THESIS

EFFECTS OF PREGNANCY STATUS ON ORGANIC ANION TRANSPORTERS AND PROSTAGLANDIN RECEPTORS IN EQUINE ENDOMETRIUM DURING MATERNAL RECOGNITION OF PREGNANCY

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY ELLANE R. CLEYS, ENTITLED "EFFECTS OF PREGNANCY STATUS ON ORGANIC ANION TRANSPORTERS AND PROSTAGLANDIN RECEPTORS DURING MATERNAL RECOGINITION OF PREGNANCY" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT OF THESIS

EFFECTS OF PREGNANCY STATUS ON ORGANIC ANION TRANSPORTERS AND PROSTAGLANDIN RECEPTORS IN EQUINE ENDOMETRIUM DURING MATERNAL RECOGNITION OF PREGNANCY

Despite being the subject of many studies, the physiological mechanism for maternal recognition of pregnancy (MRP) in the mare has yet to be discovered. Identification of the mechanism for MRP is of particular interest, not only for better understanding of equine reproductive physiology, but for the development of new treatments to suppress estrus in competitive mares. It is know that the signal(s) for MRP occur between days 12 to 16 post ovulation, when the conceptus migrates throughout the uterus. With failure of MRP, the endometrium secretes luteolytic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which travels via systemic circulation to the ovaries to induce lysis of the corpus luteum (CL). Luteolytic $PGF_{2\alpha}$ can act through an assortment of receptors and transporters, such as the solute carrier organic anion transporter family member 2A1 (SLCO2A1), also referred to as the prostaglandin transporter (PGT). Additionally, $PGF_{2\alpha}$ can be transported by multidrug resistant protein 4 (ABCC4/MRP4) or act via its seven transmembrane receptor, PTGFR/FP. While $PGF_{2\alpha}$ has a luteolytic effect, prostaglandin E_2 (PGE_2) has been shown to have a luteotrophic effect in other species, such as humans and sheep. Although PGE_2 may also be transported by SLCO2A1 and ABCC4, it can initiate signaling cascades via cell surface receptors, such as PGE_2 receptor subtypes 1 (PTGER), 2 (PTGER2), 3

(PTGER3), and 4 (PTGER4). Interestingly, up-regulation of SLCO2A1 has recently been observed during luteolysis in ovine endometrium. This suggests that SLCO2A1 may be facilitating luteolytic $PGF_{2\alpha}$ signaling in the mare. It is hypothesized that SLCO2A1 will be down-regulated in the pregnant equine endometrium to facilitate signaling for MRP. ABCC4 is also hypothesized to be down-regulated in the pregnant mare endometrium to attenuate $PGF_{2\alpha}$ luteolytic signaling. In addition, PTGFR is expected to be up-regulated in pregnant mares to inhibit $PGF_{2\alpha}$ transport through SLCO2A1. PGE_2 receptors (PTGER, PTGER2, PTGER3, and PTGER4) are hypothesized to be up-regulated in the pregnant mare endometrium. To test this hypothesis, a cross-over study was designed using endometrial biopsies collected at days 12, 14, 16 and 18 (during the period of MRP and post-luteolysis) from pregnant and non-pregnant mares. Endometrial biopsies were collected from 12 normally cycling stock-type mares, which served both as a control (non-mated) and a treatment (pregnant) with n=3 per time point. RNA was isolated and used for real time RTPCR analysis, and protein expression was examined using Western blot analysis. To identify protein localization within the endometrium, additional endometrial biopsies were taken from normally cycling mares, both during diestrus (n=1) and estrus (n=1). Biopsies were embedded in paraffin and fixed for immunohistochemistry and histology (hematoxylin and eosin staining). Real time RTPCR results showed that endometrial *SLCO2A1* expression was lower on day 14 than day 16 when pregnancy status was combined to compare the effect of day ($p<0.05$). Additionally, endometrial *PTGER3* expression was higher in day 16 pregnant mares than day 12 pregnant mares ($p<0.03$). Interestingly, *PTGER* was not expressed in equine endometrium. While fold-changes ranging from two- to eleven-fold were observed for all

genes, no difference was found between pregnant and non-pregnant mare samples per time point for any of the genes of interest. This was likely due to the variability of expression observed between mares. However, trends in fold changes suggest that increased *SLCO2A1* and *PTGER2* expression may be facilitating MRP after day 12 post-ovulation. For Western blot analysis, only PTGER2 antibody was found to have immunoreactivity under the conditions used. Western blot results showed variability in protein expression across mare samples for each time point of collection. Additionally, no difference was found between PTGER2 expression between pregnant and non-pregnant mare samples per time point, suggesting either variability in individual mare expression or in tissue localization of the protein within the biopsy samples. However, endometrial PTGER2 expression was lower in day 12 pregnant mares than day 16 ($p<0.002$) and day 18 ($p<0.007$) pregnant mares. This suggests that PTGER2 may be functioning for a previously unidentified luteotrophic PGE_2 signaling process in the endometrium near the end of MRP. Immunohistochemical localization of SLCO2A1 and PTGER2 was investigated as endometrial *SLCO2A1* and *PTGER2* expression both tended to be higher in pregnant mares after day 12 post-ovulation. Endometrial SLCO2A1 localization was observed in the basal aspect of the epithelial glands and stroma during diestrus, while minimal SLCO2A1 localization was observed during estrus. Additionally, PTGER2 had punctate localization in the apical aspect of the endometrial epithelial glands during diestrus and punctate localization throughout the epithelial glands during estrus. Basal localization of SLCO2A1 in endometrial epithelial glands could facilitate luteolytic $PGF_{2\alpha}$ transport into systemic circulation for lysis of the corpus luteum in the non-pregnant, diestrous mare. If SLCO2A1 is localized in the apical aspect of the endometrial

epithelial glands in pregnant mares, it may be functioning for metabolism of $PGF_{2\alpha}$ inside the epithelial glands. Additionally, PTGER2 may be localized to the apical epithelial glands during diestrus to facilitate possible luteotrophic PGE_2 signaling from the conceptus. By these mechanisms, localization of SLCO2A1 and PTGER2 in the endometrial epithelial glands could be functioning in the pregnant mares for signaling MRP. However, it is difficult to interpret the actual function of SLCO2A1 and PTGER2 in MRP from biopsies taken from diestrous and estrous mares. Additionally, as gene and protein expression varied widely between mares, it is difficult to determine what function the prostaglandin transporters (SLCO2A1 and ABCC4) and receptors (PTGFR, PTGER2, PTGER3, and PTGER4) serve in regulating prostaglandin signaling during MRP. To further confirm these results and accurately establish transporter and receptor expression patterns, a larger sample size should be used in future experiments to aid in establishing statistical significance and to eliminate outliers. Also, future experiments should investigate the localization of these proteins throughout MRP with immunohistochemistry analysis of both pregnant and non-pregnant mare endometrium. Once the localization and function of the prostaglandin transporters and receptors is understood, the physiological process utilized in the mare for MRP may be identified. After the physiological process utilized for MRP is known, it may be possible to develop alternative treatments for estrus suppression in mares used for competition.

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CHAPTER I

REVIEW OF THE LITERATURE

1. Introduction

Maternal recognition of pregnancy is the physiological process required by a dam to maintain early pregnancy. For maternal recognition to occur, signals from the conceptus have to be recognized by the uterus; downstream rescue and maintenance of the corpus luteum follow to ensure successful maintenance of pregnancy in mammals (reviewed by Roberts et al., 1996).

It is during the period of maternal recognition in the mare, specifically days 11 to 16 post ovulation (Leith and Ginther, 1984; McDowell et al., 1988; reviewed by Ginther, 1998), that early embryonic death primarily occurs. Due to the loss of these pregnancies, major economic impact can be seen on the equine industry (Meyers et al., 1991; Carnevale et al., 2000; Morris and Allen, 2002). As the mechanism of maternal recognition of pregnancy in the mare is not yet understood, further research should elucidate the physiological signal that occurs during days 11 to 16 (Leith and Ginther, 1984; McDowell et al., 1988; reviewed by Ginther, 1998). By identifying the physiological process required for maternal recognition, alternative methods to suppress estrus in performance mares may also be developed. Currently, the use of Regumate (Intervet, Millsboro, DE), an oral altrenogest, is the only method approved for estrus

suppression (McCue, 2003). By manipulating the physiological process for maternal recognition, a treatment to suppress estrus may be developed that is effective yet reversible.

2. *Physiological Mechanisms of Maternal Recognition of Pregnancy in the Equine*

In comparison to other domestic species, such as cow, sheep and pig, the physiological process of maternal recognition utilized by the mare is currently unknown. Studies have determined that maternal recognition needs to occur by day 15 post ovulation (Hershman and Douglas, 1979; reviewed by Sharp, 1992). If maternal recognition does not occur, luteolysis follows for continuation of the estrous cycle, which is approximately 21 to 22 days in the mare (reviewed by Sharp, 1992). Similar to other domestic species, such as the cow or ewe, $PGF_2\alpha$ functions as the luteolytic factor in the mare (Douglas and Ginther, 1972; Douglas et al., 1974; Kooistra and Ginther, 1976). Oxytocin also functions in a conserved role in the mare, although it is of endometrial origin contrary to the luteal origin in ruminants (reviewed by Flores and Barlund, 2009). Endometrial oxytocin stimulates $PGF_2\alpha$ release from the endometrium, causing luteolysis (reviewed by Melampy and Anderson, 1968; Ginther and First, 1971; Stabenfeldt et al., 1974; Starbuck et al., 1998).

During luteolysis, concentrations of $PGF_2\alpha$ in the uterine vein are increased (Douglas and Ginther, 1976). However, in the mare, $PGF_2\alpha$ does not travel directly to the ovary through the countercurrent exchange of the uteroovarian vein and ovarian artery as in

ruminants and the sow (reviewed by Allen, 2001). In the mare, $PGF_2\alpha$ effluxes into systemic circulation before arriving at the ovary (Ginther et al., 1972; reviewed by Ginther, 1998; reviewed by Gaivão and Stout, 2007). By this mechanism, endometrial $PGF_2\alpha$ can bind to receptors in the corpus luteum, leading to luteolysis (Vernon et al., 1979; Sharp et al., 1984).

Additionally, episodic increases in $PGF_2\alpha$ metabolite, PGFM, are seen in systemic circulation during luteolysis (Kindahl et al., 1982). In pregnant mares, a reduction in $PGF_2\alpha$ has been identified in both uterine venous drainage (Douglas and Ginther, 1976) and in uterine secretions (Berglund et al., 1982; Zavy et al. 1984). Despite a significant decrease in intra-uterine luminal $PGF_2\alpha$ concentrations in pregnant mares, studies have shown that concentrations of PGFM are not significantly lower in pregnant mares. This suggests that the reduction seen in $PGF_2\alpha$ concentrations in pregnant mares is not due to prostaglandin metabolism, but is more likely due to a decrease in $PGF_2\alpha$ synthesis or release in response to the signal for maternal recognition of pregnancy (Berglund et al., 1982; Sharp et al., 1984; Starbuck et al., 1998). Interestingly, oxytocin receptor density is similar in the endometrium of pregnant and open mares on day 12. However, by day 14, the oxytocin receptor is not only down-regulated in the pregnant endometrium, but it also has lower affinity for substrate binding (Sharp et al., 1997; Starbuck et al., 1998), suggesting that the signal(s) for maternal recognition of pregnancy affects oxytocin receptor expression in the pregnant mare endometrium.

Therefore, the endometrial secretion of $PGF_2\alpha$ is somehow repressed by the presence of a viable embryo, allowing for maternal recognition of pregnancy and continued progesterone secretion from the primary corpus luteum (Sharp et al., 1997;

reviewed by Fortier et al., 2008). Two popular theories are postulated about where the signal(s) for maternal recognition of pregnancy originate in the mare. One school of thought is that physical movement of the conceptus initiates the signal (McDowell et al., 1988). In another, it is suggested that signaling for maternal recognition originates from secretions from the equine conceptus (Stout et al., 2004).

2.1) Conceptus Migration

Prior to day 17, the equine conceptus remains mobile in the uterus upon first entering from the oviduct at approximately day 6 post ovulation. It continues to migrate throughout the uterus for several days and is aided in this mobility by remaining spherical in shape. The equine conceptus does not elongate during this period, but retains the spherical form due to the glycoprotein capsule (reviewed by Allen, 2001). Subsequent to the migratory phase, the conceptus becomes fixed at the site of placentation, typically at the base of either uterine horn, by day 17 (Ginther, 1983; reviewed by Ginther, 1998).

The equine conceptus mobility, which occurs primarily between days 11 to 14 (Leith and Ginther, 1984), has been shown to be necessary for corpus luteum and pregnancy maintenance. Uterine ligation studies that have limited conceptus mobility during this period resulted in luteolysis and a return to estrus (McDowell et al., 1985; 1988). Therefore, the migration of the equine embryo likely allows for necessary interaction with the uterine endometrium (McDowell et al., 1988; reviewed by Ginther, 1998), resulting in a signal for maternal recognition of pregnancy during days 11 to 16 post ovulation. Several studies have corroborated the observation that embryonic loss after

day 19 resulted in a pseudopregnancy, likely as the signal for maternal recognition had already been established (Ginther, 1983; Ginther et al., 1985). However, while migration of the embryo is necessary, it still is not certain whether the signal for maternal recognition is initiated simply by the physical migration through the uterus or whether conceptus secretions, once distributed throughout the uterus during migration, initiate maternal recognition (Sharp et al., 1997; Stout et al., 2004).

Interestingly, the myometrial contractions that facilitate embryo migration are believed to be stimulated by prostaglandin secretions from the conceptus (Watson and Sertich, 1989; Weber et al., 1991a; 1991b; 1992; 1995; Vanderwall et al., 1992; McDowell et al., 1988; Stout and Allen, 2001). Exogenous treatment with a cyclooxygenase inhibitor resulted in a significant reduction in embryo migration (Stout and Allen, 2001). Similarly, exogenous treatment with oxytocin, prostaglandin, or a prostaglandin analogue has been shown to induce myometrium contractions (Taverne et al., 1979; Jones et al., 1991; Troedesson et al., 1995), thereby supporting the suggested role of prostaglandins in myometrial contractility and conceptus migration during the period of maternal recognition.

2.2) Conceptus Secretions

While embryonic migration has been studied, researchers have also studied equine conceptus secretions to determine if they play a role in signaling to the endometrium for maternal recognition of pregnancy. Some investigations suggest that an embryo-derived factor or secretion is necessary for the reduction of endometrial $PGF_2\alpha$ secretion required

for downstream survival of the corpus luteum (reviewed by Sharp, 1992; Sharp et al., 1997; Stout et al., 2004).

Interestingly, explants from equine endometrium that have been coincubated with membranes from equine conceptus produce significantly lower concentrations of $PGF_2\alpha$ in *in vitro* studies (Berglund et al., 1982; Sharp and McDowell, 1985; Weithenauer et al., 1987; Watson and Sertich, 1989; Sissener et al., 1996), whereas endometrial explants from pregnant mares incubated without conceptus membranes produce equal amounts of $PGF_2\alpha$ as diestrous mares (Vernon et al., 1981; Berglund et al., 1982). McDowell et al. (1988) concluded that this portrays the conceptus initiating a transitory effect on the endometrium, facilitated by conceptus motility, leading to the reduction of $PGF_{2\alpha}$ production, CL and progesterone maintenance, and thereby, maternal recognition of pregnancy.

Early studies have suggested some embryo-derived products might be playing a role in endometrium signaling for maternal recognition. For instance, at embryonic day 5, when the equine conceptus is still in the oviduct, the equine embryo is known to secrete PGE_2 (Weber et al., 1991b); other studies determined that oviductal transport of the equine embryo is hastened by PGE_2 (Weber et al., 1991a, 1995). Upon entering the uterus at approximately day 6, PGE_2 secretion continues (Watson and Sertich, 1989; Weber et al., 1991b; 1992; Vanderwall et al., 1992). Additionally, Watson and Sertich (1989) described $PGF_2\alpha$ secretions by the equine conceptus. The precise role the prostaglandin secretions play in luteolysis and maternal recognition has yet to be identified. It is suggested, however, that these intrauterine prostaglandin secretions induce myometrial contractions to facilitate equine embryo migration and maternal recognition (McDowell

et al., 1985; 1988; Troedesson et al., 1995; Stout and Allen, 2001). However, as $PGF_2\alpha$ is known to have a luteolytic effect after efflux into systemic circulation (Sharp et al., 1997), the exact role of these secretions is not well understood.

In addition to conceptus prostaglandin secretions, various studies also have investigated other equine conceptus secretory products. In 2004, Stout et al. investigated low molecular weight proteins that are secreted by the equine conceptus during days 7 to 17 post ovulation. Using previously determined molecular weight restrictions of 1-6 Kda (Weithenauer et al., 1987) and 3-10 Kda (Ababneh et al., 2000) for signaling molecules from the equine conceptus, two peptides of interest were isolated: ubiquitin and the β-chain of insulin. While embryonic *ubiquitin* mRNA expression was not different between days 7, 10 and 14, embryonic insulin secretions were found to be released in greater quantities during days 10 to 18 (Stout et al., 2004). Although insulin has been suggested as a possible signal for equine maternal recognition of pregnancy, exogenous treatment with insulin has been shown to be ineffective at maintaining the corpus luteum. In a study conducted by Stout et al. (2004) and Rambags et al. (2007), administration of 0.01 IU/kg BW i.v. of short-acting insulin with 0.2 IU/kg BW i.m. of intermediate-acting insulin did not delay the return to estrus. This may be due to the method of administration utilized in these studies, which did not distribute insulin locally, such as would be facilitated with the use of a mini-osmotic pump attached to the uterus. Additionally, no studies have determined equine endometrial expression of the insulin receptor. Therefore, the possibility that conceptus secretions of insulin play a role in signaling for maternal recognition in the mare cannot be entirely discredited as yet.

Although estrogen also is secreted by the equine embryo (Heap et al., 1982; Choi et al., 1997), it does not seem likely it exerts any effect on down-regulating $PGF_2\alpha$ production. It also seems unlikely that these estrogen secretions produce an anti-luteolytic effect (reviewed by Allen, 2001), as is proposed for the estrogen secretions from the porcine conceptus (Bazer and Thatcher, 1977). However, further research is warranted to determine the exact roll of estrogen secretions as several studies have found down-regulation of *estrogen receptor 1* mRNA in pregnant equine endometrium on days 13.5 (Klein et al., 2010), 15 (McDowell et al., 1999), and 18 post ovulation (Krull et al., 2009). Additionally, future research should identify if estrogen secretions can affect a directional change in $PGF_{2\alpha}$ secretions, from luteolytic endocrine release to possibly an exocrine release for sequestering $PGF_{2\alpha}$. While the equine conceptus produces a chorionic gonadotropin, eCG, it is not produced until approximately day 40, when the conceptus forms endometrial cups (Allen and Moor, 1972). Additionally, eCG production occurs well after the period of maternal recognition and therefore does not play a role in initiating the primary anti-luteolytic signal.

Others have addressed the possibility of a conserved mechanism of interferon (IFN) secretion, similar to the signaling mechanism found in pregnant ruminants. However, in contrast to the ruminant conceptus, the equine conceptus does not produce significant amounts of alpha or omega interferon secretions (Baker et al., 1991). Additionally, while IFNT inducible Mx protein has been identified in the equine endometrium, it has not been found to be up-regulated during pregnancy as it is in other species (Charleston and Stewart, 1993; Hicks et al., 2003).

In light of these studies, and considering the important role of $PGF_2\alpha$ signaling in luteolysis, research into the possible role of the prostaglandin transporter, SLCO2A1/PGT, will help elucidate the mechanism of maternal recognition of pregnancy in the mare. It also is of interest to determine the possible role SLCO2A1 plays in the equine endometrium in regulating or redirecting $PGF_2\alpha$ secretions from the equine conceptus.

i) Prostaglandins

3.1) Cellular Synthesis and Metabolism

Well known for mediating inflammation, pain, and immune responses, prostaglandins are found throughout the body, affecting a variety of systems, including: respiratory, immune, and reproduction (reviewed by Smith, 1989; reviewed by Fortier et al., 2008). Prostaglandins are produced by several different reactions (see **Figure 1**), starting with the release of arachiodonic acid from the cell membrane through the action of phospholipases, specifically phospholipase $cPLA_2\alpha$ (reviewed by Kudo and Murakami, 2002). Arachiodonic acid is then converted to PGG_2 and PGH_2, the precursors of all prostaglandins. This is accomplished through the actions of one of the isomers of prostaglandin synthase, either cyclooxygenase COX-1 or COX-2 (reviewed by Smith and Song, 2002). PGH_2 is further converted into prostaglandins depending on the synthase present. However, there are several sythases for each prostaglandin; PTGES, PTGES2, and PGES3 are all PGE synthases while AKR1B1 and AKR1C3 are both PGF synthases

that convert PGH_2 to $PGF_2\alpha$ (reviewed by Fortier et al., 2008; reviewed by Narumiya et al., 1999).

