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Biology and Detection of Pregnanes During Late Gestation in the Mare

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Biology and Detection of Pregnanes During Late Gestation in the Mare

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THESIS

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A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By:

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Lexington, Kentucky

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ABSTRACT OF THESIS

BIOLOGY AND DETECTION OF PREGNANES DURING LATE GESTATION IN
THE MARE

Progesterone in the mare declines to almost undetectable concentrations in late gestation.

It's metabolized into several pregnanes, some circulating at very high concentrations.

Although the function of many pregnanes remains unclear, 5α-dihydroprogesterone and

allopregnanolone are bioactive. Measurements of pregnanes in late gestation are

typically by immunoassay, although results are confounded by cross-reactivity with

related pregnanes. Conversely, liquid chromatography tandem mass spectrometry (LC-

MS/MS) allows differentiation of individual pregnanes. The purposes of these studies

were: 1) to evaluate the ability of a 5α-reductase inhibitor, dutasteride, to alter pregnane

metabolism and pregnancy outcome, 2) to evaluate changes in target pregnanes in late

gestation by LC-MS/MS in mares with ascending placentitis, and 3) compare

immunoassay and LC-MS/MS detection of pregnanes in late gestation. Our findings

suggest that dutasteride significantly altered pregnane metabolism without effects on

pregnancy outcome. Pregnane measurement by LC-MS/MS resulted in a significant

($p<0.05$) increase in pregnanes metabolized in the placenta, but not those coming directly

from the fetus in mares with placentitis. Results indicate pregnane profiles of mares with

chronic placentitis mirror those of healthy pregnancies prior to parturition. A comparison of immunoassays demonstrated significant (p<0.05) differences in assay results, while correlation was observed between immunoassay measurements and actual progesterone concentrations by LC-MS/MS. These studies demonstrate the complexity of pregnane metabolism in late gestation in the mare and the necessity of LC-MS/MS to detect specific changes that immunoassays cannot differentiate.

KEYWORDS: Mare, Pregnanes, LC-MS/MS, Placentitis, Immunoassay

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Michelle Arelia Ann Wynn
6-1-2017

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BIOLOGY AND DETECTION OF PREGNANES DURING LATE GESTATION IN

THE MARE

By

Michelle Arelia Ann Wynn

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<u>6-1-2017</u>

I would like to dedicate this to my husband and son, Dave and Sam. I will never be able to replace our sacrificed time together, but the pursuit of this degree was with a bright future for all of us in mind. Sam, no dream is too big if you have the heart to follow it. May you always have the heart and ambition to realize your dreams. This is also for Dr. Silvia. My bricks were laid after you passed, but your excitement and passion for discovery was a driving force that led me to research and I consider myself lucky to have known you.

"The purpose of research is not to discover the next big thing. The purpose of research is to add another brick in the wall of knowledge."

-Dr. Bill Silvia

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Chapter One: Introduction and objectives

Late gestation in the mare is unique in relation to most other mammalian species, which rely on progesterone (P4) for pregnancy maintenance throughout gestation [1]. Progesterone in the latter half of gestation is at concentrations of <1.0 ng/mL in the mare [2, 3]. P4 is metabolized into several pregnanes in the placenta [4] which can be measured in the periphery of the mare's circulation.

Changes in total pregnanes by immunoassay have been studied to indicate possible issues with the fetus or placenta [5, 6], and immunoassays have been a useful tool in diagnosing pregnancy complications. However, these assays are most often used in late gestation (>300 days) when pregnancy compromise could result in an aborted fetus or complications for the mare. Due to the time in which they are used, cross-reactions are problematic [7]. The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) or gas chromatography mass spectrometry (GC-MS) has allowed the study of individual pregnanes throughout gestation in healthy and compromised equine pregnancies and assay results cannot give information beyond changes in total pregnane concentrations. Further, multiple P4 antibodies are available for use in assays, and discrepancies among results demonstrate a need for a standard method in hormone measurement in the mare [8].

