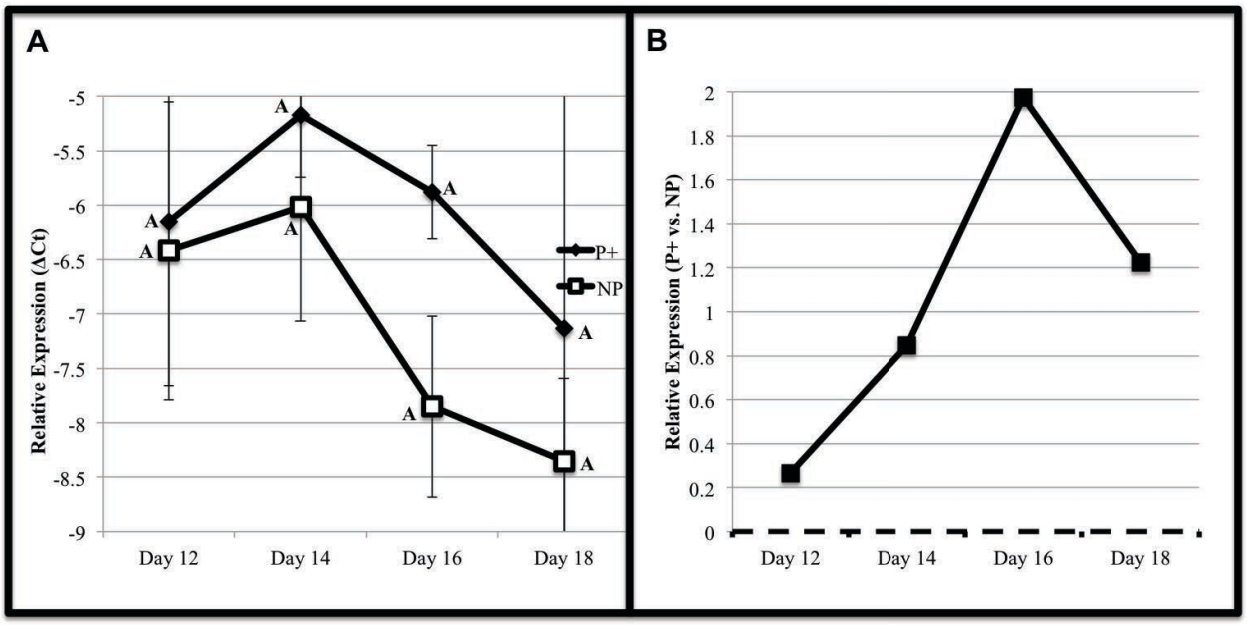

Figure 6: *JAZF1* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *JAZF1* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *JAZF1* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

sPLA2 across days 12, 14, 16, and 18, in endometrium from pregnant non-pregnant mares had expression differences. In non-pregnant mares there was no expression difference across days, but there was a difference in pregnant mares. Figure 7A shows the expression pattern of *sPLA2* across days and Figure 7B depicts the relative expression of *sPLA2* across days in endometrium from pregnant versus non-pregnant mares.

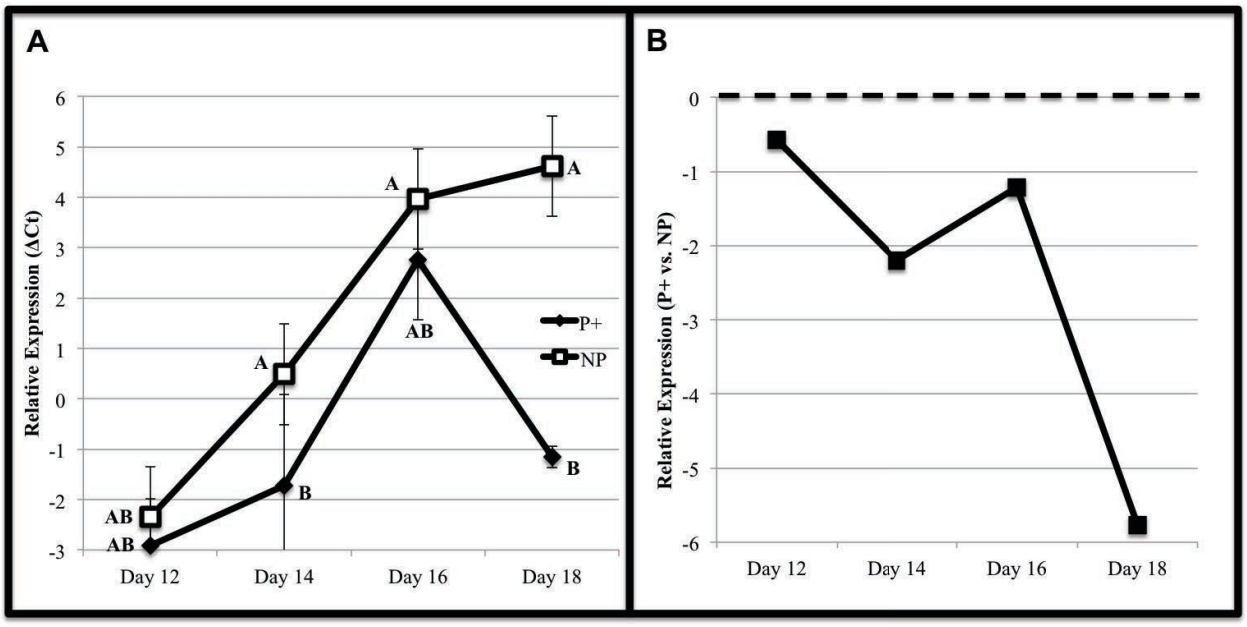

Figure 7: *sPLA2* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *sPLA2* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *sPLA2* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

S100G had a higher expression level on all days in endometrium from pregnant mares, but across days 12, 14, 16, and 18 there is no difference between pregnant and non-pregnant mares. Figure 8A shows the expression pattern of *S100G* across days and Figure 8B depicts the relative expression of *S100G* across days in endometrium from pregnant versus non-pregnant mares.

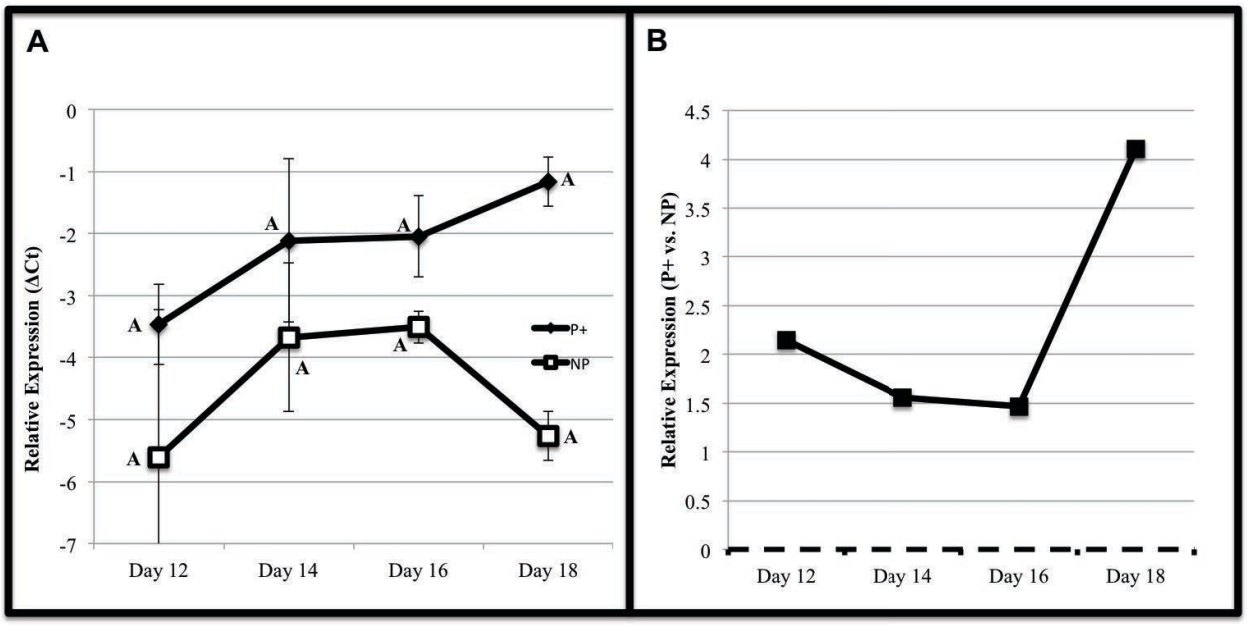

Figure 8: *S100G* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *S100G* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *S100G* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

ESR1 was consistently expressed at higher levels in the endometrium from non-pregnant mares versus pregnant mares on each day. There was no difference across days for the non-pregnant mares and for the pregnant mares, although a decline was observed in pregnant mares. Figure 9A shows the expression pattern of *ESR1* across days and Figure 9B depicts the relative expression of *ESR1* across days in endometrium from pregnant versus non-pregnant mares.

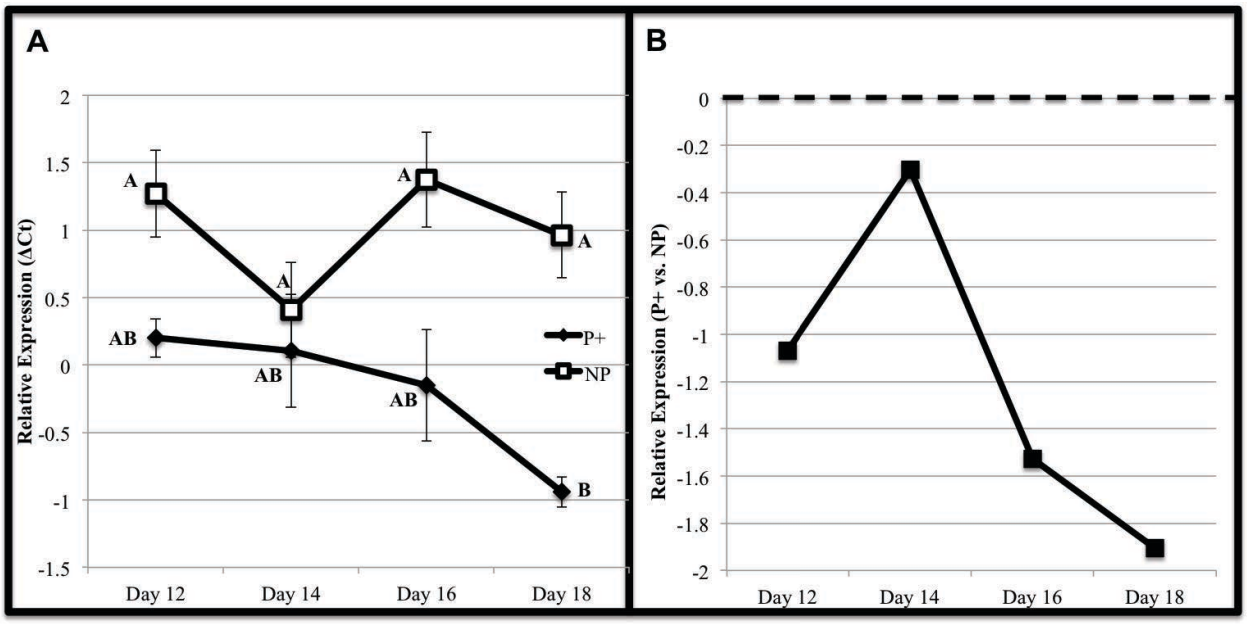

Figure 9: *ESR1* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *ESR1* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *ESR1* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

SLC36A2 was higher expressed in endometrium from pregnant mares versus non-pregnant mares at all time points. Across the time points there was no differences in pregnant mares and no differences across days for non-pregnant mares. There was a difference observed between some time points between endometrium from pregnant and non-pregnant mares. Figure 10A shows the expression pattern of *SLC36A2* across days and Figure 10B depicts the relative expression of *SLC36A2* across days in endometrium from pregnant versus non-pregnant mares.

