AN ABSTRACT OF THE THESIS OF

Jenny A. Strooband for the degree of Master of Science in Animal Science presented on August 29, 2002. Title: The Biologic Activity of 5α-Reduced Pregnanes in the Late Gestation Mare.

Abstract approved: Redacted for privacy

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Mares have an atypical hormone profile during pregnancy. Systemic progesterone (P4) levels approach zero by day 220 of gestation. Other reduced pregnanes such as 5α-pregnane-3, 20-dione (5α), 5α-pregnane-3β, 20α-diol (βα), 3β-hydroxy-5α-pregnan-3-one (3β) and 20α-hydroxy-5α-prenan-3-one (20α), increase to near μg/mL levels in the peripheral system of the mare until directly before parturition when they decrease. This unusual hormone profile during gestation indicates the possibility that other pregnanes, not P4, are responsible for uterine quiescence and gonadotropin inhibition during pregnancy. Three experiments were conducted to determine if these steroids have biologic activity. Experiment 1 consisted of jugular vein blood samples taken from mares from ten days pre-partum until the foal heat ovulation, approximately 15 days postpartum. Samples were analyzed for luteinizing hormone (LH), follicle stimulating hormone (FSH), and pregnane content. Concentrations of these hormones were analyzed for

serial correlations. There was a serial negative correlation with pregnanes and FSH ($p= 0.0138$), which were analyzed on a same day basis, day -5 to day of foaling. There also was a positive correlation with pregnanes and FSH analyzed from day of foaling to 10 days post-foaling ($p<0.001$). There was also a significant negative correlation ($p=0.0196$) between pregnanes and LH, analyzed on a lag basis, day -5 to day of foaling for pregnanes, and day -5 to day of ovulation for LH. There was also a significant negative correlation when pregnanes were analyzed from day of foaling to 10 days post foaling, and LH was analyzed from 10 days before ovulation to day of ovulation ($p=0.004$). Maximum pre-partum pregnane levels did not affect time to ovulation ($p= 0.34$). In experiment 2 equine anterior pituitary glands were harvested and the cells plated to begin a primary cell culture. After attachment, the cells were divided into treatment groups: P4, 5α, $\beta\alpha$, 20α, 3β or a control and each group subjected to a 1.0nM Gonadotropin Hormone Releasing Hormone (GnRH) challenge. Subsequently cells and medium were collected and analyzed for LH and FSH content using radioimmunoassay (RIA). The cells did exhibit a response to GnRH ($p=0.015$ between positive and negative controls) and there was a treatment effect for FSH ($p=0.0058$); only 3β resulted in significantly more FSH release than the positive control ($p=0.043$) after stimulation with GnRH. There was no treatment effect on LH ($p=0.56$). Experiment 3 analyzed the response of equine uterine myometrial tissue to pregnane treatment. Myometrial tissue was harvested and placed in a 37°C Krebs buffered saline bath, connected to a physiograph and repetitive spontaneous smooth muscle contraction was induced

with oxytocin. Tissue was then treated with P4, 5α, βα, 20α, 3β or a control (ethanol). The amplitude and frequency of the spontaneous contractions were measured and compared to the control. There were no differences between post treatment responses of the control and pregnane treated samples in either frequency ($p=0.78$) or amplitude ($p=0.63$) of myometrial contractions. From these data we conclude that *in vivo* there is a significant and differential physiologic relationship between pre-partum pregnanes and gonadotropins. Due to lack of response it is unlikely that pregnanes inhibit pituitary secretion, and thus may exert their effects elsewhere, such as at the hypothalamus. The involvement of pregnanes in modulating myometrial contractions remains unclear. It is likely that P4 does play a role in decreasing myometrial responses to OT, however, that result was inconsistent in this study.

The Biologic Activity of 5α-Reduced Pregnanes in the Late Gestation Mare

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Completed August 29, 2002
Commencement June, 2003

Master of Science thesis of Jenny A. Strooband presented on 29 August, 2002.

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Jenny A. Strooband, Author

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CHAPTER 1: DEFFINITION OF PROBLEM

Abortion occurs in a minimum of 5% of mares diagnosed as pregnant after breeding (Ginther, 1992). Early embryonic losses as well as late gestation abortion can lead to significant financial losses. A common method of abortion prevention involves treating a "problem" mare with Regumate®, a synthetic progestin treatment. Unfortunately, there is very little evidence supporting the benefits of this treatment. A portion of the confusion regarding this issue is the result of mares having a unique hormone profile during pregnancy. While most mammals have high levels of systemic 4-pregnene-3,20-dione, better known as progesterone (P4) throughout pregnancy, the concentration of P4 in the mare is basal by the end of the 2nd trimester of the pregnancy. Instead, levels of 5α-reduced pregnanes (Table 1.1), metabolites of P4, increase gradually beginning early gestation, and peak shortly before parturition.

Table 1.1: List of Abbreviations

Systemic Name	Abbreviation
3β-hydroxy-5-pregnen-20-one	P5
5-pregnen-3β,20β-diol	P5-$\beta\beta$
4-pregnen-3,20-dione	P4
5α-pregnane-3,20-dione	5α
3β-hydroxy-5α-pregnan-3-one	3β
20α-hydroxy-5α-pregnan-3-one	20α
5α-pregnane-3β,20β-diol	$\beta\beta$
5α-pregnane-3β,20α-diol	$\beta\alpha$
Oxytocin	OT
Estradiol-17β	E2
Estrone	E1
Estrone sulfate	E1SO_4

The biologic activity of these pregnanes remains unclear. However, because they appear in significant concentrations when the level of P4 is decreasing in the blood, coupled with the fact that many of them have been shown to interact with the P4 receptor, it is logical to assume that these progestins work individually, or in concert, to perform the mandatory pregnancy maintenance duties of P4 throughout gestation. This research set out to discover if the 5α-reduced pregnanes effect the secretion of gonadtropins from the anterior pituitary gland *in vivo* or *in vitro*, or modulate myometrial contractions *in vitro*.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Introduction

Mares have a unique progesterone profile during pregnancy. In most mammals, P4 is responsible for pregnancy maintenance and is found in high levels in the systemic circulation and the feto-placental unit throughout gestation. Early progesterone analysis in the pregnant mare demonstrated that P4 was just one of several progestins present in the mare during pregnancy (Holtan et al., 1975). Other progestins present are the reduced P4 metabolites which begin to appear in the peripheral blood of the mare at approximately day 50 of gestation. This coincides with the formation of the placenta. Further analysis demonstrates that P4 is undetectable in the mare jugular and uterine vein by day 220 of gestation (Holtan et al., 1979 and 1991). The lack of systemic P4 in the late gestation mare is atypical compared to other mammalian species. The prominent role of P4 in the maintenance of pregnancy in most mammals suggests that the mare has an unusual endocrinological pathway during the last trimester of gestation.

2.2 *The Origin and Function of Progesterone during Pregnancy in the Mare*

Progesterone has two distinct origins during pregnancy in the mare. The original source of P4 during approximately the first 80 days of pregnancy and possibly longer is luteal, that is, it is derived from the corpus luteum (CL). In the mare, equine chorionic gonadotropin (eCG) from the endometrial cups stimulates P4 biosynthesis in the luteal cells of the CL during the first stage of pregnancy, approximately days 35 to 80 (Roser et al, 1982). This serves to maintain high levels of luteal P4 in the early gestation mare. The CL begins to regress during mid-gestation as the feto-placental unit begins generating large amounts of 5α-reduced pregnanes, metabolites of P4 (Holtan et al,, 1991). The feto-placental unit, which begins to secrete low levels of pregnanes as early as day 30, completely takes over pregnane production by day 100.

Progesterone is important in pregnancy maintenance in nearly all mammals because it has secretory, quiescent and immunosuppressant effects on uterine tissue. In most mammals, progesterone increases secretory activity of glands in the uterine endometrium. During the estrous cycle the influence of estrogen on uterine tissue causes a proliferation of endometrial stromal gland concentration. The presence of luteal P4 results in the biosynthesis and release of

glycogen from the endometrial glands. This creates a nutrient rich environment in the uterus important for embryo implantation and fetal development. Progesterone also has been shown to cause uterine quiescence, which prevents uterine contractions and fetal expulsion. Progesterone promotes uterine quiescence through four distinct mechanisms; hyperpolarization of myometrial tissue, decrease in electrical coupling between muscle cells, reduction in the formation of gap-junctions and voltage gated calcium channels, and a decrease in α-adrenergic receptor concentration as reviewed by Griffin and Ojeda (2000). Another important function of P4 during pregnancy is suppression of the immune response of the animal. Progesterone has the ability to inhibit T-lymphocyte cell mediated responses (Griffin and Ojeda, 2000). Therefore, high levels of P4 are able to block a local immune response to foreign antigens. This prevents the immune system from rejecting the fetus as a foreign body.

Progesterone also is thought to have a significant down-regulating effect on the release of gonadotropins from the anterior pituitary gland in most mammals. It is thought to do this by decreasing the number of gonadotropin hormone releasing hormone (GnRH) receptors present in the anterior pituitary gland, as well as decreasing GnRH pulses from the hypothalamus. Garcia and Ginther (1975) demonstrated that progesterone treated mares had decreased levels of GnRH pulses versus control mares.

2.3 5-α Reduced Pregnanes

Research evaluating the effects of ovariectomy in pregnant mares has helped elucidate the mechanism of the role of P4 in maintaining pregnancy in the mare. Ovariectomizing mares during pregnancy obviously removes any CL derived P4 production. Holtan et al (1979) demonstrated that removal of the ovaries before day 50 of pregnancy resulted in abortion in 100% of mares. Pregnancy was maintained in 11 of 20 mares when the ovaries were removed between day 50 and 70. Mares ovariectomized on days 140 or 210 maintained the pregnancy 100% of the time (n=12). These data suggest that the feto-placental unit is beginning to take over progestin production and hence, pregnancy maintenance, between day 50 and 70 of pregnancy. This coincides with placental formation, indicating it has a role as a prominent endocrine organ in the mare.

The separation and identification of several progesterone metabolites became possible with the use of gas chromatography/mass spectrometry (GC/MS). The concentration of P4 in the mare increases from day 13 to approximately day 130, where it then decreases to baseline levels by approximately day 220. There is a small P4 spike immediately before parturition.

5α-Pregnane-3, 20-dione (5α), 3β-hydroxy-5α-pregan-20-one (3β), and 5α-pregnane-3β, 20β-diol (ββ) are present in increasing concentrations (5 to 35 ng/mL) from approximately day 13 to day 300 of gestation (Figure 2.1). 20α-Hydroxy-5α-pregnan-3-one (20α) and 5α-pregnane-3β, 20α-diol (βα) appear at approximately day 80, and increase to approximately 200 ng/mL, until they drop slightly immediately before parturition (Holtan et al., 1991). It is speculated that in the absence of P4 these progestins could then act locally and systemically to maintain pregnancy and participate in the parturition cascade (Schutzer et al., 1996; Schutzer and Holtan, 1996).

Schutzer et al. (1996) demonstrated that the 3β-hydroxysteroid dehydrogenase (3β-HSD) inhibitor trilostane altered steroidogenesis in the pregnant mare. 3β-Hydroxysteroid dehydrogenase is the enzyme that converts pregnenolone (P5) to P4. This is an essential enzyme for steroidogenesis. These authors found that trilostane inhibited the conversion of P5 to P4 in placental tissue *in vitro* while mediating an increase in the concentration of 5α and 3β. These data suggest that, "the pregnant mare possesses unique steroid hormone metabolic activity and suggests that another steroid, perhaps 5α, and not P4, is the important steroid precursor for the other progestin metabolites found in circulating plasma (Schutzer et al. 1996)."

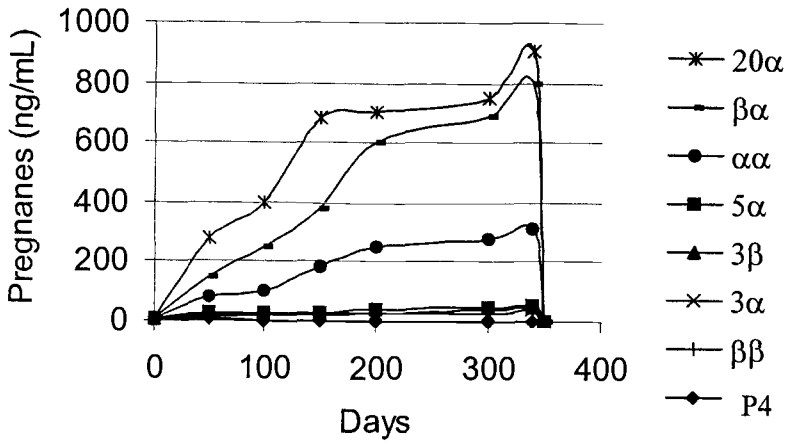

Figure 2.1: Schematic of concentrations of pregnanes throughout gestation in the mare. Adapted from Holtan et al. (1991).

2.4 Progesterone Metabolism

Although the systemic concentration of progestins increases gradually in early gestation and then begins to increase more rapidly at approximately day 300 of gestation, the origin and metabolism of these progestins is still somewhat speculative. Substantial work by Holtan et al. (1991) and Houghton et al. (1991) demonstrates that most likely they are products of both the fetus and the mother. Higher concentrations of P5 are found in the fetal artery than the fetal vein. This suggests there is a net secretion of P5 by the fetus to the uteroplacental tissues and that cholesterol side chain cleavage complex is present in the fetus (Holtan et al. 1991; Chavatte et al. 1997). Cholesterol side chain cleavage complex serves to hydroxylate cholesterol and subsequently cleave the cholesterol molecule between carbon 20 and 22. In this pathway the rate-limiting step in cholesterol synthesis

occurs by the conversion of 3β-hydroxy 3-methylglutaryl CoA (HMG CoA) to mevalonic acid by the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase).

More recent data has also suggested that cytochrome P_{450} is expressed in the fetal gonads and the equine placenta (Rogerson et al. 1993; Han et al. 1995). Schutzer et al (1996) also found that the fetal gonads produce moderate amounts of pregnenolone (P5). Cytochrome P_{450} is a mixed function oxygenase, which catalyzes steroid hydroxylations. Therefore, because fetal gonadectomy does not influence maternal progestin levels, it is thought that fetal gonads are not involved in pregnane production. However, it is very likely that the fetus (adrenal gland, and liver), as well as the placenta itself are responsible for the sustained maternal plasma concentrations of progestins (Pashen and Allen, 1979).