Figure 1: Process of Prostaglandin Production

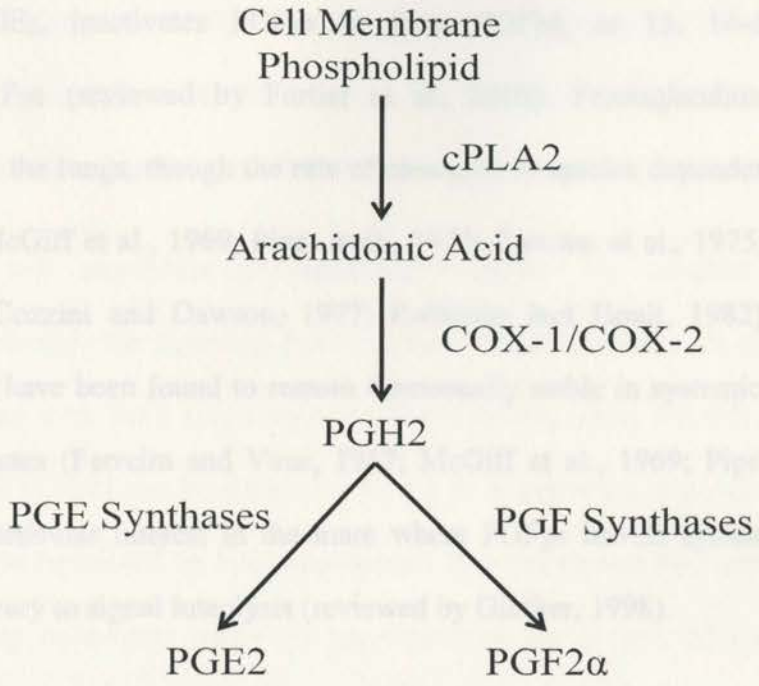

Figure adapted from Fortier et al., 2008

After release into the cytosol, prostaglandins were once believed to permeate into the extracellular space. However, at physiological pH, prostaglandins exist as charged organic anions (Avdeef et al., 1995; Roseman and Yalkowsky, 1973; Uekama et al., 1978) and in multiple studies have been shown to have low cell membrane permeability. These studies determined that the rate of prostaglandin diffusion is too slow to mediate any physiological activity (Bito and Baroody, 1975a; Baroody and Bito, 1981; Kanai et

al, 1995; Itoh et al., 1996). Therefore, other cellular mechanisms must be functioning to mediate cellular prostaglandin efflux and influx, such as the prostaglandin transporters SLCO2A1 and MRP4, discussed below (reviewed by Schuster, 1998).

Prostaglandins are inactivated by cellular metabolism, such as through the action of HPGD (15-PGDH), into specific metabolites. For instance, HPGD, which also inactivates PGE_2, inactivates $PGF_2\alpha$, to form PGFM, or 13, 14-dihydro-15-keto prostaglandin $F_2\alpha$ (reviewed by Fortier et al., 2008). Prostaglandins are primarily metabolized in the lungs, though the rate of clearance is species dependent (Ferreira and Vane, 1967; McGiff et al., 1969; Piper et al., 1970; Dawson et al., 1975; Anderson and Eling, 1976; Cozzini and Dawson, 1977; Robinson and Hoult, 1982). Additionally, prostaglandins have been found to remain functionally stable in systemic circulation for at least 2 minutes (Ferreira and Vane, 1967; McGiff et al., 1969; Piper et al., 1970), which is of particular interest in the mare where $PGF_2\alpha$ travels systemically prior to reaching the ovary to signal luteolysis (reviewed by Ginther, 1998).

3.2) Prostaglandin Cell Receptors, Signaling, and Transporters

i) Cellular Receptors and Signaling

Due to rapid conversion into inactive metabolites, prostaglandins act in autocrine, paracrine, and endocrine manner. This can be accomplished through the use of multiple cellular receptors. However, when prostaglandins bind to different receptors, diverse and even opposing actions on cellular regulation can result (reviewed by Fortier et al., 2008). Recently, multiple cell membrane receptors have been identified for prostaglandins, with eight types and subtypes in the rhodopsin-type superfamily (reviewed by Narumiya et al.,

1999). Prostaglandin receptors are G-protein coupled receptors with a seven transmembrane domain and specific prostaglandin substrate binding domain. Four receptors have been identified for PGE_2: PTGER/EP_1, PTGER2/EP_2, PTGER3/EP_3, and PTGER4/ EP_4. Other prostaglandins seem to only have a single cell membrane receptor, such as the PTGFR/FP receptor for $PGF_2\alpha$ and the PTGDR/DP receptor for PGD_2 (reviewed by Fortier et al., 2008). Interestingly, various species may have more than one variant of a receptor, such as the $PTGFR_A$ and the $PTGFR_B$ isoforms in the sheep (Pierce et al., 1997) and the six isoforms of PTGER3 in the human (Schmid et al., 1995).

These cell membrane prostaglandin receptors are coupled to secondary messengers, although the signaling pathway and cellular downstream effects of ligand binding can vary even for a single receptor. For instance, while PTGFR is known to be coupled to α_q, PTGER3 can be coupled to α_i, α_q, or α_s pathways depending on the tissue or cellular origin (reviewed by Hata and Breyer, 2004; reviewed by Fortier et al., 2008). Some secondary messenger pathways are conserved for several of the prostaglandin receptors. Adenlyl cyclase stimulation functions as a secondary messenger for PTGER2 and PTGER4, while calcium mobilization is a secondary pathway for PTGER and PTGFR signaling (Abramovitz et al., 2000). In addition to functioning as cell membrane receptors, recent research has identified PGE_2 receptors, namely receptors PTGER3 and PTGER4, in the nuclear envelope of neonatal porcine brain and adult rat liver cells (Bhattacharya et al., 1998; 1999). These studies suggest that prostaglandin receptors regulate signaling at multiple levels and can therefore elicit a variety of effects on cellular function (reviewed by Hata and Breyer, 2004).

Not surprisingly, prostaglandin receptors also have varying affinities to different prostanoids. Of all the prostaglandin receptors, PTGFR is the least selective of all, binding primarily to $PGF_{2\alpha}$, although it also can bind PGD_2 and PGE_2. Of the EP subtypes, PTGER3 has the highest affinity for PGE_2. PTGER4, PTGER2, and PTGER follow in descending order of affinity for PGE_2 in human embryonic kidney cells (Abramovitz et al., 2000). By maintaining such variety in cellular prostaglandin receptors, isoforms, secondary messenger pathways, and substrate binding affinities, a variety of physiological responses can be carried out in a cell or tissue through a complex prostaglandin signaling cascade (Schmid et al., 1995). For instance, when $PGF_2\alpha$ binds to the Ca^{2+}- coupled PTGFR receptor, an increase in intracellular IP_3/DAG is observed (reviewed by Fortier et al., 2008), along with release of PLC (reviewed by Hata and Breyer, 2004). Other studies have identified that $PGF_2\alpha$ activation of PTGFR leads to down regulation of prostaglandin transport through the transporter, SLCO2A1, discussed below. This down-regulated transport is attributed to regulation via $G\alpha_s$ rather than phosphorylation of the transporter itself (Vezza et al., 2001).

ii) Prostaglandin Transporters

While cellular receptors have recently been identified for prostaglandins, other mechanisms were suspected to play a role in transporting prostaglandins. Due to their charged anion state at physiological pH and poor membrane permeability (Bito and Baroody, 1975a; Baroody and Bito, 1981; Kanai et al, 1995; Itoh et al., 1996), prostaglandin movement was hypothesized to require an energy-dependent transporter or transporters (Irish, 1979).

Reid et al. (2003) determined that, in addition to passive diffusion, newly synthesized prostaglandins can be transported actively out of the cell through a multidrug resistance protein, ABCC4/MRP4, which is a constitutively expressed facilitated transporter (reviewed by Fortier et al., 2008). ABCC4 is known to have two membrane-spanning domains with six transmembrane helices each and has been shown to be localized to both basal and apical aspects of the polar cell membrane (reviewed by Russel et al., 2008). Determined to regulate cellular prostaglandins efflux, specifically efflux of PGE_1 and PGE_2, ABCC4 is suggested to be an ATP-dependent transporter that likely plays a significant role in prostaglandin clearance and metabolism (Reid et al., 2003).

In addition to ABCC4, another prostaglandin transporter has been identified. Originally discovered in 1995, a prostaglandin specific transporter, SLCO2A1/PGT, was isolated in the rat after identification of its cDNA (Kanai et al.). Facilitating movement of prostaglandins between successive cell layers (reviewed by Fortier et al., 2008), SLCO2A1 likely transports prostaglandins in the anion form (Chan et al., 1995; Itoh et al., 1996). Primary analysis of SLCO2A1, which predicted a transmembrane protein with 12 hydrophobic domains (Kanai et al., 1995), has aided in classifying SLCO2A1 as a member of the Solute Carrier Organic Anion Transporter family (OATP/SLCO) (reviewed in Schuster, 1998; 2002). In the transmembrane domains there are at least three charged residues for substrate binding that have been suggested in early studies: E77, R561, and K614 (Lu et al., 1996b, reviewed in Schuster, 1998). Supporting the hypothesis that these charged residues function in conserved substrate translocation and ligand binding, both R561 and K614 have been identified on the transmembrane domain of the ovine SLCO2A1 (Banu et al., 2003; 2008). Additionally, three to four N-linked

glycosylation sites are also found in other members of the OATP family, including a Na+-independent OATP in the rat (Jacquemin et al., 1994; reviewed in Schuster, 1998). Chan et al. (1999), in a later study involving rat SLCO2A1, identified four residues on the membrane-spanning segments of SLCO2A1 that likely contribute to the binding site: A526, A529, C530, H533. These findings suggest a conserved region of SLCO2A1 for ligand binding, although there appear to be variations between species.

While SLCO2A1 has been found to transport multiple prostaglandins, in the rat it has a higher affinity for PGE_1, PGE_2, and $PGF_2\alpha$ in respective order, which is similar to the rates of prostaglandin uptake (Kanai et al., 1995). In addition to PGE_1, PGE_2, and $PGF_{2\alpha}$, the human SLCO2A1 is noted to have high affinity for PGD_2 (Lu et al., 1996b). Significantly lower affinity is reported for TxB_2, 6-Keto $PGF_1\alpha$, and iloprost, with likely little to no transport of arachidonate (Kanai et al., 1995). Other substrates for SLCO2A1 have also been identified, such as 8-iso-PGE_2 and 8-iso-$PGF_2\alpha$ (Itoh et al., 1996). However, it is considered unlikely that prostaglandin metabolites, such as PGFM, are transported back into cells through SLCO2A1 given their low rate of cellular uptake (Anderson and Eling, 1976; Robinson and Hoult, 1982).

Interestingly, research determined that SLCO2A1 is coupled to cellular metabolism, specifically glycolysis, for energetically primed prostaglandin movement (Tannenbaum et al., 1979; Chan et al., 1998; 2002). Chan et al. (2002) determined that prostaglandin movement through SLCO2A1 required a lactate gradient, strongly suggesting that SLCO2A1 is a lactate-prostaglandin exchanger. Chan et al. (2002) proposed that SLCO2A1 functions both in the influx and efflux of prostaglandins due to the inverse relationship of prostaglandin movement to the lactate gradient. However, a

more recent study has identified ABCC4 as an efflux transporter of cellular prostaglandins and SLCO2A1 as primarily an influx transporter for the reuptake of newly synthesized prostaglandins (Reid et al., 2003). Bao et al. (2002) supports this theory by arguing that the coupling of SLCO2A1 to glycolysis, along with the efflux of prostaglandins from cells through simple diffusion, point to SLCO2A1 being used primarily for prostaglandin reuptake by the cell. Despite this, research has also shown that SLCO2A1, though perhaps primed for prostaglandin influx (Bao et al., 2002; Chan et al., 2002), also functions as an efflux transporter (Banu et al., 2008). More specifically, Banu et al. (2008) demonstrated that silencing of the ovine *SLCO2A1* gene with siRNA resulted in up to 95% reduction in $PGF_2\alpha$ influx and approximately 80% reduction in $PGF_2\alpha$ efflux. This suggests that SLCO2A1 mediates approximately 80% of $PGF_2\alpha$ release seen *in vivo* in the ewe.

Evidence exists that there are other transporters besides ABCC4 and SLCO2A1 that also transport prostaglandins, likely in a sodium dependent manner (Reid et al., 2003). For instance, in the rat, SLCO2A1 has been identified as being Na+ independent (Kanai et al., 1995), Na+-dependent prostaglandin transport has been described in several tissues, including; anterior uvea (DiBenedetto and Bito, 1980), choroid plexus (DiBenedetto and Bito, 1986), and ileum (Bikhazi et al., 1991). Additionally, other transporters in the OATP and MRP families have been shown to mediate prostaglandin transport (reviewed by Schuster, 2002), such as LST-1 (Abe et al., 1999), moat1 (Nishio et al., 2000), OAT1 (Sekine et al., 1997), OAT2 (Sekine et al., 1998; Pavlova et al., 2000), MRP1 (Evers, et al., 1997), and MRP2 (Evers et al., 1998). This, along with identification of species specific prostaglandin transport (Bito, 1972b), strongly suggests

the existence of other prostaglandin transporters functioning in various systems, though likely with divergent substrate affinities (reviewed by Schuster, 2002). Although, Schuster et al. (2000) has shown that different structural requirements bind prostaglandins to SLCO2A1 in comparison to the prostaglandin receptors (2000), further studies are warranted to determine if SLCO2A1 has a different affinity for prostaglandins in comparison to prostaglandin receptors and various prostaglandin transporters.

3.3) SLCO2A1 Expression

Conserved across various species, SLCO2A1 is expressed in a broad spectrum of tissues, including: testes, ovaries, uterus, kidney, brain, stomach, and small intestines (Kanai et al., 1995; Lue et al., 1996; Banu et al., 2003, 2005; Kang et al., 2005). The highly conserved expression of SLCO2A1 across species suggests that SLCO2A1 plays an important role in regulating prostaglandin signaling and cellular function. Described below is a brief overview of SLCO2A1 identification and expression in various species to date.

i) Murine

The first study to identify SLCO2A1 utilized the rat and examined several different tissues, including the eye, lung, heart, skeletal muscles, and stomach. Kanai et al. (1995) found transcripts of *SLCO2A1* mRNA in several rat tissues; highly expressed in the lung, liver, and kidney, *SLCO2A1* mRNA was also expressed in the brain, stomach, ileum, and jejunum. Interestingly, no *SLCO2A1* mRNA was found in the heart or skeletal

muscle, but was found in other tissues containing epithelia, suggesting that SLCO2A1 functions for transepithelial prostaglandin transport. Later studies found immunocytochemical localization of the rat SLCO2A1 in the prostate, seminal vesicles, bladder and penis (Itoh et al., 1998). Furthermore, *SLCO2A1* cDNA from the mouse and rat was cloned (Kanai et al., 1995; Pucci et al., 1999) and, interestingly, it has been found that the rat and mouse SLCO2A1 have varying prostaglandin substrate affinities (Pucci et al., 1999).

Bao et al. (2002) confirmed expression of SLCO2A1 within regions of the rat kidney. More specifically, SLCO2A1 was expressed only in cell types that also coexpressed cyclooxygenase, strongly suggesting that the transporter is present only in cells that synthesize prostaglandins. Interestingly, SLCO2A1 was expressed in higher concentrations in the papilla and medulla of the kidney as compared to lower expression in the cortex (Kanai et al., 1995; Bao et al., 2002). SLCO2A1 was expressed highly in rat platelets (Bao et al., 2002), which is not surprising given the regulatory role of prostaglandins in inflammation (reviewed by Smith, 1989; reviewed by Fortier et al., 2008).

ii) Primate

A human homologue to the rat SLCO2A1 was discovered originally in the kidney and later cloned for further research (Lu et al., 1996a; 1996b; Lu and Schuster, 1998). Northern blot analysis of human tissues revealed that human *SLCO2A1* mRNA is expressed in a wide spectrum of tissues. Human *SLCO2A1* expression was in several

tissues, including: skeletal muscle, colon, small intestine, and prostate (Lu et al., 1996b). The heart, brain, placenta, lung, ovary, and testis where found to have higher expression of human *SLCO2A1* mRNA (Lu et al., 1996b). SLCO2A1 is strongly expressed in human fetal tissue (Lu et al., 1996b) and in the human endometrium throughout the menstrual cycle (Kang et al., 2005). Endothelial expression of SLCO2A1 was also identified in human arterial and cardiac vasculature and in umbilical vein endothelial cells (Topper et al., 1998; McCormick et al., 2001). Interestingly, endothelial expression of human SLCO2A1 does not appear to be affected by either interferon (IFN) gamma or Tumor Necrosis Factor α (Topper et al., 1998).

iii) Bovine

SLCO2A1 also has been identified in multiple tissues in the cow. In 2005, Banu et al. identified gene and protein expression of SLCO2A1 in both maternal and fetal tissue, specifically in the intercaruncular tissue, fetal membrane tissues, placentome caruncles, and the utero-ovarian plexus. In the fetal membrane tissues, SLCO2A1 expression was confirmed by immunohistochemical localization in mononuclear, binuclear, and giant cells of the trophectoderm, as well as in the smooth muscle cells of fetal blood vessels. Similarly, SLCO2A1 was found in myometrial smooth muscle cells of the intercaruncular tissue and the endometrial luminal epithelium. Interestingly, *SLCO2A1* mRNA expression was found to be higher in the maternal tissue of the placentome caruncle. *SLCO2A1* gene and protein expression increased in both placental and endometrial tissues with increasing duration of pregnancy (Banu et al., 2005).

Mondal et al. (2009) found that treatment of bovine endometrial epithelial cell lines with interferon-τ (IFNT) actually stimulates SLCO2A1 expression. Surprisingly, this SLCO2A1 up-regulation varies from the down-regulation suggested in pregnant ovine endometrium, discussed below (Banu et al., 2008). However, an earlier study determined that IFNT treatment did not affect the expression of SLCO2A1 in either endometrial, myometrial, or luteal tissue (Arosh et al., 2004). The variation observed in SLCO2A1 expression with IFNT treatment between these two studies could reflect IFNT concentration and tissue dependent effects, which warrant further study.

iv) Ovine

After identification and cloning of ovine SLCO2A1, Banu et al. (2008) examined *SLCO2A1* mRNA and protein expression during the ovine estrous cycle. Interestingly, while SLCO2A1 was found throughout the ovine endometrium, including endometrial luminal, glandular epithelia and stromal cells, SLCO2A1 expression increased during luteolysis. In addition, it was determined that *in vivo* treatment with 100 mg DIDS, a specific SLCO2A1 inhibitor, inhibited the oxytocin-induced $PGF_2\alpha$ release, and more importantly, resulted in prolonged interestrus intervals (Banu et al., 2008). The ovine corpus luteum experienced an extended lifespan and plasma progesterone concentrations remained higher, above the basal levels reported in the control ewes. When SLCO2A1 was inhibited, results suggested that SLCO2A1 potentially regulates luteolysis in the ewe (Sontineni et al., 2005; Banu et al., 2008).

v) Equine

Currently, neither expression of SLCO2A1, prostaglandin receptors, nor other prostaglandin transporters, have been identified in the horse. However, given the high conservation of SLCO2A1 across species, as well as the conservation of other prostaglandin transporters and receptors, it is probable that SLCO2A1 will be found in a variety of tissues types in the horse. Additionally, regulation of SLCO2A1 expression may facilitate signaling for maternal recognition of pregnancy in the mare. As stated, binding of $PGF_2\alpha$ to the PTGFR receptor leads to down-regulation of SLCO2A1 (Vezza et al., 2001). Since $PGF_2\alpha$ is secreted from the equine conceptus (Watson and Sertich, 1989), it could be binding to endometrial PTGFR receptors to down-regulate SLCO2A1 in a similar pattern suggested by Banu et al. (2008) in the pregnant ovine endometrium. Insulin secretions from the conceptus (Stout et al., 2004) also may play a role in inhibiting cellular metabolism necessary to create the lactate gradient required for the prostaglandin-lactate exchange mechanism proposed for SLCO2A1 (Chan et al., 2002). Therefore, if such mechanisms are functioning in the mare, down-regulation of SLCO2A1 expression could be functioning as a mechanism for preventing luteolysis. Further investigation into the expression and role of SLCO2A1 in the equine endometrium is necessary. Additionally, correlating changes in SLCO2A1 expression, as well as changes in prostaglandin receptor and ABCC4 expression during days 12 to 16 post ovulation, could lead to insights into the physiological processes used in the mare for maternal recognition of pregnancy.

3.4) Possible Regulatory Roles of SLCO2A1

While it has been suggested that SLCO2A1 regulates prostaglandin reuptake and intracellular oxidation (reviewed by Schuster, 1998; 2002), the exact role of SLCO2A1 is still unclear. Bao et al. (2002) suggests several possibilities, but cautions that the role is likely specific to the cell-type in which SLCO2A1 is expressed. SLCO2A1 may be functioning in prostaglandin reuptake to facilitate a negative-feedback loop, to regulate prostanoid synthesis or to retain a basal level of prostaglandins in a quiescent cell. This seems possible as the results from Reid et al. (2003) confirmed that cellular concentrations of prostaglandin reach equilibrium, possibly due to the prostaglandin efflux from ABCC4 and influx from SLCO2A1.