The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) or gas chromatography mass spectrometry (GC-MS) has allowed the study of individual pregnanes throughout gestation in the healthy and compromised equine pregnancy [9-12]. In addition to individual pregnane measurement, tissue incubations have demonstrated

that at least three pregnanes, 5α-dihydroprogesterone (DHP), 20α-hydroxy-5α-pregnan-3-one (20α5P), and 5α-pregnane-3β,20α-diol (βα-diol) bind the equine progesterone receptor [9, 12, 13]. This suggests their bioactivity in late gestation when P4 is nearly undetectable. Allopregnanolone has also demonstrated bioactivity, acting through GABAA receptors to maintain fetal quiescence [14, 15]. Other quantitatively important pregnanes include 3β-hydroxy-5α-pregnan-20-one (3β5P) and 5α-pregnane-3β,20β-diol (ββ-diol), and these can reach concentrations of approximately 30-300 ng/mL in late gestation [11].

Studies attempting to inhibit enzyme activity, necessary for pregnane metabolism, have benefited from the ability to measure specific pregnanes and report changes associated with enzyme disruption [16, 17]. Additionally, GC-MS has been used to identify changes in individual pregnanes, as they relate to pregnancy disease or other pregnancy complications from clinical cases [11]. To date, however, establishment of changes in pregnanes which may be associated with a specific pregnancy complication has not been reported in a controlled experiment.

The objectives for the studies in this thesis are as follows; 1) to investigate the effects of a type 1 5α-reductase inhibitor (dutasteride) on pregnane metabolism and pregnancy outcome in a set of healthy mares in late gestation, 2) to identify changes in a set of target pregnanes (P5, P4, DHP, allopregnanolone, 20α5P, 3β5P, βα-diol, and ββ-diol) from mares with experimentally induced ascending placentitis compared to a group of mares with normal pregnancies 3) to compare results from multiple progesterone immunoassays to actual P4 and DHP results by LC-MS/MS, and 4) to evaluate cross-reactions of P4, P5, DHP, allopregnanolone, and altrenogest with multiple immunoassays.

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Chapter two: Literature review

This review will focus on pregnanes (steroid hormones within the progesterone family) and progestogens (21-carbon, steroid hormones that have progesterone-like activity and are able to bind progesterone receptors) with an emphasis on normal and abnormal profiles in late gestation in the mare. A healthy pregnancy in the mare is dependent on a number of factors; one of them being the production and equilibration of steroid hormones, including pregnanes [10, 18]. During the typical 340 day gestation (range 320-360 days) [19], a unique pregnane profile has been characterized in the mare [10]. The best known pregnane, progesterone (P4), begins to rise shortly after ovulation [20], and continues to rise until approximately 80 days of gestation [10]. However, a healthy pregnancy in the mare does not depend upon P4 alone [12, 16]. In fact, P4 levels begin to decline around 100 days of gestation and are often undetectable in late gestation [2, 3, 10]. During this time, other pregnanes increase in concentration, including 5α-dihydroprogesterone (DHP) and allopregnanolone which are believed to play a role in pregnancy maintenance [9, 12, 15]. Production of these pregnanes ultimately depends on a number of enzymes in the feto-placental unit [4, 21]. In cases of compromised pregnancies, pregnane concentrations may be altered, making progesterone assays a possible diagnostic tool [7]. However, the most common P4 assay, the enzyme linked immunosorbent assay (ELISA), fails to give specific information on individual pregnanes because there is a high incidence of cross-reactivity between different pregnanes within these assays [22]. Therefore, results from liquid chromatography tandem mass spectrometry (LC-MS/MS) or gas chromatography mass spectrometry (GS/MS) are

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useful to better understand changes in specific pregnane in both normal and abnormal pregnancy [11].