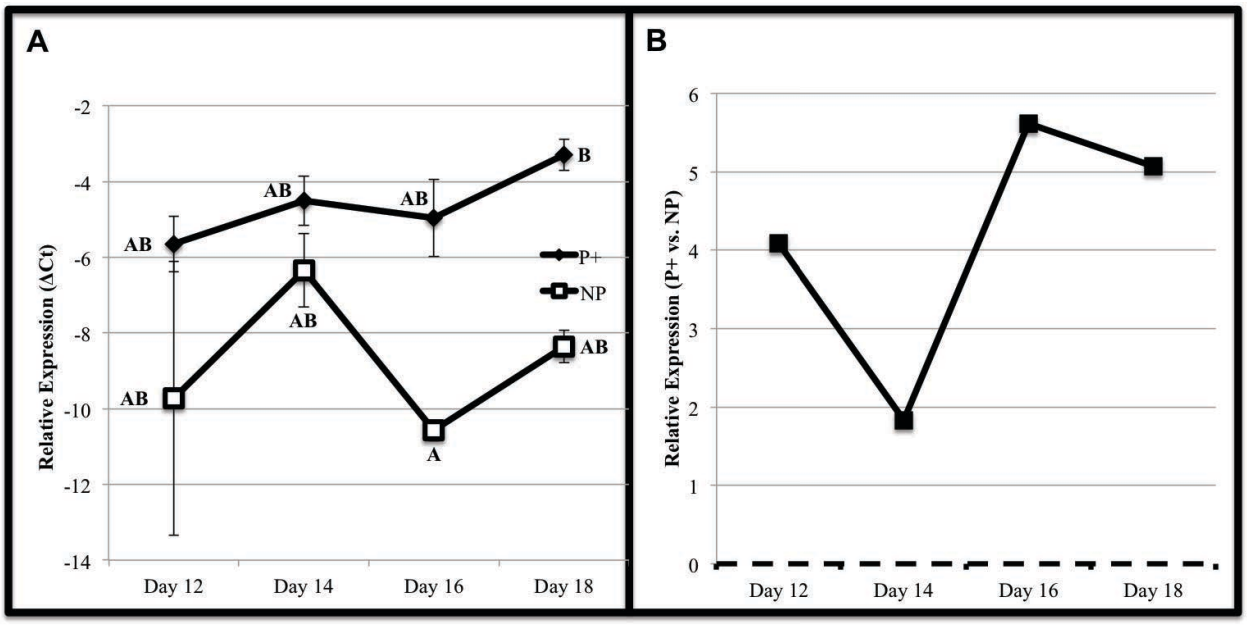

Figure 10: *SLC36A2* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *SLC36A2* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *SLC36A2* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

METTL7A had a higher expression level in endometrium from a non-pregnant mare on day 12 post-ovulation, but on days 14, 16, and 18 post-ovulation had a higher expression level in endometrium from a pregnant mare. Across days 12-18 post-ovulation there was a significant decrease in *METTL7A* expression in endometrium from non-pregnant mares, while in pregnant mares the expression pattern did not change. Figure 11A shows the expression pattern of *METTL7A* across days and Figure 11B depicts the relative expression of *METTL7A* across days in endometrium from pregnant versus non-pregnant mares.

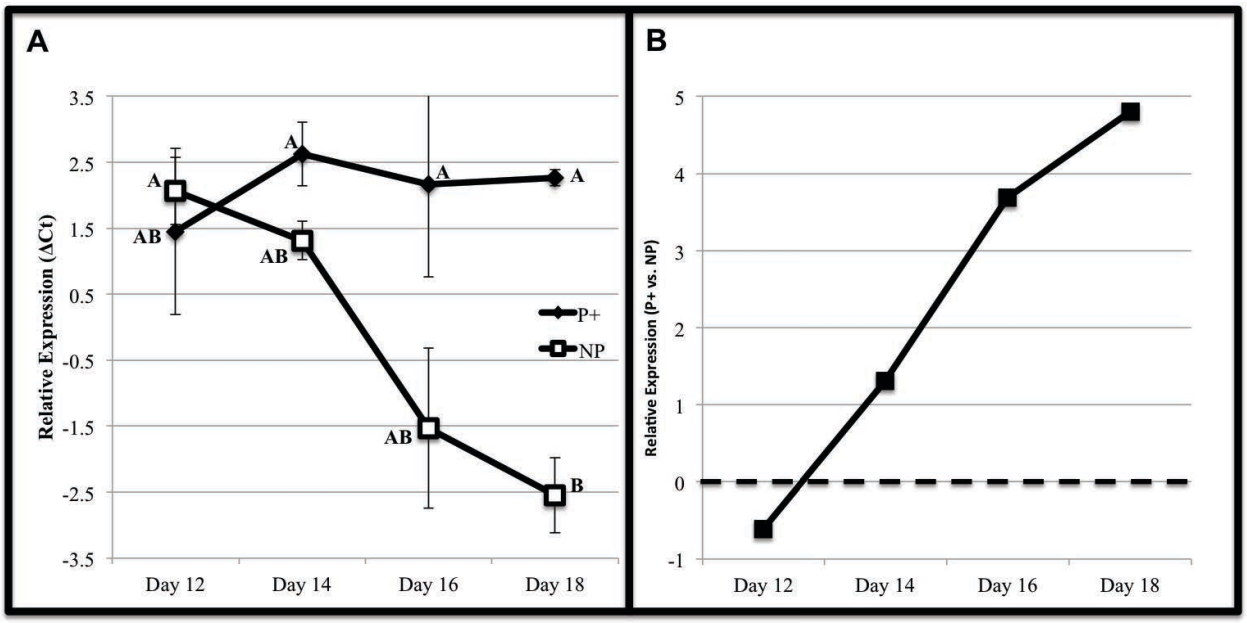

Figure 11: *METTL7A* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *METTL7A* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *METTL7A* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

RALDH1 had a higher expression level in endometrium from pregnant mares on day 12 post-ovulation, but then had a higher expression level in endometrium from non-pregnant mares on days 14-18 post-ovulation. When evaluated across days there was no difference observed in endometrial samples from both pregnant and non-pregnant mares. Figure 12A shows the expression pattern of *RALDH1* across days and Figure 12B depicts the relative expression of *RALDH1* across days in endometrium from pregnant versus non-pregnant mares.

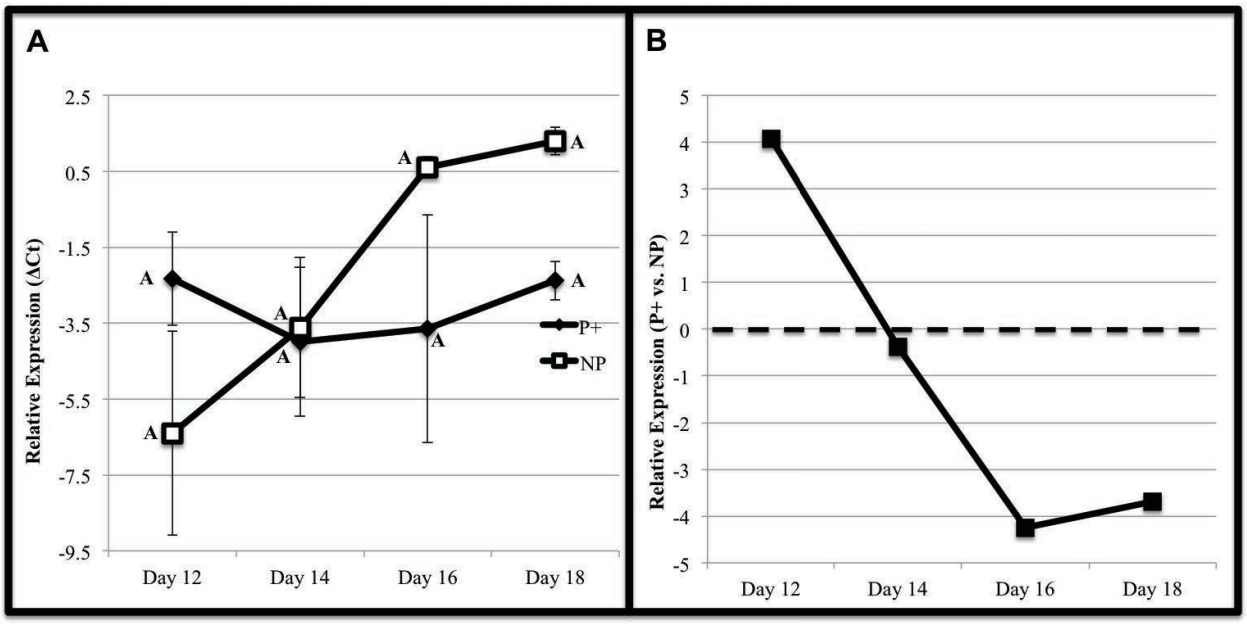

Figure 12: *RALDH1* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *RALDH1* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *RALDH1* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

EIF2AK3 had a higher expression level in endometrium from non-pregnant mares on day 12 post-ovulation, but had a higher expression level in endometrium from pregnant mares on days 14-18 post-ovulation. When evaluated across days there was a significant difference in endometrium from non-pregnant mares as the days progressed. In endometrium from pregnant mares there was no difference across days in *EIF2AK3* expression levels. Figure 13A shows the expression pattern of *EIF2AK3* across days and Figure 13B depicts the relative expression of *EIF2AK3* across days in endometrium from pregnant versus non-pregnant mares.