In general, it is believed that some progestins are maternally derived, and others originate in the fetus. This is highly dependent on the stage of pregnancy. Holtan et al. (1991) demonstrated that 5α and 3β may be related to corpus luteum function because they closely mimic the pattern of P4 secretion during early pregnancy (Figure 2.1). In 1996, Schutzer and Holtan speculated that P5 is converted to P4 in both the placenta and the maternal and fetal adrenal glands. They also proposed that P5 was 5α-reduced to 3β in the endometrium. This was further oxidized to 5α in the placenta. Placental derived P4 was also thought to be metabolized to 5α in the placenta and endometrium. The 5α could then be

further β-reduced at C-3 in the fetal unit (Schutzer and Holtan, 1996). In conclusion, the proposed metabolic pathway for these progestins is that P5 is 5α-reduced to 3β, and 3β is oxidized to 5α (Figure 2.2).

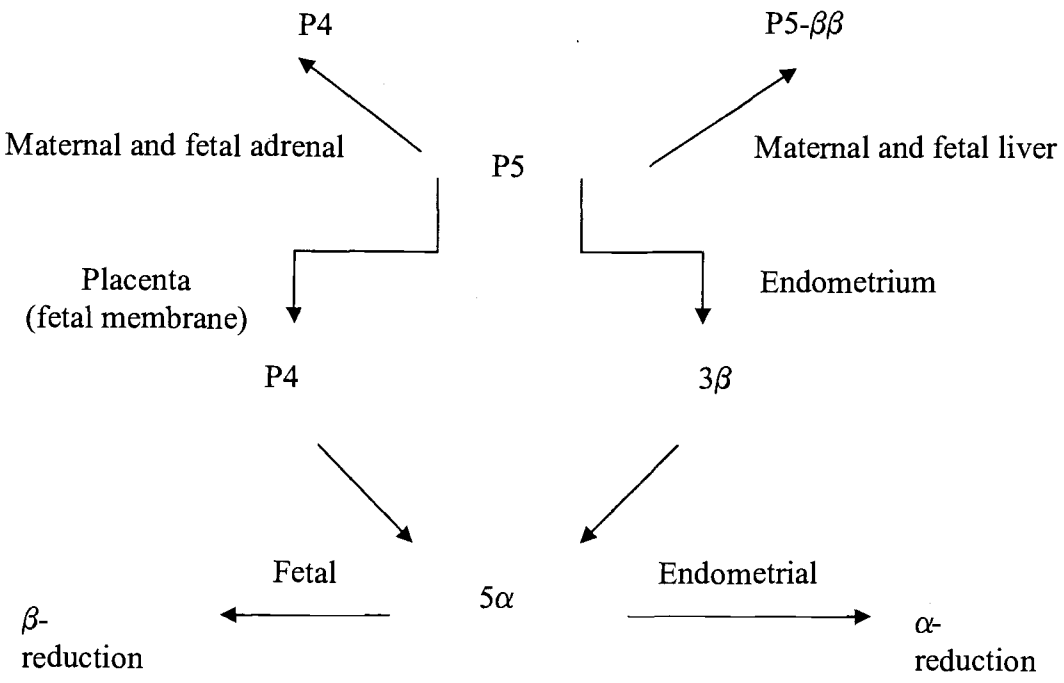

Figure 2.2: Proposed progestin metabolic pathways found in the mare. Adapted from Schutzer and Holtan, 1996.

2.5 The Role of Maternal and Fetal Adrenal Glands

There is ample evidence to suggest that the fetal adrenal gland is a direct or indirect source of progestins. Maternal plasma progestin concentrations increase significantly following a clinically induced fetal stress (Rossdale et al., 1991). Fetal injections of corticotropic releasing hormone (CRH), adrenocorticotropin (ACTH) and betamethasone between 248 and 335 days of gestation also resulted in a significant increase in maternal plasma progestin concentrations (Rossdale et al., 1992). The presence of cholesterol side chain cleavage complex in the fetal adrenal is controversial. Rogerson et al (1993) did not locate it using a Western immunoblot and immunohistochemistry. However, Han et al. (1995) located it throughout the adrenal cortex using immunohistochemistry. He noted that the expression of cholesterol side chain cleavage complex significantly increased after birth.

2.6 Progesterone Receptor Localization and Metabolite Affinity

In order for progestins to have a biologic effect, they must be able to interact with receptors located throughout reproductive tissue. Chavatte-Palmer et al. (2000) demonstrated that there was a trend in increasing concentration of P4

receptors from day 150 to parturition in the uterus. These authors also demonstrated that the P4 and estrogen receptor ratios were significantly lower in pregnant mares than non-pregnant mares as a result of increasing estrogen receptor concentrations in pregnant mares. Further, they demonstrated that mares have significantly higher P4 receptor concentrations in the mammary gland from day 150 to day 180 of gestation compared with the rest of gestation, and that these then decrease. This decrease of P4 receptors in the mammary gland is coincident with an increase in P4 receptor concentration in the uterus. The difference could be related to highly active 5α-reductase activity in the endometrium, as demonstrated by increasing concentrations of 5α in the fetal vein (Chavatte-Palmer et al., 2000, Holtan, 1991); 5α is undetectable in the maternal artery. This suggests that 5α could influence P4 receptor concentration (Chavatte-Palmer et al., 2000).

Progesterone receptors have a very high affinity for 5α, and a moderately high affinity for the other 5α-reduced pregnanes. The IC_{50} value is the steroid concentration that reduces specific binding by 50% (Table 2.1, adapted from Chavatte-Palmer et al., 2000). Individual IC_{50} (Table 2.1) values indicate that 5α has a sufficiently high affinity for the P4 receptor to affect steroidogenesis. 5α-Pregnane-3,20-dione has the potential to be either a P4 receptor agonist or an antagonist. As an antagonist it may compete with P4 and other progestins, preventing inhibition of OT and therefore stimulating myometrial activity when

5α concentrations increase before parturition. As an agonist it could promote myometrial quiescence at the time when P4 is no longer present to do so. Perhaps its agonistic and antagonistic abilities are concentration dependent. Therefore, the observed decrease in overall progestins at parturition may remove the inhibition of myometrial contractions (Chavatte-Palmer et al., 2000). 5α-Pregnane-3,20-dione could also have other, unexplored effects at the level of the anterior pituitary gland or hypothalamus.

Table 2.1: IC_{50} values of progestins. Adapted from Chavatte-Palmer et al., 2000

Pregnane	IC_{50} Value
P_4	4.7×10^{-8}
5α	3.8×10^{-8}
3β	5.3×10^{-5}
ββ	5.7×10^{-5}
20α	3.6×10^{-3}
βα	$> 10^{-3}$
P5-ββ	$> 10^{-3}$

While it is very likely that some progesterone metabolites exert their effects through the P4 receptor, there is tissue and species specific variability. For instance, there is little or no binding of metabolites to the rat anterior pituitary gland, (Iswari et al., 1986), the chick oviduct (Scott, 1974), the guinea pig uterus (Saffran et al., 1978) or the rat uterus (Gee et al., 1988). However, researchers have shown that some of the 5β-reduced progesterone metabolites, but not progesterone, are able to bind and potently modulate the gamma amino butyric

acid$_A$ ($GABA_A$) receptors in many species (Gee et al., 1988). Additionally, others have shown that there is a relatively high density of $GABA_A$ receptor sites in the rat uterus and these can be activated by the progesterone metabolite 3α-hydroxy-5α-pregnan-20-one (Erdo, 1984; Majewska et al., 1989). This observation is especially noteworthy considering that the $GABA_A$ agonist, muscimol, has been reported to inhibit uterine contractility in the rat (Riesz and Erdo, 1985). Putnam et al. (1991) reported that both the progesterone receptor and $GABA_A$ receptor systems can mediate the uterine-quieting effects of progestins, with the particular receptor system employed depending on the progestin administered in the rat. For example, the effects of 5β-pregnane-3, 20-dione was not blocked by the P4 receptor antagonist RU486 but were blocked by $GABA_A$ receptor antagonist picrotoxin. In contrast, 3α-hydroxy-5β-pregnan-20-one and 3α-hydroxy-5α pregnane-20-one effects were blocked by RU486, but not blocked by picrotoxin (Putnam et al., 1991). It is not known if the 5α-reduced progesterone metabolites found in the pregnant mare, rather than the 5β-reduced metabolites, have the same stimulatory or inhibitory effect on $GABA_A$ receptors.

2.7 The role of a non-genomic Progesterone Receptor

In general, P4 interacts with a genomic steroid receptor, which exerts its effects by altering the transcription of genes. The effects of P4 are not usually

observed for several hours after treatment. However, recently it has been demonstrated that P4 can interact at the level of the plasma membrane in the uterus of the rat. Grazzini et al (1998) demonstrated that a P4 dose-dependent reduction of specific oxytocin binding to the oxytocin receptor occurred in parturient rat uterus (Grazzini et al., 1998). These authors also observed that P4 mediated the activity of oxytocin induced second messengers such as inositol phosphate and calcium. Interestingly, they also observed that oxytocin receptors in human cells were unaffected by P4, but oxytocin binding and stimulation of 2nd messengers was altered by the P4 metabolite 5β-pregnane-3,20-dione. These authors effectively provided the first demonstration of a functional interaction between a steroid and a G-protein linked receptor. Recent work at Oregon State University has provided evidence that in the ewe, P4 binds to the oxytocin receptor monomer, preventing receptor dimerization and subsequent activation (Dunlap, 2002).

A study done a few years earlier suggests that the mare has plasma membrane P4 binding sites in the CL. Researchers discovered that a P4 tracer was bound *in vitro* to fractions with a buoyant density similar to that of luteal cell surface membrane markers (Bramley et al, 1995). Further, the classical P4 receptor antagonist, RU-486 did not compete with P4 for these binding sites. This effectively distinguishes the classical genomic, and particulate binding sites for P4 in the mare (Bramley et al., 1995). The presence of a non-genomic steroid

receptor could indicate that P4 metabolites may elicit much more rapid responses than typical of a genomic response system.

2.8 Control of Pituitary Gland Peptide Hormones

Luteinizing hormone is an anterior pituitary gland peptide hormone with a non-covalently linked α and β subunit. Luteinizing hormone is released from the anterior pituitary gland upon stimulation by luteinizing hormone releasing hormone (LHRH), also called gonadotropin hormone releasing hormone (GnRH), which is stored in the hypothalamus.

Luteinizing hormone has luteotropic effects and could possibly maintain the CL through the diestrous phase of the estrous cycle of the mare, especially since the mare has a unique, slow increase in LH, which reaches a maximum on or shortly after ovulation. Luteinizing hormone also plays a key role in follicular development and ovulation. The luteotropic function of LH declines as the luteal phase progresses. There are LH-receptors located in thecal, granulosa and luteal cells in the ovary. The LH-receptor is a seven membrane spanning, G-protein coupled receptor. Stimulation of these receptors by their ligand results in an increase in intracellular cAMP and consequently, an increase in the production of P4 by the CL in the mare (Roser et al., 1982).

Several researchers have demonstrated that the anterior pituitary gland is an important site of both positive and negative feed back of steroid hormones affecting the synthesis and release of LH. Krummen and Baldwin (1988) evaluated the direct effect of testosterone (T) on LH subunit apoprotein synthesis, glycosylation, and release by the human male pituitary. These authors demonstrated that not only did T suppress GnRH-stimulated LH release in males by direct action on the pituitary and indirectly altered GnRH secretion, but also acts at the pituitary to specifically inhibit LH subunit apoprotein synthesis and differentially suppresses LHα glycosylation. They also demonstrated that this effect of T was specific to LHα because ^{35}S methionine incorporation was not altered in total proteins.

Schoenemann et al. (1983) were able to demonstrate that the estrogens, estriol and estradiol-17β, modulate pituitary responsiveness to GnRH, and P4 exerts an inhibitory effect at the hypothalmic level in ovariectomized cows. These authors found that GnRH receptor concentration increased after an estrogen stimulus in cows treated with estrogens alone. Interestingly, LH concentration in blood only increased in animals not treated with P4. This study demonstrates that in the cow, the negative effect of P4 occurs at the level of the hypothalamus, not the pituitary gland (Schoenemann et al., 1983). This could be pertinent to horses as well, because as of yet, there is no published findings of P4 receptors in the equine pituitary gland.

Ramey et al. (1987) investigated the effects of GnRH and estradiol on LH biosynthesis and release using cultured rat anterior pituitary cells. These authors determined that the major effect of estradiol-17β (E2) on LH biosynthesis was to lower the effective physiological concentration of GnRH needed to stimulate LH glycosylation and LH apoprotein synthesis. They suggest that estradiol sensitizes the gonadotroph to GnRH to enhance both LH biosynthesis and release. However, the mechanisms by which this occurs are still not known, although it is logical that E2 would up-regulate GnRH receptors. Some authors suggest that it involves action on the generation of cAMP (Tang and Tang, 1981), while others suggest it involves increased activity of specific glycosyltransferases (Nelson et al, 1975).

Girmus and Wise (1991) hypothesized that P4 directly inhibits pituitary LH release in ovariectomized ewes exposed first to E2. Luteinizing hormone release from intact ewe anterior pituitary glands was decreased in E2 and P4 treated groups, when compared to separate E2 and P4 treatments. This disagrees with a study done by Hamernik et al. (1987) who determined that P4 significantly decreased LH release in ovariectomized ewes. DiGregorio and Nett (1995) suggested that a direct effect at the level of transcription of the gonadotropin subunit genes may be the primary mechanism of action by P4. They found that P4 treated ovariectimized, hypothalamic-pituitary-disconnected ewes had decreased amounts of FSH α and β subunit mRNA when compared to control

ewes (DiGregorio and Nett, 1995). Moss et al (1980) found a negative correlation between pituitary concentrations of LH and serum progesterone 30 days after parturition in the ewe, suggesting that P4 might have a delayed effect on LH. Finally, Al-Gubory et al (1989) demonstrated that removal of the CL on day 70 of pregnancy in the ewe resulted in a significant LH spike. In this regard, the ewe is representative of most domestic agricultural animals.