In addition, SLCO2A1 also may function as a mechanism for clearing prostaglandins from circulation (Kanai et al., 1995). Indication that SLCO2A1 may be functioning to mediate vascular clearance of prostaglandins is noted in the rate of prostaglandin metabolism reported in the lungs, which is equivalent to the transport of prostaglandins by SLCO2A1 (Pitt et al., 1983; Kanai et al., 1995). This is of particular significance as SLCO2A1 has been reported at higher concentrations in the lungs of several species, including the rat (Kanai et al., 1995). Considering the transport reported in tissues that do not actively metabolize prostaglandins (Holmes and Horton, 1968; Bito, 1972a; Bito and Salvador, 1972; Nakano et al., 1972; Bito and Baroody, 1974; Bito et al.; 1976a; 1976b; Hagen et al., 1977; DiBenedetto and Bito, 1980), Schuster (1998) proposed that SLCO2A1 additionally may be functioning in a tissue specific manner to terminate prostaglandin signaling. SLCO2A1 may be transporting unmetabolized prostaglandins from specific tissues, such as the choroid plexus (Bito and Baroody,

1974), and releasing them into the blood for final metabolism by the lungs (reviewed in Schuster, 1998). Banu et al. (2005) suggested a similar role for SLCO2A1 in cattle for the intrauterine metabolism of prostaglandins during pregnancy. SLCO2A1 also is suggested to function in terminating prostaglandin signaling locally, to avoid unintended physiological effects that could occur with the escape of local prostaglandins into systemic circulation or neighboring tissue (reviewed by Schuster, 1998; 2002).

Bao et al. (2002) postulated that SLCO2A1 is inducing intracellular signaling events, possibly different and even opposing cell-surface prostaglandin receptor signaling pathways. By this method, SLCO2A1 may be functioning to mediate prostaglandin signaling to nuclear membrane receptors, such as nuclear envelope receptors PTGER3 and PTGER4 (Bhattacharya et al., 1998; 1999; reviewed by Schuster, 2002). Additionally, SLCO2A1 may be functioning in a tissue specific manner in the pulmonary system to regulate vasodilation and blood pressure. For instance, once PGE enters arterial circulation, it has a vasodilatory effect and causes a significant reduction in systemic blood pressure (Pitt et al., 1983). Additionally, use of indocyanine green, a SLCO2A1 inhibitor, in anesthetized rabbits inhibited pulmonary PGE_1 removal when administered at concentrations equivalent to the inhibition constant (K_i) of SLCO2A1, suggesting obstruction of the transporter (Pitt et al., 1983).

Identification of SLCO2A1 in bovine fetal and maternal tissues during pregnancy further suggests that SLCO2A1 is functioning to regulate exchange and possibly signaling between the dam and fetus during pregnancy (Banu et al., 2005). Additional findings have correlated a reduction in *SLCO2A1* mRNA and protein expression with prolonged interestrous intervals and retained corpa lutea in the ewe. These results support

the proposal that SLCO2A1 functions to regulate prostaglandin signaling and possibly even luteolysis in the ewe (Banu et al., 2008). This leaves open the possibility that SLCO2A1 may be functioning in regulating luteolysis or maternal recognition of pregnancy in other species, such as in the mare.

With the suggestion that SLCO2A1 functions to induce cell signaling, the reuptake of extracellular prostaglandins through SLCO2A1 could either be inhibiting (Schror and Weber, 1997) or signaling cell proliferation (Kawamura et al., 1999; Chan et al., 2002). This cellular regulation is of particular interest, especially in the case of tumors and cancers (Lu et al., 1996b), as a SLCO2A1 inhibitor, DIDS, has recently been identified and used *in vivo* without toxic side effects (Banu et al., 2008).

Others have proposed that SLCO2A1 may be functioning to direct the flow of prostaglandins either towards or away from other receptors (reviewed in Schuster, 2002). As stated, SLCO2A1 binds to different structural determinants in prostaglandins than those bound by the receptors (Chan et al., 1999; Schuster et al. 2000); however, the relative affinities of prostaglandin receptors versus SLCO2A1 for their substrates have yet to be identified. Interestingly, SLCO2A1 may be functioning in specific cells and tissues for the directional release of prostaglandins, and thereby regulate prostaglandin receptor binding. For instance, correlation of basolateral PGE_2 release to SLCO2A1 presence in the apical membrane of MDCK cells suggests a directional release of prostaglandins *in vitro* (Nomura et al., 2005). Additionally, SLCO2A1 has been suggested to function in the directional release of prostaglandins in the renal collecting duct (Nomura et al., 2005).

3.5) Conclusions

Further research is needed to determine what role SLCO2A1 is playing in various tissues and species. More specifically, as SLCO2A1 has been suggested to play a role in regulating luteolysis and maternal recognition in other species (reviewed by Schuster, 2002; reviewed by Fortier et al., 2008), it is of considerable interest to determine if SLCO2A1 is playing a similar role in equine endometrium. Additionally, as SLCO2A1 has been inhibited *in vivo* in the ovine endometrium without toxic side effects (Banu et al., 2008), inhibition of SLCO2A1 may be a potential treatment to suppress estrus in performance mares. To better understand the possible regulatory role of SLCO2A1 in equine maternal recognition of pregnancy, it is also of interest to study the expression of prostaglandin receptors in the mare (specifically PTGFR, PTGER, PTGER2, PTGER3, and PTGER4) as well as another prostaglandin transporter, ABCC4. Therefore, it was hypothesized that equine endometrial SLCO2A1, in addition to endometrial ABCC4, will be up-regulated in the non-pregnant mare during the period of maternal recognition to facilitate luteolytic $PGF_{2\alpha}$ signaling. Additionally, I hypothesize that endometrial PTGFR will be up-regulated in pregnant mares to facilitate down-regulation of prostaglandin transport by SLCO2A1 (Vezza et al., 2001). Endometrial PGE_2 receptors are also hypothesized to be up-regulated in the pregnant mare during maternal recognition, possibly to facilitate PGE_2 luteotrophic signaling (Weber et al., 1992; Arosh et al., 2004), as PGE_2 is already known to function for oviductal release of the embryo for the first step of maternal recognition in the mare (Weber et al., 1991a; 1991b).

CHAPTER II

EFFECTS OF PREGNANCY STATUS ON ORGANIC ANION TRANSPORTERS AND PROSTAGLANDIN RECEPTORS IN THE EQUINE ENDOMETRIUM: INSIGHTS INTO MATERNAL RECOGNITION OF PREGNANCY IN THE MARE

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Introduction

In contrast to ruminants and swine, the mechanism for equine maternal recognition of pregnancy is still unknown. However, maternal recognition is known to occur between days 11 to 16 post ovulation (Hershman and Douglas, 1979; Leith and Ginther, 1984; McDowell et al., 1988) and requires embryonic migration throughout the uterus during that period (Leith and Ginther, 1984; McDowell et al., 1998). The antiluteolytic signal that ensues rescues the corpus luteum prior to day 18. In the absence of an embryo or with failure of maternal recognition, luteolysis is initiated by pulsatile release of endometrial $PGF_{2\alpha}$ on approximately day 14 post ovulation (Ginther, 1983; Sharpe et al., 1984; reviewed by Ginther, 1998).

Once luteolytic $PGF_{2\alpha}$ is secreted from the equine endometrium (Douglas and Ginther, 1972; Douglas et al., 1974; Kooistra and Ginther, 1976), it reaches the corpus

luteum only after traveling through general circulation (reviewed by Allen, 2001). This varies greatly from ruminants and swine, where the ovarian artery and uterine vein are closely associated, allowing for counter-current exchange of $PGF_{2\alpha}$ to be directed toward the ovary (Ginther et al., 1972; reviewed by Ginther, 1998; reviewed by Gaivão and Stout, 2007). Interestingly, while oxytocin functions in a feedback mechanism to promote endometrial $PGF_{2\alpha}$ release (reviewed by Melampy and Anderson, 1968; Ginther and First, 1971; Stabenfeldt et al., 1974; Starbuck et al., 1998), endometrial oxytocin receptors are found to be down-regulated and have lower substrate affinity by day 14 post ovulation in pregnant mares (Sharp et al., 1997; Starbuck et al., 1998). Additionally, uterine venous plasma contains lower concentrations of $PGF_{2\alpha}$ on day 14 post ovulation in pregnant mares than non-pregnant mares (Douglas and Ginther, 1976). Uterine luminal $PGF_{2\alpha}$ and PGFM content in pregnant mares is lower than non-pregnant mares during maternal recognition (Sharp et al., 1984). Endometrial $PGF_{2\alpha}$ production also is lower in pregnant mares than in non-pregnant mares when evaluated *in vitro* (Sharp et al., 1984). Therefore, the signal for maternal recognition of pregnancy in the mare must reduce synthesis or suppress the release of $PGF_{2\alpha}$ up-stream of oxytocin induced signaling pathway (Sharp et al., 1997; reviewed by Fortier et al., 2008).

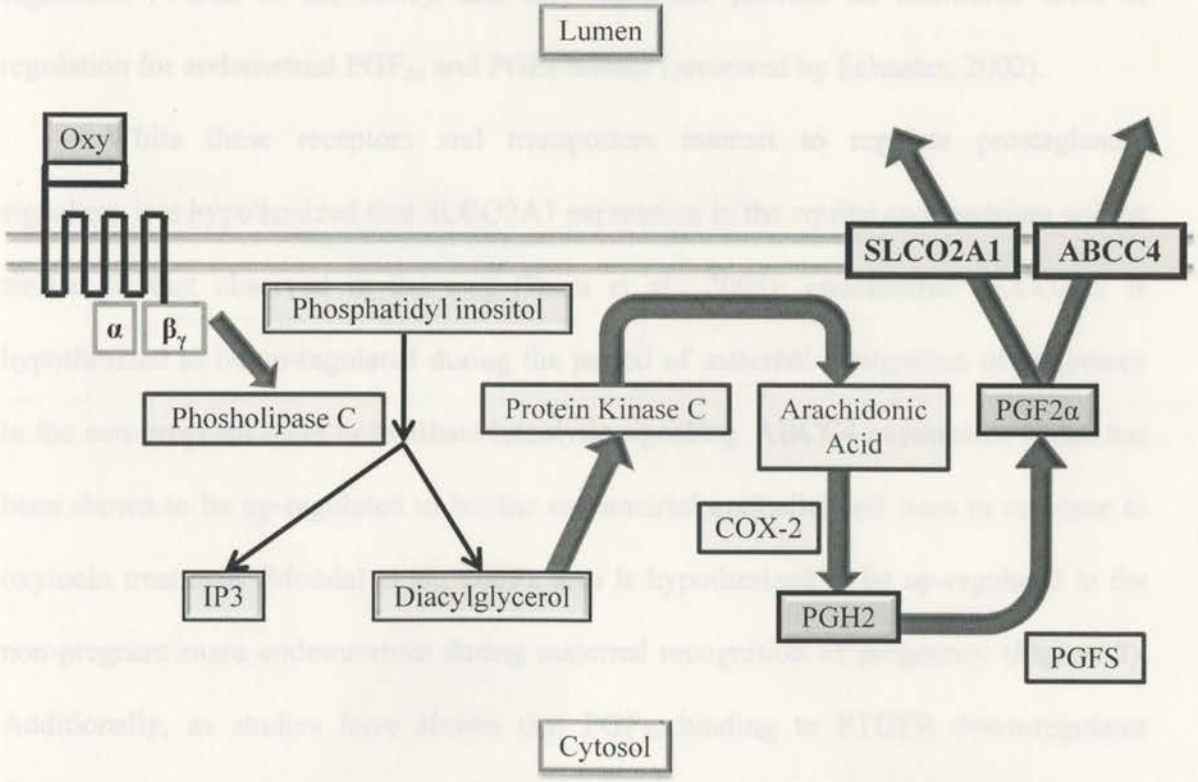
While the antiluteolytic signal(s) is still unknown in the mare, early studies have suggested that interferon secretions are unlikely to function for maternal recognition of pregnancy as interferons alpha and omega are not produced in significant amounts by the equine conceptus (Baker et al., 1991; Charleston and Stewart, 1993; Hicks et al., 2003). Additionally, while estrogen functions for porcine maternal recognition, intrauterine infusion of estrogen does not inhibit luteolysis (reviewed by Allen, 2001). Insulin

secretions are also unlikely to act as the embryonic signal for maternal recognition of pregnancy in the mare as systemic insulin administration does not affect cycle length (Stout et al., 2004; Rambags et al., 2007). Unfortunately, early embryonic loss during the period of maternal recognition, specifically days 11 to 16 post ovulation (Hershman and Douglas, 1979; McDowell et al., 1988; reviewed by Sharp, 1992), results in significant financial losses to the equine industry (Meyers et al., 1991; Carnevale et al., 2000; Morris and Allen, 2002). Therefore, identification of the mechanism for maternal recognition of pregnancy will aid in identifying early pregnancies (days 11 to 16) that are likely to be lost. Additionally, by identifying the mechanism for maternal recognition of pregnancy, alternative methods could be developed to suppress estrus in mares used for competition.

Recent research has suggested that a prostaglandin specific transporter, SLCO2A1/PGT, may play a conserved role in regulating luteolysis in several species, including the cow (Mondal et al., 2009) and ewe (Banu et al., 2008). SLCO2A1, which is a twelve-transmembrane transporter belonging to the organic anion transporter family (reviewed by Schuster, 1998; 2002), has been found throughout a variety of tissues in various species, suggesting it is highly conserved and may be regulating several cellular functions (Kanai et al., 1995; Lu et al., 1996a; 1996b; Lu and Schuster, 1998; Topper et al., 1998; Pucci et al., 1999; McCormick et al., 2001; Bao et al., 2002; Banu et al., 2005; Kang et al., 2005; Sontineni et al., 2005; Banu et al., 2008). Changes in expression of *SLCO2A1* mRNA and protein have been documented in ovine endometrium throughout the estrous cycle, specifically during luteolysis. This has led to the suggestion that down-regulation of SLCO2A1 may play a role in preventing ovine luteolysis and thereby facilitating signaling for maternal recognition of pregnancy (Banu et al., 2008).

Additionally, treatment of bovine endometrial epithelial cell lines with interferon-τ (IFNT) stimulates SLCO2A1 expression, suggesting that SLCO2A1 expression also is regulated during maternal recognition of pregnancy in cows (Mondal et al., 2009). Given the high conservation of SLCO2A1 across mammalian species, we predict that SLCO2A1 is present in equine tissue as well, possibly functioning in prostaglandin signaling such as by facilitating luteolytic $PGF_{2\alpha}$ release from the endometrium (**Figure 2**).

Figure 2: Proposed Role for SLCO2A1 and ABCC4 in Non-Pregnant Mare Endometrium



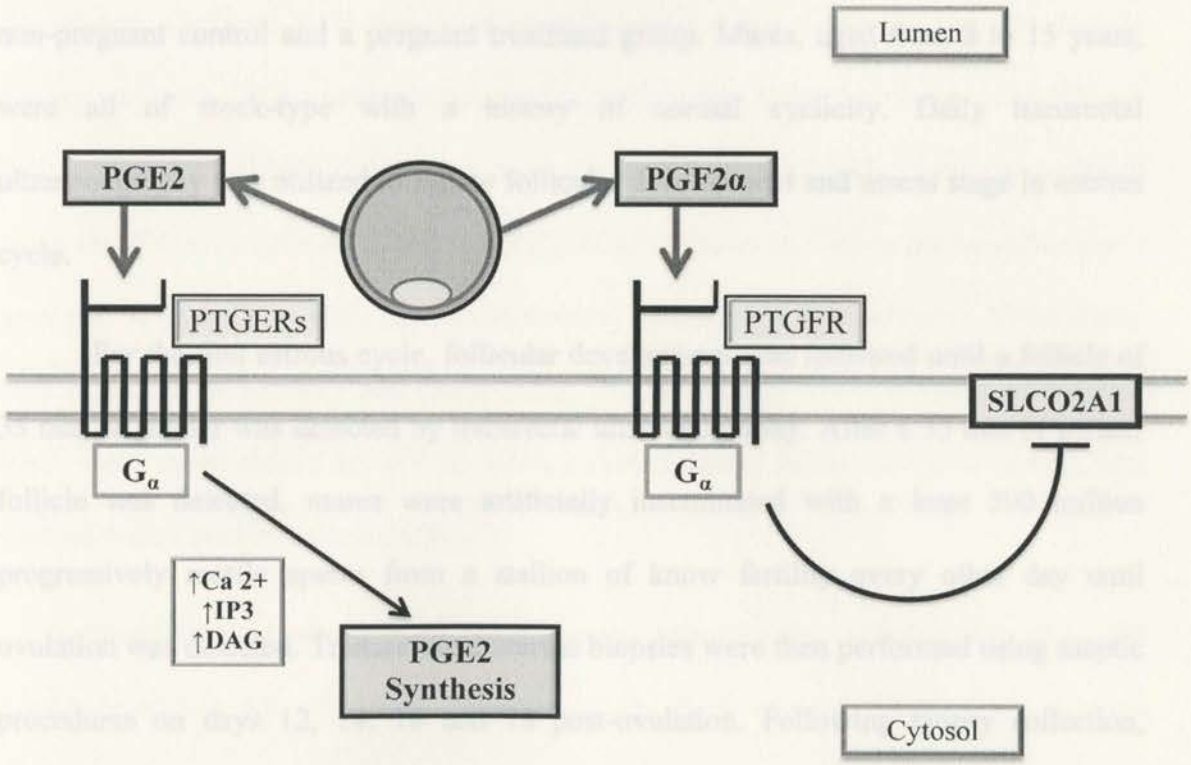
Due to the conserved expression of SLCO2A1 in tissue and its suggested function in regulating luteolysis (reviewed by Schuster et al., 2002), it is of interest to determine

equine endometrial SLCO2A1 expression. In particular, it is important to determine endometrial SLCO2A1 expression as it relates to pregnancy status and luteolysis in the mare. To aid in understanding the role of SLCO2A1 in maternal recognition of pregnancy, we examined the endometrial expression pattern of another prostaglandin transporter, multidrug resistant protein ABCC4/MRP4. Additionally, we evaluated the expression pattern of several prostaglandin receptors, PTGFR/FP, PTGER/EP_1, PTGER2/EP_2, PTGER3/EP_3, and PTGER4/EP_4, to determine if any significant changes in expression are correlated to SLCO2A1 expression and pregnancy status. These receptors have been shown to facilitate prostaglandin signaling, such as for SLCO2A1 regulation (Vezza et al., 2001), and may therefore provide an additional level of regulation for endometrial $PGF_{2\alpha}$ and PGE_2 release (reviewed by Schuster, 2002).

While these receptors and transporters interact to regulate prostaglandin signaling, it is hypothesized that SLCO2A1 expression in the equine endometrium will be similar to that observed in the ewe (Banu et al., 2008); endometrial SLCO2A1 is hypothesized to be up-regulated during the period of maternal recognition of pregnancy in the non-pregnant mare to facilitate luteolytic signaling. ABCC4 expression, which has been shown to be up-regulated in bovine endometrial epithelial cell lines in response to oxytocin treatment (Mondal et al., 2009), also is hypothesized to be up-regulated in the non-pregnant mare endometrium during maternal recognition of pregnancy (**Figure 2**). Additionally, as studies have shown that $PGF_{2\alpha}$ binding to PTGFR down-regulates SLCO2A1 prostaglandin transport (Vezza et al., 2001), PTGFR expression in the non-pregnant mare endometrium was hypothesized to be down-regulated. An increase in endometrial PGE_2 receptor expression, in particular PTGER2, also was hypothesized to

be observed in the pregnant mare (**Figure 3**) as similar up-regulation has been documented in the pregnant bovine endometrium, possibly for facilitating luteotrophic PGE_2 signaling (Arosh et al., 2004). Therefore, our first research objective was to determine equine endometrial gene expression for *SLCO2A1*, *ABCC4*, *PTGFR*, *PTGER*, *PTGER2*, *PTGER3*, and *PTGER4* during pregnancy and the estrous cycle. Our second research object was to determine endometrial protein expression and, thirdly, to determine localization of transporters and receptors within the endometrium.

Figure 3: Proposed Role for PTGFR and PGE_2 Receptors in Pregnant Mare Endometrium



Materials and Methods

Experiment 1

Mare Management

The Colorado State University Animal Care and Use Committee approved all experiments utilizing mares in Experiment 1. Endometrial biopsies were obtained at the uterine bifurcation on days 12, 14, and 16 post ovulation, covering the period of maternal recognition of pregnancy; day 18 biopsies also were obtained to compare expression after luteolysis in the non-pregnant mare (Ginther, 1983; reviewed by Ginther, 1998). Three mares were used per time point in a crossover study, with each mare serving both as a non-pregnant control and a pregnant treatment group. Mares, aged from 8 to 15 years, were all of stock-type with a history of normal cyclicity. Daily transrectal ultrasonography was utilized to follow follicular development and assess stage in estrous cycle.

For the first estrous cycle, follicular development was followed until a follicle of 35 mm or greater was detected by transrectal ultrasonography. After a 35 mm or greater follicle was detected, mares were artificially inseminated with a least 500 million progressively motile sperm from a stallion of know fertility every other day until ovulation was detected. Transcervical uterine biopsies were then performed using aseptic procedures on days 12, 14, 16 and 18 post-ovulation. Following biopsy collection, terminal uterine lavage was performed and embryos were collected to confirm pregnancy status. Endometrial biopsies were snap-frozen in liquid nitrogen and then stored at -80° C for RNA and protein extraction.