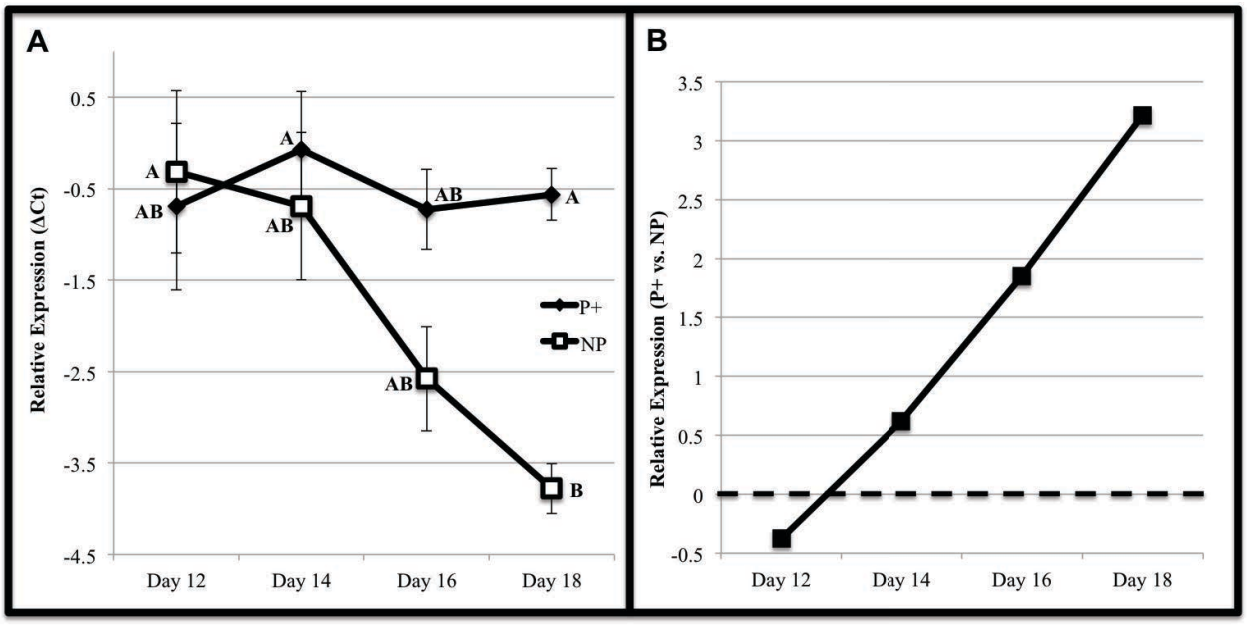

Figure 13: *EIF2AK3* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *EIF2AK3* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *EIF2AK3* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

DKK1 had a higher expression level in endometrium from pregnant mares on days 12-18 post-ovulation. When evaluated across days there were differences between days in the endometrium from both pregnant and non-pregnant mares. Interestingly, *DKK1* on day 12 post-ovulation in endometrium from pregnant mares was expressed significantly higher than endometrium from day 18 post-ovulation non-pregnant mares. Figure 14A shows the expression pattern of *DKK1* across days and Figure 14B depicts the relative expression of *DKK1* across days in endometrium from pregnant versus non-pregnant mares.

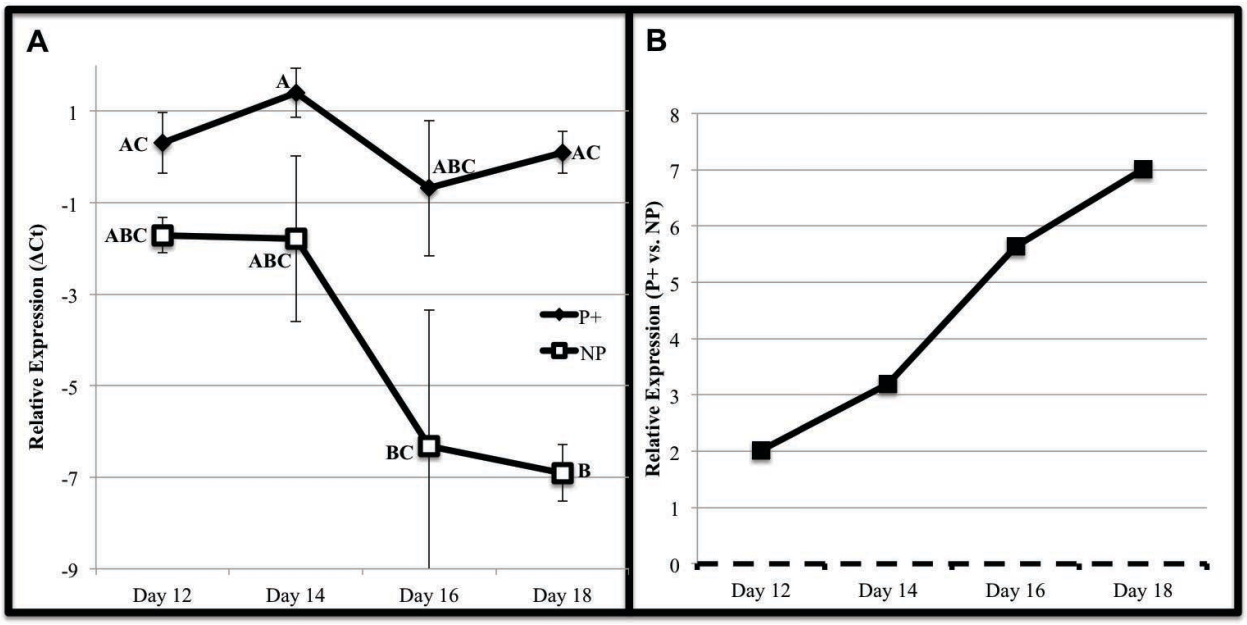

Figure 14: *DKK1* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *DKK1* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *DKK1* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

ADM was significantly higher expressed in endometrium from non-pregnant mares on day 12 post-ovulation, but higher expressed in endometrium from pregnant mares on days 14-18 post-ovulation. When evaluated across days there was no significant differences found. Figure 15A shows the expression pattern of *ADM* across days and Figure 15B depicts the relative expression of *ADM* across days in endometrium from pregnant versus non-pregnant mares.

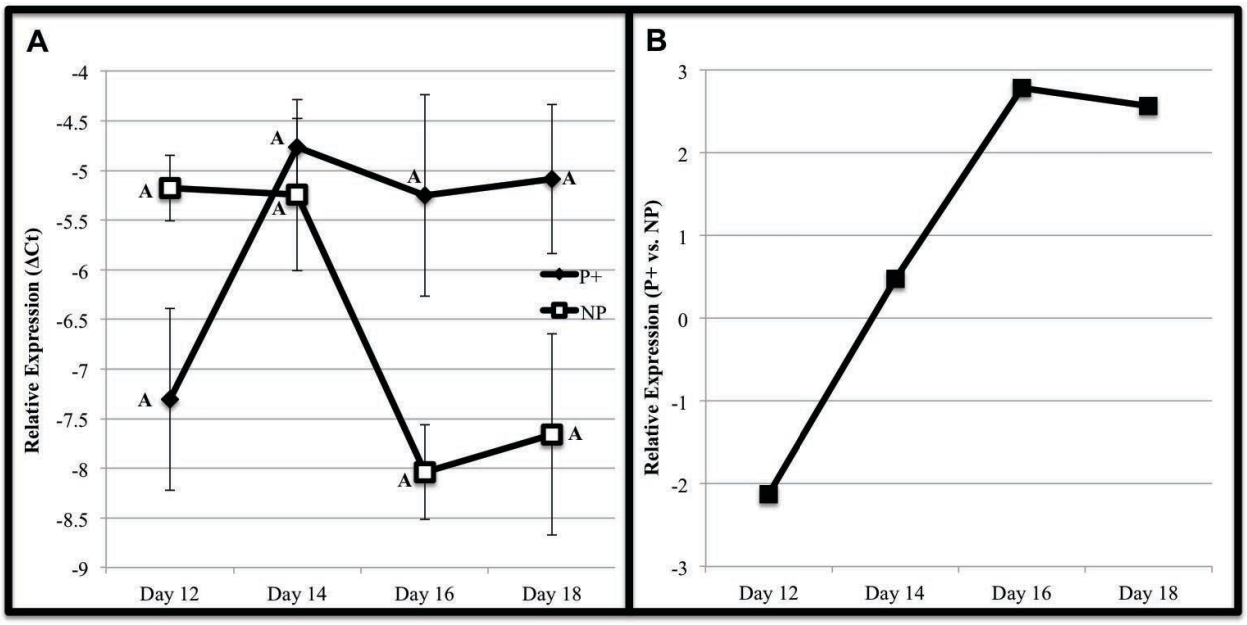

Figure 15: *ADM* Endometrial mRNA Relative Expression Across Days

This figure demonstrates the expression pattern of *ADM* in endometrium from pregnant and non-pregnant mares. Panel A shows the comparison of the relative expression across days 12, 14, 16, and 18 post-ovulation, with significance being demonstrated with differing letters. Panel B demonstrates the expression of *ADM* in endometrium from pregnant mares compared to endometrium from non-pregnant mares.

Real-time PCR results for days 12, 14, 16, and 18 confirm the previously obtained expression patterns in endometrium from pregnant and non-pregnant mares, although not all genes at all time points showed higher or lower expression levels. Day 12 results were consistent with the later days for 6 of the genes, *JAZF1, sPLA2, S100G, ESR1, SLC36A2,* and *DKK1*, but *METTL7A, RALDH1, EIF2AK3,* and *ADM* levels showed an opposite expression pattern. These results are summarized in Table 5.

Table 5: Microarray and qRT-PCR Results Summarized

This table summarizes the results from the microarray and qRT-PCR. One star denotes P<0.05 and two stars denotes P<0.01.

Gene	Microarray Day 14, 16, 18	PCR Day 12 Pregnant vs. Non-Pregnant	PCR Day 14 Pregnant vs. Non-Pregnant	PCR Day 16 Pregnant vs. Non-Pregnant	PCR Day 18 Pregnant vs. Non-Pregnant
JAZF1	HIGHER	HIGHER	HIGHER*	HIGHER	HIGHER
sPLA2	LOWER	LOWER	LOWER	LOWER	LOWER**
S100G	HIGHER	HIGHER	HIGHER*	HIGHER	HIGHER**
ESR1	LOWER	LOWER**	LOWER**	LOWER*	LOWER*
SLC36A2	HIGHER	HIGHER	HIGHER**	HIGHER*	HIGHER**
METTL7A	HIGHER	LOWER	HIGHER*	HIGHER	HIGHER*
RALDH1	LOWER	HIGHER	LOWER	LOWER	LOWER**
EIF2AK3	HIGHER	LOWER	HIGHER**	HIGHER	HIGHER
DKK1	HIGHER	HIGHER*	HIGHER	HIGHER	HIGHER*
ADM	HIGHER	LOWER*	HIGHER	HIGHER*	HIGHER

Western Blots

Western Blot analysis was conducted to confirm the specificity of the antibodies to DKK1 or sPLA2 in equine endometrium because they were not designed with the equine peptide, but have been confirmed in various species. Endometrium from day 13 pregnant and non-pregnant mares was utilized for this experiment. Figure 6 shows the western blot with the endometrial protein from pregnant and non-pregnant mares for sPLA2, which has a predicted size of 14 kDa, and Figure 7 shows the western blot with the endometrial protein from pregnant and non-pregnant mares for DKK1, which has a predicted size of 38 kDa.