Pregnanes in late gestation in healthy pregnancies

Measurable pregnanes and progestogens in the mare's blood are the products of a complex relationship with the fetus and the utero-placental tissues, commonly called the feto-placental unit [4]. Pregnenolone (P5) is the primary precursor for downstream pregnanes [23, 24], and P5 is produced by the fetus. Though some speculation exists regarding its exact origin [25], P5 is believed to be primarily synthesized within the fetal adrenal cortex with likely contributions from the fetal gonad. It is known that P5 is synthesized from cholesterol by cytochrome P450scc, and immunostaining for P450scc in the equine fetal adrenal gland indicated localization in the zona glomerulosa, zona reticularis, and zona fasciculata of the adrenal cortex, with increasing abundance through gestation [26]. Further evidence for the production of P5 in the fetal adrenal cortex is the increase in maternal pregnane concentrations with intra-fetal administration of ACTH [27]. Early work from MacArthur et al., suggested the presence of P450scc in the fetal gonad by incubating the testis with sodium acetate in vitro, ultimately observing androgens dependent on the enzyme [28]. However, studies in which fetal gonads were removed during pregnancy did not yield changes to concentrations in maternal pregnanes by immunoassay [29], leading to the suggestion that the fetal adrenal gland is the primary source for P5 synthesis.

Pregnenolone concentrations rise steadily from approximately 0.5 ng/mL in week 18, peaking at approximately 3.4 ng/mL by week 30 in the mare's circulation, then steadily

decrease [10]. Though concentrations are low in maternal circulation, Ousey et al., demonstrated the uptake of P5 into uteroplacental tissue from the fetus by measuring blood flow and P5 concentrations to and from the fetus and placenta via the umbilical artery and vein [4]. Results indicated concentrations of approximately 5-10 μmol/min flowing from the fetus to the uteroplacental tissue, while less than 1 μmol/min of P5 entered maternal circulation from the uteroplacental tissue, with concentrations rising throughout gestation. This uptake from the utero-placental tissue, with low output into the mare's circulation, suggests P5 is metabolized in the placenta and serves as a precursor for downstream pregnanes.

While the majority of P5 is directed to the placenta for metabolism (Figure 2.1) [3], fetal P5 metabolism results in pregnanes such as 3β-hydroxy-5α-pregnan-20-one (3β5P), 5-pregnene-3β,20β-diol (P5ββ), and 5a-pregnane-3β,20β-diol (ββ-diol) [30]. In vitro work showed that the fetal liver possesses the ability to metabolize P5 to P5ββ and ββ-diol by the enzyme 20β-reductase [30]. Further work by Schutzer and Holtan also showed conversion of P5 to P5ββ and ββ-diol in the fetal liver with tissue incubations, as well as conversion of 3β5P to ββ-diol [24]. These pregnanes are preferentially directed to the uteroplacental tissue, although they are also present in the mare's circulation [4], with maternal concentrations of 3β5P, P5ββ, and ββ-diol reaching 100 ng/mL, 10 ng/mL, and 100 ng/mL, respectively, in late gestation (326-350 days) [11]. The fetus is also capable of converting placental metabolites, delivered to the fetus by the umbilical vein; 5α-dihydroprogesterone (DHP) is the progestogen which enters fetal circulation in the largest quantities, while P4 has also been shown to do the same but in smaller concentrations [4].

Of the metabolites of P5, P4 is the hormone most commonly associated with pregnancy. However, the mare possesses a number of progestogens and pregnanes which allow the continuation of pregnancy in late gestation in the absence of circulating P4. Schutzer demonstrated evidence of this when he inhibited 3β-hydroxysteroid-dehydrogenase (3β-HSD) in pregnant mares in mid-gestation. Three β-hydroxysteroid-dehydrogenase is a placental enzyme necessary for the conversion of P5 to P4 in the placenta. Inhibition of 3β-HSD increased P5 and the fetal metabolites (3β5P, P5ββ, and ββ-diol), with a decrease in P4 metabolites (P4 was unmeasurable in this study). Inhibition of 3β-HSD did not result in abortion with the presumed decrease in P4 production, leading to the presumption that other pregnanes must be bioactive in mid to late gestation in the place of P4 [16].