In the subsequent estrous cycle, mares were left unmated while daily transrectal ultrasonography was used to determine the day of ovulation. Trans-cervical uterine endometrial biopsies were taken on days 12, 14, 16, and 18 post-ovulation to serve as each mare's non-pregnant control sample. All samples were snap frozen in liquid nitrogen and stored at -80° C for RNA and protein isolation.

1.1 Gene Expression in Pregnant and Non-Pregnant Mare Endometrium

A tissue sample of approximately 0.1 gram was severed from frozen biopsy samples for RNA isolation. The tissue was homogenized and RNA isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. To improve sample quality, genomic DNA contamination was removed from several samples using RNase-Free DNase Set (QIAGEN, Valencia, CA). These RNA samples also were concentrated using the RNeasy MinElute Cleanup Kit (QIAGEN, Valencia, CA) as per manufacturer's protocol. A NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to quantify and check purity of the isolated RNA. Aliquots of isolated RNA were again stored at -80° C. RNA concentrations, collection date, quality, and treatment are listed in **Appendix I**.

Isolated RNA was processed using reverse transcription, utilizing the qScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Each reverse transcription reaction contained 2 µl of 5x qScript reaction mix, 0.5 µl of qScript reverse transcriptase, 5 µl of molecular grade water, and 2.5 µl of 55 ng/µl RNA template; the total reaction volume was 10 µl. The resulting cDNA was used as a template for biological replicates in real

time RTPCR quantification. For real time PCR analysis, 2.5 µl of nanopure water was added to every 5µl of Lightcycler 480 SYBR Green I Master (Roche, Basel Switzerland). Primer sets were added at 1.5µl [5µM] to each master mix. Additionally, 1µl of cDNA was added to each reaction for a final cDNA concentration of 34.4 ng/µl in a total volume of 10µl. Real time PCR plates (Roche, Basel, Switzerland) were analyzed using a Light Cycler 480 (Roche, Basel, Switzerland), with equine *GAPDH* and *tubulin* serving as the internal housekeeping genes.

Primers used for *SLCO2A1*, *ABCC4*, *PTGFR*, *PTGER2*, *PTGER3*, *PTGER4*, *GAPDH*, and *Tubulin* are listed in **Table 1**. All primers were blasted to ensure equine specificity (NCBI BLAST) to predicted equine sequences if the actual sequence is not known. DNA was isolated from 2% agarose bands using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) and sequenced by the Colorado State University Proteomics Laboratory; equine gene of interest specificity was confirmed for all primers. Additionally, primers for *PTGER3* and *GAPDH* were designed to span introns to identify genomic DNA contamination. Although no sequence for *PTGER* is reported for the horse (Ensemble), three separate sequences were used to design primers for *PTGER* using a conserved region from the human, bovine, and rat genome.

Statistical Analysis

Raw Ct values, $2^{-\Delta Ct}$ values, and fold changes are listed in **Appendix II**, **III**, and **IV**, respectively. $2^{-\Delta Ct}$ values were calculated using the comparative C^t method (Schmittgen and Livak, 2008) with data normalized to equine *Tubulin* expression. Only biological replicates with real time RTPCR results for all genes of interest and *Tubulin* were used

for calculating average expression per mare sample (**Appendix II**). Statistical analysis of real time RTPCR results used $2^{-\Delta Ct}$ values. The Student's paired, two-tailed *t*-test was used to compare gene expression within each day of tissue collection. To compare gene expression across days of tissue collection and pregnancy status, a two-sample *t*-test for samples with equal variance was utilized. A *p*-value less than 0.05 was considered statistically significant. Standard curve analysis of primer set efficiency was determined from serial RNA dilutions from a pool of RNA samples. Efficiency values from the standard curve analysis are listed in **Appendix V** while the dynamic range of standard curves is given in **Appendix VI**.

1.2 Protein Expression in Pregnant and Non-Pregnant Mare Endometrium

Analysis of SLCO2A1, ABCC4, PTGFR, PTGER2, PTGER3, and PTGER4 protein expression was completed to compare to mRNA expression observed in the previous experiment. To extract protein, approximately 0.2 gram was severed from frozen endometrial samples and homogenized on ice in 2mL of RIPA lysis buffer (**Appendix VII**). Samples were then sonnicated on ice for approximately one minute and centrifuged at approximately 10,000 rpm for 10 minutes at room temperature. Supernatant was extracted and frozen prior to concentration analysis (NanoDrop 1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE). Supernatant was diluted in 6x loading dye-DTT (200µl 6xDTT:1ml sample) and further diluted in 6x loading dye-DTT to reach a concentration of 5µg/µl to facilitate loading of gels. Protein lysates were heated for 10 minutes at approximately 75°C and then 50 ng (10 µl) of sample was loaded and resolved

on 10% Tris-HCL SDS-PAGE gels (BioRad, Hercules, CA). Protein was transferred to Protran nitrocellulose membranes (Whatman, Dassel, Germany) for 1 hour at 4°C.

Primary antibodies and blocking peptides were obtained from the following companies: SLCO2A1, SLCO2A1 Blocking Peptide, PTGFR, PTGFR Blocking Peptide, PTGER2, PTGER2 Blocking Peptide, PTGER3, PTGER4, and PTGER4 Blocking Peptide were supplied by Cayman Chemicals (AnnArbor, MI) and ABCC4 antibody was supplied by AbD Serotec (Raleigh, NC). Secondary antibodies used include: goat anti-rabbit (Cayman, AnnArbor, MI), goat anti-rabbit (SantaCruz Antibodies, Santa Cruz, CA), and goat anti-mouse (Promega, Madison, WI). Primary antibody concentrations were optimized in 1-5% dry milk-TBST, by varying incubation times from approximately 2-12 hours, varying incubation temperature between either room temperature or 4°C, and by varying secondary antibody concentrations in dry milk-TBST. However, under the experimental conditions used, only PTGER2 antibody had specific immunoreactivity.

For Western blot analysis of PTGER2 expression, nitrocellulose membranes first were blocked for approximately two hours at room temperature in a 5% dry milk-TBST to prevent non-specific binding. Blots were then washed in TBST and PTGER2 antibody was added at 1:1000 dilution in 1% dry milk-TBST overnight in 4°C. Blots were then washed in TBST and HRP-conjugated goat anti-rabbit antibody (Cayman, AnnArbor, MI) was added at 1:5000 dilution in 3% dry milk-TBST for 6 hours at 4°C. After agitation with secondary antibody, blots again were washed in TBST. Chemiluminescence was then assessed using ECL Plus Western Blotting Detection System (Amersham, Buckinghamshire, UK) and immunoreactive banding was analyzed using the Storm 860 (Amersham Biosciences, Sweden). HRP-conjugated β-actin (Santa

Cruz Antibodies, Santa Cruz, CA) was used as a loading control at 1:1000 dilution in 5% dry milk-TBST for 6 hours at 4°C. To confirm PTGER2 banding specificity, PTGER2 blocking peptide was used in a 1:1 ratio with PTGER2 primary antibody as per manufacturer's protocol.

PTGER2 expression was quantified with ImageQuant TL software (Amersham Biosciences, Sweden) and normalized to β-actin expression. Mares randomly assigned to one of three groups to be analyzed on a series of three Western blots so each blot shows non-pregnant and pregnant PTGER2 expression across all time points. The mean PTGER2:β-actin expression was analyzed with a Student's paired, two-tailed *t*-test to compare statistical significance between pregnant and non-pregnant mare expression for each time point. A two-tailed *t*-test for samples with equal variances was used to compare PTGER2 expression between pregnant samples at different time points. A *p*-value less than 0.05 was considered statistically significant.

Experiment 2

2.1 SLCO2A1 and PTGER2 Immunohistochemical Localization in Equine Endometrium

Mare Management

To determine localization of SLCO2A1 and PTGER2 protein in the equine endometrium, endometrial biopsies were collected from mares in diestrus and estrus. Mares were examined by transrectal ultrasonography to determine follicular activity and

stage in the estrous cycle. Diestrus was designated by the presence of a corpus luteum, a tight cervix, absence of large follicles (> 20mm) on either ovary, and lack of endometrial edema (n=1). Estrus was designated by the presence of an irregular, thick-walled follicle (45mm), endometrial edema, and an open cervix (n=1).

Tissue Preparation

Endometrial biopsies were fixed in 4% PFA overnight at 4°C. Samples were washed in PBS and embedded in paraffin by Colorado State University Diagnostics Laboratory. Sections were cut at 5μm and fixed to charged slides. Hematoxylin and eosin staining was performed on several slides for histology comparison of endometrial structures. Immunohistochemistry (IHC) was performed in technical replicates to determine SLCO2A1 and PTGER2 immunoreactivity and localization. Primary antibodies were added at 1:1000 dilution in Super Block Blocking Buffer (Thermo Scientific, Waltham, MA) overnight at 4°C. Control samples followed the same IHC protocol, but lacked primary antibody. Blocking peptides for SLCO2A1 and PTGER2 (Cayman, AnnArbor, Michigan) were added at a 1:1 ratio to primary antibodies as per manufacturer's protocol. VectaStain ABC peroxidase rabbit IgG (Vector Labs, Burlingame, CA) was used as a secondary antibody for all treatments. A 3,3'-Diaminobenzidine (DAB) peroxidase substrate kit (Vector Labs, Burlingame, CA) was used for HRP immunoreactivity detection.

Results

1.1 Gene Expression in Pregnant and Non-Pregnant Mare Endometrium

For RTPCR analysis, no primer sets for *PTGER* showed amplicon banding when run on a 2% agarose gel. Experiments were therefore continued without the inclusion of *PTGER* as it does not appear to be expressed in the horse. Additionally, due to poor efficiency and primer-dimer formation with *GAPDH* (**Appendix V** and **VI**), *Tubulin* expression was used to normalize data. When all time points of tissue collection were combined for each treatment (**Figure 4**), no difference was observed between non-pregnant and pregnant treatments for any of the genes of interest. However, when the treatment groups were combined for each time point of tissue collection (**Figure 5**), *SLCO2A1* expression was lower on day 14 than day 16 post-ovulation ($p<0.05$). No other difference was found in gene expression across all time points when pregnancy status was pooled to test the effect of day. When each treatment and day of tissue collection was separated (**Figures 6**, **7**, **8**, **9**, **10**, and **11**), *PTGER3* expression was higher in day 16 pregnant mare samples in comparison to day 12 pregnant mare samples ($p<0.03$). No differences were observed in gene expression between pregnant and non-pregnant mare samples for each time point despite large fold changes (**Appendix IV**). For example, endometrial *SLCO2A1* tended to have two-fold increased expression in day 14 pregnant mares in comparison to day 14 non-pregnant mares. Additionally, a trend of four-fold increase in endometrial *SLCO2A1* expression was seen in day 16 and 18 pregnant mares in comparison to day 16 and 18 non-pregnant mares, respectively. Endometrial *ABCC4* also tended to have a two-fold increased expression in day 12 non-pregnant mares. A trend of two-fold increased endometrial *ABCC4* expression was observed in day 18

pregnant mares in comparison to non-pregnant mares. Endometrial *PTGFR* showed a similar trend in expression as *ABCC4*, with approximately a two-fold increase in expression in day 12 non-pregnant and day 18 pregnant mares. The endometrial expression of *PTGER2* showed a trend toward the highest fold-change observed, with a trend of approximately eleven-fold increase in expression in day 18 pregnant mares. A trend of approximately three-fold increase in endometrial *PTGER2* expression was observed in day 14 and 16 pregnant mares. Additionally, a trend of approximately two-fold increase in expression was observed in endometrial *PTGER3* in day 12 non-pregnant mares and in days 16 and 18 pregnant mares. Endometrial *PTGER4* also tended to be three-fold higher expressed in day 16 pregnant mares. However, while a trend of two- to eleven-fold changes in gene expression were observed, changes in expression between treatments for each time point were not statistically significant. This is likely due to the small sample size (n=3 per time point) and the wide variation in expression observed between mares (**Appendix II** and **III**).

1.2 Protein Expression in Pregnant and Non-Pregnant Mare Endometrium

For Western blot analysis, only the antibody for PTGER2 was immunoreactive with equine endometrial samples. Specificity of PTGER2 immunoreactivity was determined by use of a PTGER2 blocking peptide. PTGER2 specific bands were detected at the expected molecular weight of 52 kD (**Appendix VIII**). Interestingly, Western blot results showed inconsistent PTGER2 expression across the treatment groups (pregnant versus non-pregnant) and all time points (days 12, 14, 16, and 18), despite even loading of protein samples (**Appendix VIII**). No difference was found between pregnant and non-pregnant PTGER2 expression for any time point. However, endometrial PTGER2

expression was lower in day 12 pregnant mares than day 16 ($p<0.002$) and day 18 ($p<0.007$) pregnant mares (**Figure 12**).

2.1 SLCO2A1 and PTGER2 Immunohistochemical Localization in Equine Endometrium

Both the SLCO2A1 and PTGER2 antibodies appeared to have immunoreactivity with equine samples when used for IHC; additionally, the use of both antibodies in the horse for IHC was validated by the use of blocking peptides (**Figures 13.B**, **13.F**, **14.B**, and **14.F**), which effectively blocked immunoreactivity.

Interestingly, SLCO2A1 immunoreactivity was localized differently between diestrous and estrous mare endometrium (**Figure 13**). In the diestrous mare endometrium, SLCO2A1 appeared to have greater immunoreactivity while minimal immunolocalization was observed the estrous mare endometrium. Additionally, in the diestrous mare SLCO2A1 immunoreactivity was observed in the stromal tissue as well as in the endometrial epithelial glands. Specifically, SLCO2A1 localization appeared to be higher in the basal aspect of the endometrial epithelial glands in the diestrous mare while minimal SLCO2A1 immunoreactivity was observed in the estrous mare endometrium.

In contrast, PTGER2 immunoreactivity was observed in both the diestrous and estrous mare endometrium (**Figure 14**). PTGER2 immunoreactivity was primarily localized to the apical aspect of the endometrial epithelial glands in the diestrous mare endometrium. However, PTGER2 immunoreactivity was observed throughout the endometrial epithelial glands in the estrous mare. Additionally, PTGER2 immunoreactivity had highly punctate localization in the epithelial glands of both the estrous and diestrous mare endometrium.

Discussion

Real time RTPCR results showed that endometrial *SLCO2A1* expression was lower on day 14 than day 16 post-ovulation when pregnancy status was combined to test the effect of day ($p<0.05$) (**Figure 5**). This suggests that endometrial *SLCO2A1* expression is dynamic and is regulated differently between days 14 and 16. Additionally, it is of interest that endometrial *SLCO2A1* was lower on day 14, at the time when endometrial $PGF_{2\alpha}$ reaches its peak in non-pregnant mares (Sharp et al., 1984). Interestingly, no difference was seen in endometrial *SLCO2A1* expression between day 14 pregnant and non-pregnant mares. However, this is likely due to the variability in expression observed between mares (**Appendix II** and **III**), suggesting that a larger sample size might show significant changes in *SLCO2A1* expression. As pregnancy status did not have any effect on endometrial *SLCO2A1* expression (**Figure 6**), the presence of an embryo may not effect *SLCO2A1* expression. However, while no statistical significance was found, endometrial *SLCO2A1* tended to be expressed two-fold higher in pregnant mares on day 14 than non-pregnant mares (**Appendix IV**). Additionally, endometrial *SLCO2A1* also tended to be expressed four-fold higher in day 16 and 18 pregnant mares in comparison to non-pregnant mares. Trends in fold-changes in expression of *SLCO2A1* may suggest that the presence of an embryo can up-regulate *SLCO2A1* expression after day 12. Since the presence of an embryo is known to significantly suppress $PGF_{2\alpha}$ production on day 12 (Sissener et al., 1996), the trends in endometrial *SLCO2A1* expression may function down-stream of an initial antiluteolytic signal on day 12 in the pregnant mare. Additionally, the trend for increased *SLCO2A1* expression in pregnant mares varies from the hypothesized model (refer to **Figure 5**) in which endometrial SLCO2A1 was

hypothesized to facilitate transport of luteolytic $PGF_{2\alpha}$ and be down-regulated in pregnant mares. The trend for up-regulation of *SLCO2A1* in pregnant mares varies from the observed down-regulation of *SLCO2A1* during luteolysis in the ewe (Banu et al., 2008). However, the trend in *SLCO2A1* expression is similar to results observed by Mondal et al. (2009), where treatment of bovine endometrial epithelial cell lines with IFNT stimulated *SLCO2A1* expression. These results may suggest that the regulatory role of SLCO2A1 may vary with species.

The trend for increased expression of endometrial *SLCO2A1* in pregnant mares after day 12 (**Appendix IV**) suggests that SLCO2A1 may be functioning to sequester luteolytic $PGF_{2\alpha}$ inside the cell. By sequestering luteolytic $PGF_{2\alpha}$ inside the cell, it can be metabolized in a similar process to that described by Nomura et al. where *in vitro* co-expression of SLCO2A1 and prostaglandin 15 dehydrogenase in HeLa cells increased metabolism of exogenous PGE_2 and $PGF_{2\alpha}$ (2004). However, to confirm this role for SLCO2A1, immunohistochemistry analysis of SLCO2A1 should be conducted to determine localization throughout the period of maternal recognition of pregnancy in both non-pregnant and pregnant mare endometrium in a larger sample size. If endometrial SLCO2A1 expression is different between pregnant and non-pregnant mares, identification of what factor(s) control SLCO2A1 expression could aid in recognizing the original up-stream signal(s) for maternal recognition. However, additional research is needed to determine what substrate affinities control competitive prostaglandin binding to endometrial prostaglandin receptors in comparison to endometrial SLCO2A1.

Interestingly, endometrial *PTGER3* was found to be expressed higher in day 16 pregnant mare samples in comparison to day 12 pregnant mare samples ($p<0.03$),

suggesting that PTGER3 may be functioning to regulated PGE_2 signaling from the embryo (Vanderwall et al., 1993). In comparison to endometrial *SLCO2A1* and *PTGER3* expression, no differences were observed in endometrial *ABCC4*, *PTGFR*, *PTGER2*, and *PTGER4* expression (**Figures 4**, **5**, **7**, **8**, **9**, and **11**). However, gene expression was highly variable between mares (**Appendix II** and **III**). Taken in combination with the small sample size, the variability in expression may obscure statistically significant differences in gene expression between pregnant and non-pregnant mare samples. This can be seen in particular with endometrial *PTGER2* expression, which tends to be approximately eleven-fold higher in day 18 pregnant mares than non-pregnant mares, yet no difference was found in expression. However, the trends seen in fold-changes (**Appendix IV**) may suggests the regulatory function ABCC4, PTGFR, PTGER2, PTGER3, and PTGER4 serve in the equine endometrium during maternal recognition. For instance, endometrial *ABCC4* expression tended to be almost three-fold higher in non-pregnant mares on day 12, yet two- to three-fold higher in pregnant mares on days 16 and 18, respectively. This suggests that ABCC4 may play an important role in transporting prostaglandins during maternal recognition. As ABCC4 functions as an efflux transporter (Reid et al., 2003), and as endometrial luminal content of $PGF_{2\alpha}$ is lower in pregnant mares during maternal recognition of pregnancy, this would suggest that ABCC4 may be localized different in pregnant mares to facilitate the efflux of $PGF_{2\alpha}$ away from the uterine lumen and systemic circulation to prevent luteolysis. Further confirmation of ABBC4 localization, however, should be confirmed with immunohistochemistry.

Endometrial *PTGFR* expression also showed trends in fold-changes (**Appendix IV**), with approximately two-fold higher expression in non-pregnant mares on day 12 and in

pregnant mares on day 14 and 16. However, the trend observed for endometrial *PTGFR* expression did not seem to be correlated with down-regulation of *SLCO2A1* as could be suggested by the results from Vezza et al. (2001) where $PGF_{2\alpha}$ binding to PTGFR inhibited SLCO2A1 prostaglandin transport. As *PTGFR* expression tended to change throughout maternal recognition, it might suggest that PTGFR may also function for embryonic $PGF_{2\alpha}$ signaling in the endometrium. Additionally, the trend for increased endometrial *PTGER2*, *PTGER3*, and *PTGER4* expression in pregnant mares after day 12 (**Appendix IV**) suggests that the PGE_2 receptors may be facilitating luteotrophic or embryonic PGE_2 signaling (Ginther, 1983; reviewed by Ginther, 1998). Additionally, prostaglandin transports and receptors also may be constitutively expressed in the endometrium for other prostaglandin signaling pathways, such as inflammation or contractile signaling (reviewed by Schuster et al., 2002; reviewed by Fortier et al., 2008).

Interestingly, endometrial *PTGER2* expression shows a similar trend to *SLCO2A1* expression, with increased expression in pregnant mares after day 12. This again suggests that endometrial *PTGER2* expression is being affected by the presence of an embryo after day 12. Therefore, another mechanism may be functioning up-stream of both endometrial *SLCO2A1* and *PTGER2* on day 12 in the pregnant mare to suppress luteolytic $PGF_{2\alpha}$ release (Sissener et al., 1996) for maternal recognition of pregnancy. However, the trend of up-regulation observed for both endometrial *SLCO2A1* and *PTGER2* in pregnant mares may be functioning after day 12 to facilitate additional signaling for maternal recognition of pregnancy. It is therefore of interest to determine not only what signal is occurring up-stream of endometrial *SLCO2A1* and *PTGER2* expression in the pregnant

mare on day 12, but also what mechanisms are then signaling regulatory changes in endometrial *SLCO2A1* and *PTGER2* expression.