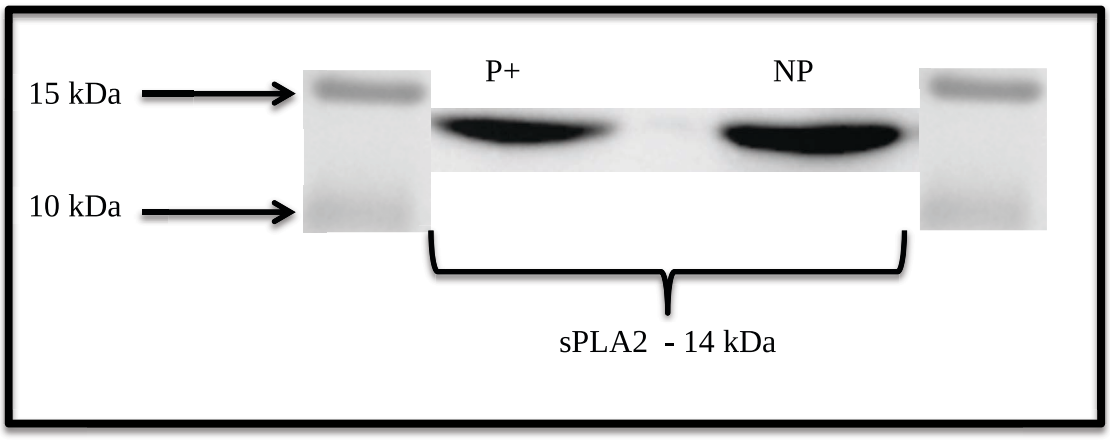

Figure 16: Western Blot Analysis for sPLA2

Western Blot analysis on day 13 endometrial protein in pregnant and non-pregnant endometrium confirmations that the rabbit polyclonal sPLA2 antibody is specific for sPLA2 in equine endometrium. sPLA2 is 14 kDa in size and is detected in pregnant and non-pregnant endometrium in the above figure.

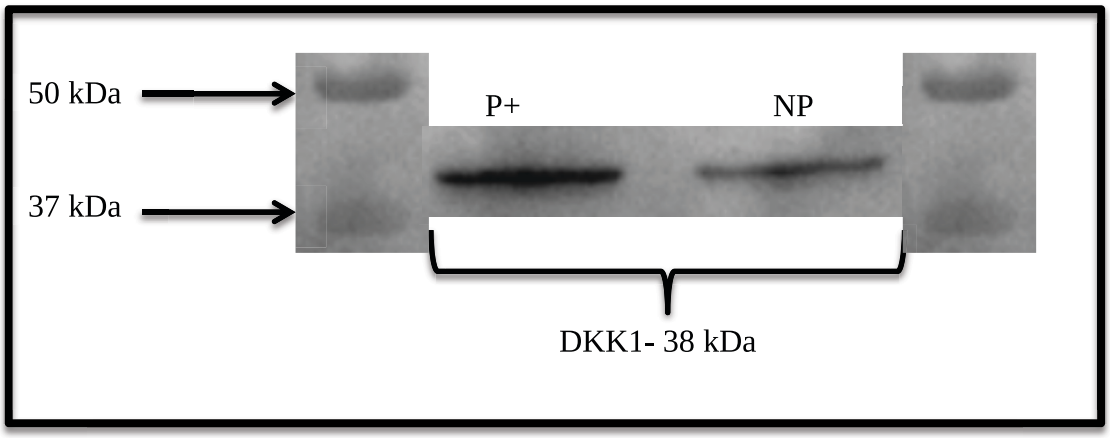

Figure 17: Western Blot Analysis for DKK1

Western Blot analysis on day 13 endometrial protein in pregnant and non-pregnant endometrium confirmations that the rabbit polyclonal DKK1 antibody is specific for DKK1 in equine endometrium. DKK1 is 38 kDa in size and is detected in pregnant and non-pregnant endometrium in the above figure.

Immunohistochemistry

Localization of sPLA2 and DKK1 was determined by immunohistochemistry. ESR1 was included as a positive control and in the endometrium from pregnant mares localized to the basal region of the glandular epithelium. Very little staining for ESR1 was found in the luminal glandular region and the endometrial epithelium. In the endometrium from non-pregnant mares, ESR1 localization was throughout the endometrium, and detected in the endometrial epithelium and throughout the glandular epithelium. This localization can be seen in Figure 8.

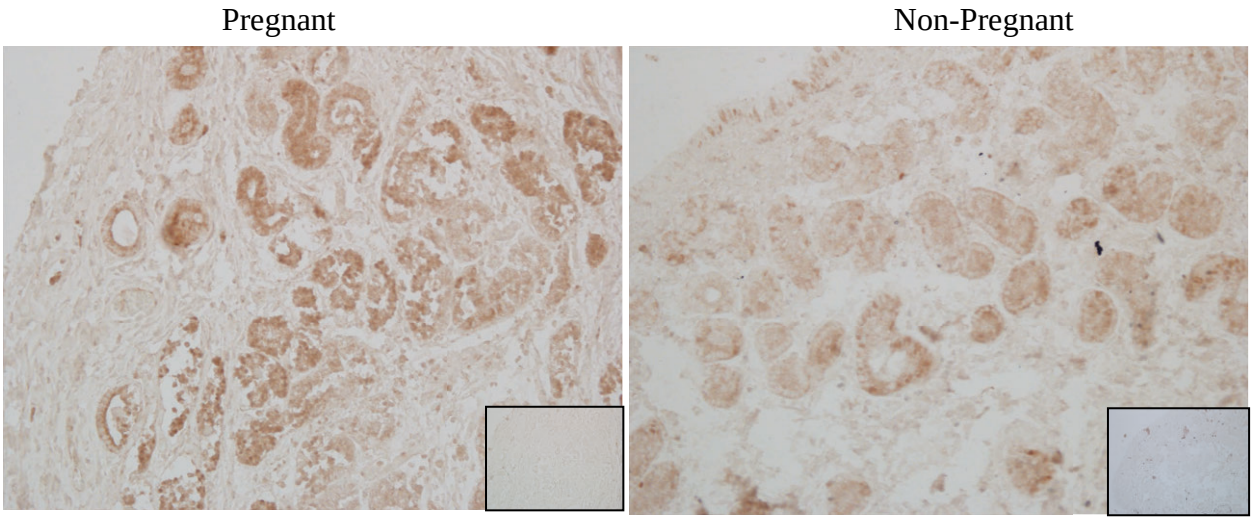

Figure 18: ESR1 Localization in the Endometrium from Pregnant and Non-Pregnant Mares

Immunohistochemistry on day 13 endometrium from pregnant and non-pregnant mares for ESR1 identifies the cellular location of this protein. The negative control for each tissue section is in the lower right region of each picture.

sPLA2 was localized to the endometrial epithelium and the glandular cells. This localization pattern did not vary between the endometrium from pregnant and non-pregnant endometrium on day 13. Figure 9 shows the localization of this protein.

Pregnant Non-Pregnant

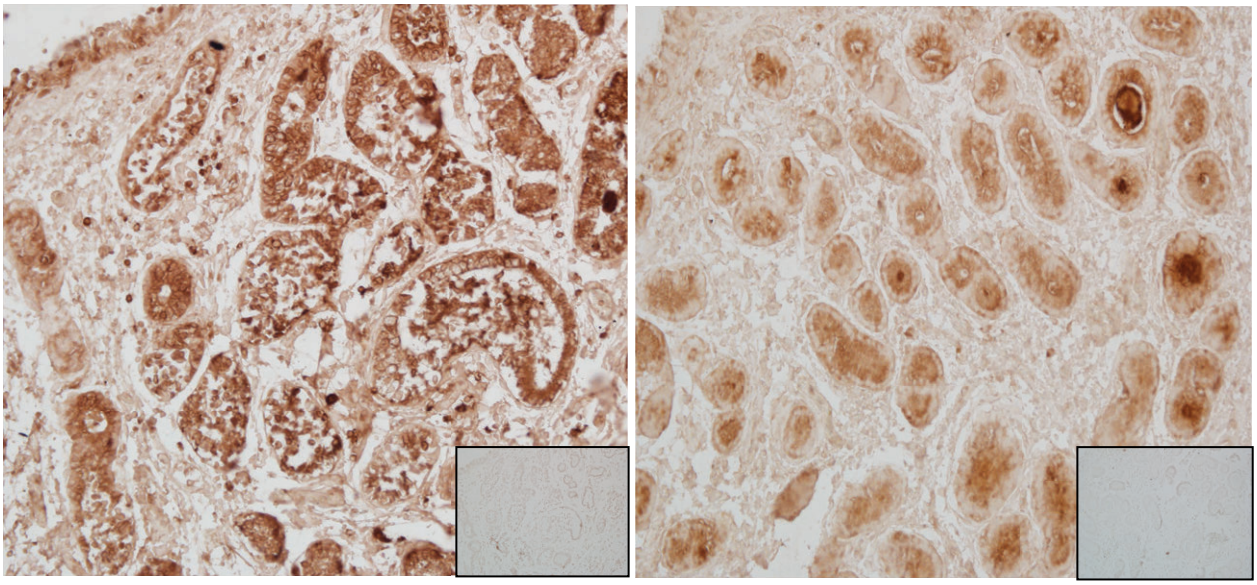

Figure 19: sPLA2 Localization in the Endometrium from Pregnant and Non-Pregnant Mares

Immunohistochemistry on day 13 endometrium from pregnant and non-pregnant mares for sPLA2 identifies the cellular location of this protein. The negative control for each tissue section is in the lower right region of each picture.

The localization of DKK1 endometrium from pregnant and non-pregnant mares was determined by immunohistochemistry on day 13 endometrium. In pregnant endometrium, DKK1 is localized to the endometrial epithelium and in the glandular cells in the glandular region. In the endometrium from non-pregnant mares, very little staining is seen in both the endometrial epithelium and the glandular cells. The localization of this protein can be seen in Figure 10.