Interestingly, this has not necessarily been shown to be true in the mare. A report by Kelly et al. (1988) stated that mares having elevated P4 levels as a result of exogenous hCG treatment had similar LH levels as control mares, indicating that P4 might not have a negative feedback effect at the level of the anterior pituitary gland as it does in most mammals. Perkins et al (1993) found no temporal relationship to exist between P4 and LH pulses in pregnant and non-pregnant mares. However, it is probable that progesterone has a delayed, or lag, effect on pituitary LH release. This is especially valid if P4 affects the synthesis of LH α-subunit mRNA synthesis.

Mares experience a seasonal anestrous in the late fall and early winter. Seasonal anestrous is directly correlated with a decreasing photoperiod, which increases melatonin production by the pineal gland, thereby modifying GnRH secretion by the hypothalamus (Ginther, 1992). Strauss et al (1979) demonstrated that ovariectomized mares have a lower hypothalamic GnRH content during the winter than during the summer months, indicating that the decreased

concentration of GnRH during winter anestrous is not solely dependent on the presence of luteal P4. Intact mares have baseline LH and GnRH levels, coupled to elevated melatonin levels during winter anestrous. Hyland et al (1987) demonstrated that mares in shallow seasonal anestrus are more likely to respond to constant GnRH infusion than mares in deep seasonal anestrus. Gonadotropin hormone releasing hormone infusion resulted in LH pulses and ovulations in treated mares, followed by an increase in plasma P4. They demonstrated that during seasonal anestrus, systemic P4, LH and FSH are at baseline levels, and are thus not stimulatory to follicular development and ovulation. This indicates the presence of an alternative negative feedback loop, involving the pineal gland suppressing gonadotropin release rather than P4 being the inhibitor of release (Hyland et al., 1987).

Muyan et al. (1993) has researched the effect of modulation of GnRH stimulated LH release in cultured stallion anterior pituitary cells by gonadal steroids such as testosterone (T), dihydrotestosterone (DHT), Estrone (E1), Estradiol-17β (E2) and estrone sulfate ($E1SO_4$). These authors found that GnRH significantly increased LH release in a dose dependent manner, with optimal doses being 10^{-8}M GnRH. In contrast to studies conducted in human males by Krummen and Baldwin (1988) mentioned earlier, testosterone and DHT did not alter GnRH stimulated LH release. However, E2 increased LH significantly over E1, T, DHT and $E1SO_4$. These authors concluded that in the stallion, the

negative feedback effect of the testicular androgens on LH release does not appear to involve a direct effect at the pituitary level, but may involve actions at the hypothalamus (Muyan et al., 1993).

To determine if mare reproductive hormones might alter this result, Baldwin et al (1991) researched the effects of E1, E2 and $E1SO_4$ on LH release in mare pituitary cells in culture after receiving a GnRH challenge. These authors found that E2 significantly increased the release of LH after a GnRH challenge, as in the stallion. The concentration of E1 necessary to imitate the LH response of E2 was 100 times greater. Estrone sulfate demonstrated no estrogenic activity. They conclude the pituitary is an important site of action of E2's positive feedback effects on LH release in the mare (Baldwin et al., 1991) and that E2 may be the major estrogen component responsible for regulating the physiological events associated with GnRH stimulated LH release. However, they also stated that the mechanisms involved in this process are not fully understood. The positive feedback of estradiol on LH release at the level of the anterior pituitary is similar to that of females of other species such as the rat (Drouin et al., 1976), the sheep (Huang and Miller, 1980), the cow (Padmanabhan et al., 1978) and the monkey (Frawley and Neill, 1984).

Interestingly, Pinaud et al. (1991) demonstrated that the response of the equine pituitary gland to a GnRH stimulus is dependent on stage of the reproductive cycle. These authors employed a perfusion media system, and

measured LH output from culture mare pituitary cells after a GnRH stimulus. Mares in estrus had significantly higher LH release than mares in diestrus in perfusion media and in pituitary content (Pinaud et al., 1991). This indicates that mares under the influence of estrogen are more responsive to GnRH than mares not under the influence of estrogen.

McCue et al. (1992) also demonstrated that manipulating the gonadotropin output at the level of the hypothalamus and anterior pituitary gland had beneficial effects *in vivo* on fertility rates. These authors increased the rates of pregnancy in mares undergoing seasonal transition by treating them with a regimen of GnRH twice daily and a single injection of hCG when a follicle of > 35mm was present. They noted increased ovulations, as well as increased fertile ovulations when compared to mares given only hCG. They hypothesized that GnRH increased systemic LH concentrations sufficiently to induce ovulation, while hCG prevented premature luteolysis.

It is well documented that pregnant mares have nearly undetectable levels of LH during the last 250 days of pregnancy (Miller et al., 1980). Treatment of mares with GnRH on day 240 and day 320 of pregnancy did not result in a marked LH response in comparison to mares treated with GnRH on day 3 of estrus (Nett et al., 1989). Therefore, there must be a negative feedback effect occurring at the level of the pituitary gland or hypothalamus which prevents the release of LH upon GnRH stimulation during pregnancy. Recall that this late in

pregnancy, there is very little P4 present in the mare, but very high concentrations of the 5α-pregnanes. It is speculated that pituitary content of LH is replenished very rapidly in the mare after parturition, possibly leading to the presence of the unusual day 10 post-parturition ovulation, or "foal heat," (Ginther, 1992). Irvine and Evans (1978) observed LH at basal levels until approximately 2 days before the foal heat ovulation when levels began to escalate above 2 ng/mL. Interestingly, two follicle stimulating hormone (FSH) surges occurred 24 and 12 days before the foal heat ovulation.

The effect of other progestins besides P4 on gonadotropins has not been extensively researched in most agricultural and domestic animals. Much of the work done revolves around investigating the effects of synthetic progestins, used to synchronize the cycles of cows, on *in vivo* fertility rates. Kojima et al. (1993) found that synthetic progestins influenced secretion of LH and timing of ovulation. These authors found that cows treated with norgestomet and Melenorgestrol (MGA) had increased LH pulses, a shortened estrus, and a lengthened estrous cycle, thus stimulating rather than inhibiting pituitary secretions. They concluded that treatments of low doses of P4 or synthetic progestins may cause reduced fertility via alteration in the secretion pattern of reproductive hormones (Kojima et al., 1993). Stevenson and Pursley (1994) observed that concentrations of FSH increased *in vivo*, but concentrations of LH

decreased in dairy cows treated with exogenous norgestomet, a synthetic progestin.

Gilles and Karavolas (1981) studied the effect of 20α-hydroxy-4-pregnen-3-one, P4, 5α and αα on serum gonadotropin levels and hypothalmic LHRH content in the rat. The rat is interesting in that P4 and the 5α reduced pregnanes have a biphasic effect on gonadotropin release. An original injection of the pregnane leads to an actual increase in LH and FSH in rats that have been primed with estrogen for 3 days. A second injection 12 hours later down-regulates gonadotropin levels to baseline levels. It is interesting to note that the presence of E2 is mandatory to obtain this result. Pregnane treatment has no effect on the release of GnRH from either the medial basal or preoptic hypothalamic area.

Follicle stimulating hormone (FSH) is another peptide hormone released from the anterior pituitary gland. Follicle stimulating hormone promotes the formation of the small, fluid filled lumen of Graafian follicles known as the antrum. Follicle stimulating hormone, like LH, consists of α and β subunits. Follicle stimulating hormone β-subunit synthesis is controlled by GnRH via both a protein kinase C (PKC) pathway and a mitogen-activated protein kinase (MAPK) pathway (Griffin and Ojeda, 2000). Follicle stimulating hormone also serves to promote estradiol synthesis in the granulosa cells of the ovary. Progesterone inhibits FSH-stimulated E2 production *in vivo* and *in vitro*. Follicle stimulating hormone interacts with a membrane spanning G-protein coupled

receptor and activation of this receptor results in cAMP production and calcium mobilization (Griffin and Ojeda, 2000).

Evans and Irving (1975) were some of the first researchers to develop a reliable radioimmunoassay for equine FSH. They observed that FSH surged rather than spiked, on 10 to 11 day increments throughout the estrous cycle in the mare. The early diestrous FSH surge initiated the development of up to 20 follicles, and the mid-diestrous surge proved to be important for further development of follicles destined to ovulate. These authors noted an inverse relationship between P4 and LH levels in the mare, but observed no such relationship between P4 and FSH (Evans and Irvine, 1975).

2.9 Uterine Quiescence during Pregnancy

Oxytocin (OT) is a small peptide hormone (9 amino acids) secreted from the posterior pituitary gland containing two internal disulfide bonds at cysteine residues one and six. Oxytocin stimulates milk ejection by contracting myoepithelial cells surrounding the alveoli and ducts in the mammary gland. More important to this research, OT stimulates rhythmic myometrial contractions in the uterus, aiding in expulsion of the fetus. A positive feedback mechanism exists between OT and prostaglandin-F2α (PGF2α), which is important in the parturition cascade. Acetylcholine or dopamine is thought to be the stimulatory

neurotransmitter which promotes OT release from the posterior pituitary (Griffin and Ojeda, 2000). Interestingly, exogenous E2 has been shown to stimulate OT release. Oxytocin is frequently used *in vitro* to stimulate myometrial contractions.

Progesterone plays a key role in maintaining uterine quiescence during pregnancy in most mammals. Vallet et al. (1990) researched the effect of P4 and E2 on OT receptors in the ewe. They found that P4 pre-treatment decreased OT receptor concentrations in ewe myometrium from ewes treated subsequently with either P4 for 5 days or P5 for 5 days plus E2 on days 4 and 5 of P4 treatment. This effectively left the ewe uterus unresponsive to OT. They also found that P4 treatment alone for 12 days, mimicking the luteal phase, is sufficient to induce OT receptors and increase OT-induced PGF2α release. These data are important in implementing P4 in control of luteolysis and estrous cycle length in the ewe.

In the mare, authors have analyzed the response of uterine muscle treated with progestins. Ousey et al. (1998) used force transducers to measure oxytocin-challenged progestin-treated uterine strips *in vitro*. She found that in the mare, progesterone, 5α, $\beta\beta$, $\beta\alpha$, and 20α did not mediate the effects of oxytocin challenged tissue. However, this study did not use physiological concentrations of these progestins, and only used a few different amounts of these hormones in each analysis. Also, these authors did not verify that the hydrophobic steroids were adequately suspended in the polar medium. These authors were also unable

to demonstrate a correlation between time of gestation and myometrial sensitivity to oxytocin. It is unclear if further analysis would clarify these results. Ousey et al. (1998) suggests the poor clinical condition of the mares (low average body condition score) might have impacted these results. Other authors have used similar methods to research the effect of the synthetic estrogen DDT on stimulated contraction frequency in the rat uterus *in vitro* (Juberg et al., 1991), and the *in vitro* sensitivity of ovine myometrium and mesometrium to oxytocin (OT) and prostaglandins E2 and $F_{2\alpha}$ (Baguma-Nibasheka et al. 1998). The authors found that P4 decreased the response of the tissue to OT.

Interestingly, Alm et al. (1975) found that mares treated with exogenous P4 from day 318 of pregnancy until foaling, foaled an average of 12 days earlier than control mares (n=9). These authors also discovered that mares treated simultaneously with both P4 from day 318 until foaling, and dexamethasone from days 321 to 324, foaled an average of 16 days earlier than control mares. In this study no pre-or post-partum problems were encountered, and all foals were born alive and healthy. This is especially interesting in light of data from Holtan et al. (1976) which demonstrates that P4, along with all the other reduced pregnanes, have a significant spike directly before parturition, and then decrease. This introduces the possibility that in the late gestation mare, P4 and its reduced metabolites could possibly prime the uterus in preparation for labor, and then decrease to remove any inhibition they might have on OT.

The mechanism for myometrial contraction most likely resides from the agonist stimulated increase in free intracellular calcium as a result of stimulation by oxytocin (Adelstein and Eisenberg, 1980). Recent data (Grazzini et al., 1998) demonstrating that P4 could mediate the action of OT receptors suggests that P4 could play a prominent role in the parturition cascade. Bound calcium is stored predominantly in the mitochondrial matrix and sarcoplasmic reticulum (SR) in smooth muscle cells (Rasmussen, 1986). These organelles release calcium into the cytosol during muscle contraction. The calcium is re-sequestered following the complete contraction (Bolton, 1979). During pregnancy, the uterine artery retains its ability to contract, but the arterial tone progressively decreases as the diameter increases, resulting in a weaker contraction (Ford, 1995). Most likely OT induces the breakdown of phosphatidylinositol 4, 5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol triphosphate (IP_3). Inositol triphosphate binds to receptors in the SR releasing calcium into the cytosol. This calcium binds to calmodulin, which is then responsible for the activation of myosin light chain kinase. The activation of this kinase ultimately leads to the movement of myosin along the actin chain. The resulting contraction is phasic because the calcium is rapidly removed from the cytosol (Van Breeman et al., 1986; Rasmussen, 1987; Cartsen and Miller, 1987).

Haluska et al (1987) studied the electomyographic (EMG) properties of the myometrium correlated with the endocrinology of the pre-partum, partum and

post-partum periods in pony mares. These authors found that EMG were elevated the week before parturition, decreased 2-4 hrs pre-partum, then increased abruptly with the rupture of the chorioallantois. It is unknown if this myometrial activity corresponds to increasing progestin levels in the days before parturition, or the decrease that can be observed directly before parturition. If there was a direct correlation, it would support the hypothesis of some of the progestins being P4 receptor antagonists, while others are P4 receptor agonists. These same authors noted that pony mare myometrium was most active during the first 240 days of pregnancy, and became increasingly quiescent thereafter. After day 220, myometrial contractile bursts evolved from long to medium, and then short bursts (Haluska et al, 1987a). This is the time of decreasing estrogens and increasing progestins. These data indicate that myometrial activity occurs even under the influence of physiologic levels of progestins 7 days pre-partum until parturition.

2.10: Objectives

This information raises questions about the possibility of progestin metabolites performing the mandatory duties of LH and FSH inhibition, as well as uterine quiescence in the absence of P4 in the late gestation mare. Therefore, the main objectives of this study are: To correlate the concentrations of LH, FSH and pregnanes from ten days before parturition through the foal heat ovulation; to

analyze the effects of the progestins P4, 5α, 20α., 3β, and βα on the release of LH and FSH from mare pituitary glands in culture after a GnRH challenge; to research the effect of these progestins on myometrial tissue *in vitro* under the influence of an oxytocin challenge.