Western blot analysis revealed great variability in PTGER2 expression, both between mares and across all time points (**Appendix VIII**). The variability in PTGER2 expression observed may be due to individual mare variability in expression. Additional, variability in PTGER2 also may be due to localization of the protein within the endometrium and biopsy sample, which would affect the observed expression pattern depending on the tissue composition used for protein isolation. It may be due to these inconsistencies that no difference was found in PTGER2 expression between pregnant and non-pregnant mares. Endometrial PTGER2 expression was, however, found to be lower in day 12 pregnant mares than day 16 ($p<0.002$) and day 18 ($p<0.007$) pregnant mares (**Figure 12**). This might suggest that PTGER2 may be functioning to facilitate a luteotrophic PGE_2 signaling pathway during maternal recognition of pregnancy, particularly after day 12 post-ovulation, in a process that has not yet been described in the mare. For instance, PTGER2 may respond to PGE_2 secretions from the embryo (Weber et al., 1991a; 1991b) to signal other cellular pathways for maternal recognition of pregnancy, such as increased SLCO2A1 expression for cellular $PGF_{2\alpha}$ metabolism.

However, as IHC results showed that SLCO2A1 and PTGER2 had varying localization at different stages of the estrous cycle (**Figures 13** and **14**), any variability in protein localization between pregnant and non-pregnant mare biopsy samples could have affected the expression observed in both the real time RTPCR and Western blot analysis. Additionally, localization of the endometrial biopsy sample and localization of SLCO2A1 and PTGER2 within the biopsy sample could also have affected the results.

Therefore, to confirm real time RTPCR and Western blot results, a similar study should be conducted to determine tissue localization of SLCO2A1, ABCC4, PTGFR, PTGER2, PTGER3, and PTGER4 in pregnant and non-pregnant mare endometrial biopsies. Additionally, a range of variability of gene expression in individual mares was observed (**Appendix II** and **III**). As individual mare variability in mRNA and protein expression could be a factor affecting results, future studies utilizing endometrial biopsies should use a larger sample size to help exclude outliers and perhaps show statistically significant differences that were otherwise not determined in this study

As mentioned, SLCO2A1 and PTGER2 localization at different phases of the estrous cycle varied as determined by IHC (**Figures 13** and **14**). Again, this would suggest that SLCO2A1 and PTGER2 localization may vary between non-pregnant and pregnant mare endometrium, possibly affecting their regulatory role in prostaglandin signaling during maternal recognition of pregnancy. While SLCO2A1 antibody had no specific immunoreactivity for Western blot analysis, its immunoreactivity for IHC may due to maintenance of isotopes with IHC tissue preparation in comparison to Western blot protein denaturing or loss of secondary structure. Interestingly, PTGER2 was localized primarily to the apical aspect of the epithelial glands while SLCO2A1 was localized to the basal aspect of the glands in diestrous mare endometrium. This might suggest that PTGER2 localization in the apical aspect may be functioning to receive PGE_2 secretions from the conceptus for luteotrophic signaling during diestrus. Also, the punctate localization of PTGER2 may suggest that vesicular trafficking of the receptor throughout the estrous cycle regulates its function. For example, the punctate localization of PTGER2 throughout the estrous mare endometrial epithelial glands suggests cellular

receptor recycling or transport when PGE_2 signaling should be minimal. Additionally, SLCO2A1 localization in the basal aspect of the endometrial epithelial glands may be functioning to transport luteolytic $PGF_{2\alpha}$ into systemic circulation during diestrus when no conceptus is present. As *SLCO2A1* expression tended to be higher in pregnant mares (**Appendix IV**), SLCO2A1 may be localized to the apical aspect of epithelial glands in pregnant mares during maternal recognition of pregnancy for $PGF_{2\alpha}$ influx for metabolism. Future immunohistochemical analysis should clarify the localization of SLCO2A1 and PTGER2 in pregnant mares to determine their function during maternal recognition of pregnancy as they may be similarly regulated.

Correlation in SLCO2A1 and PTGER2 expression and possibly even localization is further supported by the similar trends in expression noted between endometrial *SLCO2A1* and *PTGER2* (**Figures 6** and **9** and **Appendix IV**). These results suggest that future research investigate mechanisms regulating endometrial *SLCO2A1* and *PTGER2* expression, such as by using an *in vivo* endometrial explant culture system with equine embryo explants/secretions. Discovery of the up-stream mechanism(s) that function as the initial signal for regulating *SLCO2A1* and *PTGER2* expression may aid in identification of the physiological process used for maternal recognition of pregnancy in the mare. Additional immunohistochemical analysis of ABCC4, PTGFR, PTGER3, and PTGER4 localization between pregnant and non-pregnant mares should clarify their role in prostaglandin signaling during maternal recognition of pregnancy. Once the physiological signal(s) for maternal recognition are identified, new treatments to suppress estrus in mares used for competition could be developed.

Conclusions

Results showed that endometrial *SLCO2A1* was expressed lower on day 14 than day 16 when gene expression was compared by day regardless of pregnancy status ($p<0.05$). Additionally, endometrial *PTGER3* expression was higher in day 16 pregnant mares than day 12 pregnant mares ($p<0.03$). *PTGER* was not found to be expressed in the endometrium of the mare. Pregnancy status did not appear to affect endometrial *SLCO2A1*, *ABCC4*, *PTGFR*, *PTGER2*, *PTGER3*, or *PTGER4* expression when comparing pregnant to non-pregnant mare samples from each time point. While no statistically significant differences were found, fold-changes in gene expression were observed for all genes ranging from two- to eleven-fold changes in expression. However, lack of statistically significant differences is likely due to the variability observed in individual mare gene expression. Trends in fold-changes, however, can suggest the function of the transporter and receptors. For instance, results showed a trend for increased *SLCO2A1* expression in pregnant mares on days 14, 16 and 18. This varies from the hypothesized decreased *SLCO2A1* expected in pregnant mares. This suggests that SLCO2A1 may be functioning to sequester luteolytic $PGF_{2\alpha}$ secretions for metabolism in the endometrium as opposed to facilitating its release. A trend for increased *PTGER2* expression in pregnant mares on days 14, 16, and 18, as well as increased *PTGER3* expression on days 16 and 18 and *PTGER4* expression on day 16 in pregnant mares, also suggests that PGE2 may be functioning through its receptors for luteotrophic signaling after day 12. Interestingly, endometrial *ABCC4* expression tended to be two-fold higher in non-pregnant mares on day 12, but tended to be two-fold higher in day 18 pregnant mares. Again, this suggests that prostaglandin transport is dynamically

regulated during maternal recognition of pregnancy. Western blot results showed variability between endometrial PTGER2 expression between individual mares across all time points. While no difference was seen in PTGER2 expression between pregnant and non-pregnant mares for each time point, this may be due to variability in individual mare expression, protein localization, or biopsy sample tissue composition. However, PTGER2 was expressed higher in day 16 ($p<0.002$) and day 18 ($p<0.007$) pregnant mares than day 12 pregnant mares. This may suggest that PTGER2 is functioning after day 12 post-ovulation for luteotrophic PGE_2 signaling from the embryo. Immunohistochemical analysis showed SLCO2A1 localization to the basal aspect of the endometrial epithelial glands and stroma during diestrus. Additionally, PTGER2 had punctate localization in the apical aspect of the endometrial epithelial glands in diestrous mare. During estrus, minimal SLCO2A1 immunoreactivity was observed. Therefore, SLCO2A1 and PTGER2 localization throughout the estrous cycle possibly is associated with their function in luteolytic $PGF_{2\alpha}$ signaling. For instance, basal localization of SLCO2A1 in the epithelial glands of non-pregnant, diestrous mare endometrium could facilitate luteolytic $PGF_{2\alpha}$ transport into systemic circulation for lysis of the corpus luteum. If SLCO2A1 is localized in the apical aspect of the endometrial epithelial glands in pregnant mares, it may be functioning to metabolize $PGF_{2\alpha}$ inside the epithelial glands. Additionally, PTGER2 may be localized to the apical epithelial glands during diestrus to facilitate possible luteotrophic PGE_2 signaling from the conceptus. However, in the estrous mare, PTGER2 may undergo cellular recycling or vesicular transport to the basal aspect as punctate PTGER2 localization was observed throughout the endometrial epithelial glands. By this mechanism, PTGER2 localization may be regulated to facilitate PGE_2

signaling during maternal recognition of pregnancy. Therefore, localization of SLCO2A1 and PTGER2 in the endometrial epithelial glands could be functioning in the pregnant mares for signaling maternal recognition of pregnancy. However, due to the variability in endometrial gene and protein expression observed between mares, it is difficult to interpret the actual expression and function of SLCO2A1, ABCC4, PTGFR, PTGER2, PTGER3, and PTGER4. Therefore, future experiments should investigate the localization of these proteins throughout maternal recognition with immunohistochemistry in both pregnant and non-pregnant mares. Additionally, future experiments should utilize a larger sample size to help determine statistical significance and eliminate outliers. Once the endometrial localization of SLCO2A1, ABCC4, PTGFR, PTGER2, PTGER3, and PTGER4 in known in pregnant mares, the primary signal(s) effecting their expression, and possibly maternal recognition of pregnancy, can be identified. Once the signal(s) for maternal recognition are know, it may be possible to develop alternative treatments for estrus suppression in mares used for competition.

Table 1

PCR Primer Sequences

Gene	Forward Primer	Reverse Primer	Amplicon Size
SLCO2A1	GCCCCACAGTAAATGCCACA	GTTGGGCTCCGAGAAGTCGT	156
ABCC4	GATGGTGCAAAAGGGGACCT	TCCTCAGCGTGGGAGTTCCT	122
PTGFR	TTTGGCCACCTCATCAATGG	GGCCATTGCACTGCCTAGAA	155
PTGER2	CAGTACTGCCCTGGGACGTG	GAGGTGGAGGATGACGCTGA	128
PTGER3	GTCGCCACTGCTGATTATGA	CCAAGATCTGGTTCAGTGACG	139
PTGER4	CGTACATGAAGGGCGAGTGG	CGTGGTTGATGGCCAGGTAG	136
GAPDH	CTCAAAGGGAAGCTGACAGG	GTAGGCAAGGATGCCAGCTA	158
TUBULIN	ACGTGGTTCCCAAAGATGTC	CACAGTGGGAGGCTGGTAAT	122

Table 1. Primer sequences used for real time RTPCR analysis.

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Weber, J.A., Woods, G.L., Lichtenwalner, A.B. Relaxatory effect of prostaglandin E_2 on circular smooth muscle isolated from the equine oviductal isthmus. *Bio Reprod Monograph Series* 1995; 1:125-130.

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Zavy, M.T., Vernon, M.W., Asquith, R.L., Bazer, F.W., Sharp, D.C. Effect of exogenous gonadal steroids and pregnancy on uterine luminal prostaglandin F in mares. *Prostaglandins* 1984; 27: 311-320.

Figure 4

Changes in Relative Gene Expression for all Time Points Combined by Pregnancy Status

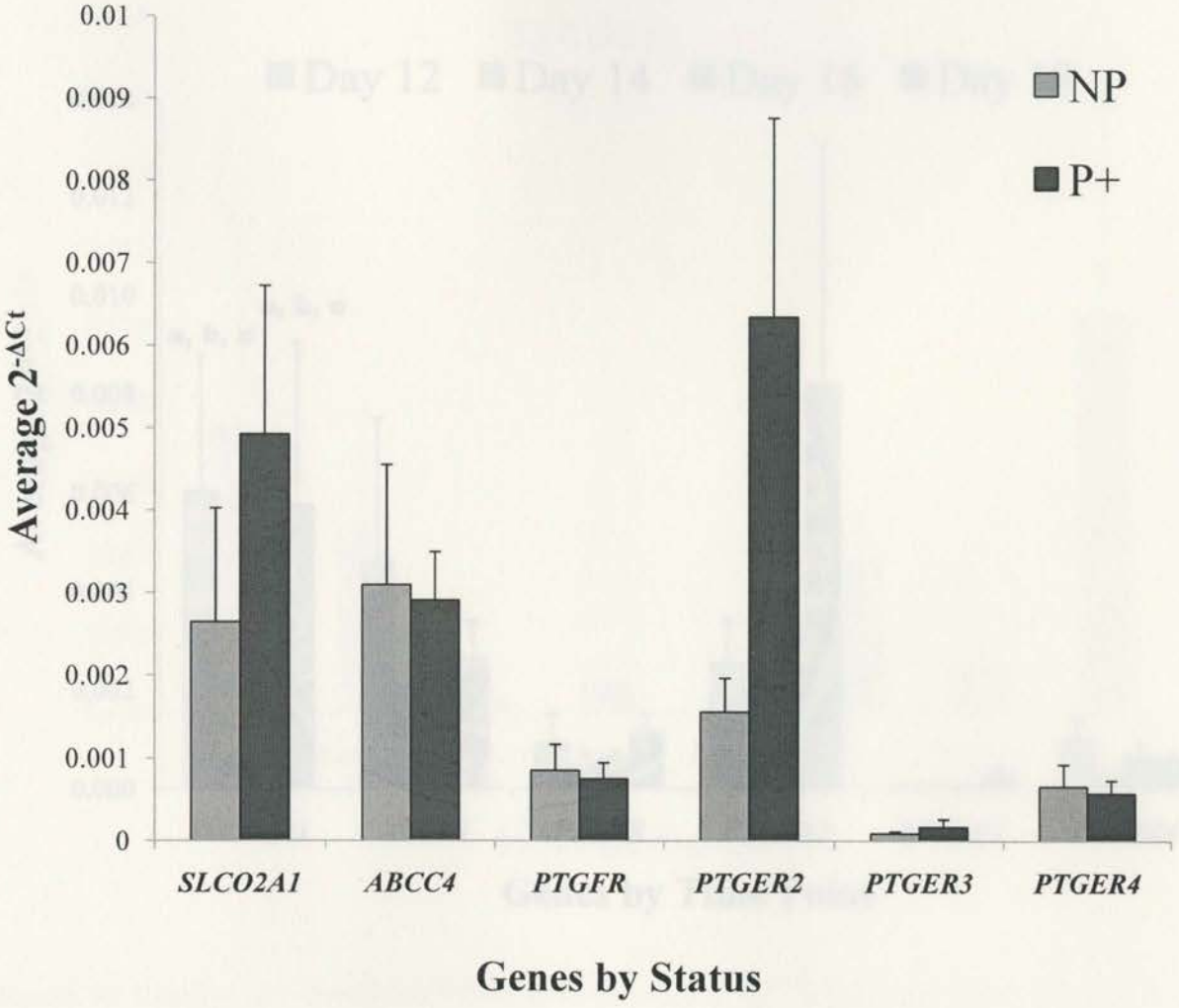


Figure 4) Changes in relative gene expression ($2^{-\Delta Ct}$) with all time points combined per pregnancy status. Day of biopsy collection was pooled to test for the effect of pregnancy status. NP denotes non-pregnant. P+ denotes pregnant. Error bars represent SEM values.

Figure 5

Changes in Relative Gene Expression by Day Regardless of Pregnancy Status

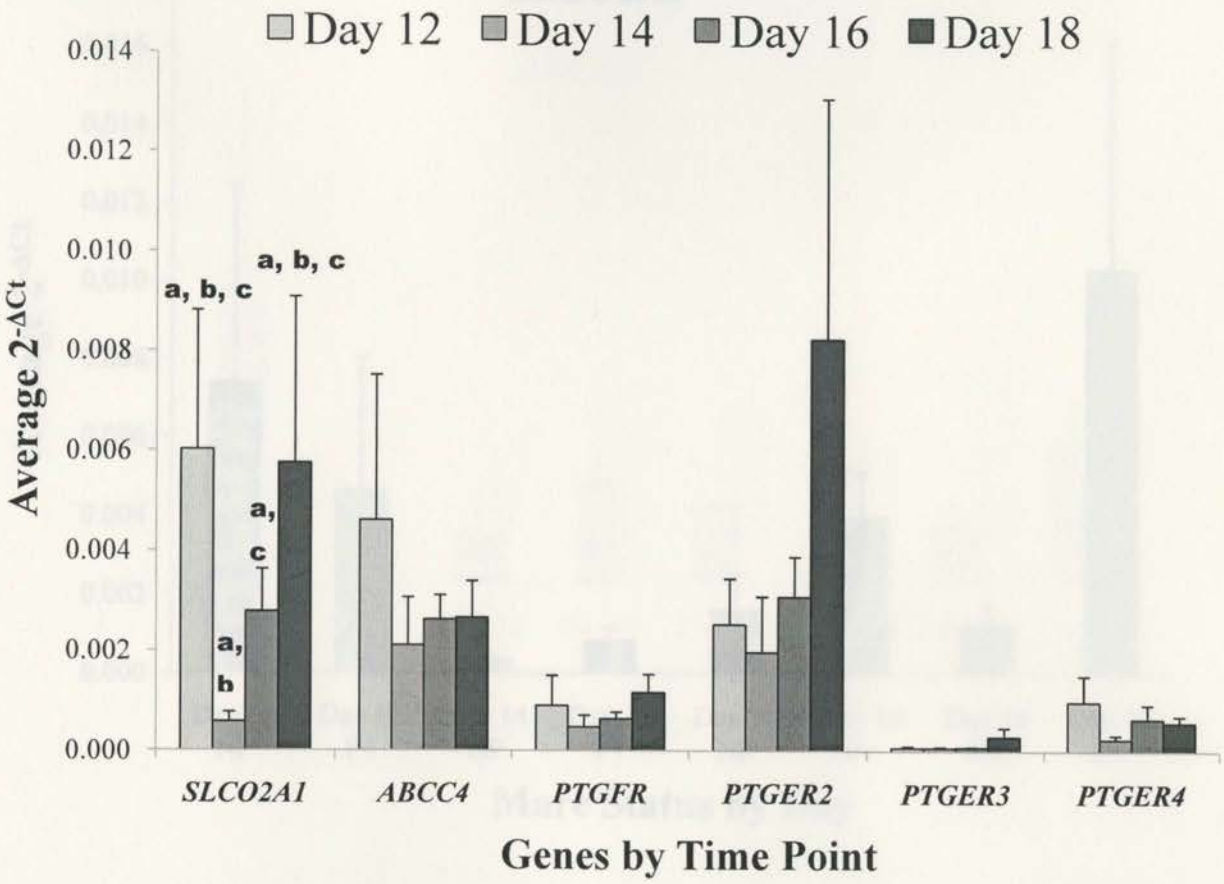


Figure 5) Changes in relative gene expression ($2^{-\Delta Ct}$) regardless of pregnancy status per day of biopsy collection. Pregnancy status was pooled to test effect of day. Error bars represent SEM values. Different letters denote statistically significant differences in expression ($p<0.05$)

Figure 6

Changes in Relative *SLCO2A1* Gene Expression by Day and Pregnancy Status

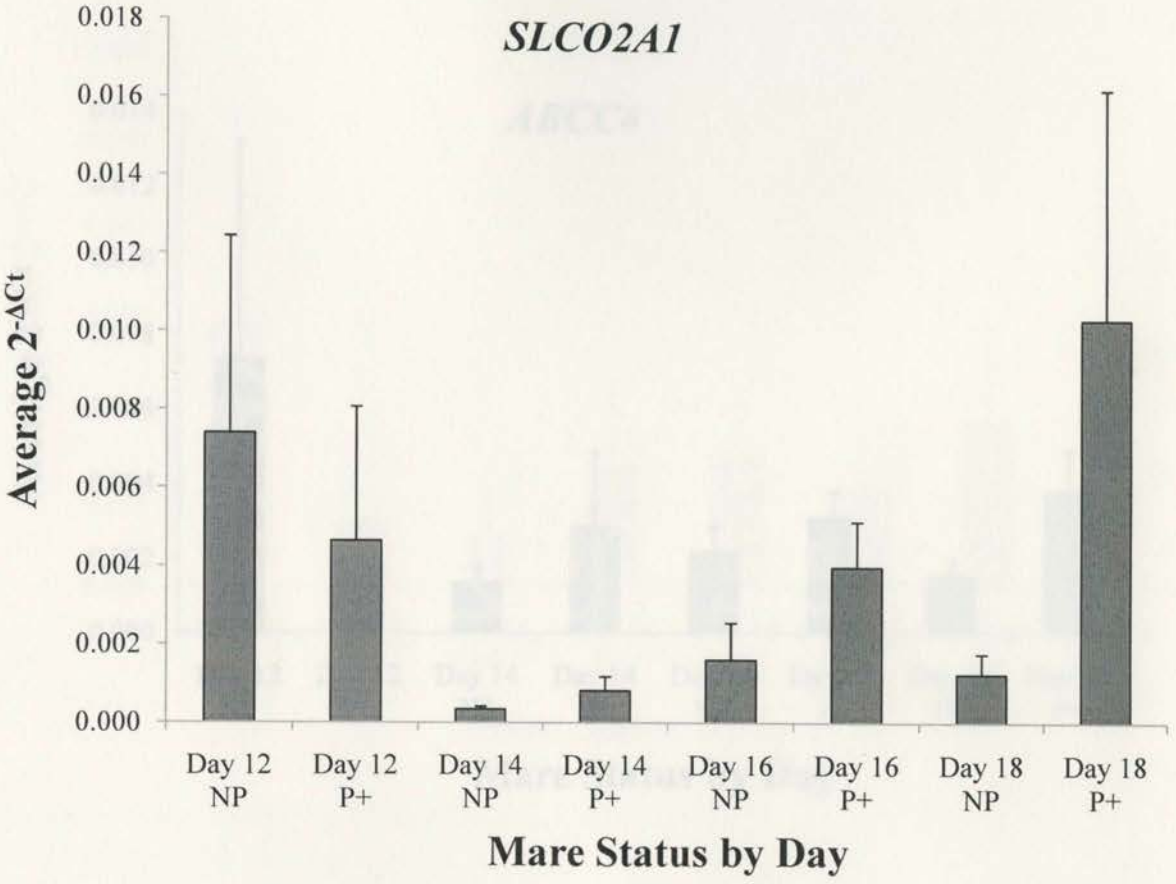


Figure 6) Changes in relative *SLCO2A1* gene expression ($2^{-\Delta Ct}$) per time point of tissue collection and mare pregnancy status. NP denotes non-pregnant. P+ denotes pregnant. Error bars represent SEM values.