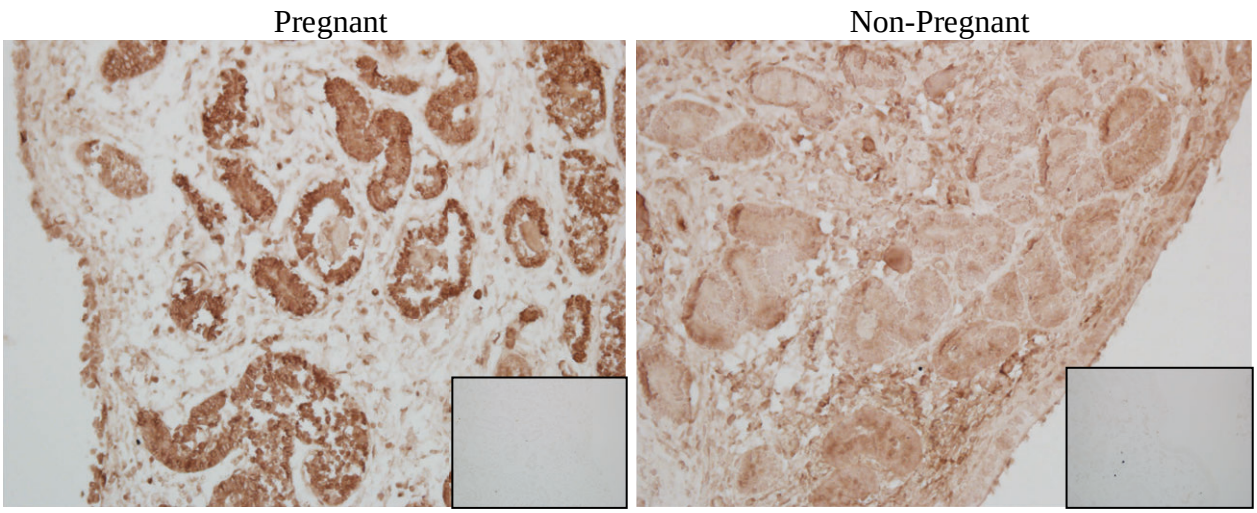

Figure 20: DKK1 Localization in the Endometrium from Pregnant and Non-Pregnant Mares

Immunohistochemistry on day 13 endometrium from pregnant and non-pregnant mares for DKK1 reveals localization of this protein. The negative control for each tissue section is in the lower right region of each picture.

Discussion

This experiment was performed to confirm and extend a previously performed equine specific microarray analysis on days 14, 16, and 18 post-ovulation in endometrium of pregnant and non-pregnant mares (Bruemmer et al. 2010). qRT-PCR was performed on days 12, 14, 16, and 18 to determine the relative expression level of *JAZF1, sPLA2, S100G, ESR1, SLC36A2, METTL7A, RALDH1, EIF2AK3, DKK1,* and *ADM*. Interestingly, six out of the ten genes were regulated in a similar manner on day 12 as they were on days 14, 16, and 18, but 4 genes switched relative expression levels in samples from pregnant versus non-pregnant mares between days 12 and 14 post-ovulation. qRT-PCR confirmed the results of the microarray across days 14, 16, and 18.

On day 12 post-ovulation, *JAZF1, sPLA2, S100G, ESR1, SLC36A2,* and *DKK1* were differentially expressed the same (higher or lower expression in endometrium from pregnant mares) as in days 14, 16, and 18. By day 12 post-ovulation, *ESR1* had a significantly lower expression level in endometrium from pregnant mares. This corresponds to previous literature stating that *ESR1* decreases during mid-estrus (Watson et al. 1992) and is down-regulated in early pregnancy due to the presence of progesterone (Spencer and Bazer 1995). Gene ontology for ESR1 revealed that is involved in uterus development and steroid hormone receptor activity. Immunohistochemistry for ESR1 on day 13 also corresponded with previous literature about its localization (Hartt et al. 2005). This experiment showed that in endometrium from pregnant mares ESR1 is localized in the lower stroma and glandular epithelium, but in endometrium from non-pregnant mares it is localized to the luminal and glandular epithelium.

On day 12 post-ovulation, *DKK1* was also significantly higher expressed in endometrium from pregnant mares. Gene ontology of DKK1 revealed that it is involved in signal transducing activity, cell morphogenesis and is located in the extracellular region. *DKK1* is a Wnt-signaling antagonist in the endometrium during pregnancy (Kawano and Kypta 2003) and may play an active role in preventing differentiation of the endometrium during early pregnancy. It has also been found to inhibit the attachment of the embryo to the endometrium (Liu et al. 2010). In early pregnancy preventing attachment is critical in order for the embryo to continue to be mobile throughout the entire uterine lumen, signaling maternal recognition of pregnancy. *DKK1* is also up-regulated by high progesterone levels in the circulation (Atli et al. 2011). Immunohistochemistry revealed that DKK1 in endometrium from pregnant mares is located in the luminal epithelium and glandular cells, but very little localization of this protein was seen in these areas in endometrium from pregnant mares. This may indicate how DKK1 prevents attachment of the embryo to the endometrium during early pregnancy.

By day 14 post-ovulation, qRT-PCR analysis of all of the genes confirmed the microarray results in their expression levels in endometrium from pregnant mares. *ESR1* continued to be significantly lower expressed on this day. Interestingly, *EIF2AK3,* and *METTL7A* both switch from having lower expression in endometrium from pregnant mares on day 12 post-ovulation to significantly higher expression on day 14 post-ovulation. Both of these genes are involved in gene transcription and modifications. EIF2AK3 spans the endoplasmic reticular (ER) membrane and is a major stress sensor in the ER (Li et al. 2003). When under stress, EIF2AK3 initiates the unfolded protein response so the cell can react to the stressor (Rutkowski and Kaufman 2004). This stress response may be triggered by the mobile conceptus in the uterine lumen. METTL7A is a methyltransferase that may be involved in the modification of specific genes and proteins.

Gene ontology identifies that METTL7A is involved in metabolic processes and transferase activities. Little research has been done to evaluate its role in the reproductive cycle.

S100G and *SLC36A2* are significantly higher expressed on day 14 post-ovulation in endometrium from pregnant mares. Both genes are involved in nutrient transport from the endometrium to the conceptus. *S100G* encodes for calbindin-D9k (*CaBP9k*), which is a calcium binding protein. S100G is involved in calcium and vitamin D binding. It is regulated by progesterone levels, so as progesterone increases *S100G* expression increases (Hoffmann et al. 2009b). It is involved in providing the conceptus with necessary nutrients and a decrease in *CaBP9k* results in insufficient nutrients reaching the conceptus and conceptus death (Hoffmann et al. 2009b). The up-regulation of *S100G* may be critical in order for the conceptus to survive through early pregnancy prior to attachment. *SLC36A2* is an H+ coupled amino acid transport system (Edwards et al. 2011). Gene ontology indicates that it is involved in ion and amino acid transport across the plasma membrane. It is thought to also be necessary for conceptus survival by providing the conceptus with the necessary amino acids during early pregnancy prior to attachment. Previous research identified *SLC36A2* also as being up-regulated in day 13.5 pregnant equine endometrium compared to endometrium from a non-pregnant mare (Klein et al. 2010). *RALDH1* also switches from being higher expressed on day 12 post-ovulation in endometrium from non-pregnant mares to lower expression on day 14 and *JAZF1* is significantly higher expressed on day 14 post-ovulation in endometrium from pregnant mares. RALDH1 is involved in the retinol metabolic process and retinal dehydrogenase activity. Gene ontology of JAZF1 indicates that it is involved in the regulation of transcription by negative regulation of transcription from RNA polymerase II promoter. *JAZF1* is a tumor suppressor that is found in the endometrium (Li et al. 2007). Mutations in this gene are implicated with endometrial stromal

tumors surrounding the endometrial glands under the surface epithelium (Li et al. 2007). *JAZF1* may be critical during the time of maternal recognition in order to support the endometrium during this time of many endometrial changes that will be occurring.

On day 16 post-ovulation, the genes all followed the same trend of expression as they did on day 14. Only *ESR1, SLC36A2, EIF2AK3,* and *ADM* had significantly different expression levels between endometrium of pregnant and non-pregnant endometrium on day 16. Day 16 also corresponds to the day of fixation of the embryo. At this point maternal recognition of pregnancy has occurred, so endometrial gene expression should be directed towards maintaining a successful pregnancy. Gene ontology of ADM indicates it is a positive regulater of angiogenesis and involved in the progesterone biosynthetic process. ADM is a vasodilator that causes an increase in uterine blood flow in the sheep (Cameron et al. 2002). It is also thought to regulate fetal and maternal blood pressure throughout pregnancy (Macri et al. 1996). In contrast to these results, *ADM* levels were increased when progesterone was declining and estrogen was increasing in rodents and humans (Cameron et al. 2002; Maybin et al. 2011). Data from this experiment suggest the opposite regulation is occurring in equine endometrium. More research needs to be done to determine the function of *ADM* in equine endometrium.

On day 18 post-ovulation, multiple genes became significantly differentially expressed. These genes included *sPLA2, S100G, ESR1, SLC36A2, METTL7A, RALDH1, EIF2AK3,* and *DKK1*. Each of these genes may play a critical role in maintaining the endometrium, allowing it to support a viable pregnancy. Day 18 post-ovulation is the first day that *sPLA2* becomes significantly lower expressed in endometrium from pregnant mares. Gene ontology reveals that sPLA2 in involved in the phospholipid metabolic process, phospholipase A1 activity, and lipid catabolic processes. sPLA2 is involved in the synthesis of prostaglandin $F_{2\alpha}$ (PGF) by releasing

arachadonic acid, the precursor to PGF, from the phospholipid bilayer (Needleman et al. 1986). Maximal sPLA2 expression is observed during maximal PGF synthesis and in the horse corresponds with the increased capacity to synthesize PGF in response to oxytocin stimulation (Sharp et al. 1997). The expression of *sPLA2* negatively correlates with progesterone levels indicating that progesterone is a key regulator of *sPLA2* expression (Ababneh et al. 2011). It has also been found bound to equine embryonic capsules, increasing in response to PGF (Hayes et al. 2008). This may contribute to the breakdown of embryonic capsules resulting in early embryonic loss (Hayes et al. 2008). These results indicate that *sPLA2* may play a critical role in PGF synthesis so it must be closely regulated during and after maternal recognition of pregnancy. On day 13 post-ovulation, sPLA2 was also identified to be located in the luminal epithelium and the glandular epithelium in the endometrium from pregnant and non-pregnant mares.

RALDH1 also became significantly lower expressed on day 18 post-ovulation in endometrium from pregnant mares. It is a key regulator in retinoic acid synthesis (Duester et al. 2003). Excess or deficiency of retinoic acid can result in abortion or embryonic malfunction (Mohan et al. 2001). In rodents excess retinoic acid can also induce blastocyst apoptosis (Huang et al. 2003). In rodents and humans *RALDH1* is regulated by estrogen (Bucco et al. 1997; Deng et al. 2003). It has also been found to play a role in blastocyst-endometrium interaction (Duester et al. 2003). Due to the negative effects that excess retinoic acid may have on the blastocyst, *RALDH1* expression must be closely regulated during and after maternal recognition of pregnancy.

Each of the ten genes may have a critical role in maternal recognition of pregnancy in the mare. These results indicate that some of the genes become increasingly more expressed in endometrium from pregnant mares as the number of days post-ovulation increases. The higher or

lower expression of these genes may lead to the identification of the signal for maternal recognition of pregnancy in the mare by identifying what is causing the expression of these genes.

CHAPTER III: DISCUSSION

The results from this experiment confirm and extend the results from a previously performed microarray experiment on days 14, 16, and 18 post-ovulation in endometrium from pregnant and non-pregnant mares. The results from day 12 post-ovulation for the ten previously identified genes, *JAZF1*, *sPLA2*, *S100G*, *ESR1*, *SLC36A2*, *METTL7A*, *RALDH1*, *EIF2AK3*, *DKK1*, and *ADM*, revealed that some of the genes were expressed in the same manner across days 12, 14, 16, and 18 post-ovulation and some genes that were expressed in the opposite manner and then switched by day 14 post-ovulation. Each of the previously identified genes may play a key role in maternal recognition of pregnancy in the mare due to their roles in regulation of transcription, nutrition transport, metabolic processes, biosynthetic processes, regulation of translation, angiogenesis, cell morphogenesis, and regulation of apoptosis.