CHAPTER 3: MATERIALS AND METHODS

3.1 Animals

Pregnant light horse mares (n = 5) and pregnant pony mares (n = 3) were maintained and fed according to appropriate husbandry standards. They were housed in stalls at Oregon State University Animal Sciences Horse Center, and spent approximately 2 hrs per day in groups in pastures. Mares used for pituitary and uterine tissue experiments were euthanized and necropsied for other, unrelated reasons at Oregon State University College of Veterinary Medicine.

3.2 *Follicles stimulating hormone, luteinizing hormone and progestin concentrations pre-partum through the post-partum ovulation*

Five pregnant light horse mares and three pregnant pony mares were sampled daily to analyze pregnane, FSH and LH concentrations from April through June in the years 2001 and 2002. Samples from the jugular vein were taken beginning on day 315 of pregnancy if possible, and ending the day after the first ovulation post-partum. Ovulation was detected with rectal palpation and ultrasound. Samples were collected in a heparinized tube and centrifuged at approximately 100 x g. Plasma samples were frozen at -20°C until assayed. Samples from -20, -15 and -10, then every day until the post-partum ovulation, or

"foal heat" were analyzed. Follicle stimulating hormone and luteinizing hormone were analyzed by radioimmunoassay at The Endocrine Diagnostic Laboratory at Colorado State University and progestins were analyzed by gas chromatography/mass spectrometry at Oregon State University.

3.3 LH and FSH Radioimmunoassay

Luteinizing hormone and follicle stimulating hormone were analyzed by a competitive binding radioimmunoassay as described by Nett et al. (1975 and 1979). The assay utilized an anti-ovine LH and radioiodinated LH (Colorado State University, Fort Collins, CO), and an equine LH standard (University of California, Davis, Davis, CA). Preparation of the antiserum to ovine FSH and radioiodination of human FSH was described by L'Hermite et al. (1972). The average intra-assay coefficient of variation (CV) for LH was 3.28% +/- 1.36% (mean +/- SE). The inter-assay CV was 7.98%. The average intra-assay CV for FSH was 4.63% +/- 1.80% (mean +/- SE) and the inter-assay covariance was 9.11%.

3.4 Gas Chromatography/Mass Spectrometry

Gas chromatography/mass spectrometry was performed similar to published work by Holtan et al (1991). Total volume of the plasma sample (3-6 ml) was added to a conical centrifuge tube with 3 ml of deionized water (diH_20). A standard

curve was created with gelding plasma lacking endogenous progestins. The standards used to create a standard curve were the following: 5α, P4, 3α, P5, 3β, 20α, αβ, βa, αα and ββ, all in ethanol. All steroids were purchased from Sigma Chemical Co., St. Louis, MO. Deuterium labeled internal standards (500ng, $^2D_4$) were added to both the samples and the standards. The following $^2D_4$ internal standards were used: $^2D_4$-5α quanitified 5α and P4, $^2D_4$ -P5 quantified P5, $^2D_4$-3β quantified 3β, 3α and 20α, and $^2D_4$-ββ quantified αα, ββ, and βα. All deuterium lables were prepared as described by Dehennin et al (1980). A ratio of standards to $^2D_4$ internal standards was created for each standard with the following ratios: 0.25:1, 0.5:1, 1:1, 2:1 and 4:1. Samples were mixed gently for 20 min on a Thermolyne Speci-mix test tube mixer (Sybron, Dubuque, IW).

Solid phase extraction was used to purify and concentrate pregnanes. A t-C18 Sep-Pak (Waters, Milford, MA) connected to a vacuum manifold was activated and charged with 2 mL of methanol (Fisher, Pittsburg, PA), followed by 2 mL of di$H_2$0. The sample was then passed through the Sep-Pak, followed by a 3 mL rinse of di$H_2$0. One mL of hexane (Fisher) was then passed through the Sep-Pak, and all reagents were collected in a waste beaker. The sample was extracted from the Sep-Pak with 4 mL of diethyl ether (J.T. Baker, Phillipsburg, N.J.), and collected into clean, dry 4 mL vials. These vials were then dried down in a laminar flow fume hood at 80°C with nitrogen (N_2).

The samples were then derivatized in a two step process with 3% methoxyamine HCL (MOX, Sigma) in pyridine (Regis Chemical Company, Morton Grove, IL) and N-methyl-N-t-butyldimethylsilyl-trifluoroacetamide (MtBSTFA, Regis) because the derivatization processes is necessary for accurate and sensitive gas chromatography. The ketones on the steroids are derivatized with MOX to form methoxime groups, while the hydroxyl moieties are derivatized with MtBSTFA to form tertbutyldimethylsilyl (tBDMS) groups on the steroids. The derivatized steroids will fragment in a predictable fashion which helps in the identification and improves sensitivity, in that underivatized products will often yield low molecular weight ions. It is important to note that tBDMS is a large molecule, and therefore is unable to interact with 17-hydroxy pregnanes as a result of stearic hindrance.

First the samples are incubated in 50µL MOX for 30 min at 60°C and dried at 80°C with N_2 in a laminar flow fume hood. Next, 25 µl of MtBSTFA and 50 µl of the catalyst, 5% diethylamine (DEA, Regis) in dimethlyformamide (DMF, Regis) were added. This solution was covered, vortexed, and incubated for 45 min at 80°C. One half mL of diH_20 was added to the samples to stop the reaction, and the vials were filled with diethyl ether. They were then covered, and shaken aggressively for 30 seconds so that the hydrophobic steroids would diffuse to the ether layer. The ether layer was transferred to a 15 mm x 85 mm culture tube with a Pasteur pipette: the sample was extracted twice. Total recovery of steroids was

above 95% as evidenced by internal standard recovery in the GC/MS. These tubes were dried at 80°C with N_2 and the sides rinsed with ether. Finally, 30 µl of undecane was added to the culture tubes, vortexed, and heated. The steroids in undecane were then transferred to a plastic gas chromatography micro-vial with a 25 µl micropipette (VWR Scientific, West Chester, PA).

One µl of each sample was injected into a Hewlett Packard 5890 series II gas chromatograph (GC) interfaced with a model 5971A mass spectrometer (MS) equipped with an automatic sample injector (model 7673 Hewlett Packard, Wilmington, DE). The GC/MS contained a DB-5MS column (30m x 0.25mm i.d., x 0.25um film, J&W Scientific, Folsom, CA). Samples were separated under a temperature program in the GC. The injection port temperature was 250°C and the initial oven temperature was 160°C. Upon injection of the sample the oven temperature increased at 25°C/minute to 310°C and remained at that temperature for 10 min. Total sample analysis was 20 min per sample. Hewlett Packard ChemStation software was used to analyze the data. This software program works by calculating the least squares linear regression for each steroid within each assay from peak area ratios, i.e., steroid area vs. $^2D_4$ internal standard area. The $^2D_4$ labeled internal standards were used to create a standard curve, and since each sample had a concurrent internal standard, these levels were compared to the standard curve and actual steroid levels were quantitated in standard ion mode. Ion identification was performed in full scan mode.

Previous work in this laboratory reported the mean inter-assay coefficient of variation to be 2.6%, the intra-assay coefficient of variation to 6.2%, and the mean coefficient of variation for the machine and quantitation system to be 2.0% (Miller, 2000).

3.5 Equine Pituitary Cell Culture

Equine pituitary cell culture was performed similar to methods described by Baldwin et al. (1991) and Muyan et al. (1993). Light horse mares (n=3) were humanely euthanized without regard to stage of cycle at Oregon State University Veterinary Hospital between May, 2001 and July, 2002 and their pituitary glands were collected. The pituitary glands were immediately transported to the cell culture facility in room temperature sterile complete Dulbecco's Minimum Essential Medium (cDMEM, Gibco BRL, Grand Island, NY) containing 10% charcoal stripped horse serum, and 0.1% of the following: streptomycin, penicillin, amykacin, and 2mM L-glutamine and HEPES buffer. The pituitary glands were placed in a Petri dish containing 4mL cDMEM and were cut into quarters. Connective tissue was dissected away with a sterile surgical (#10) blade. They were then placed in 9mL of 0.2M Trypsin/EDTA solution and incubated on a shaker bath for 20 min. to break up connective tissue. The pituitary glands were then centrifuged for 5 min at 900 x g, the Trypsin solution was discarded, and the pituitaries were re-suspended in approximately 5mL of cDMEM in a conical

centrifuge tube. The pituitary glands were then dispersed first with an insulin syringe by aspirating the tissue clumps approximately 50 times, then with a small bore, sterilized flame polished, disposable Pasteur pipette (Fisher). The tissue was again pipetted approximately 40-50 times to break up tissue blocks. Remaining tissue blocks were allowed to settle to the bottom, and the remaining medium (containing the cells) was transferred to a separate conical centrifuge tube. The remaining tissue blocks were treated again with the Trypsin/EDTA solution. The collected cells were centrifuged for 5 min at 300 x g and the medium was discarded. The cells were re-suspended in 10mL cDMEM and the concentration of cells in this solution was estimated with a Coulter Counter. Initial cell viability was estimated with Trypan blue (Sigma) staining. The method for the trypan blue staining was from Sigma (1997) catalog. An average of 92% of cells were alive and viable before being plated.

Cells were plated at a concentration of 500,000 cells/well on 24-well plates (Gibco) that had been incubated for at least two hours with cDMEM (100µl/well). The cells were incubated for 3 hrs at 37°C and 5% CO_2 and 95% air to promote attachment to each plate, and then flooded with 400µl of cDMEM, or until the final volume was 500µl/well. The cells were allowed to incubate for 48 to 72 hrs, or until they appeared to be maximally attached. Final pre-treatment cell viability as determined with a Trypan blue staining was approximately 85%.

Once cells were attached the medium was removed and replaced with fresh medium containing treatments. Each 24-well plate contained 4 wells treated with only 1% ethanol in cDMEM (control), and 4 wells containing each of the following: P_4, 5α, βα, 3β, and 20α at a final concentration per well of 1000 ng/ml steroid. The steroids were dissolved in 1% ethanol in cDMEM. Cells were incubated for 3 hrs and then treated with 100nM GnRH (Sigma, human LHRH) final concentration. Of the 4 control wells two were treated with GnRH as positive controls and two were untreated and used as negative controls. This resulted in information on the GnRH response from a plate of cells. Cells were incubated for an additional 3 hrs after GnRH treatment, and then the medium was collected and frozen at -20°C until assayed for LH and FSH content at The Endocrine Diagnostic Laboratory, Colorado State University.

3.6 Uterine Myometrial Studies

Equine uterus (n=3) was collected from humanely euthanized mares and transported to the lab in gassed Krebs-Ringer bicarbonate buffer (Sigma) containing 1800 mg/L glucose. Sodium bicarbonate (0.02M) and $CaCl_2$ (1.0 mM) was added to the buffer to make complete buffer (cBuffer), pH = 7.2 +/- 0.2 at room temperature. Uterine tissue was cut into 1cm x 2cm strips and the endometrium and mesometrium were carefully dissected away from the myometrium. Suture loops were carefully attached to each end of the myometrial strip, and the tissue

was placed in a double tissue bath (Harvard Applications Limited, Edenbridge, KY) containing 50 ml cBuffer at 37°C. The tissue bath was gently aerated. The bottom suture strip was secured to the bottom of the tissue bath, while the top suture strip was attached to a 5/6 Hz physiograph, (Gilson Medical Electronics, Middleton, WI) containing a calibrated displacement transducer (VFI, Moro Bay, CA). Force applied to the transducer was calibrated in grams and recorded on the strip chart.

Unlike many species, mare myometrium does not exhibit spontaneous contractions after removal from the mare. Therefore, contractions were induced in myometrial strips upon stimulation with 0.04 IU/mL oxytocin. If no contractions were observed in a fifteen minute time period, the tissue was discarded and fresh was used. If oxytocin simulated contractions were judged consistent, the tissue was treated with 1μg/mL pregnane, final concentration. Treatments were 2.5% ethanol (positive control), P4, 5α, 20α, 3β, and $\beta\alpha$, with 2 to 4 replications per treatment. The steroids were suspended in 2.5% ethanol solution in cBuffer.

After treatment myometrial contractions were monitored for differentiation from the pre-treatment pattern. Effects of treatment showed high levels of variation, and strips were monitored until it was empirically clear that the myometrial tissue was contracting in a consistent manner, post treatment. Changes in both the frequency and amplitude of the contraction were measured with digital calipers. Post-treatment responses were compared to pre-treatment responses by percentage change, as a result of the high levels of variation observed.

3.7 Statistical Analysis

All hormone concentrations were analyzed after transformation to the natural logarithm. The relationship between pregnanes and LH was analyzed for two time periods (Figure 3.1). Pregnane concentrations from 5 days pre-partum to foaling were compared to LH concentrations from 5 days pre-ovulation to ovulation. These data were modeled as a linear mixed effects model. Serial correlation was taken into account using a Gaussian spatial covariance structure with day of study as the basis for distance (SAS online document, 1999). The random intercept was deemed significant and included in the model. Pregnanes were also analyzed for 10 days post foaling and compared to the 10 days pre-ovulation for LH. This was also modeled as a linear mixed effects model and serial correlation was taken into account using an exponential spatial covariance structure, with day as the basis for distance.

The relationship between pregnanes and FSH was also analyzed for two time periods (Figure 3.1). Pregnanes and FSH were modeled on a same day basis from 5 days pre-partum to day of foaling. These data were also modeled as a linear mixed effects model but in this model, serial correlation was taken into account using a symmetric covariance structure. The random intercept was significant and

included in the model. The second time point analyzed was the interval from day of foaling to 5 days post-partum. These data were modeled as a linear mixed effects model and serial correlation was taken into account using an exponential spatial covariance structure with day as the basis for distance. The random intercept was significant and included in the model.

Difference between total pregnane levels for pony and light horse mares was analyzed with repeated measures analysis of variance from day -10 to foaling. Day and day by mare interactions were excluded from the analysis as a result of being insignificant.