Progesterone itself begins to decline rather early in gestation. The peak of P4 concentrations can be seen by approximately 12 weeks of gestation with peak concentrations of approximately 11 ng/mL [10]. Progesterone begins to decline to concentrations of ≤1 ng/mL [2, 3, 10] throughout late gestation. In early pregnancy, P4 is essential to maintain myometrial relaxation [1, 31-33] by reducing the number of gap junctions and receptors for oxytocin and prostaglandin F2α. Though circulating P4 can be below detectible levels in late gestation, this is not to say it ceases to be produced. Production of P4 continues throughout gestation; however, it becomes a major substrate for other pregnanes which are metabolized in the placental tissue, and is therefore not easily detected in the maternal circulation [4].

Perhaps the most notable metabolite of P4 is DHP. This progestogen is converted by the enzyme 5α-reductase, primarily found in the placenta and endometrium of the mare [12,

23]. This progestogen increases from approximately 1.5 ng/mL in the early weeks of pregnancy and peaks at approximately 37 ng/mL shortly before parturition [10]. It parallels P4 concentrations by less than half, then surpasses P4 concentrations by approximately fifteen weeks of gestation [10, 12]. Fifteen weeks is also the time that marks the luteo-placental shift, in which the primary site of P4 production ceases to be the corpus luteum, and placental metabolism takes over [10]. Until relatively recently, there was only speculation that DHP was a bioactive progestogen [10, 12]. However, work by Scholtz et al., showed that DHP is not only a quantitatively important P4 metabolite, but also sufficient for the maintenance of pregnancy in the absence of P4 [12]. DHP binds the progesterone receptor (PR) with similar affinity as P4 [13], suggesting that DHP could function in late gestation to maintain myometrial relaxation. Administration of DHP to a group of early pregnant mares which had undergone luteolysis via administration of PGF2α demonstrated that DHP alone could maintain pregnancy with seven of the nine mares with pregnancy maintenance without P4. This work was central to understanding how equine pregnancy can be maintained in late gestation when P4 concentrations are often undetectable.

Other bioactive progestogens include allopregnanolone and potentially 20α-hydroxy-5α-pregnan-3-one (20α5P) and 5α-pregnane-3β,20α-diol (βα-diol) [9, 14, 15]. Allopregnanolone, a direct DHP metabolite converted by the enzyme 3α-hydroxysteroid dehydrogenase (3αHSD) [14] is neuroactive, having the capability of crossing the blood-brain barrier and allosterically binding GABA$_A$ receptors [14, 29]. This elicits a calming response on the central nervous system and is considered a protective hormone [34]. Allopregnanolone suppression has been linked to cell death in the brain as well as

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delayed myelination of white matter, [28] indicating its importance to brain development. Further, allopregnanolone is credited for suppressing the activation of the hypothalamic-pituitary-axis (HPA) of the dam and reducing fetal exposure to maternal glucocorticoids [35, 36]. In an in-vivo study by Madigan et al., allopregnanolone infusion in a healthy foal resulted in behavioral suppression [15]. This gives evidence of the possible role allopregnanolone plays in fetal quiescence during mid-to late gestation, when concentrations rise. Allopregnanolone concentrations rise from approximately 1 ng/mL in week nine of gestation, to approximately 20 ng/mL near parturition [10].

In vitro work has demonstrated the possible bioactivity of 20α5P and βα-diol [9]. Identified by Holtan in 1991 [3], these metabolites of DHP both reach concentrations greater than 200 ng/mL in late gestation. They were found to be able to bind PRs using a luciferase reporter assay [9], although they did so at less than half the affinity of P4. Potential exists for these metabolites to elicit a response in later gestation; however, they are not considered key players in pregnancy maintenance. Concentrations mirror DHP, increasing by 15 weeks to approximately 300 ng/mL for 20α5P and approximately 480 ng/mL for βα-diol by week 50 [10].