Juxtaposed with another zinc finger protein 1-like (JAZF1)

Until recently not much information was known about the function of *JAZF1*. *JAZF1* is present in many different tissues throughout the body but is found in highest levels in adipose and testicular tissue and moderate levels in the colon, placenta, prostate, and ovary (Nakajima et al. 2004). Recently, *JAZF1* was found to be identical to TAK1-interacting protein 27 (*TIP27*). *JAZF1* represses TGF-beta activated kinase 1 (*TAK1*) mediated transactivation and more specifically DR1-dependent transcriptional activation by *TAK1*, but the direct effect of this repression remains unknown (Nakajima et al. 2004).

JAZF1 mutations have also been implicated in endometrial stromal tumors. These tumors occur in the mesenchymal tissue under the surface epithelium and surrounding the endometrial glands that are formed by invaginations of the surface epithelium (Li et al. 2007). In these tumors

JAZF1 is down regulated and instead the fusion of JAZF1-JJAZF1 (JAZF1-SUZ12) is noted in abundance. This fusion is seen only in endometrial stromal tumors and not normal endometrium (Koontz et al. 2001). The unmutated version of JAZF1 is thought to be a tumor suppressor (Li et al. 2007). *JAZF1* has also been shown to be a transcriptional repressor of a gene that causes growth retardation in mice and height variation in humans (Johansson et al. 2009). Most recently, *JAZF1* was found to show a potential role in glucose metabolism. Overexpression of *JAZF1* results in an increase in *GLUT1*, a glucose transporter (Li et al. 2011). *JAZF1* is a novel gene whose complete functions and roles remain unknown. It has been found to have an effect on tumor suppression, glucose metabolism, and growth. Interestingly it is also found to be present in endometrium and when fused to *JJAZF1* seems to play a role in endometrial stromal tumors showing its role in the regulation of transcription.

In this experiment, *JAZF1* was higher expressed in endometrium from pregnant mares on all days (days 12-18 post-ovulation). This may be due to the role that *JAZF1* may play in endometrial remodeling for pregnancy. It also acts as a tumor suppressor, which may be critical during early pregnancy to support the endometrium while it is undergoing functional changes.

Secretory Phospholipase A2 (sPLA2)

Secretory phospholipase A2 (*sPLA2)* is a member of the phospholipase A2 enzyme family that is involved in the biosynthesis of prostaglandin $F_{2\alpha}$ (PGF). sPLA2 binds to the anionic phospholipid bilayer and cleaves the phospholipid releasing it from the membrane. (Beers et al. 2003; Birts et al. 2008; Hayes et al. 2008). The phospholipid that is released from the lipid bilayer is arachadonic acid, the precursor to PGF. Once released, cystolic phospholipase A2 (*cPLA2*) mobilizes the arachadonic acid that is converted to prostaglandin H2 (PGH) and

eventually becomes PGF (Needleman et al. 1986). The PLA2 family are the rate limiting enzymes in the biosynthesis of prostaglandins (Ababneh et al. 2011). Maximal PLA2 activity has been found to correlate to the period of maximal PGF secretion in luteolysis, suggesting that PLA2 may have a direct effect on luteolysis. It also corresponds with the increased capacity to synthesize PGF in response to oxytocin stimulation (Sharp et al. 1997).

Interestingly, a negative correlation between PLA2 expression levels and progesterone concentrations was described, inferring the control progesterone may have control over PLA2 expression levels (Ababneh et al. 2011). This relationship was confirmed by progesterone supplementation to ovariectomized mares resulting in a decrease in the PLA2 family enzymes (Ababneh and Troedsson 2013). Throughout the estrous cycle, expression levels of the PLA2 family vary in horses. There are high levels of expression during estrus, but they decline after ovulation. In the same study results showed that on day 15 expression levels of *cPLA2* were similar between pregnant mares and non-pregnant mares that had not undergone functional luteolysis, indicating that cPLA2 specifically does not vary throughout the cycle (Ababneh et al. 2011). This implies that sPLA2 may be the member of the PLA2 family that is varying based upon pregnancy status and progesterone concentration.

sPLA2 is also present bound to equine embryonic capsules. The amount bound to the capsule increases substantially in response to PGF (Hayes et al. 2008). The yolk sac walls of these embryos were also evaluated to determine the level of *sPLA2*, and they contained very low expression levels. This implies that bound sPLA2 originates in the endometrium (Hayes et al. 2012). It is proposed that binding of sPLA2 to the capsule in the response to PGF contributes to the removal and breakdown of the capsule and the conceptus, possibly resulting in early embryonic loss (Hayes et al. 2008). While the function of sPLA2 is well understood, such as its

involvement in hormone biosynthesis, its role and regulation during maternal recognition of pregnancy remains to be understood.

sPLA2 is lower expressed in endometrium from pregnant mares on all days in the this experiment with it being significantly lower expressed on day 18 post-ovulation. Since it plays a key role in PGF synthesis, it needs to be down regulated in endometrium from pregnant mares in order to decrease PGF synthesis in the endometrial cells. Localization of sPLA2 protein also revealed it is concentrated in the endometrial epithelium and the glandular cells. The location did not change between pregnant and non-pregnant endometrium. This may indicate that some *sPLA2* is constitutively present in order to aid in the PGF pathway when properly signaled to be activated.

S100 calcium binding protein G (S100G)

S100 calcium binding protein G (*S100G*) is the gene that encodes for calbindin-D9k (*CaBP9k*), which is a calcium binding protein. In general, S100G is involved in calcium and vitamin D binding. CaBP9k has been evaluated in the endometrium in many different species, but the regulation of expression levels varies among species (Inpanbutr et al. 1994). In the pregnant rat uterus *CaBP9k* is localized in the glandular epithelium, myometrium, and endometrial stroma (Bruns et al. 1988). Expression levels vary throughout the estrous cycle but are higher in estrogen dominated phases and lower in progesterone dominated phases (Inpanbutr et al. 1994). This implies that in the rat uterus, CaBP9k is controlled by estrogen and/or inhibited by progesterone. *CaBP9k* expression in the rat also coincides with myometrial contractility (Mathieu et al. 1989). It is controlled in an opposite manner in other species. In humans, there are higher expression levels in progesterone dominated periods, such as the luteal phase, with

expression levels of *CaBP9k* peaking during the menstrual phase. Elevated *CaBP9k* levels have also been found during pregnancy and maintained until labor (Inpanbutr et al. 1994). The bovine uterus regulates *CaBP9k* expression in the same manner as humans. It is more present in the luteal phase than the follicular phase, indicating it is also regulated by progesterone. The localization is also specialized in cattle. CaBP9k is localized to the glandular epithelium, with lower expression levels seen closer to the uterine lumen (Inpanbutr et al. 1994).

In the mare, *CaBP9k* expression levels are controlled by progesterone in the same manner as the human and the cow. As serum progesterone concentration increases, there is an increase in *CaBP9k* (Hoffmann et al. 2009a). In equine endometrium CaBP9k is localized to the glandular epithelium with maximal staining and maximal expression observed during early to mid-estrus (Hoffmann et al. 2009a). Since *CaBP9k* is a calcium binding protein there has been speculation that expression levels also have an effect on the nutritional supply to the conceptus. Recent studies have shown that decreased levels of *CaBP9k* lead to an insufficient nutrient supply reaching the conceptus, ultimately leading to conceptus death (Hoffmann et al. 2009a). This information suggests that *CaBP9k* expression is important for embryo survival in the equine endometrium and that progesterone levels are necessary for the regulation of *CaBP9k*.

In this experiment *S100G* is higher expressed in endometrium from pregnant mares across all days. This confirms that it is regulated by progesterone because across all the time points progesterone is the dominating hormone. If its role is to supply nutrition to the conceptus, higher expression in endometrium from pregnant mares is critical for the survival of the conceptus.

Estrogen Receptor 1 (ESR1)

Estrogen receptor 1 (*ESR1*), previously known as estrogen receptor α (*ERα*), is a steroid hormone receptor that has been well characterized in many species throughout pregnancy and the estrous cycle. It is involved in the regulation of apoptotic processes, uterus development, and steroid hormone activity, indicated by its gene ontology. In a few species, such as pigs and cows, *ESR1* remains present in early pregnancy (Robinson et al. 1999; Knapczyk-Stwora et al. 2011). Although ESR1 remains present in the cow, the mRNA levels of *ESR1* decrease significantly from day 0-18 of pregnancy (Fürst et al. 2012). In sheep, *ESR1* is down-regulated during early pregnancy, due to the presence of progesterone. In sheep, estrogen up-regulates steroid hormone receptors and progesterone down regulates steroid hormone receptors (Spencer and Bazer 1995). The regulation of *ESR1* behaves in the same manner in primates. Progesterone down-regulates *ESR1* in the secretory phase compared to high expression levels in the late-proliferative phase (Okulicz et al. 1993).

Research in equine endometrium for expression of *ESR1* has shown different results. ESR1 is localized to the luminal and glandular epithelium during estrus, but in diestrus and early pregnancy it is not present in the luminal epithelium, only in the lower stroma and glandular epithelium (Hartt et al. 2005). ESR1 concentration is highest during estrus and early diestrus, but lowest during mid-estrus (Watson et al. 1992). Regulation of the receptors is the same in the equine endometrium as it is in ovine and primate endometrium, which is progesterone down-regulates steroid hormone receptors and estrogen up-regulates steroid hormone receptors (Hartt et al. 2005). In early pregnancy the conceptus derived estrogens also down-regulate ESR1 receptors in the endometrium (Klein et al. 2010). Interestingly fetal membranes also show ESR1 expression (Wilsher et al. 2011). While the localization and presence of ESR1 has been shown in

multiple studies, varying results have been shown regarding mRNA levels in the endometrium. One study shows that *ESR1* mRNA decreases during days 11-20 of pregnancy, while another study shows there is no difference in mRNA levels between days 8-15 of pregnancy (McDowell et al. 1999; Hartt et al. 2005).While localization and expression of ESR1 is well characterized in horses, expression levels and how the expression is controlled is still yet to be defined.

On days 12, 14, 16, and 18 post-ovulation, *ESR1* was lower expressed in endometrium from pregnant mares. This also corresponds with previous results for *ESR1* in endometrium being decreased during days 11-20 of pregnancy (McDowell et al. 1999). Localization of ESR1 also corresponded with previous data. In the endometrium from pregnant mares it is localized to the lower stroma and glandular region, but not the endometrial epithelium. In non-pregnant endometrium it was localized throughout the epithelium and glandular region.