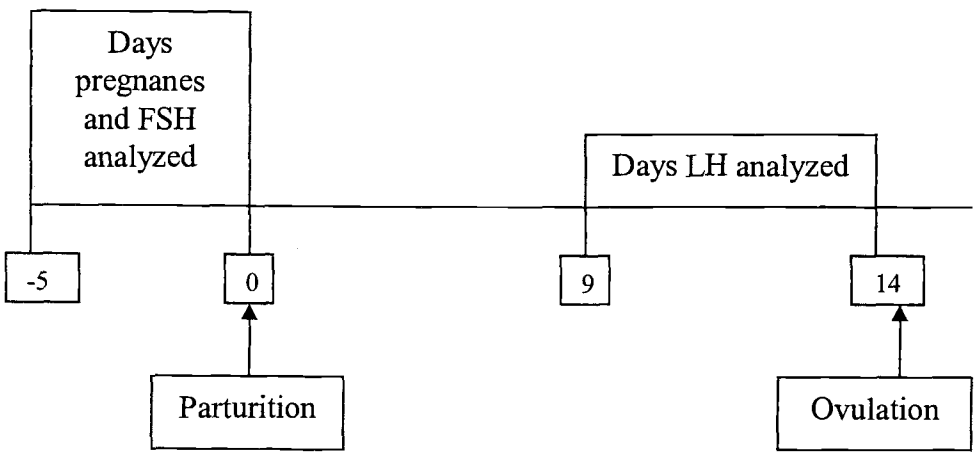

Figure 3.1: Schematic of days used to analyzed pre-partum pregnanes and gonadotropin correlation.

Equine pituitary cell cultures data and myometrial data were both analyzed with one way analysis of variance (S-Plus, MathSoft Inc, Cambridge, MA). All LH and FSH data were transformed using the natural logarithm.

CHAPTER 4: RESULTS

4.1 *Follicles stimulating hormone, luteinizing hormone and pregnane concentrations pre-partum through the post-partum ovulation*

There was a negative temporal relationship between total pregnanes (n=9) and LH (p =0.0196) when analyzed from 5 days pre-partum to foaling for pregnanes, and 5 days pre-ovulation to ovulation for LH (Figure 4.1). A summary of the relationship is as follows: a one unit increase in pregnanes is estimated to have a median LH hormone level at the corresponding day that is 99.98% (95%CI: 99.96% – 99.996%) of the median LH hormone level at the original total pregnane level. A clear way to look at the model is to consider that a 100 ng/mL increase in pregnane level would decrease LH levels by 2.1% when analyzed on a time lag basis. There was also a relationship between pregnanes and LH when analyzed from the day of foaling to 10 days post-foaling for pregnanes, and 10 days pre-ovulation to ovulation for LH (p=0.0004). This relationship is summarized as follows: a one unit increase in pregnanes is estimated to have a median LH hormone level at the corresponding day that is 99.94% (95% CI: 99.91% to 99.98%) of the median LH hormone level at the original total pregnane level. If pregnane concentrations were to increase 100 ng/mL there would be an estimated 5.9% decrease in LH for this model.

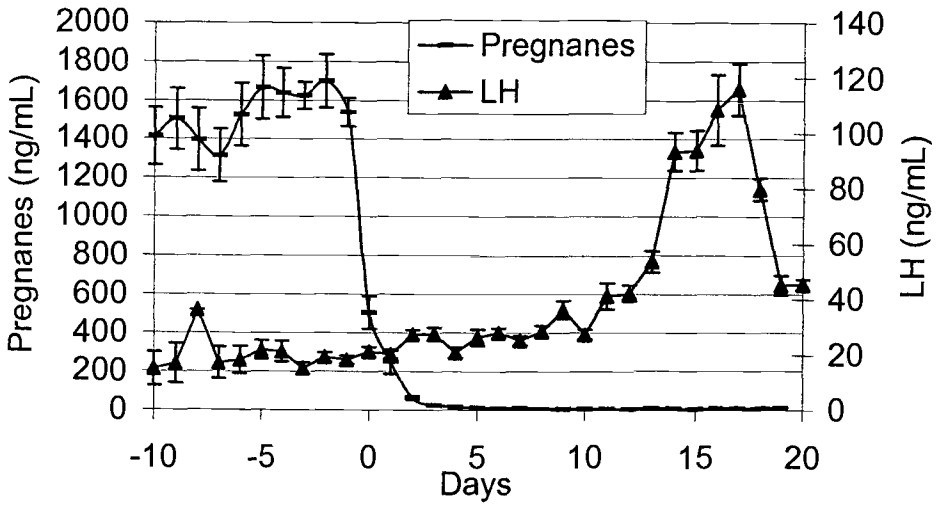

Figure 4.1: Total pregnane and LH concentrations (mean +/- SE) in mares (n=8) pre and post-foaling. Day 0 = parturition. Luteinizing hormone is normalized for day of ovulation, D = 14. There is a significant temporal relationship (p=0.0196) of total pregnanes and LH.

There was also a temporal relationship between total pregnanes and FSH (p =0.0138) when analyzed from five days pre-partum to foaling for both pregnanes and FSH (Figure 4.2). A summary of the relationship is as follows: a one unit increase in pregnanes is estimated to have a median FSH hormone level on the same day as the measurement of the pregnanes, that is 99.97% (95%CI: 99.95% – 99.99%) of the median FSH hormone concentration at the original total pregnane level. More simply stated if pregnane concentrations were to increase by 100 ng/mL, it is estimated FSH levels would decrease by approximately 3% on a same day basis. There also is a relationship between total pregnanes and FSH concentrations when measured on a same day basis from birth to 10 days post-partum ($p<0.0001$). During this time period, a one unit increase in the

concentration of pregnanes is estimated to have a median FSH response on the same day that is 100.04% (95% CI: 99.998% to 100.10%) of the median FSH concentration at the original total pregnane level. This model estimates that if pregnane levels were to increase 100ng/mL, FSH would also increase by 4.1%. Individual pregnanes were not analyzed for correlation with LH or FSH because the secretion patterns for all pregnanes are nearly identical.

The temporal relationship between LH and FSH is shown in Figure 4.3 where day 0 = ovulation. Representative temporal relationships among pregnanes, LH and FSH for individual mares 6 and 7 are shown in Figures 4.4 and 4.5 respectively. Individual pregnanes are shown in Figures 4.6, 4.7 and 4.8. Individual pregnanes from all mares transformed by $\log_{10}$ from day -10 to foaling is shown in Figure 4.9 to demonstrate the relationship in rate of change between individual pregnanes. As a result of the nearly identical slope of each individual pregnane (except P4) all statistical analysis were performed on the total pregnane concentration. Light horse mares (n = 5) had higher total pregnane levels (mean = 1897.2 +/- 150.6, S.E.) compared to pony mares (n = 3, mean = 1101.5 +/- 122.0) when analyzed from day -10 to parturition ($p < 0.001$).

There was no correlation between maximum concentrations of pregnanes and duration from the pregnane peak to the ovulatory event (mean = 1921.2 ng/mL pregnanes +/- 738.2 S.E., mean = 14.4 days post-partum ovulation +/- 1.50 S.E., p = 0.338, Figure 4.10).

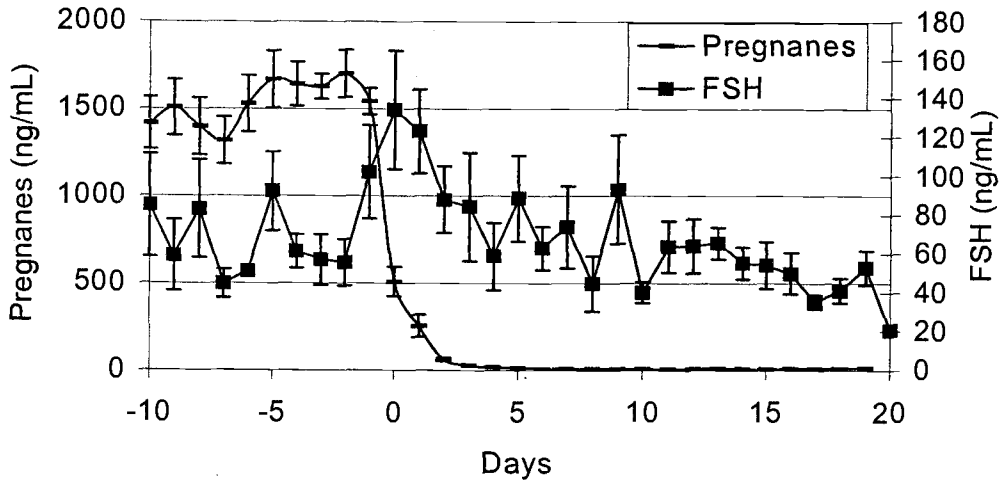

Figure 4.2: Total pregnanes and FSH concentrations (mean +/- SE) in mares (n=8) pre and post-foaling. Day 0 = parturition. There is a significant temporal relationship of total pregnanes and FSH (p=0.0138).

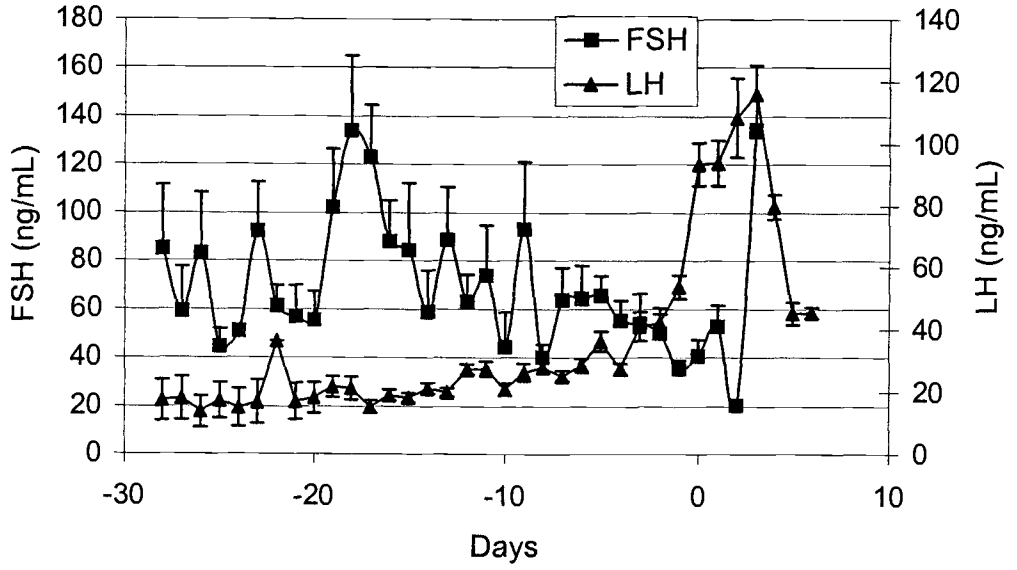

Figure 4.3: Temporal relationship between LH and FSH (mean +/- SE) in mares (n=8) normalized to day of ovulation, D = 0.

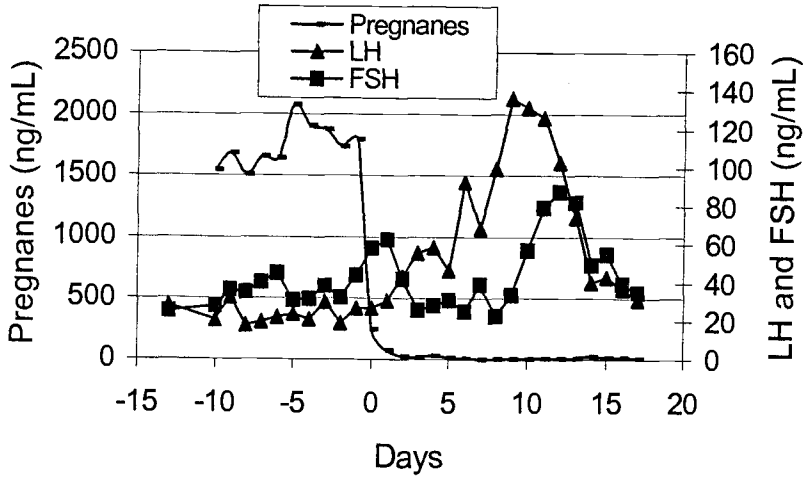

Figure 4.4: Temporal relationship of total pregnanes, LH and FSH for mare 6 (Day 0 is day of parturition, day of ovulation is day 8).

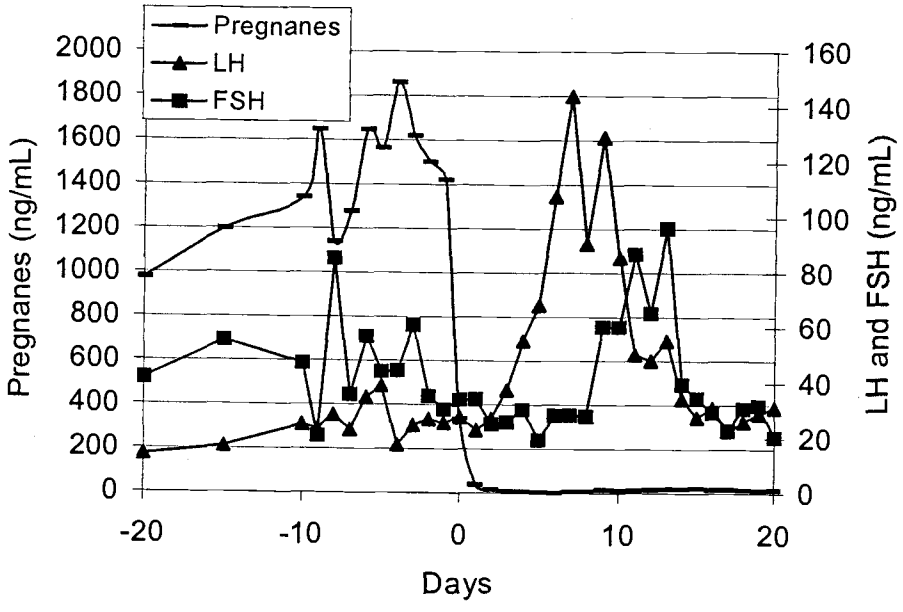

Figure 4.5: Temporal relationship of total pregnanes, LH and FSH for mare 7 (Day 0 is day of parturition, day of ovulation is day 6).