Figure 7

Changes in Relative *ABCC4* Gene Expression by Day and Pregnancy Status

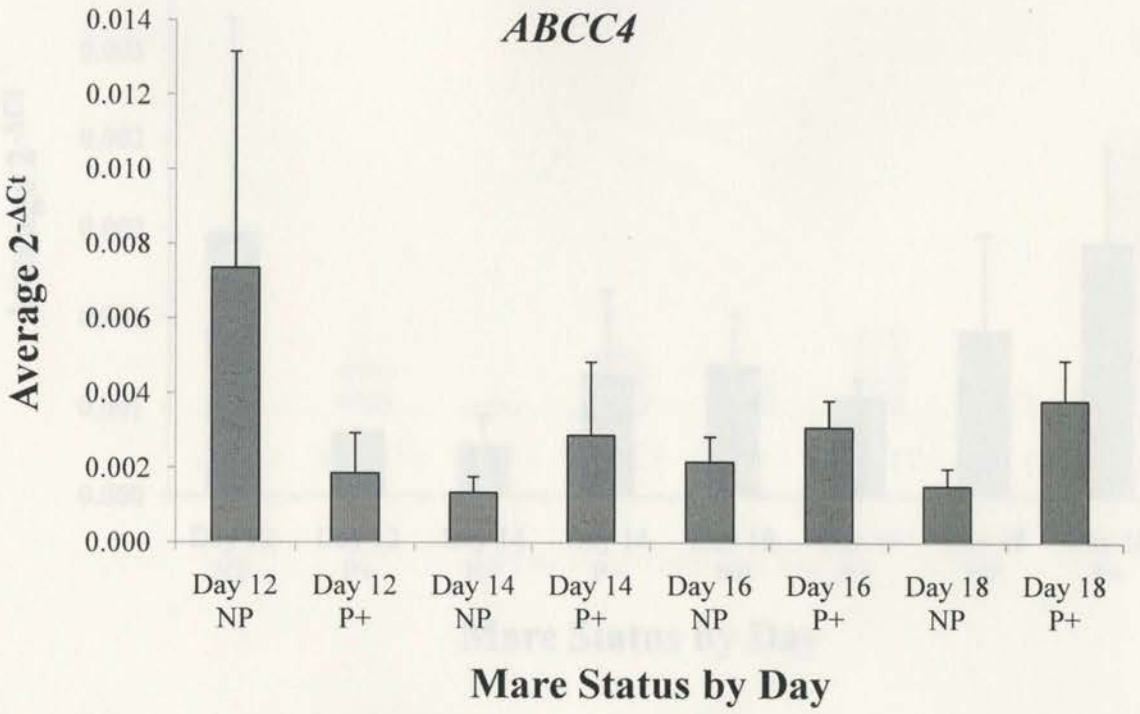


Figure 7) Changes in relative *ABCC4* gene expression ($2^{-\Delta Ct}$) per time point of tissue collection and mare pregnancy status. NP denotes non-pregnant. P+ denotes pregnant. Error bars represent SEM values.

Figure 8

Changes in Relative *PTGFR* Gene Expression by Day and Pregnancy Status

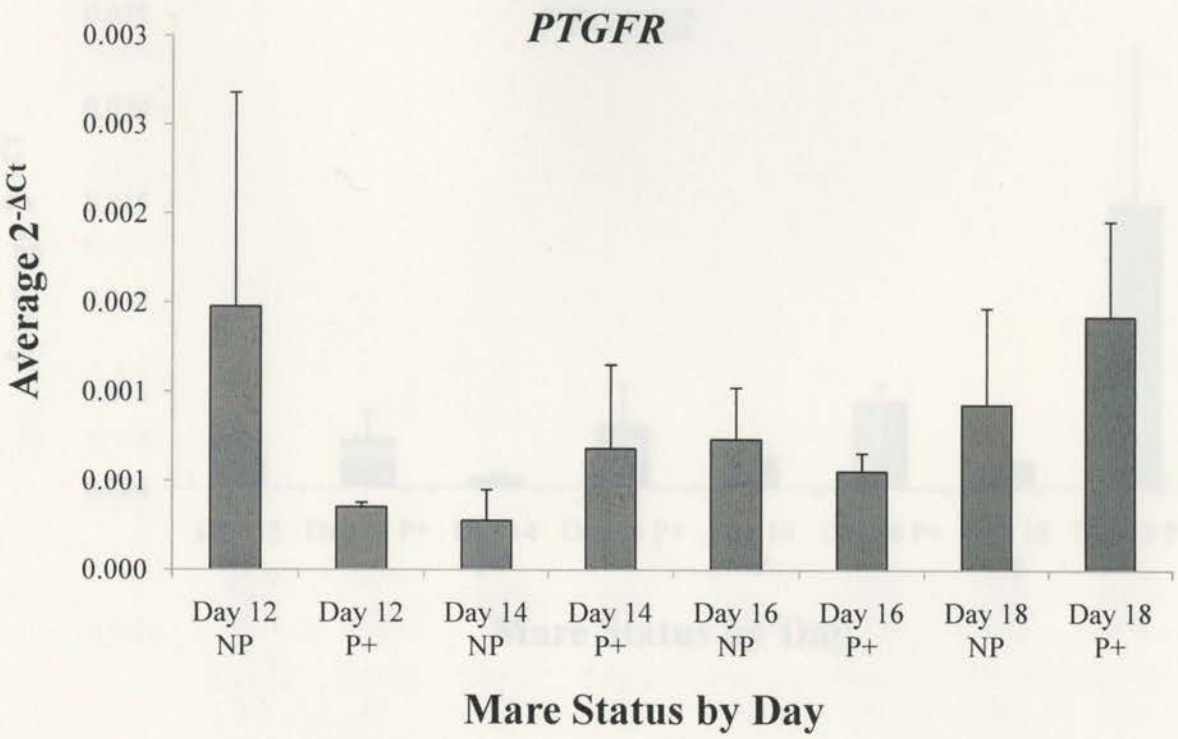


Figure 8) Changes in relative *PTGFR* gene expression ($2^{-\Delta Ct}$) per time point of tissue collection and mare pregnancy status. NP denotes non-pregnant. P+ denotes pregnant. Error bars represent SEM values.

Figure 9

Changes in Relative *PTGER2* Gene Expression by Day and Pregnancy Status

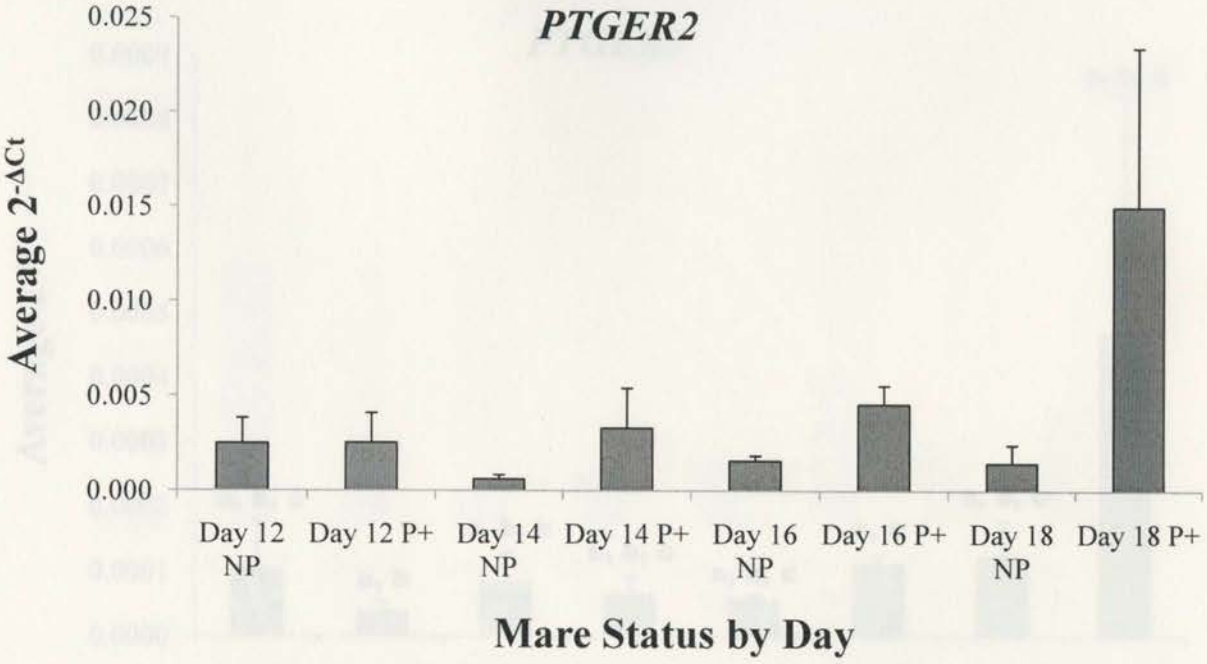


Figure 9) Changes in relative *PTGER2* gene expression ($2^{-\Delta Ct}$) per time point of tissue collection and mare pregnancy status. NP denotes non-pregnant. P+ denotes pregnant. Error bars represent SEM values.

Figure 10

Changes in Relative *PTGER3* Gene Expression by Day and Pregnancy Status

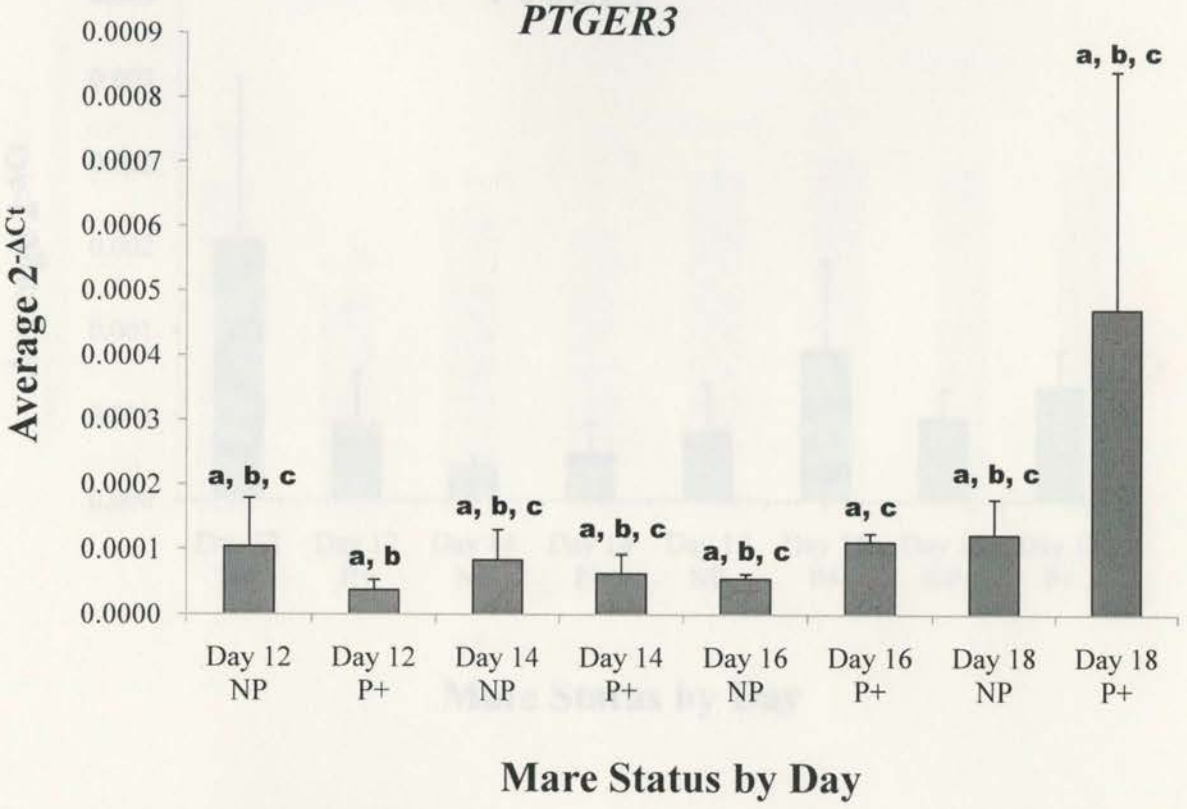


Figure 10) Changes in relative *PTGER3* gene expression ($2^{-\Delta Ct}$) per time point of tissue collection and mare pregnancy status. NP denotes non-pregnant. P+ denotes pregnant. Error bars represent SEM values. Different letters denote statistically significant differences in expression ($p<0.03$)

Figure 11

Changes in Relative *PTGER4* Gene Expression by Day and Pregnancy Status

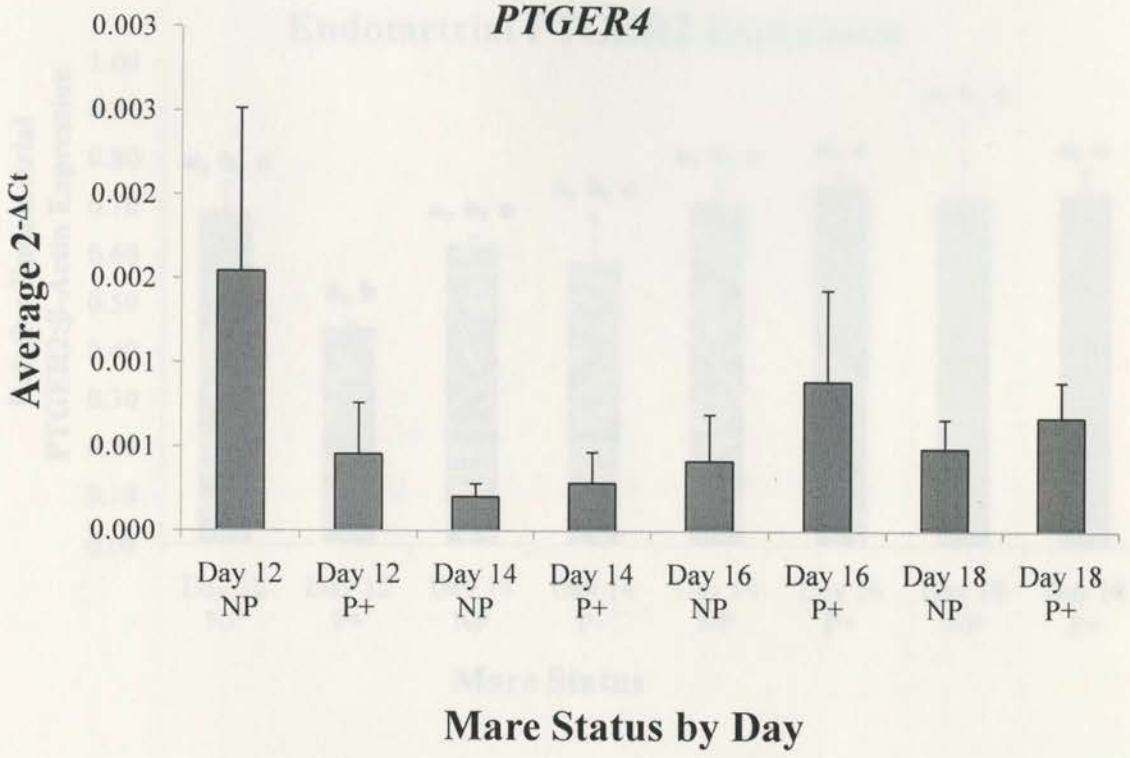


Figure 11) Changes in relative *PTGER4* gene expression ($2^{-\Delta Ct}$) per time point of tissue collection and mare pregnancy status. NP denotes non-pregnant. P+ denotes pregnant. Error bars represent SEM values.

Figure 12

Endometrial PTGER2 Protein Expression

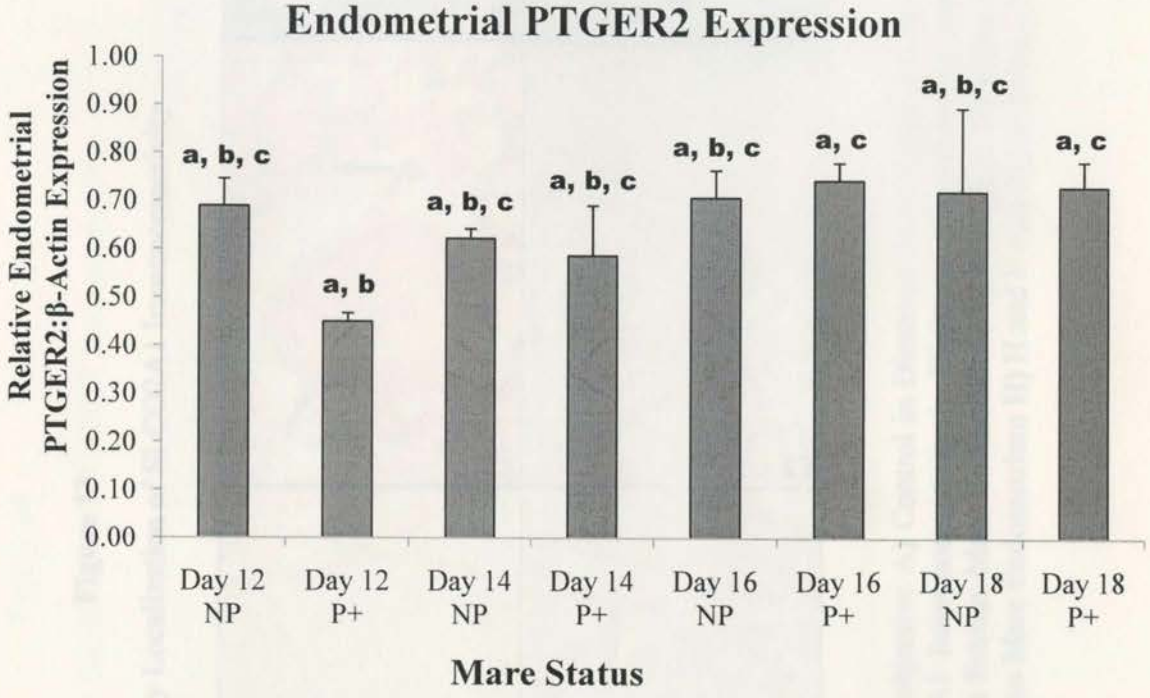


Figure 12) Endometrial PTGER2 protein expression was calculated as a ratio to β-actin expression. Results are the average of three independent Western blots (**Appendix VIII**) with error bars representing SEM values. Different letters denote statistically significant differences in expression ($p<0.05$).