Solute carrier family 36 (proton/amino acid symporter), member 2 (SLC36A2)

Solute carrier family 36 (proton/amino acid symporter), member 2 (*SLC36A2*) is a member of the SLC36 family of transporter-related genes, consisting of four members (Edwards et al. 2011). It is also known as phosphate acetyl transferase, *PAT2*. Not much is known regarding *SLC36A2* or *PAT2*, except that it is involved in the transport of amino and fatty acids (Foltz et al. 2004). Gene ontology also reveals that it is involved in glycine trnsmembrane transporter activity. It is a H+ coupled amino acid transport system that has been identified in a multiple locations throughout the body (Edwards et al. 2011). It is localized to myelinating neuronal Schwann cells, suggesting a role in amino acid supply in differentiation of those cells (Bermingham et al. 2002). *SLC36A2* has also been identified to be involved in amino acid movement in neuronal tissue (Rubio-Aliaga et al. 2004). Recently it has been shown to be

specifically involved in glycine, proline, and hydroxyproline reabsorption. When a mutation is present in this gene it has major effects on the reabsorption of these amino acids (Bröer et al. 2008).

Very little research has been done with the involvement of *SLC36A2/PAT2* in the reproductive system. Recently *SLC36A2* has been shown to be present in equine endometrium. It was up-regulated on day 13.5 of pregnancy compared to endometrium from non-pregnant mares on day 13.5 (Klein et al. 2010). It is though to be involved in the appropriate delivery of nutrients to the conceptus before attachment occurs. *SLC36A2/PAT2* is a transporter that has been identified, but needs to be evaluated more to determine its involvement with the conceptus and pregnancy.

In this experiment, *SLC36A2* is higher expressed in endometrium from pregnant mares across all time points. These results also confirm a previous study showing the up-regulation of *SLC36A2* in endometrium from pregnant mares on day 13.5 (Klein et al. 2010). SLC36A2 is important in amino acid and proton transport. This is necessary in order to provide the conceptus with the necessary nutrients for survival.

Methyltransferase-like protein 7A-like (METTL7A)

Methyltransferase-like protein 7A-like (*METTL7A*) is a member of the super family of methyltransferases. A methyl transferase removes the methyl group from a donor and puts it on an acceptor. Little research has been done on the function of METTL7A, but it has been identified in many different tissues throughout the body. During pregnancy, METTL7A is found in the chorion of human placentas (Nhan-Chang et al. 2010). It has also been identified in podocytes that were excreted in urine from the kidney (Prunotto et al. 2013). Most notably it has

been identified to be in lipid droplets and to be a part of lipid metabolism (Brasaemle and Wolins 2012). No research has been done to date on *METTL7A* in the endometrium and it's involvement in pregnancy in humans or horses.

In this experiment *METTL7A* is lower expressed on day 12 post-ovulation in endometrium from pregnant mares, but then has higher expression on days 14-18 post-ovulation. As a methyltransferase it is involved in the regulation of genes. This higher expression in endometrium from pregnant mares on day 14 may be a result of maternal recognition of pregnancy and the need to modify additional genes in order to prevent luteolysis.

Retinaldehyde dehydrogenase 1-like (RALDH1)

Retinaldehyde dehydrogenase 1-like (*RALDH1*) is also known as *ALDH1A1*. It is a key regulator in retinoic acid synthesis, which becomes Vitamin A (Duester et al. 2003). The main function in of RALDH1 in retinoic acid synthesis is to catalyze retinaldehyde in to retinoic acid. RALDH2 and CYP26A1 are also critical enzymes in retinoic acid synthesis (Deng et al. 2003). Retinoic acid and vitamin A have a role in pregnancy and embryo balance. There is a very delicate line between deficiency and excess of vitamin A. Both extremes can lead to abortion or embryonic malfunction (Mohan et al. 2001). Research has been done looking at retinoic acid and *RALDH1* in the mouse. Retinoic acid was found to suppress decidualization, differentiation, and development of the blastocyst. It can also induce blastocyst cell apoptosis (Huang et al. 2003). From this the authors concluded that excess retinoic acid can decrease the viability of mammalian blastocysts and can regulate cell death (Huang et al. 2003).

RALDH1 has also been identified in high concentrations in the murine oocyte, blastocyst, and hatched blastocyst. This suggests that RALDH1 and retinoic acid plays a role in blastocyst-

endometrium interaction (Duester et al. 2003). The regulation of *RALDH1* has been an active area of research. Expression levels of *RALDH1* fluctuate throughout the estrous cycle. When rodents were administered pregnant mare serum gonadotropin (PMSG), which increases estrogen levels, retinoic acid production was increased (Bucco et al. 1997). This suggested that RALDH1 expression is regulated by estrogen. In humans *RALDH1*, *RALDH2,* and *CYP26A1* expression levels are also regulated by estrogen (Deng et al. 2003). While research has been done looking at humans and rodents, little research regarding RALDH1 expression and its role in equine pregnancy has been performed.

RALDH1 in this experiment is higher expressed on day 12 post-ovulation in endometrium from pregnant mares, but then has a lower expression on days 14-18. This expression change may be due to the endometrium recognizing the presence of a mobile conceptus. This down regulation is critical because of *RALDH1*'s role in retinoic acid and vitamin A synthesis. Too little or too much vitamin A and retinoic acid can result in embryonic death (Mohan et al. 2001). The lower expression also becomes more significantly different by day 18 post-ovulation leading to the necessity to regulate this gene.

Eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3)

Eukaryotic translation initiation factor 2-aplha kinase 3 (*EIF2AK3*) is also known as pancreatic endoplasmic reticulum eIF2α kinase (*PERK*) (Li et al. 2003). EIF2AK3 spans the endoplasmic reticulum (ER) membrane and is one of the major sensors in the ER that activates the unfolded protein response when the cell is under stress so that it can react to the stressor (Li et al. 2003; Rutkowski and Kaufman 2004). *EIF2AK3* is also a major regulator of *IGF-1* dependent neonatal growth (Li et al. 2003). Recently *EIF2AK3* has been linked to Wolcott-

Rallison syndrome. Deficiency of EIF2AK3 in humans leads to permanent neonatal diabetes associated with Wolcott-Rallison syndrome (e et al. 2004). A mutation in the *EIF2AK3* gene also leads to Wolcott-Rallison syndrome (Delépine et al. 2000). EIF2AK3 is also involved in the regulation of translation, protein kinase activity, and nucleotide binding. While the role *EIF2AK3* plays in causing Wolcott-Rallison disease is well understood, research has not been done on the role it may play in the endometrium and/or during pregnancy.

In this experiment, *EIF2AK3* is lower expressed on day 12 post-ovulation in endometrium from pregnant mares, but is then higher expressed for days 14-18. On day 14 post-ovulation there is a dramatic, significant switch to higher expression in endometrium from pregnant mares. *EIF2AK3* is a major regulator of stress sensory in cells, so the mobile conceptus may be creating a stress response in the endometrial cells. The mobility of the conceptus is critical for maternal recognition of pregnancy and initiating the stress response in cells may be part of the maternal recognition mechanism.

Dickkopf 1 homolog (DKK1)

Dickkopf 1 homolog (*DKK1*) has been described to be a Wnt signaling antagonist during pregnancy (Kawano and Kypta 2003). Wnt signaling is crucial to estrogen mediated uterine growth, but has also been described to have many other functions (Hou et al. 2004). These functions include cell proliferation, differentiation and polarity, epithelial-mesenchymal communication, and embryogenesis (Wodarz and Nusse 1998). *DKK1* is also unique in that it's function, regulation, and presence is conserved across many species. In rodents it is expressed in preimplantation embryos and the stromal cells of the uterus (Li et al. 2008). It is secreted by the decidual cells and induces trophoblast cell invasion, playing an essential role in embryo

implantation. This was further confirmed by injecting antisense oligonucleotides of *DKK1* into the uterine horn on day 3 of pregnant murine endometrium. This resulted in inhibition of embryo implantation (Li et al. 2008). In humans DKK1 has been found to inhibit the attachment of the embryo in a dose-dependent manner (Liu et al. 2010). Interestingly it is up regulated in human endometrium during the midsecretory phase, corresponding to the implantation period, of the menstrual cycle. This also corresponds to when circulating progesterone levels are at their highest (Tulac et al. 2003). These results were confirmed in four other human studies showing that *DKK1* is up-regulated in this phase (Bauersachs and Wolf 2012). Further research revealed that in human endometrium *DKK1* is activated by progesterone in the endometrial stromal cells (Tulac et al. 2006). In contrast, *DKK1* is also up-regulated in human endometrium when estrogen and progesterone serum concentration levels are high, as seen in humans being over stimulated for *in vitro* fertilization procedures (Liu et al. 2010).

Research in other species has confirmed that *DKK1* regulation is conserved across species. In sheep, *DKK1* expression levels are dependent upon progesterone. It is hypothesized that its role is to inhibit Wnt signaling pathways in the trophoblast giant binucleate cells in the endometrial stroma and prevent their migration into the stroma (Hayashi et al. 2007). In bovine endometrium *DKK1* is up-regulated during the luteal phase and at day 18 of pregnancy (Bauersachs and Wolf 2012). This confirmed that in cattle *DKK1* is also regulated by progesterone. Recently, *DKK1* was evaluated in equine endometrium. Studies have shown that it is also up-regulated during periods of high progesterone (Atli et al. 2011). While *DKK1* has been studied extensively in human endometrium, its role in other species is not as well defined. It is known that it inhibits the Wnt signaling pathway and prevents attachment, but the down stream

effects of this inhibition still remain unknown. Gene ontology revealed that DKK1 is also involved in cell morphogenesis in differentiation, protein binding, and signal transducing activity.

In this experiment *DKK1* was higher expressed across all time points in endometrium from pregnant mares. This corresponds to previous research stating that *DKK1* expression is regulated by progesterone in the endometrium. When determining the localization pattern of DKK1, there were differences between endometrium from pregnant and non-pregnant mares. In the endometrium from pregnant mares it was localized to the glandular epithelium and the glandular cells. In the endometrium from non-pregnant mares, DKK1 staining is less present in the endometrial epithelium. This may be due to the function of DKK1 in the endometrium. The equine conceptus is mobile until day 16 but does not attach to the endometrium until day 36. DKK1 has been found to inhibit attachment in other species (Liu et al. 2010), so in the mare it may be serving the same function, leading to its up regulation on days 12, 14, 16, and 18.

Adrenomedullin (ADM)

Adrenomedullin (*ADM*) is a versatile peptide that has been found performing multiple functions throughout the body. Its functions include a vasodilator, bronchodilator, regulator of hormone secretion, neurotransmitter, antimicrobial agent, and controller of renal function. It has also been implicated in tumor biology, such as tumors of pulmonary and neural lineage (Zhao et al. 1998; Julián et al. 2005). Due to all of its functions it is located in many different tissue types in the body. It plays a large role in the female reproductive tract in tissue remodeling, but its regulation is not well known. It is found in the endometrium of many non-pregnant animals including rat, human, and sheep (Zhao et al. 1998; Cameron et al. 2002). In the sheep uterus it is a potent vasodilator causing increased uterine blood flow (Cameron et al. 2002). It has also been

found in amniotic fluid and the epithelium of human amnion, and the chorioallantoic membrane of the chicken (Macri et al. 1996; Zhao et al. 1998). Due to its location throughout the reproductive system it implies that *ADM* plays a vital role in pregnancy and embryogenesis. It is also thought to regulate fetal and maternal blood pressure throughout pregnancy (Macri et al. 1996).