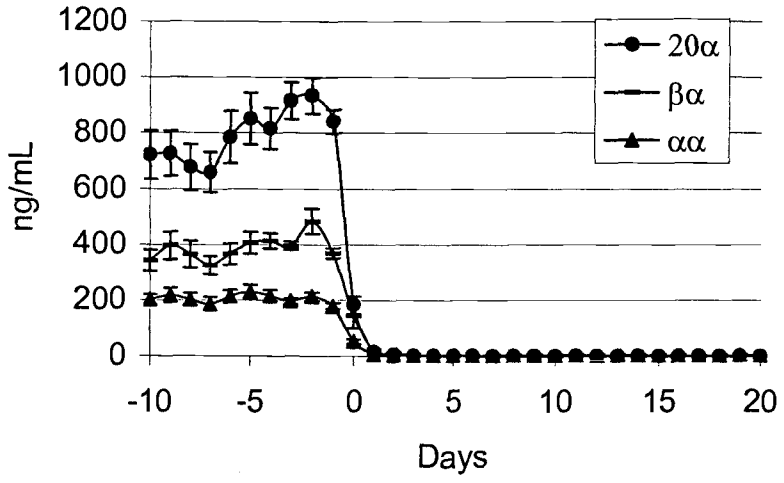

Figure 4.6: Individual pregnanes 20α, $\alpha\alpha$ and $\beta\alpha$ in pregnant periparturient mares (mean +/- SE, n = 8, Day 0 = parturition).

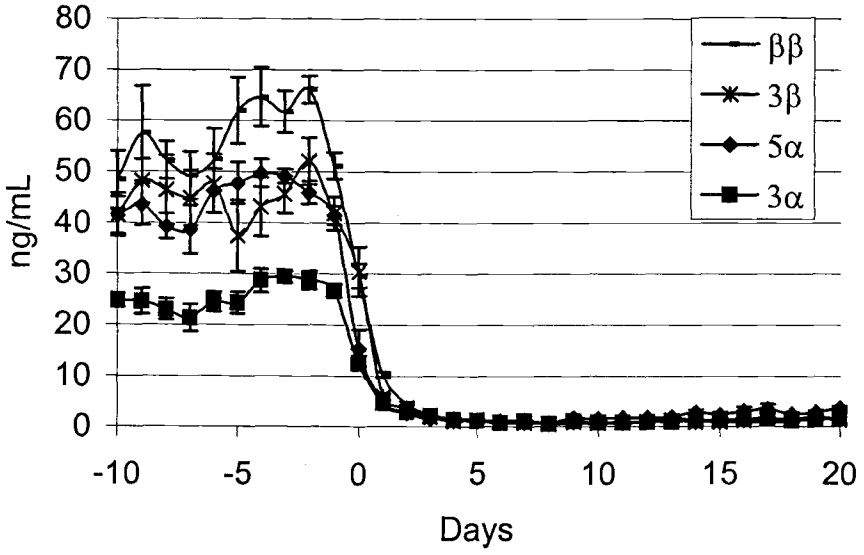

Figure 4.7: Individual pregnanes $\beta\beta$, 3β, 5α and 3α in pregnant, periparturient mares (mean +/- SE, n = 8, Day 0 is day of parturition).

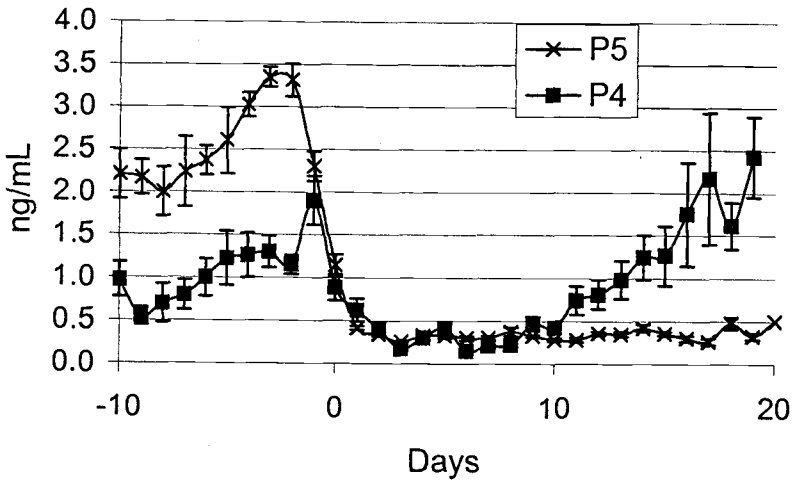

Figure 4.8: Individual pregnanes P5 and P4 in pregnant, periparturient mares (mean +/- SE, n = 8, Day 0 is day of parturition).

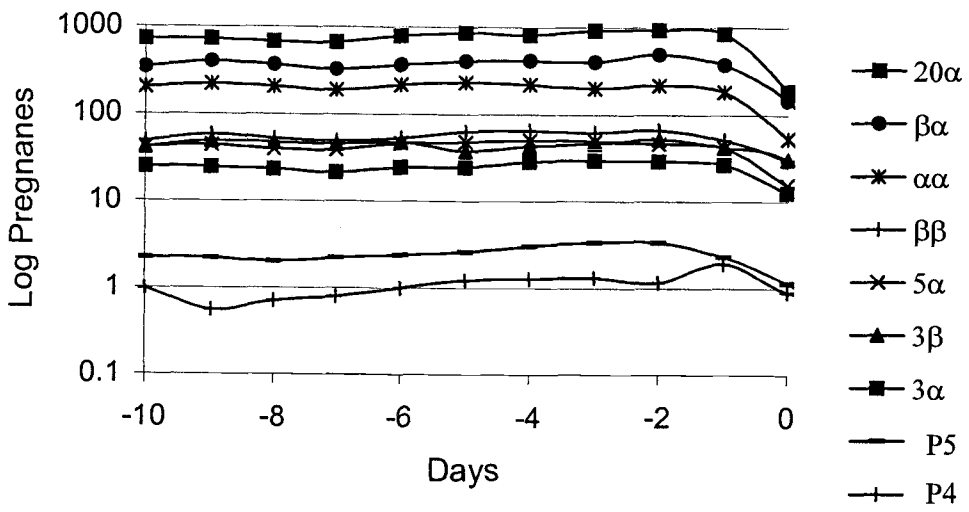

Figure 4.9: Log (mean total pregnanes +/- SE) from all mares (n=8) day -10 to day of foaling. All pregnanes exhibit the same pattern, with the exception of P4.

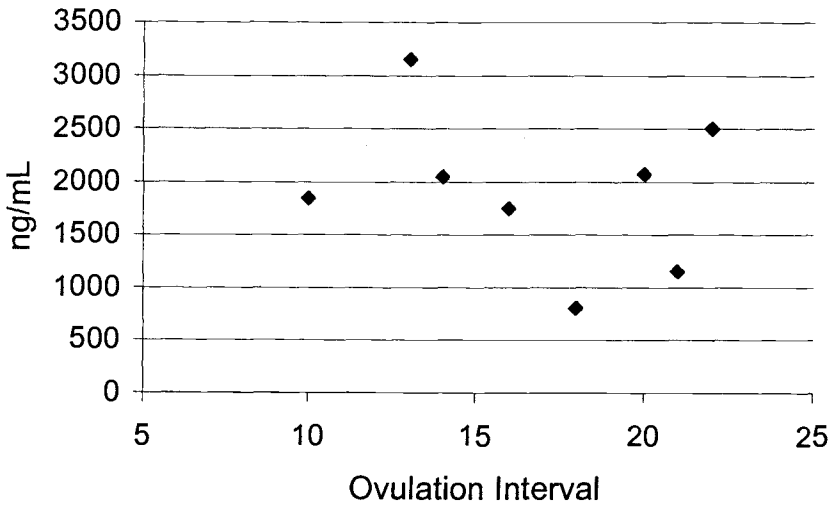

Figure 4.10: Relationship between pre-partum total pregnane maximum and post-partum ovulation interval (p= 0.338, n = 8).

4.2 Equine Pituitary Cell Culture

The LH and FSH responses for cells treated with a pregnane and then stimulated with GnRH are shown in Figures 4.11 and 4.12 respectively. These figures are shown as a percent of the positive control (ETOH + GnRH) because of biologic variability. They were analyzed with one way analysis of variance after transforming them with the natural logarithm. There were no significant interactions in the LH analysis, however, there was a treatment by mare effect in the FSH analysis. There was a significant 70% increase in LH after GnRH treatment (positive control) compared to vehicle alone (negative control, p= 0.015) as

determine by a two tailed distribution equal variance students t-test (Microsoft Excel). This indicates a sufficient GnRH response. There was not as pronounced a response following GnRH treatment (53%, p=0.113) in FSH with vehicle only and cells treated with vehicle and GnRH. There was no difference between treatment groups and the positive control for LH ($p = 0.557$, Table 4.1) as determined by fixed effects one-way analysis of variance and Fisher's LSD. The model for LH was: LH = treatment + plate + mare. There was a difference between treatments for FSH ($p = 0.0058$, Table 4.2). The model for FSH was: FSH = mare + plate + treatment + mare x treatment. When compared to the FSH positive control using a 2-distribution paired students t-test the only treatment group that was significantly different was 3β ($p=0.042$). There was also a significant mare x treatment interaction in this model ($p=0.0027$). Both the LH and FSH analysis resulted in a significant mare and plate effect. This is not problematic however, because each treatment was compared to a control from the same mare and the same plate.

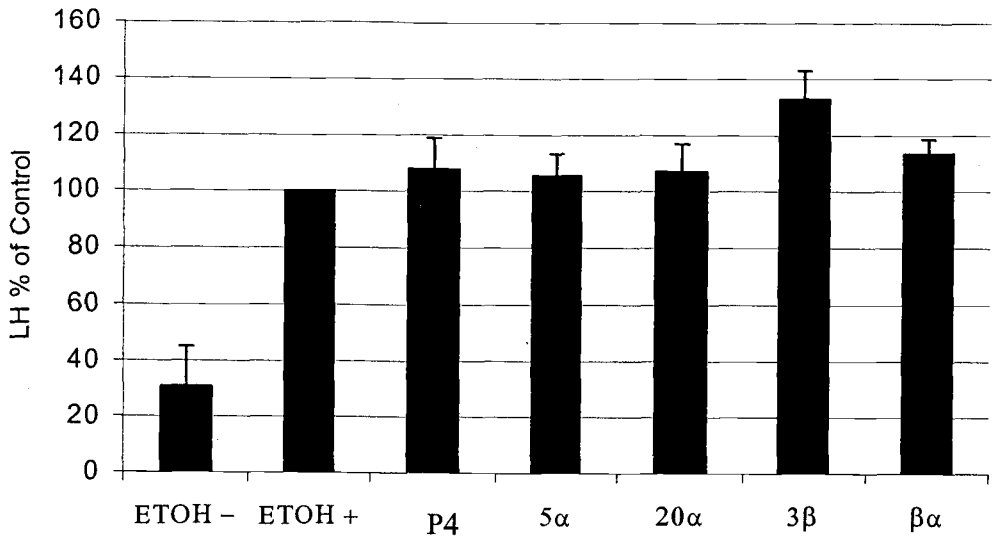

Figure 4.11: Effect of pregnane treatment on LH in equine pituitary cells stimulated with GnRH in culture (mean +/- SE, n = 3). There was no difference among treatment groups (p = 0.557).

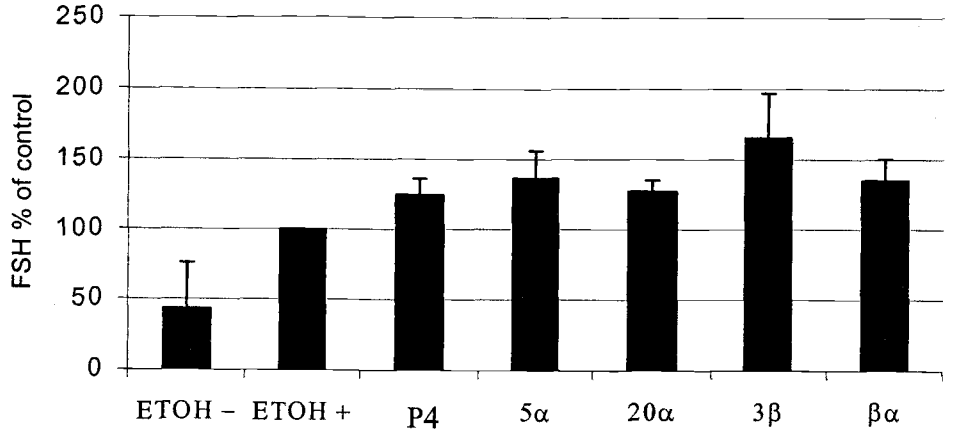

Figure 4.12: Effect of pregnane treatment on FSH in equine pituitary cells stimulated with GnRH in culture (mean +/- SE, n = 3). There was significant treatment effect (p=0.0058). 3β is significantly higher than ETOH + (p=0.043), all other pregnanes are not.

Table 4.1: Analysis of Variance Model for Equine Pituitary LH Analysis*.

	D.F.	Sum of Squares	Mean Square	F-Value	p-value
Mare	2	112.72	56.36	1079.62	<0.001
Plate	2	0.402	0.201	3.852	0.030
Treatment	5	0.201	0.040	0.770	0.557
Residuals	38	1.984	0.052		

*All data has been transformed using the natural logarithm.

Table 4.2: Analysis of Variance Model for Equine Pituitary FSH Analysis*.

	D.F.	Sum of Squares	Mean Square	F-Value	p-Value
Mare	2	145.5	72.77	1395.5	<0.001
Plate	2	0.405	0.203	3.884	0.032
Treatment	5	1.089	0.218	4.177	0.0058
Treatment:Mare	10	1.959	0.196	3.756	0.0027
Residuals	28	1.460	0.052		

*All data has been transformed using the natural logarithm.

4.3 Uterine Myometrial Studies

Percent change between pre and post steroid treatments for frequency and amplitude of myometrial contractions are shown in Figures 4.13 and 4.14 respectively. There was no difference in the treatment group and the (ethanol treated) control for either the amplitude ($p = 0.78$) or the frequency ($p = 0.63$). There was no difference among mares in the amplitude ($p = 0.10$) or frequency ($p = 0.35$) studies. Table 4.3 depicts the analysis of variance table for the frequency analysis, and Table 4.4 depicts the analysis of variance table for the amplitude analysis. The average pre-treatment frequency was 5.57nm +/- 0.25nm S.E. The average pre-treatment amplitude was 0.31 grams +/- 0.19 grams S.E.