Figure 13

Immunohistochemistry Localization of SLCO2A1 Immunoreactivity

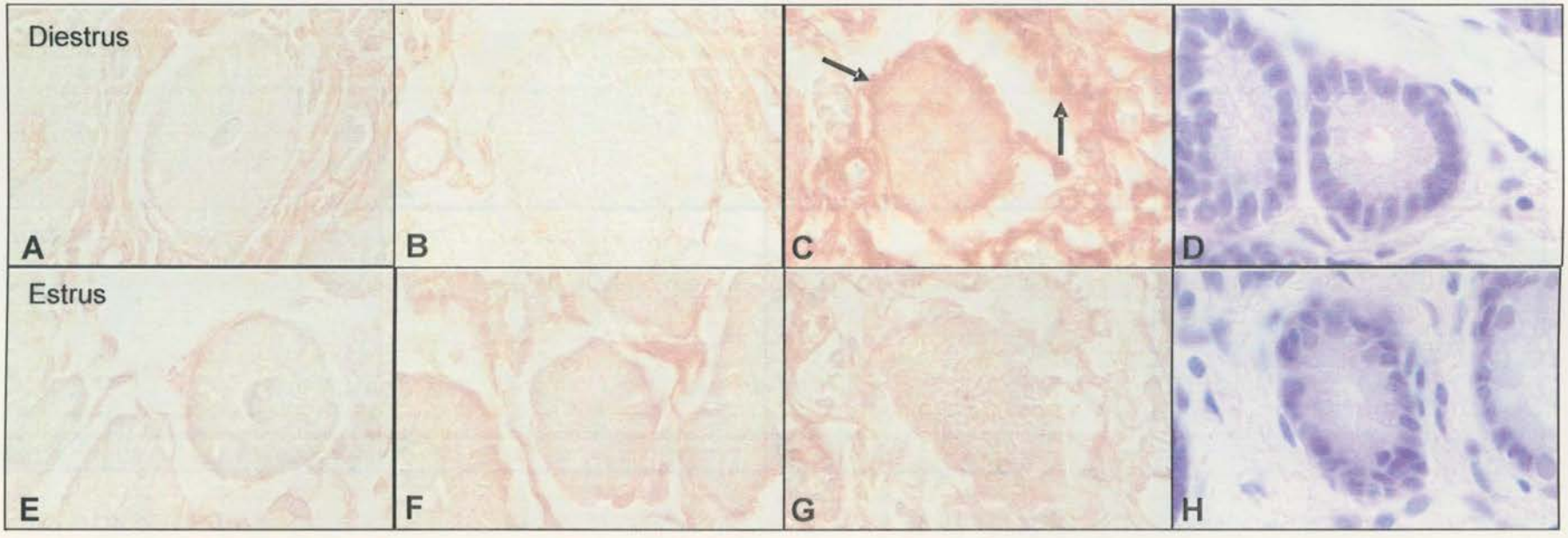


Figure 13. Immunohistochemistry analysis with 60 x objective **A)** Control in Diestrous Mare Endometrium **B)** SLCO2A1 Blocking Peptide in Diestrous Mare Endometrium **C)** SLCO2A1 Immunoreactivity in Diestrous Mare Endometrium (arrows) **D)** H and E staining of Diestrous Mare Endometrium **E)** Control in Estrous Mare Endometrium **F)** SLCO2A1 Blocking Peptide in Estrous Mare Endometrium **G)** SLCO2A1 Immunoreactivity in Estrous Mare Endometrium **H)** H and E staining of Estrous Mare Endometrium

Figure 14

Immunohistochemistry Localization of PTGER2 Immunoreactivity

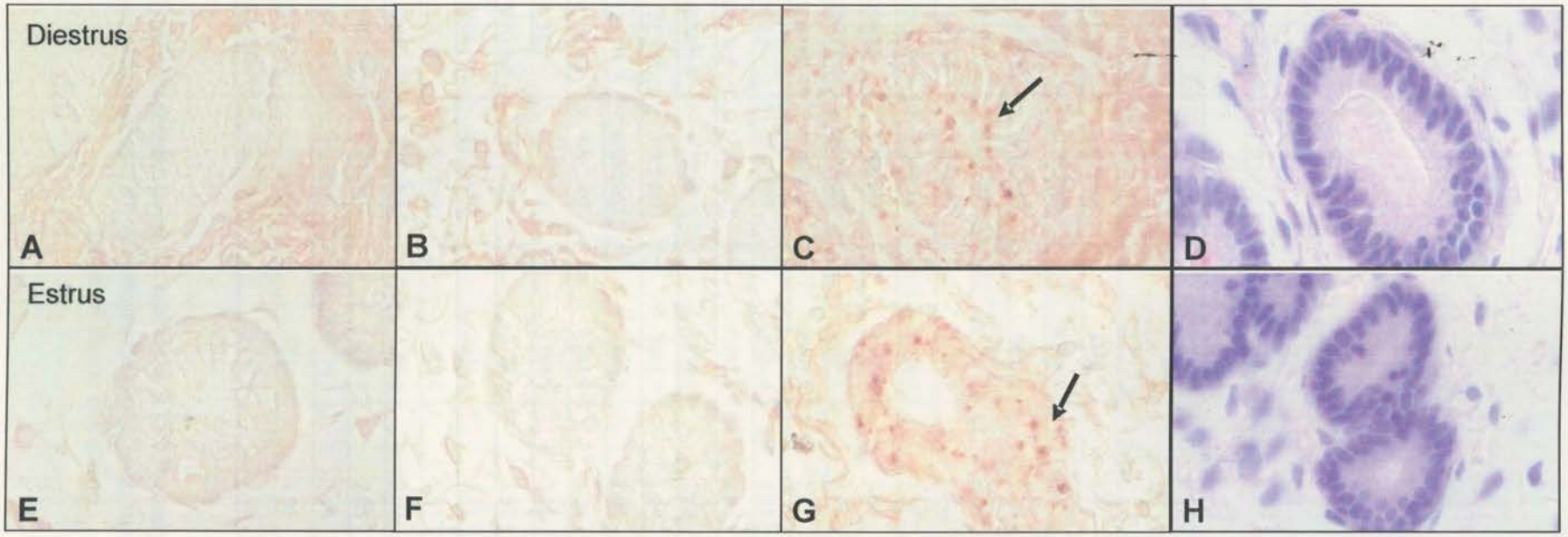


Figure 14. Immunohistochemistry analysis with 60 x objective **A)** Control in Diestrous Mare Endometrium **B)** PTGER2 Blocking Peptide in Diestrous Mare Endometrium **C)** PTGER2 Immunoreactivity in Diestrous Mare Endometrium (arrow) **D)** H and E staining of Diestrous Mare Endometrium **E)** Control in Estrous Mare Endometrium **F)** PTGER2 Blocking Peptide in Estrous Mare Endometrium **G)** PTGER2 Immunoreactivity in Estrous Mare Endometrium (arrow) **H)** H and E staining of Estrous Mare Endometrium

Appendix I

List of Isolated RNA Samples: Concentrations, Quality, and Treatment

Day	Mare ID	Collection Date	Concentration [ng/µl]	260/280	260/230	DNase and Clean Up Treatment
Day 12	Mare 455 NP	7/26/2007	3368.2	1.85	1.99	
	Mare 455 P+	6/19/2007	1258.6	2.11	1.84	Yes
	Mare 2145 NP	7/18/2007	1233.5	2.10	2.09	Yes
	Mare 2145 P+	6/28/2007	521.5	2.10	0.77	Yes
	Mare 7106 NP	7/16/2007	1107.3	2.12	0.99	Yes
	Mare 7106 P+	6/25/2007	2638.9	1.93	1.73	
Day 14	Mare 299 NP	7/24/2007	1535.6	2.07	1.96	Yes
	Mare 299 P+	6/29/2007	1262.1	2.05	2.27	Yes
	Mare 708 NP	5/28/2007	2493.0	1.95	1.68	
	Mare 708 P+	7/9/2007	2604.6	1.93	2.01	
	Mare 887 NP	6/15/2007	281.9	2.04	1.63	Yes
	Mare 887 P+	5/27/2007	2539.8	1.95	2.01	
Day 16	Mare 151 NP	7/3/2007	3003.7	1.89	2.09	
	Mare 151 P+	6/10/2007	2203.3	1.93	2.13	
	Mare 4230 NP	6/9/2007	1881.8	1.95	1.76	
	Mare 4230 P+	6/30/2007	398.6	2.03	1.54	Yes
	Mare 8265 NP	7/11/2007	2322.6	1.92	2.09	
	Mare 8265 P+	6/13/2007	2234.4	1.91	2.10	
Day 18	Mare 513 NP	5/26/2006	989.6	1.97	2.20	
	Mare 513 P+	7/9/2006	228.8	2.07	0.60	Yes
	Mare 2001 NP	6/30/2006	270.0	2.05	0.97	Yes
	Mare 2001 P+	6/2/2006	1539.3	1.99	2.00	
	Mare 8212 NP	6/25/2006	1573.2	1.95	2.19	
	Mare 8212 P+	5/27/2006	1744.8	1.98	1.98	

Appendix I) RNA samples used for real time RTPCR analysis. Mare status is designated as NP for non pregnant and P+ for pregnant. Note: each mare was used for only one time point of tissue collection.

Appendix II

Raw Real Time RTPCR Ct Values:

Raw Ct Values									
Day	Sample	*SLCO2A1*	*ABCC4*	*PTGFR*	*PTGER2*	*PTGER3*	*PTGER4*	*GAPDH*	*TUBULIN*
Day 12	Mare 455 NP	24.85	26.48	29.88	26.94	29.13	27	27.43	17.18
		23.81	25.61	29.07	24.14	28.45	26.61	35.7	
	Mare 455 P+	28.72	28.09	29.02	27.84	32.66	31.1	33.13	17.61
		27.47	27.03	27.02	27.23	32.75	30.57	34.81	
	Mare 2145 NP	24.57	24.42	26.71	26.29	33.08	26.9	30.31	18.7
		21.78	22.71	25.34	23.24	32.72	26.85	35.93	35.04
	Mare 2145 P+	26.25	27.48	28.99	24.77	31.13	27.19	29.47	17.31
		24.57	26.48	25.6	26.25	31.23	26.72	35.51	34.89
	Mare 7106 NP	30.35	27.3	29.2	27.66	34.2	30.89	34.21	17.94
		28.62	26.48	26.74	26.17	33.67	30.22	35.51	
	Mare 7106 P+	23.9	25.41	28.78	27.25	33.4	29.61	25.04	17.45
		21.33	24.23		27.03	32.75		34.82	
Day 14	Mare 299 NP	27.59	24.32	26.15	25.73	29.17	27.03	33.54	15.49
		26.8	24.28	24.73	23.95	28.82	26.67	33.08	
	Mare 299 P+	25.89	23.73	25.81	23.6	29.52	27.13	32.52	16.52
		24.6	23.57	24.56	21.82	26.71	26.89	35.93	35
	Mare 708 NP	29.46	28.09	31.91	30.34	30.42	31.66	35.27	17.88
		28.42	27.94	29.99	27.88	30.14	31.94	34.58	
	Mare 708 P+	30.59	30.18	33.86	30.23	33.95	34.13	34.88	18.89
		29.88	29.55	30.87	28.96	32.88	33.63	34.96	40
	Mare 887 NP	30.59	29.54	32.19	29.69	36.41	31.85	35.91	19.56
		28.84	29.43	29.97	27.82	35.51	30.65	35.5	40
	Mare 887 P+	26.13	24.95	26.79	24.42	30.16	27.97	32.75	15.57
		24.21	24.28	24.13	21.57	28.3	26.98	34.56	36.06
Day 16	Mare 151 NP	29.72	25.04	27.15	26.12	31.17	29.67	34.16	16.65
		27.73	25.17	25.12	26.15	30.25	28.97	35.19	
	Mare 151 P+	24.33	24.81	27.64	24.27	30.3	25.98	32.09	17
		22.55	24.58	26.2	21.68	28.54	25.52	34.94	36.03
	Mare 4230 NP	24.83	25.14	28.55	25.48	30.46	26.62	31.52	16.61
		23.22	24.71	25.62	23.56	29.17	25.76	30.52	37.59

	Mare 4230 P+	26.09	26.48	29.26	26.96	32.02	29.41	33.51	17.77
		24.96	26.05	28.22	24.21	29.24	28.3	30.31	16.82
	Mare 8265 NP	25.47	25.77	25.54	26.48	30.02	27.74	32.29	15.06
		23.69	24.78	23.84	22.9	28.27	27.62	35.21	15.05
	Mare 8625 P+	25.77	25.33	27.45	25.73	29.72	27.49	32.7	16.25
		23.99	23.9	25.88	22.48	28.22	27.71	34.29	16.05
Day 18	**Mare 513 NP**	25.98	25.54	26.25	26.09	29.09	26.5	29.22	16.18
		23.82	24.09	23.86	22.51	27.32	26.23	35.22	16.02
	Mare 513 P+	25.81	26.23	28.57	25.9	31.15	28.1	32.42	17.72
		23.13	25.12	27.13	22.69	29.57	28.09	34.66	17.52
	Mare 2001 NP	28.85	27.24	29.3	29.17	32.45	28.94	31.52	17.83
		26.95	26.75	28.06	26.16	30.96	28.86	34.43	35.32
	Mare 2001 P+	27.84	26.57	27.57	26.43	31.55	28.81	29.73	17.18
		26.24	25.45	26.54	23.74	30.65	28.65	34.56	16.85
	Mare 8212 NP	26.45	26.79	28.07	27.73	29.84	27.72	34.76	15.51
		24.9	25.5	25.78	24.83	28.11	28.12	30.98	15.91
	Mare 8212 P+	23.68	25.17	27.66	24.44	27.63		33.72	16.57
		22.05	23.94	25.14	21.48	26.18	26.41	26.53	16.49

Appendix II) Mare status is designated as NP for non-pregnant and P+ for pregnant.

Appendix III

Real Time RTPCR $2^{-\Delta Ct}$ Values:

Average 2-ΔCt Values ± SEM						
	SLCO2A1	*ABCC4*	*PTGFR*	*PTGER2*	*PTGER3*	*PTGER4*
Day 12 NP	0.00740 ± 0.00356	0.00736 ± 0.00411	0.00148 ± 0.00085	0.00251 ± 0.00095	0.00010 ± 0.00005	0.00154 ± 0.00069
Day 12 P+	0.00464 ± 0.00242	0.00186 ± 0.00076	0.00035 ± 0.00002	0.00255 ± 0.00111	0.00004 ± 0.00001	0.00046 ± 0.00022
Day 14 NP	0.00034 ± 0.00005	0.00134 ± 0.00030	0.00028 ± 0.00012	0.00063 ± 0.00016	0.00008 ± 0.00003	0.00020 ± 0.00005
Day 14 P+	0.00083 ± 0.00025	0.00289 ± 0.00139	0.00068 ± 0.00033	0.00331 ± 0.00149	0.00006 ± 0.00002	0.00028 ± 0.00013
Day 16 NP	0.00161 ± 0.00067	0.00218 ± 0.00048	0.00073 ± 0.00021	0.00160 ± 0.00019	0.00006 ± 0.00001	0.00042 ± 0.00020
Day 16 P+	0.00397 ± 0.00082	0.00309 ± 0.00051	0.00056 ± 0.00007	0.00457 ± 0.00070	0.00011 ± 0.00001	0.00089 ± 0.00039
Day 18 NP	0.00124 ± 0.00037	0.00152 ± 0.00034	0.00093 ± 0.00038	0.00148 ± 0.00068	0.00012 ± 0.00004	0.00049 ± 0.00012
Day 18 P+	0.01028 ± 0.00417	0.00381 ± 0.00077	0.00142 ± 0.00038	0.01499 ± 0.00596	0.00047 ± 0.00003	0.00068 ± 0.00015

Appendix III) $2^{-\Delta Ct}$ values for each group of mares per time point and status. Values given as mean ± SEM. NP designates non-pregnant mare status; P+ designates pregnant mare status. (Data normalized to tubulin)

Appendix IV

Real Time RTPCR Fold Changes ($2^{-\Delta\Delta Ct}$)

Fold Changes for Genes Higher Expressed in Non-Pregnant Mare Endometrium						
	SLCO2A1	*ABCC4*	*PTGFR*	*PTGER2*	*PTGER3*	*PTGER4*
Day 12	1.14	2.66	1.76	1.10	1.67	2.87
Day 14	0.49	0.77	0.65	0.28	0.09	1.16
Day 16	0.22	0.65	1.13	0.36	0.50	0.43
Day 18	0.18	0.39	0.53	0.09	0.45	0.71

Appendix IV.I) Fold changes ($2^{-\Delta\Delta Ct}$) for genes up-regulated in the non-pregnant mare endometrium

Fold Changes for Genes Higher Expressed in Pregnant Mare Endometrium						
	SLCO2A1	*ABCC4*	*PTGFR*	*PTGER2*	*PTGER3*	*PTGER4*
Day 12	0.88	0.38	0.57	0.91	0.60	0.35
Day 14	2.04	1.30	1.53	3.61	1.10	0.86
Day 16	4.52	1.55	0.89	2.80	2.00	2.33
Day 18	4.46	2.56	1.87	10.99	2.23	1.40

Appendix IV.II) Fold changes ($2^{-\Delta\Delta Ct}$) for genes up-regulated in the pregnant mare endometrium.

Appendix V

Standard Curve Efficiency Values for Primer Sets

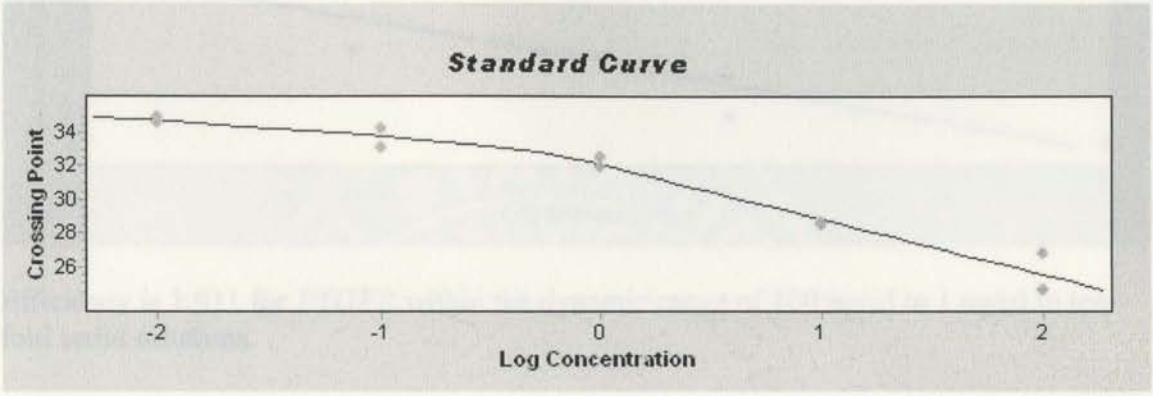
Standard Curve Analysis of Primers	
Gene	**Efficiency**
SLCO2A1	2.475
ABCC4	2.837
PTGFR	2.171
PTGER2	2.599
PTGER3	2.356
PTGER4	3.242
*GAPDH**	9.014
TUBULIN	2.016

Appendix V) Standard curve analysis was performed from RNA dilutions and analyzed on Light Cycler 480. Efficiency was calculated as $10^{(-1/\text{slope})}$, with values of 2 equal to 100% PCR efficiency. *GAPDH was not use for an internal control due to poor efficiency.

Appendix VI

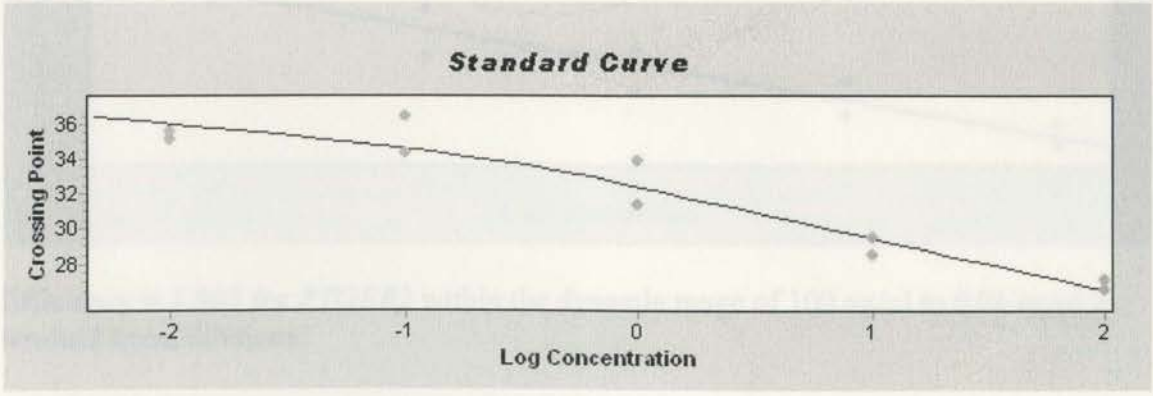
Dynamic Range of Standard Curves

A) *SLCO2A1*



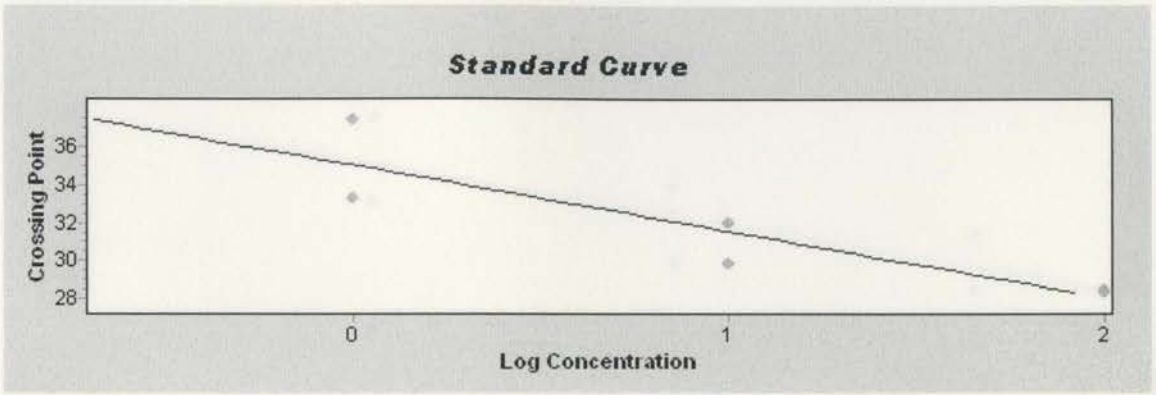
Efficiency is 2.017 for *SLCO2A1* within the dynamic range of 100 ng/µl to 0.01 ng/µl in ten-fold serial dilutions.