Other quantitatively important pregnanes include 20α-hydroxyprogesterone (20αOH-P4), 20β-hydroxyprogesterone (20βOH-P4), and 17α-hydroxyprogesterone [10, 11, 18]. With the exception of 17α-hydroxyprogesterone, which is a necessary metabolite leading to the production of cortisol [37, 38]; little is known about the function of these pregnanes in the mare. These P4 metabolites are synthesized in the placenta and available in maternal circulation. Concentrations for these pregnanes vary in the times in which they peak,

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with 17α-hydroxyprogesterone peaking in week 8 of gestation [10], while 20αOH-P4 and 20βOH-P4 are not detectible until after 300 days of gestation [11].

Parturition in the mare is marked, in part, by a distinct and reliable rise and decline in circulating pregnanes, along with production of fetal cortisol [3, 9, 23, 39, 40]. This pattern is linked to the activity of the fetal adrenal, the gland responsible for the production of P5. It is thought that activation of the hypothalamic-pituitary axis (HPA) is responsible for the surge in circulating pregnanes found in the mare's circulation [18]. Previous studies show that administering ACTH to the fetus results in increased pregnanes in the mare's circulation before 300 days of gestation. After 300 days, a progressive shift from P5 production to cortisol production in the fetal adrenal occurs [41, 42]. Approximately 4-5 days before parturition, there is a surge in fetal cortisol [18, 43]. The enzyme necessary for the synthesis of cortisol, $P450_{C17}$, has been shown to be present in the fetal adrenal by immunohistochemistry [26]. More importantly, immunohistochemical staining was very limited in early gestation with increases noted rapidly prior to parturition. This shows that the fetal adrenal is likely producing cortisol just prior to parturition, in agreement with the surge of cortisol observed. Thus, with the shift in production of cortisol, a decrease in pregnanes can be seen.

Pregnanes in late gestation in the compromised pregnancy

Pregnane profiles are frequently used as a diagnostic tool for potentially compromised pregnancies in late gestation [6, 7, 44]. When taken in account with other clinical signs, these profiles can provide information regarding the health of the fetus and placenta. As has been previously discussed, the fetus and placenta work together to produce pregnanes

found in circulating maternal blood. Therefore, abnormal changes can be attributed to disturbances to the normal synthetic pathway for these pregnanes. Typically, these patterns are a measure of total pregnanes by immunoassay, with three main patterns common in cases of abnormal pregnancy [5].

The first pattern is identified by a rapid decline in total pregnanes. This pattern is typically seen with acute dysfunction resulting in fetal demise, such as uterine torsion, colic, maternal stress, or acute cases of placentitis [45-47]. Another potential cause is the demise of a single fetus in a twin pregnancy; in this situation, the adjoining area between the placentas is already avillous [48], and when the placenta supporting the deceased fetus necrotizes, a substantial loss in pregnane metabolism occurs [49]. As the fetus is responsible for production of P5, any case in of fetal demise will result in a loss of precursor and therefore a decline in measurable pregnanes in the mare's circulation.

The second pattern includes cases where pregnanes are relatively high for the given stage of gestation. While such elevated concentrations are typically observed just prior to parturition, chronic cases of placentitis, placental edema, and placentas with poorly developed or sparse microvilli can result in this pattern of measured pregnanes [5, 6, 50]. This is believed to be associated with fetal stress which result is an increase in P5 production from the fetal adrenal, ultimately leading to an increase in total pregnanes [27, 41]. Though foals born to mares which display this pattern are often born prematurely, they tend to fair better than those born prematurely to mares which did not display this increase. This is thought to be due to the increased activation of the HPA axis [5, 51].

The third pattern can be observed when the normal prepartum rise in pregnanes fails to occur. This is most often seen in prolonged gestation caused by ingestion of tall fescue containing endophyte fungus [5]. Fescue toxicosis is thought to inhibit fetal cortisol-releasing hormone (CRH) by the ergot alkaloids present in the fescue, therefore inhibiting the normal function of the adrenal gland to produce the cortisol surge and associated changes in pregnane metabolism [8].