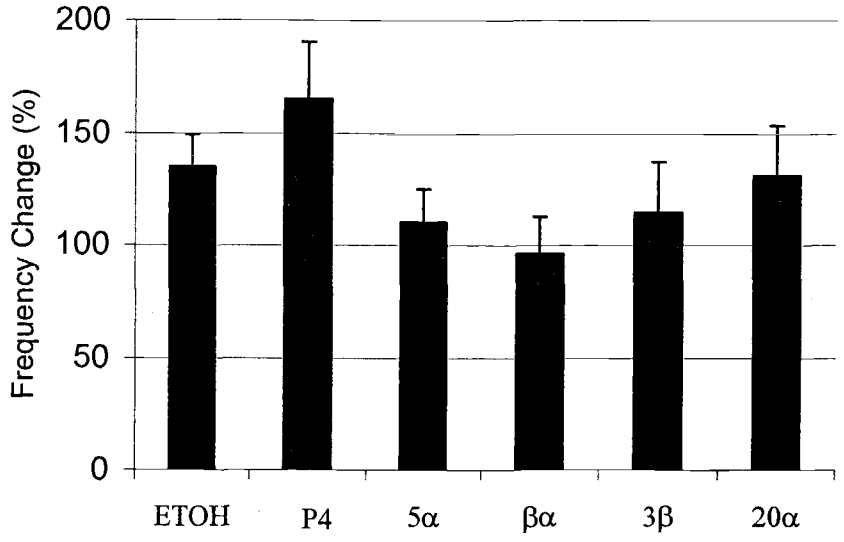

Figure 4.13: Percent change in frequency of contraction in mare myometrium (n=3) after treatment with a pregnane. ETOH is the control, and pre-treatment is 100%. No significant difference exists among groups ($p = 0.79$).

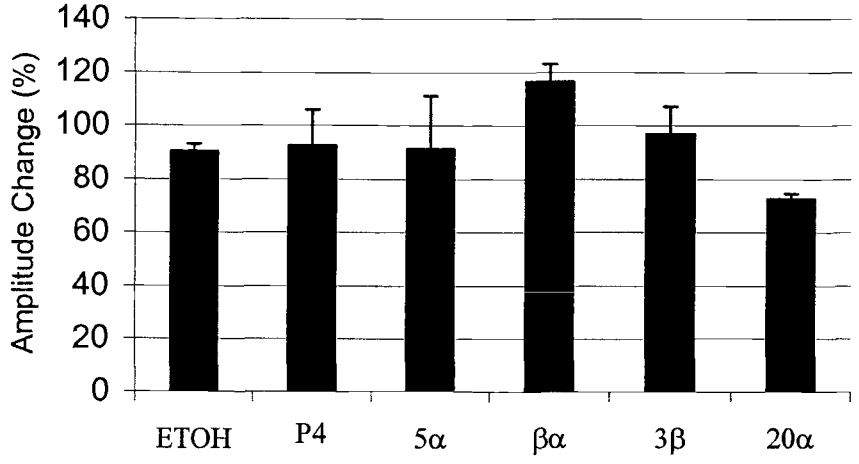

Figure 4.14: Percent change in amplitude of contraction in mare myometrium (n=3) after treatment with a pregnane. ETOH is the control, and pre-treatment is 100%. No significant difference exists among groups ($p = 0.63$).

Table 4.3: Analysis of variance table for mare uterine contraction frequency analysis.

	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	P-Value
Mare	2	12097.7	6048.9	3.02	0.0992
Treatment	5	4606.5	921.3	0.460	0.797
Residuals	9	18025.7	2002.9		

Table 4.4: Analysis of variance table for mare uterine contraction amplitude analysis.

	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	P-value
Mare	2	2324.2	1162.1	1.18	0.351
Treatment	5	3501.4	700.3	0.710	0.631
Residuals	9	8870.7	985.6		

CHAPTER 5: DISCUSSION

The objectives of this project were to determine if the 5α-reduced pregnanes found in the mid and late gestation mare possess any biologic activity. These pregnanes are found in very high concentrations late gestation in the mare, while P4, which is typically the hormone responsible for the maintenance of pregnancy in mammals, is at baseline levels at this time (Holtan, 1991). Therefore, in order to determine if the 5α-reduced pregnanes were partially or fully responsible for pregnancy maintenance in the mare, we researched the relationship between pregnanes and gonadotropins *in vivo* and *in vitro*, and observed the effects of pregnanes modulating myometrial contractions in the mare.

5.1 Follicle Stimulating Hormone, Luteinizing Hormone, and Progestins pre-Partum through the post-Partum Ovulation

The pattern of individual pregnanes remained consistent for all mares throughout this study (Figure 4.9) because the log transformations of the individual pregnanes are parallel. However, note that their concentrations range three orders of magnitude. Because of the lack of apparent differences in secretion patterns between groups, total pregnane, rather than individual pregnane concentrations were reported. The total pregnane curve is representative of the pattern of each individual pregnane (Figure 4.1) and is similar to previous work (Holtan et al., 1991). The pregnane found in highest concentration is 20α, followed by the diols

and 5α (Figure 4.9). This is consistent with previous data (Holtan et al, 1991). The exception to this is the pattern of P4 (Figures 4.8), which demonstrated slight increases directly before birth compared to the other pregnanes (Figures 4.6 and 4.7). This is similar to a result obtained by Miller (2000) in the ewe. The slight increase seen in P4 directly before birth is not represented by the total pregnane curve because it is found at significantly lower concentrations at this time than the other 5α-reduced pregnanes.

Post-partum luteinizing hormone concentrations were variable for all mares as a result of different ovulation times for each mare. When LH was normalized to day of ovulation, LH peaks were usually 0 to 48 hrs post-ovulation (Figure 4.3), consistent with published literature (Sharp et al., 1979). Individual mare examples are shown in Figures 4.4 and 4.5 for mares 6 and 7 respectively. Mare 6 ovulated day 8 post-partum and peak LH levels were day 10. Mare 7 ovulated 6 days post-partum and peak LH levels were day 8. There was a significant negative correlation between LH 5 days before ovulation to ovulation, and total pregnanes 5 days pre-partum to birth ($p = 0.0196$). A clear way to look at the model is to consider that a 100 ng/mL increase in pre-partum pregnane level is associated with a 2.1% decrease in LH levels. Interestingly, there was also a significant correlation ($p<0.001$) between total pregnanes and LH when the comparison for the 10 days prior to ovulation for LH, and the 10 days post-partum for pregnanes was analyzed. If pregnane concentrations were to increase 100 ng/mL there would be an estimated

5.9% decrease in LH for this model. These data present evidence that in the mare, the 5α-reduced pregnanes pre-partum effect post-partum LH levels. This is not altogether surprising in light of the reports indicating that P4 down-regulates LH *in vivo* in many species (Al-Gubory et al., 1989; DiGregorio and Nett, 1995; Evans and Irvine, 1975; Kojima et al., 1993) and *in vitro* in the mare (Pinaud et al., 1991).

The pattern of FSH secretion appeared to be similar for all mares (Figures 4.2 and 4.3). It was typical to see a FSH spike within 48 hrs of parturition, usually directly after the pregnane decrease. A second FSH peak 5 to 8 days post-partum was also common as seen in Figures 4.2, 4.4 and 4.5. Turner et al. (1979) also reported dual FSH peaks, one 4 to 7 days pre-partum, and one around the day of foaling. The differences between reported FSH secretion and FSH concentrations in the current study are not of concern because they are relatively similar, and the sample size in the current study was small enough so that small changes are not surprising. There was a significant negative correlation between FSH and pregnanes when modeled on a same day basis from 5 days pre-partum to birth ($p=0.0138$). If pregnane concentration were to increase by 100 ng/mL, it is estimated FSH levels would decrease by approximately 3% on a same day basis. Surprisingly, when pregnanes and FSH were analyzed from day of foaling to 10 days post foaling there was a significant positive relationship between the hormones ($p<0.001$). Interestingly, this model estimates that if pregnane levels were to increase 100ng/mL, FSH would also increase by 4.1%. Figures 4.4 and 4.5 give

interesting graphical representations of the negative relationship between total pregnanes and FSH for individual mares. There is an increase in FSH directly after the decrease in pregnanes that occurs around the day of foaling in Figure 4.4. Interestingly, mare 7 (Figure 4.5) had an unexplained drop in pregnane concentrations 9 days pre-partum. Concurrent with the decrease in total pregnanes, there is a corresponding increase in FSH. This seems to demonstrate that there is an approximate same day (+1 to 2 days) negative correlation between FSH and pregnanes in the mare.

Our concentrations of LH and FSH in the peripartum period were significantly higher than levels found by Turner et al. (1979). While their study also noted dual FSH peaks, our FSH levels were approximately one order of magnitude greater. This is most likely the result of different standards used in the assays. These authors also found LH to be at baseline levels pre-partum, to increase gradually before ovulation, and peak approximately 48 hrs post-ovulation. Interestingly, our LH values were also approximately an order of magnitude greater than levels found be Turner et al. (1979). Again, this is most likely the result of standard differences.

Evidently, there is a significant inverse relationship between pregnanes, and LH and FSH pre-partum in the mare. It should be stated that the post-foaling models are not as reliable as the pre-foaling models, because pregnane levels are nearly undetectable (<0.5ng/mL to 4ng/mL post-partum versus >1000ng/mL pre-

partum). While the exact mechanism behind this inverse relationship remains unclear, it appears that the 5α-reduced pregnanes might truly be participating in the down-regulation of the pituitary gonadotropins during pregnancy. This is not unprecedented, in that Gilles and Karavolas (1981) observed that P4, 5α, 20α-hydroxy-4-pregnen-3-one, and αα all down regulted LH and FSH concentrations in the rat *in vivo* after 3 days of E2 treatment and 2 treatments of pregnane. It is clear that P4 has a role in decreasing LH release from the pituitary gland in the ewe (Algubory et al., 1989, DiGregorio et al., 1995, and Girmus and Wise, 1991), as well as many other species (Griffin and Ojeda, 2000). It is unfortunate that the literature is lacking in research related to biologic effects of these pregnanes, for not much is known about their mode of action in any species.

In light of the apparent relationship between total pregnanes and LH, and total pregnanes and FSH, it is curious that there did not appear to be a significant relationship between maximum total pregnane concentration pre-partum and time to ovulation (Figure 4.10, p=0.338). The mares in this study ovulated an average of 14.1 days post-partum, while others report >90% of mares ovulate 7 to 9 days post-partum (Ginther, 1992). This difference may be the result of a comparatively small samples size for the current study (n=8) compared to the previous study (n=40). It is quite possible that other biological activity is influencing time to post-foaling ovulation, that is, from the event of foaling to ovulation it is very likely that more than just pregnane levels, LH, and FSH influence the duration to ovulation. These

data did not take into account other hormone levels known to be responsible for the ovulatory event in the mare, such as estrogen (Pattison et al., 1974). It has been demonstrated that in normally cycling, ovary intact mares, estradiol has a positive effect on circulating LH concentrations, while P4 has a negative effect (Burns et al., 1981). In the rat, E2 priming is mandatory for P4 to down-regulate pituitary gonadotropins (Gilles and Karavolas, 1981). In the mare there is E2 priming *in vivo* because E2 concentrations reach a maximum in the pregnant mare between day 210 and 250, and they are still quite elevated at birth. This estrogen is thought to originate from the fetal gonads (Lovell et al., 1975). These levels decrease immediately post-partum with expulsion of the fetus. More recent *in vitro* work has demonstrated the E2 treated equine pituitary cells in culture secrete significantly higher concentrations of LH than control cells (Baldwin et al., 1991). Perhaps incorporating E2 analysis in the mare would further clarify the relationship between steroid and peptide hormones.

It has been shown in past studies that progesterone can have significantly different physiologic effects during the peripartum period in the mare. For example, Alm at al.(1975) found that mares treated with P4 pre-partum foaled significantly earlier than control mares. Loy et al. (1975) demonstrated that daily administration of P4 post-partum lengthens the duration to ovulation by approximately 5 days and improves fertility at the first ovulation. While data on LH and FSH were not available from this study, some pituitary or hypothalamic

inhibition must be occurring to delay ovulation for this extended amount of time. Perhaps P4 has different effects on the mare during the drastically physiologically separate times of pre versus post-foaling, being first stimulatory and then inhibitory. This may be the result of estrogen levels.

Recall that all pregnanes, except P4, had the same secretion pattern during the last ten days of gestation. The differences in P4 secretion directly pre-partum, when compared to the concentrations of other pregnanes, suggests the possibility of a separate metabolic pathway for P4. Recall that until directly before parturition levels of P4 are consistently baseline (< 0.5 ng/mL). It is unclear why there is a slight increase in P4 before foaling while other pregnanes decrease, but it is not unfeasible that either the maternal or fetal adrenal gland (or perhaps both) is metabolizing P5 to P4 at an increased rate. Perhaps this P4 then enters the circulatory system of the mare. Meanwhile, the placenta and endometrium are converting P4 and 3β to 5α, which may then undergo reduction by the fetus or endometrium (Schutzer and Holtan, 1996). Another possibility for the difference in P4 secretion directly pre-partum could be the result of initial placental separation from the uterine epithelium. This separation could result in the placenta not reducing P4 further to 5α, leading to increased concentrations of P4 in the mare.

Regardless of the cause, the differential secretion of P4 directly pre-partum could be a clear indication of preparation for foaling. It is interesting to note that in a study performed by Alm et al. (1975) they observed that mares treated with

exogenous P4 foaled an average of 12 days earlier than control mares (n=9). This result has always been considered to be anomalous in light of the classical role of uterine quiescence P4 plays in most mammals (Griffin and Ojeda, 2000). However, perhaps increases in P4 directly pre-partum actually serves as a signal for the onset of labor in the mare.

Although the GC/MS is an excellent tool for analyzing steroid hormones, there is one class of steroids that can not be adequately detected with the method used for this study. The 17-hydroxy pregnanes, such as the corticosteroids, have a hydroxyl group present on carbon 17. Part of the derivatization process involves treating the steroids with tBDMS, a large molecule that can not interact with 17-hydroxy groups because of stearic hindrance. Although there is no indication that these steroids have any potential to participate in pregnancy maintenance, they are secreted by the fetus and therefore an unexplored candidate for participating in the maintenance of pregnancy in the late gestation mare.