B) *ABCC4*



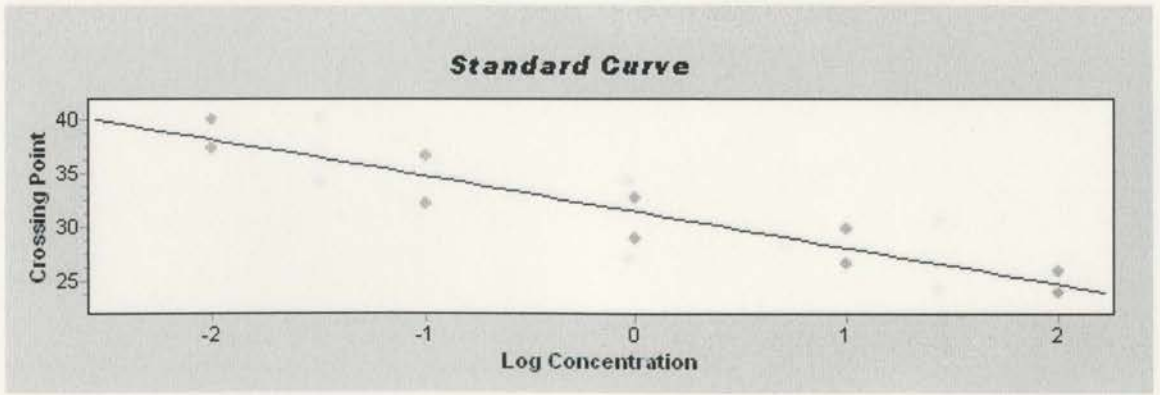
Efficiency is 2.174 for *ABCC4* within the dynamic range of 100 ng/µl to 0.01 ng/µl in ten-fold serial dilutions.

C) *PTGFR*



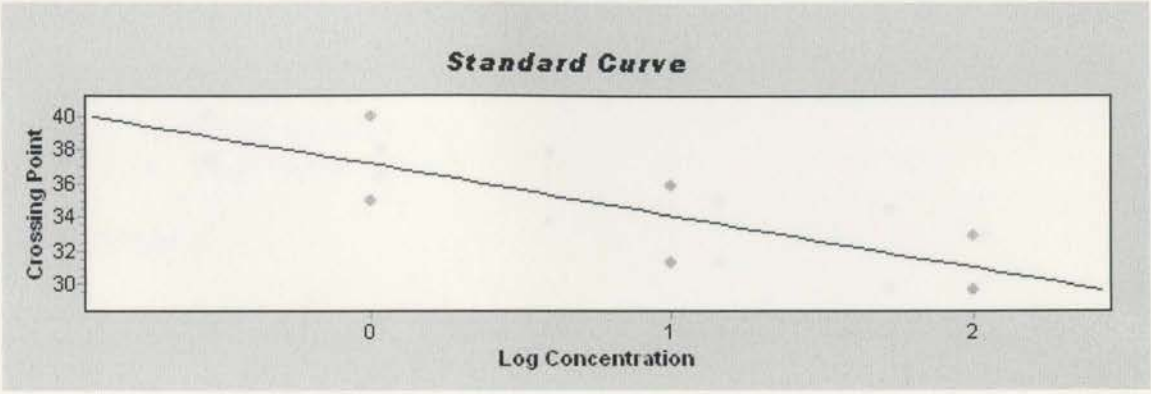
Efficiency is 1.931 for *PTGFR* within the dynamic range of 100 ng/µl to 1 ng/µl in ten-fold serial dilutions.

D) *PTGER2*



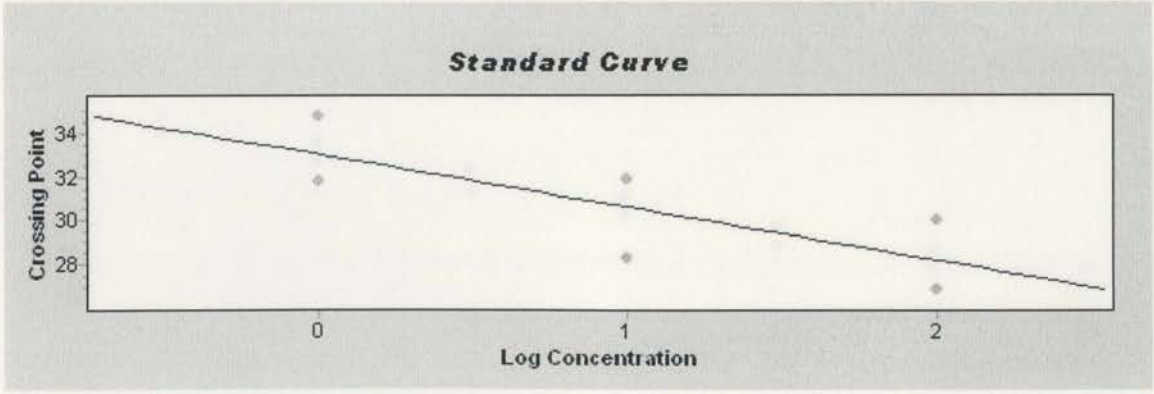
Efficiency is 1.983 for *PTGER2* within the dynamic range of 100 ng/µl to 0.01 ng/µl in ten-fold serial dilutions.

E) *PTGER3*



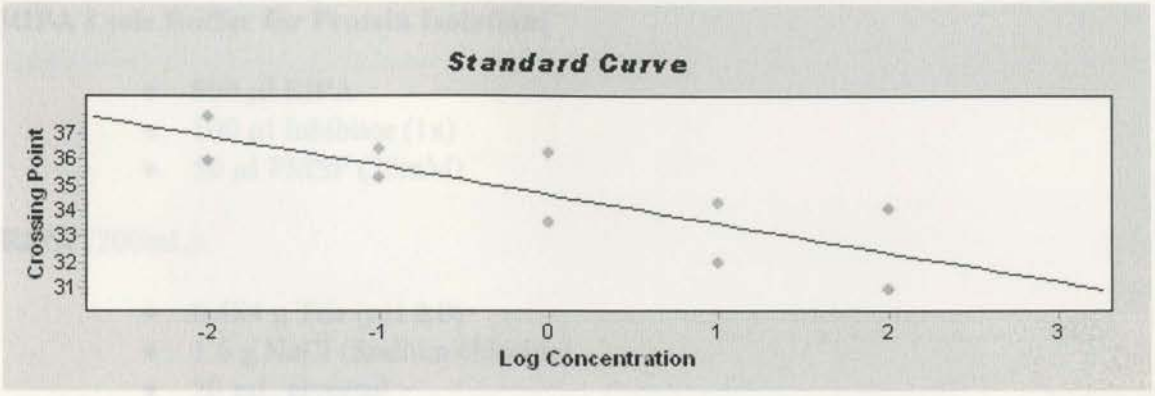
Efficiency is 2.112 for *PTGER3* within the dynamic range of 100 ng/µl to 1 ng/µl in ten-fold serial dilutions.

F) *PTGER4*



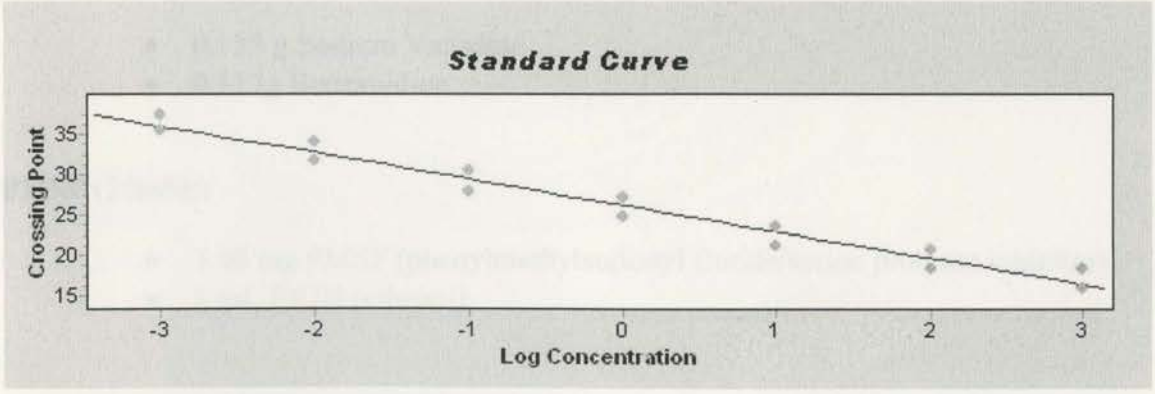
Efficiency is 2.557 for *PTGER4* within the dynamic range of 100 ng/µl to 1 ng/µl in ten-fold serial dilutions.

G) *GAPDH*



Efficiency is 7.629 for *GAPDH* within the dynamic range of 100 ng/µl to 0.01 ng/µl in ten-fold serial dilutions.

H) *TUBULIN*



Efficiency is 2.016 for *Tubulin* within the dynamic range of 100 ng/µl to 0.01 ng/µl in ten-fold serial dilutions.

Appendix VII

RIPA Lysis Buffer

RIPA Lysis Buffer for Protein Isolation:

- 900 µl RIPA
- 100 µl Inhibitor (1x)
- 50 µl PMSF (20mM)

RIPA (200mL)**:**

- 0.484 g Tris (pH 8.0)
- 1.6 g NaCl (Sodium chloride)
- 20 mL glycerol
- 2 mL NP-40 (Nonidet P-40)
- 0.2 g SDS (sodium dodecyl sulfate)
- 1.0 g deoxychlorate (deoxycholic)
- 0.117 g EDTA (ethylenediamine tetraacetic acid)
- Lower to pH 8 with HCl

Inhibitor (15 mL of 10x)**:**

- 0.138 g Sodium Vanadate
- 0.117g Benzanidine

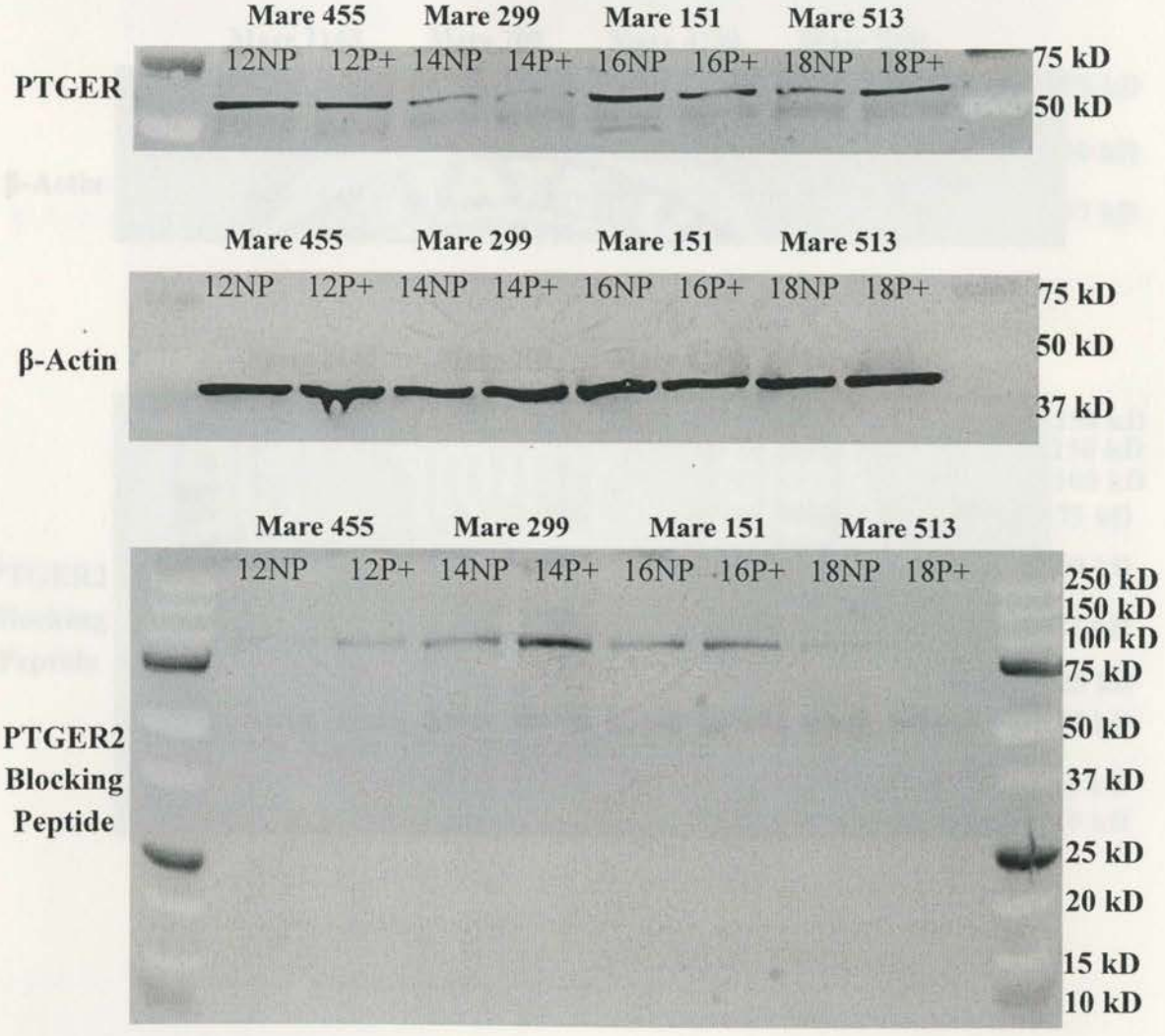
PMSF (20mM)**:**

- 3.48 mg PMSF (phenylmethylsulfonyl fluride/serine protease inhibitor)
- 1 mL EtOH (ethanol)

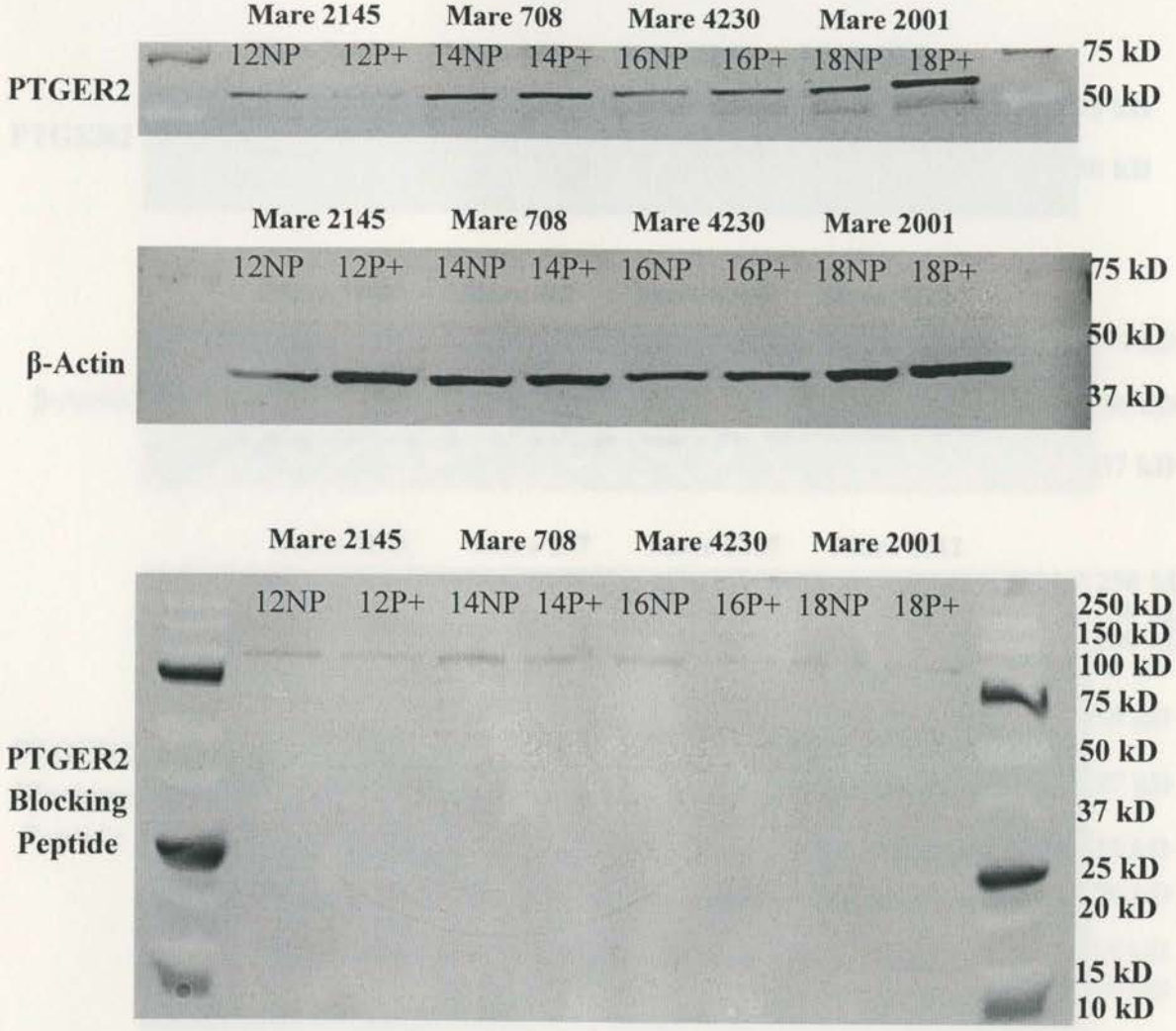
Appendix VIII

Western Blot Results for PTGER2 and β-Actin Expression

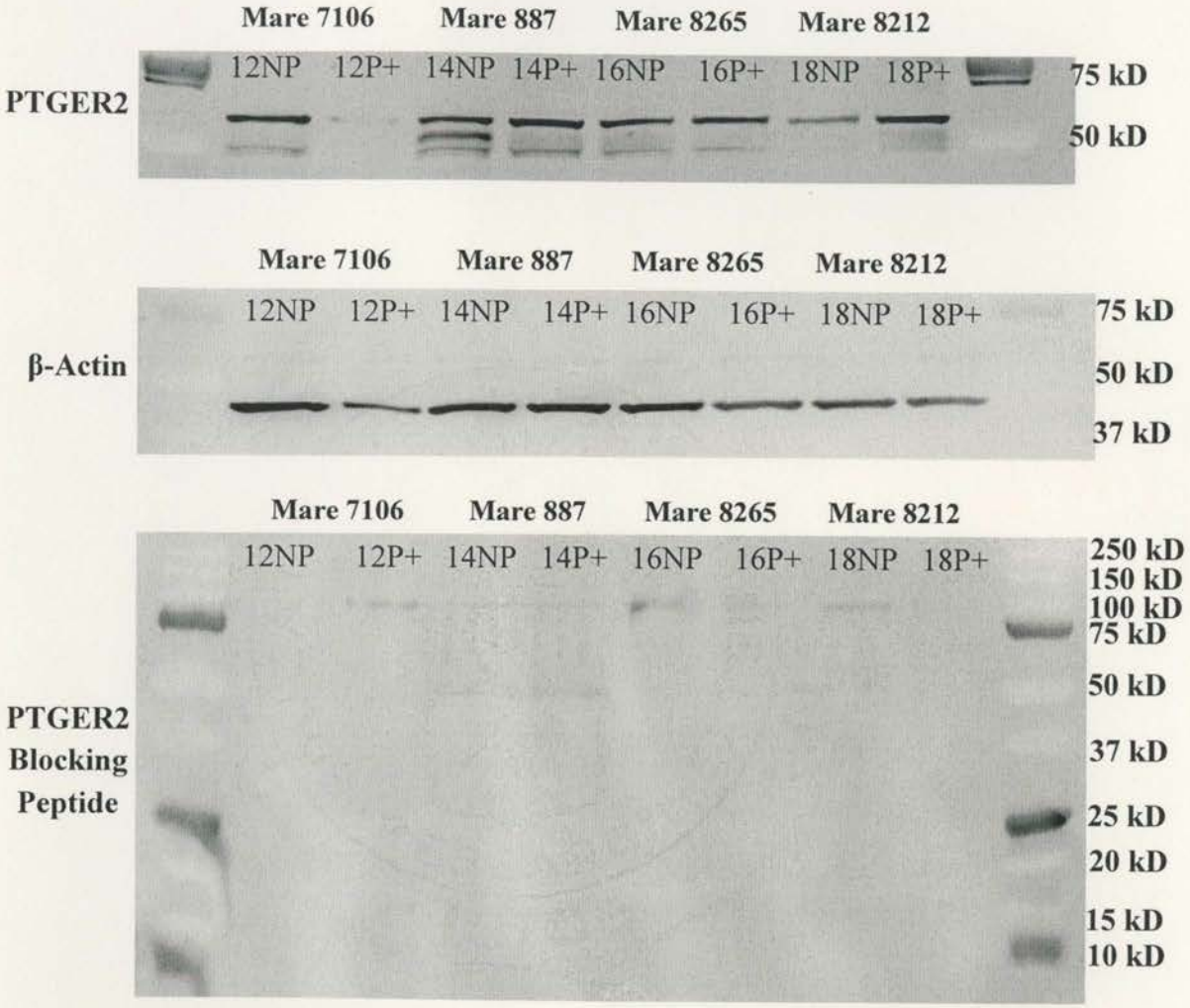
Appendix VIII.I: Group 1 Mare Samples



Appendix VIII.II: Group 2 Mare Samples



Appendix VIII.III: Group 3 Mare Samples



Appendix VIII) Western blot results for PTGER2 with mares randomly assigned to one of three groups so each blot shows non-pregnant and pregnant PTGER2 expression at all time points. PTGER2 specific banding is seen at 52 kD, and β-actin banding is seen at 43 kD. Western blot results for PTGER2 blocking peptide shows non-specific banding at approximately 100 kD. NP designates non-pregnant mare samples; P+ designates pregnant mare samples.