Mass spectrometry, either LC-MS/MS or GC/MS, have been used to better understand specific changes related to abnormal pregnancies that would not be discernable with immunoassay. Utilizing GC-MS, Ousey et al., reported changes in ten specific pregnanes (P5, P4, 3β5P, P5ββ, ββ-diol, βα-diol, DHP, 20α5P, 20α-hydroxyprogesterone, and 20β-hydroxy progesterone) from healthy mares compared to a set of mares with clinical issues or placental abnormalities. Mares with pregnancy complications were grouped based upon diagnosis; placentitis, placental pathology without placentitis, and non-placental problems. Results for the placentitis group showed increases in all measured pregnanes, including P4 and P5. This suggested an increase in precursors due to fetal stress and increased metabolites as a result. For the group with placental pathology but no placentitis, (including placental edema and avillous placenta), an increase in total pregnanes was noted with P4, specifically, also increased. Most of the individual pregnanes measured, however, were either in a normal range or decreased. As this group had issues with the placenta, it appears as though the placental function was affected and its ability to metabolize pregnanes was reduced. For the group of mares with problems unrelated to the placenta, (including colic, uterine rupture, uterine torsion, laminitis foot surgery, and a mare with premature mammary development) the majority had a decrease

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in total pregnanes. These mares also showed a decrease in most of the individual pregnanes. These fetuses were significantly compromised or dead, therefore precursor was reduced, or non-existent, with downstream metabolites reduced as well [11]. This study, coupled with the diagnosis, was useful to understand how differential issues with a pregnancy could present as different pregnane profiles. Further work and use of GC-MS or LC-MS/MS in the future may prove beneficial in predicting pregnancy outcome in cases of suspected pregnancy compromise. However, this technology is expensive and requires a skillset beyond that required of immunoassays and will unlikely be used regularly by clinics in the near future.

Attempts to better understand the pathogenesis of diseases and to better understand the function of certain pregnanes, related to feto-placental metabolism have ranged from inducing disease to inhibiting key enzymes. The most studied disease related to the placenta is ascending placentitis, as this represents the major cause of pregnancy loss or complication in late gestation [44, 52]. Ascending placentitis can be experimentally induced by removing the cervical mucus plug and introducing bacteria into the cervix [6, 53]. One of the most common causes of ascending placentitis is the bacteria *Streptococcus equi* subspecies *zooepidemicus* [54], and this bacteria is commonly used in cases of experimentally induced placentitis. The infection establishes itself at the cervical star and ascends down the placenta, causing inflammation and placental separation [52]. By knowing the interval of time from inoculation to abortion, researchers were able to distinguish between acute and chronic pregnane profiles. Morris et al., described a pattern of decreasing plasma pregnanes in cases of acute induced placentitis where the interval from inoculation to abortion was less than seven days;

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conversely, a pattern of increasing pregnanes was observed in chronic cases (>7 days) [6]. Canisso et al., also described this pattern in unpublished reports from experimentally induced placentitis [55].

Enzyme inhibition studies have also been useful to better understand necessary pathways in equine pregnancy. Work to inhibit 3β-HSD, the enzyme responsible for the conversion of P5 to P4, gave evidence that other progestogen or progestogens may be responsible for pregnancy maintenance in late gestation besides P4. Fowden et al., attempted to inhibit the action of 3β-HSD with epostane, a competitive 3β-HSD inhibitor. Late pregnant mares (292-330 days gestation) received epostane, which resulted in a decrease in plasma progesterone concentrations by immunoassay, but no effects on gestation length [43]. Schutzer et al., conducted a similar study utilizing trilostane, another 3β-HSD inhibitor, during late gestation (277-282 days gestation). In this study, GC-MS was used to measure specific pregnanes. While P4 was undetectable during the study, an increase in P5, ββ-diol, and 3β5P was noted in maternal plasma; while DHP, 20α5P, and βα-diol were decreased. Further, trilostane inhibited the conversion of P5 to P4 while increasing DHP and 3β5P concentrations when placental tissue was exposed to labeled P5 or P4. Again, there was no effect of 3β-HSD inhibition on gestation length [16]. Both studies demonstrated the ability of mares to maintain pregnancies in the face of apparent decreases in P4 and its metabolites, as well as emphasizing the unique hormone metabolizing ability of the feto-placental unit. In a third study, Chavatte et al., demonstrated the natural inhibition of 3β-HSD activity caused by pregnanes close to term with an *in vitro* study using placentas from pregnancies spanning 250-320 days of gestation [21]. These results suggest the possibility of the prepartum rise in pregnanes to