Surprisingly, there was a significant difference between the total pregnanes of ponies and light horse mares when analyzed by repeated measures analysis of variance ($p=0.0004$) from day -10 to day of foaling. This is similar to results obtained by Mackie-Baumholtz (1998) who found a difference in total pregnanes between light horse mares, and pony and miniature horse mares. In the current study there was no significance in the type (pony versus light horse) by day

interaction, or in day as a main effect. Therefore, these factors were excluded from the analysis.

5.2 Equine Pituitary Cell Culture

Cultured equine pituitary cells responded to a GnRH stimulus as evidenced by a 70% increase in LH secretion between negative and positive (GnRH treated) control groups (Figure 4.11, p=0.015). The current study had comparable increases in LH after GnRH treatment compared to Baldwin et al (1991), who observed 80% increases in LH after GnRH treatment. It is not as directly apparent that FSH responded to GnRH exposure (Figure 4.12, p=0.113). However, FSH increased 53% from control to GnRH treated cells, so this p-value might be misleading as a result of the first mare having significantly higher FSH values than the subsequent two mares. It was impossible to confirm this mare as an outlier because of the small number of horses used in this study. It is probable that if the content of gonadotropins in the interior of the cells had been analyzed much higher concentrations of gonadotropins would have been discovered. It is also possible that the rate of FSH synthesis and degradation differed from the rate of LH synthesis and degradation. Possibly much of the FSH had already been broken down two hrs after the GnRH stimulus. Other authors have judged pituitary

response to a GnRH stimulus only on the basis of an increase in LH secretion (Frawley and Neill, 1984; Muyan et al., 1993).

The models used for both the LH and FSH analysis included log transformations of LH and FSH concentrations. This was done because of the unequal variance observed among mares for LH and FSH. This is not surprising as the mares were necropsied without regard to stage of cycle. There was a significant cell culture plate effect ($p=0.030$) for both LH and FSH. The plate effect presents no problem for the validity of the study because each plate had a positive and negative control within it. Therefore, all treatments were compared to the positive control within the same plate. There was no treatment effect for LH ($p=0.557$), but there was a treatment effect ($p=0.0058$) and a treatment x mare interaction ($p=0.0027$) for FSH. When analyzed in more detail, it became conclusive that 3β was significantly greater than the positive control ($p=0.043$) when analyzed by 2-tailed distribution paired students t-test. This was likely the cause of the significant treatment and treatment x mare effects. The direct comparison can be made between the control and 3β because this comparison was planned prior to data analysis. It is unclear why 3β resulted in such an increase in FSH secretion in cultured equine pituitary cells compared to the positive control. There does exist a possibility of an artifactual response, or perhaps one of the mares was an outlier. It would be more realistic if P4 also exhibited an increase in FSH in cultured pituitary cells. Further study of 3β is necessary to determine its potency as a P4 agonist.

In general, both LH and FSH increased rather than decreased after pregnane treatment. In numerous experiments in many species, P4 has been shown to inhibit ovulation in the presence of E2 *in vivo*. Perhaps in order for P4 to have a significant effect modulating LH and FSH secretion from the anterior pituitary gland E2 must be present. Alternatively, perhaps the culture conditions in this study were inappropriate for observing a pregnane treatment effect. This study used published values of GnRH for equine pituitary cell culture stimulation (Baldwin et al., 1991 and Muyan et al., 1993). However, perhaps P4 concentrations, although physiologic, were too low. It is interesting to note that Gilles and Karavolas (1981) administered two treatments of pregnanes in order to see a decrease in LH and FSH in rats *in vivo*. The first treatment actually resulted in an increase in LH and FSH upon GnRH stimulation, while the second treatment decreased levels to below baseline concentrations. In the current study, perhaps a subsequent pregnane treatment, along with E2 priming and a 24 hour incubation period would have resulted in a significant LH and FSH decrease upon GnRH stimulation.

It is clear that E2 and E1 can increase LH release when stimulated with GnRH in cultured mare pituitary cells (Baldwin et al., 1991). This is in agreement with other species such as the rat (Drouin et al., 1976), the sheep (Huang and Miller, 1980) the cow (Padmanabhan et al., 1978), and the monkey (Frawley and Neill, 1984). It is logical therefore to assume that the pituitary is an important site for negative, as well as positive, feedback by steroids in the mare. Because it is

apparent that pituitary secretions are being inhibited throughout gestation in the mare, we hypothesized that progestins were responsible for this negative feedback. We observed a negative correlation between LH and total pregnanes pre and post-partum, and a negative pre-partum and positive post-partum correlation between FSH and total pregnanes. The *in vivo* data is contradictory to the *in vitro* cell culture data, and perhaps the presence of E2 is the key difference between the two studies.

There is evidence in other species that P4 has a negative effect on LH concentrations. For example, Al-Gubory et al. (1989) reported that intact ewes had significantly lower LH 5 to 20 days post-partum than ewes who had the CL removed at day 70 of pregnancy. This indicates that there was not sufficient circulating P4 to inhibit pituitary LH secretion. Interestingly, there was no difference in FSH concentrations between these two groups. Another study done by Schoenemann et al. (1983) in the cow indicated that P4 had an inhibitory effect on gonadotropin release, but it occurred at the level of the hypothalamus not the anterior pituitary gland. Consequently, this may be a problem with the current data. These authors found that cows treated with E2 had increased LH secretion from the pituitary gland, and cows treated with E2 and P4 had decreased GnRH pulses, as well as decreased LH concentrations when compared to cows treated with only a vehicle. This information could be pertinent in the mare, because as of yet, no P4 receptors have been localized in the mare pituitary gland. Perhaps the pituitary

gland in the mare is the site of E2's positive feedback, and the hypothalamus is the site of P4's negative feedback. This is different than the rat because no changes in GnRH secretion from the hypothalamus were observed after treatment with P4, 5α, 20α and $\alpha\beta$ (Gilles and Karavolas, 1981).

5.3 Uterine Myometrial Studies

Treatment of mare myometrium with certain pregnanes did not significantly alter ($p > 0.05$) frequency or amplitude of contractions. It should be immediately stated that there was a huge amount of biologic variability in the tissue used, and the repeatability of the current study is in question. In preliminary studies, we found that cat, llama, cow and mouse uterine tissue contract spontaneously and consistently without first being treated with OT. Unfortunately, not only did mare myometrial tissue not contract without an OT stimulus, but it also showed a considerable amount of variability. Tissue samples that appeared to be identical and that were taken from the same locale from the same mare exhibited remarkable variability in the presence of oxytocin. Ousey et al. (1998) experienced similar difficulties in a nearly identical experiment (personal communication, July, 2002). Physiologic concentrations of oxytocin were not always sufficient to induce spontaneous contractions in mare myometrium. Therefore, although the problems with this technique are obvious, we elected to use the minimal concentration of

oxytocin necessary to induce spontaneous contractions in each individual piece of tissue. Perhaps if tissue response to oxytocin was more consistent, we would have obtained a more consistent result.

Our results are surprising in light of results obtained by similar studies on other species. Vallet et al (1990) observed a significant decrease in oxytocin receptors *in vivo* in ewe myometrium that had been pretreated with P4. This rendered the tissue nearly unresponsive to OT. Juberg et al (1991) observed a decrease in response of rat myometrium to oxytocin when treated with P4 alone, but this effect was not observed when treated with P4 and the synthetic estrogen, DDT. Perhaps one of the problems with the current study, and the study performed by Ousey et al. (1998) was the amount of time the tissue was observed for a response. Progesterone is generally thought to act through a genomic receptor, and it would not be uncommon for a response to take up to 24 hrs. However, in light of other studies it is apparent in the rat uterus (Grazzini et al., 1998) and the ewe ovary (Dunlap, 2002) that P4 acts by interfering with OT receptor action. However, if P4 has a different mode of action in the mare than in the rat and ewe, this might explain the nebulous results obtained by this study. Unfortunately, due to the difficulties with maintaining consistent contractions in myometrial tissue in this study, it would be difficult to study the tissue reliably using this method over an extended period of time.

It is unlikely that the inability of P4 and the other metabolites to inhibit uterine contractions in the mare in the presence of oxytocin is a result of the absence of P4 receptors in the equine myometrium. Chavatte-Palmer et al. (2000) demonstrated that non-pregnant mares in various stages of the estrous cycle had 150-250 fmol/mg protein P4 receptors in homogenized uterine tissue. These authors did not distinguish between endometrium and myometrium however, so the final conclusions as to the implications of this study are limited. Regardless, the inability of P4 and metabolites to modulate uterine responses to oxytocin is most likely not due to the inability of P4 to bind to a receptor, but either to P4 having no ability to modulate uterine contractions in mare myometrium, or to faulty experimental design. In light of the clear effect of P4 modulating OT response in many mammals, it would be unlikely that in the mare, P4 had no ability to decrease OT induced myometrial contractions.

Ousey et al. (1998) reported similar results to this study during a nearly identical experiment; no pregnanes reduced the contractility of the tissue. The experiment was repeated in this study because we thought it unlikely that P4 would have no effect on oxytocin induced contractions in the mare. These authors make no mention of differential responses of tissue to oxytocin. This could perhaps be because these authors used pregnant mares, between day 68 and 340 of gestation. In the current study mares were necropsied without regard to stage of estrous cycle. Stull and Evans (1997) researched the changes in concentration of oxytocin

receptors throughout the estrous cycle in normal cycling mares. They found that the number of oxytocin binding sites was greater in the myometrium than the endometrium, and that the receptor concentration was greater during days 14 to 17 post-ovulation compared to days 2 to 5, 6 to 13 and 18 to 1 post ovulation. There is little data published indicating variation in oxytocin receptor concentrations in pregnant mares. Perhaps the differential response of myometrial tissue to oxytocin is a result of low oxytocin receptor concentration throughout certain days of the cycle.

It is interesting to note that at least qualitatively, *in vivo* myometrial contractions during pregnancy appear to be correlated to the time when pregnane concentrations would be changing. Haluska et al. (1987b) recorded electromyographic readings from mares beginning 7 days pre-partum, through parturition and the early post-partum period. These authors found that activity increased for 7 to 13 hrs 24 hrs before delivery. This approximately corresponds to the period where pregnane levels are decreasing before the onset of labor. It would be fascinating to repeat this study with a larger group of mares, while taking blood or fecal samples to monitor pregnane levels during this time period.

Another interesting aspect of this study was the results obtained when we attempted to perform a myometrial study on a mare euthanized in December. This mare was in winter anestrous and had no follicular activity. The myometrial tissue did not exhibit an ability to respond to OT, that is, it appeared to lack consistent

uterine tone and could not contract in response to an OT stimulus. We hypothesize that estrogen is necessary to "prime" the uterus. More than likely, E2 acts to indirectly up-regulate OT receptors in the myometrium of the mare because it is thought to directly increase PGF2α concentrations. The positive feedback mechanism between PGF2α and OT is well documented in the mare (Ginther et al., 1967).

It is interesting to observe that even ethanol treated tissue seemed to show a slight change in response after treatment (Figures 4.11 and 4.12). We observed that even slight agitation of the tissue bath associated with applying the treatment could cause a change in the frequency or amplitude of the contraction. As a result, all statistical analysis involved comparing the responses of post-treated pregnane groups to that of the post-treated ethanol group. This compensates for the artifactual treatment response of the tissue to the physical act of treating it.

CHAPTER 6: CONCLUSIONS AND FUTURE STUDIES

6.1 Conclusions

It is evident from these results that the 5α-reduced pregnanes play a significant role in gonadotropin inhibition pre-partum. Although it appears that there is also a significant relationship between pregnanes and gonadotropins post-partum, it must be slightly discounted as a result of the very low levels of pregnanes at that time. The absence of a correlation of peak pregnane concentrations and time to ovulation is surprising and most likely explained by the involvement of other factors in the ovulatory event.

The *in vitro* work performed was less conclusive than the *in vivo* work. There appeared to be no pregnane effect on GnRH stimulated LH and FSH secretion in equine pituitary cells in culture. It is feasible that pregnanes do regulate LH and FSH secretion, but it may occur at the level of the hypothalamus. That is, pregnanes may regulate GnRH pulses. There also appeared to be no pregnane effect on OT stimulated myometrial contractions. This result is more nebulous. Perhaps this study would be clearer if greater consistency in the reaction of tissue to oxytocin could be achieved. It is likely that P4 and the other pregnanes regulate uterine contractility in the mare. The phenomena of a slight increase in P4 while the 5α pregnanes decrease directly pre-partum suggests a very complex relationship between pregnanes in the late gestation mare. Perhaps the 5α-

pregnanes have a seperate hormonal action from that of P4. For example, 5α-pregnanes inhibit myometrial contractility, and the increase in P4 directly pre-partum could prepare the uterus for the onset of labor.

If pregnanes truly do modulate gonadotropin concentrations *in vivo* in the mid and late gestation mare it is good evidence that these P4 metabolites play a large role in maintaining pregnancy in the mare. It would also be further evidence to suggest that supplementing mares with Regumate® throughout gestation could be beneficial in maintaining pregnancy. However, the *in vitro* results suggest pregnanes do not decrease the myometrial response to OT, or decrease gonadotropin secretion from the anterior pituitary gland in the mare. If these results are repeatable it would indicate that costly supplementation of problem mares with Regumate® is unnecessary. It should be noted however that the *in vitro* models are not an accurate description of the complex series of events that occurs during gestation in the mare.

6.2 Future Studies

It would be interesting to further study the cause, as well as the biologic effect of the increase in P4 directly pre-partum. It also would be interesting to perform P4-receptor localization studies in the anterior pituitary gland as well as the hypothalamus of the mare to research if they are present. If P4 receptors are present in the pituitary gland it is evidence that pregnanes truly do not decrease

gonadotropin secretion in the mare. However, if P4 receptors are found in the hypothalamus, it would be a good indication that the P4 effect is actually "upstream" of the pituitary gland. That is, P4 could possibly modulate GnRH release from the hypothalamus. After developing a more reliable model, further studies clarifying the role of the 5α-reduced pregnanes, as well as P4, in uterine quisence in the mare, should also be performed.

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