In the murine uterus it is located in the stroma of the endometrium and the myometrium (Cameron et al. 2002). *ADM* levels have been directly correlated to estradiol concentrations in serum, with the highest levels of both being in proestrus and estrus. It is proposed that it's role is to inhibit muscular tone in the myometrium and suppress uterine contractility (Cameron et al. 2002). *ADM* levels are also raised throughout pregnancy, supporting it's role in suppressing contractility (Cameron et al. 2002). Due to pregnancy causing *ADM* levels to be high it is thought that the endometrium produces the *ADM* (Upton et al. 1997). Interestingly, when ADM is given as a treatment in late gestation to a mice with preeclampsia, which causes hypertension and pup mortality, the hypertension is reversed and there is reduced pup mortality (Makino et al. 1999).

In the human, ADM is also localized to the stroma and is also an endothelial cell growth factor (Zhao et al. 1998). It shares homology with calcitonin gene related peptide and is thought to be able to bind to the calcitonin receptor (Zhao et al. 1998). In humans, *ADM* is thought to be involved in endometrial repair. It has the highest expression level in the menstrual phase and protein levels are highest in the menstrual and early proliferative phases (Maybin et al. 2011). In humans, two pathways have been identified to regulate *ADM*. The first pathway is a HIF-1-mediated hypoxic induction and the other is a HIF-1 independent PGF pathway. Both of these pathways are triggered by the decline in progesterone (Maybin et al. 2011). Overall ADM has

many functions, but it plays an important role in the estrous cycle and pregnancy by increasing vasodilation and blood flow to the endometrium.

In this experiment, *ADM* had lower expression on day 12 post-ovulation in endometrium from pregnant mares, but then higher expression on days 14, 16, and 18. This expression change after day 12 may be due to recognition of a mobile conceptus in the uterus. Interestingly this regulation does not correspond with human and mouse data, where *ADM* is regulated by the decrease in progesterone. The up regulation of *ADM* in the endometrium during early pregnancy may be critical in order to increase the blood flow to the endometrium.

Pathway Analysis

Ingenuity systems was used to evaluate the pathway of the ten genes of interest. The top molecular and cellular functions were lipid metabolism and small molecule biochemistry. Five of the molecules are directly involved in tissue morphology and the other five are involved in organ development. The major upstream regulator of the molecules is progesterone, which is the main hormone during pregnancy. The main genes regulated by progesterone are *ADM, RALDH1, DKK1, ESR1*, and *S100G*. Figure 21 shows the network analysis of *JAZF1*, *sPLA2*, *S100G*, *ESR1*, *SLC36A2*, *METTL7A*, *RALDH1*, *EIF2AK3*, *DKK1*, and *ADM*, their locations within the cell, and their connections to the other genes.

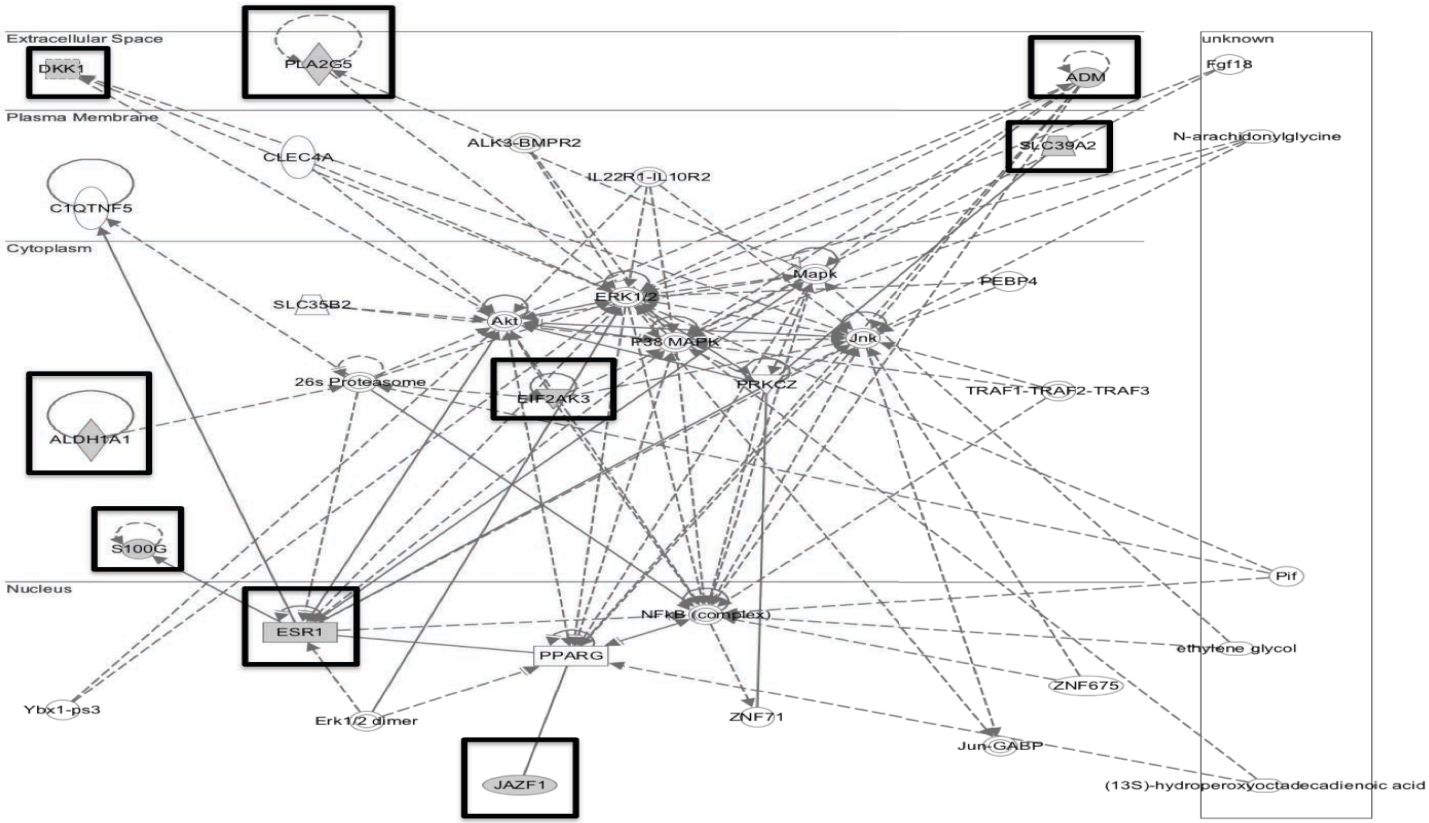

Figure 21:Pathway Analysis of Candidate Genes

Pathway analysis from Ingenuity Systems for nine of the ten candidate genes. METTL7A is not included in this image but it is located in the endoplasmic reticulum and the plasma membrane.

Conclusion

This is the first experiment to confirm and expand upon the results of an equine specific microarray previously performed (Bruemmer et al. 2010). Each of the ten genes, *JAZF1*, *sPLA2*, *S100G*, *ESR1*, *SLC36A2*, *METTL7A*, *RALDH1*, *EIF2AK3*, *DKK1*, and *ADM*, identified by the microarray may be critical in maternal recognition of pregnancy and in attenuating the production of PGF, ultimately preventing luteolysis of the corpus luteum and sustaining a viable pregnancy. These genes need to be continued to be evaluated for protein levels varying across time points throughout early pregnancy. The exact mode of regulation of these genes also needs to be identified, whether it is the mobile conceptus contacting the endometrium or secretions from the conceptus during maternal recognition of pregnancy. The identification of the mechanism of maternal recognition of pregnancy in the mare is critical in furthering the equine reproduction industry and preventing early embryonic loss.

CHAPTER IV: REFERENCES

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APPENDICES

APPENDIX I: RIPA LYSIS BUFFER RECIPES

RIPA Lysis Buffer

150mL of ddH_2O
0.484g Tris (pH 8.0)
1.6 NaCl
20mL Glycerol
2mL NP-40 (Nonidet P-40)
0.2g SDS (sodium dodecyl sulfate)
1.0g Deoxychlorate
0.117g EDTA (ehtylenediamine tetraacetic acid)
Lower to pH 8.0 with HCl
Bring up to 200mL with ddH_2O

Proteinase Inhibitor Cocktail- PIC (10X)

15mL of ddH_2O
0.138g Sodium Vanadate
0.117g Benzanidine

Phenylmethanesulfonyl Fluoride- PMSF (20mM)

3.48mg PMSF (phenylmethanesulfonyl fluoride)
1mL EtOH

APPENDIX II: PCR PRIMERS

Table 6: Designed PCR Primers for Identified Genes

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
JAZF1	GCTCTCGCTGACCTTGTCCA	AAGGAAGAGGAGGGGGTGATG
SPLA2	CAGGGGCATTTGCTGGATTT	CATCCTTGGGGGATCCTTTG
S100G	TGAGCTTGAAAAAGTCTCCTGAAG	AAGCGGGGAGTTCATTCTGG
ESR1	AGAACAGCCCCGTCTTGTCC	ACCCATCATGGAAGCCTCGT
SLC36A2	CCAGACCTTGGTTCACCTGCT	GCCAATTGCCAGCAAACTGA
METTL7A	TGTATTGACCCCAACCCCAAC	GCCACCTGGTGCATGTTCTC
RALDH1	CCACCAAGGCCAATGTTGTG	GGGTCAGCGGATTTCCAAGA
EIF2AK3	CGTGCGCAGACTTTTCCATC	CGTCATTCGAGCCACTGTCA
DKK1	CCTTGGATGGGTACTCCAGAAGA	GGACCAGAAGTGTCTGGCACA
ADM	GCTGGTTCCCGTAACCCTCA	TTCCCTCTTCCCACGACTGA
GAPDH	CACCCAGAAGACCGTGGATG	AGGGATGACCTTGCCCACAG
TUBA1B	ACGTGGTTCCCAAAGATGTC	CACAGTGGGAGGCTGGTAAT

APPENDIX III: 12% SDS-PAGE POLYACRYLAMIDE GEL PREPARATION

Running Gel

0.9mL H_2O
2.0mL 1M Tris pH 8.8
2.0mL 30% Acrylamide Solution 37.5:1 (Bio-Rad, Hercules, CA, Catalog #161-0158)
50µL 10% SDS
2.5µL TEMED (Bio-Rad, Hercules, CA, Catalog #161-0800)
25µL 10% APS

Stacking Gel

2.8mL H_2O
0.5mL 1M Tris pH 6.8
0.66mL 30% Acrylamide Solution 37.5:1 (Bio-Rad, Hercules, CA, Catalog #161-0158)
40µL 10% SDS
10µL TEMED (Bio-Rad, Hercules, CA, Catalog #161-0800)
50µL 10% APS