inhibit 3β-HSD, thereby affecting pregnane concentrations as they relate to the onset of parturition. These studies, focusing on 3β-HSD activity, demonstrated the importance of the enzyme for pregnane metabolism in the pregnant mare, but were unable to significantly affect gestational outcome. This suggests the importance of other enzymes present during this time of gestation which work together to maintain pregnancy.

Schutzer's work also focused on pregnane synthesis during gestation, and resulted in a proposed alternate pathway for metabolism of DHP beyond the 5α-reduction of P4. Incubation of labeled P5 showed conversion of P5 to 3β5P in endometrial tissue while labeled 3β5P was converted to DHP in placental tissue incubations. Further, infusion of P5 and 3β5P in a pregnant mare both resulted in elevated concentrations of DHP [24]. This suggested the possibility of 5α-reduction of P5 to 3β5P and then 3-oxidation to DHP. However, recent work disputes this pathway by demonstrating an inability of 5α-reductase to metabolize P5 [56]. Taken together, these studies highlight the complexity of pregnane metabolism in the mare.

As DHP has been found to be an important progestogen in late gestation, a study attempting to inhibit the enzyme responsible for its metabolism, 5α-reductase, was conducted. Utilizing the 5α-reductase inhibitor finasteride, Ousey et al., administered the inhibitor to mares beginning at 300 days of gestation with the objective of inhibiting the enzyme and monitoring changes to timing of parturition and changes in pregnane profiles. Results indicated that the treated mares had significantly shorter gestation lengths than the control mares. Further, total pregnanes by immunoassay were elevated in the peripheral circulation of treated mares and P4 was increased as measured by GC-MS; however, no change was seen in DHP or other measured pregnanes [57]. This study

demonstrated the possible effects of finasteride *in vivo* by shortening gestation and altering P4 concentrations. This study also suggested the importance of 5α-reductase in late gestation.

While studies to induce disease and inhibit steroidogenic enzymes have helped us learn more about the pathways and how disease can affect pregnane metabolism, there is still a significant amount of work needed to fully understand the biosynthesis of pregnanes by the equine feto-placental unit.

Methods of detection

Assays have been used to identify and quantify hormones for many years. Despite early difficulties, breakthroughs in sensitivity, specificity, automation, and commercialization have allowed hormone detection to become easier and more accessible [9-11, 17, 58, 59]. Radioimmunoassays (RIA), developed in the sixties [60] allowed the researcher to detect hormones with greater sensitivity and specificity and with greater ease than previous methods. Prior to RIAs, quantification of hormones was a laborious process which required large amounts of sample [61] and were not highly specific [62]. Radioimmunoassays depend on antibodies raised to an antigen of interest and on radioactive signal from a radioisotope, typically iodine-125, which is attached to the antigen of interest and competes for the binding site of the antibody used. A gamma counter then detects the amount of free radiolabeled antigen in the supernatant after incubation with a sample; serum for example. During the early stages of RIAs, non-specificity was a common problem. This was due, in large part, to lack of standardization of reference preparations. Standardization and development of more specific antibodies

improved agreement [63]. While this offered researchers an easier and more reliable form of detection, it came with its own set of issues. This technology required specific equipment and proper disposal of radioactive material. This assay method is still used today, though many labs have stopped using it, in favor of other assay methods.

Non-isotopic immunoassays are common in many clinics and labs, having taken the place of RIAs for hormone detection with no need for special disposal. Like RIAs, this assay method relies on the development of the antibody used to capture the antigen of interest. Detection is achieved with color intensity using enzyme linked immunosorbent assays (ELISA) [11], intensity of light with electrochemiluminescence (ECL) [59], or intensity of fluorescence with enzyme linked fluorescence assays (ELFA) [64] . Multiple methods for detection using the antibodies include: indirect, direct, sandwich, competitive, or some combination of methods to optimally capture the antigen of interest [65]. Measuring pregnanes in the late pregnant mare has been mostly dependent on P4 immunoassays for their ease of use and cost effectiveness. Though P4 is low to undetectable in late gestation, these assays rely on the ability of the progesterone antibody to cross-react with pregnanes that are present in the mare's blood during this time [7, 11]. There are a number of P4 antibodies available, and their ability to cross-react with other pregnanes varies.

Polyclonal antibodies (PABs) tend to cross-react to a higher degree than monoclonal antibodies which target a single epitope. An antigen, P4 in this case, is injected into an animal and the animal's immune system produces antibodies to the foreign P4 [63, 66]. The animal, typically a rabbit, is then bled by exsanguination; however, a larger animal may be used if a large volume of antibody is required. These antibodies tend to be less

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specific as antibodies to all epitopes of the antigen will have been produced and will be present in the blood [67]. Purification and separation of antibodies can be accomplished using columns to help eliminate unwanted antibodies [68]. An advantage of PABs is they take less time to develop, (4-8 weeks) [66] and can use the cross-reactivity to benefit measurements of total pregnanes when certain pregnanes (P4) are in such low concentrations [69]. The obvious disadvantage is they tend to have higher cross-reactions and antibodies will differ from lot to lot.

Monoclonal antibody (MAB) production is more intensive and takes much longer to produce that PABs (3-6 months) [66], but yields a more specific antibody. In 1975, Köhler and Milstein published research that would earn them a Nobel Prize by fusing two cells which could ultimately produce an antibody that could be reproduced from that same line of fused cells [70]. Like PAB development, an animal is injected with the antigen of interest. These antibodies are typically developed in mice and the spleen is harvested, as opposed to blood collection [66]. Antibody-secreting B cells are taken and fused with myeloma cells to produce hybridoma cells. These cells take advantage of the proliferation of cancer cells to be able to produce (clone) unlimited amounts of the same antibody [63, 67]. Further, as individual cells are separated, selection for antibody specificity is possible [67]. These antibodies have the advantage of higher specificity, though MABs may be produced with high cross-reactivity [71], and the ability to produce unlimited amounts of a single antibody lot. Polyclonal and monoclonal antibodies can cross-react to differing degrees, and while this can be advantageous when analyzing total pregnanes, it has potential to cause confusion due to the variations in results.

The comparisons of immunoassay results from lab to lab, or clinic to clinic, can cause discrepancies in interpretation when two or more different antibodies or assays are used to measure target hormones [72, 73]. Further, antibodies used in research are not as regulated as antibodies used in human clinical medicine [74]. For this reason, a reference range of normal values and thresholds for an assay should be established for a given time in gestation when samples would typically be analyzed [75], and interpretations should consider the cross-reactivities of the antibody used. It is also helpful to analyze serial samples from a mare in question, as variations in profiles can be seen even in healthy pregnancies [76], and single samples have not been shown to be diagnostic [47]. Immunoassays have become increasingly commercialized and sample results are available within the same day [58, 59]. Assays have come a long way since the use of bioassays, but an even more reliable method of detection for individual pregnanes can be found in mass spectrometry. Immunoassays will likely continue to dominate as the method of choice due to ease of use and cost. However, GC-MS and LC-MS/MS have become the gold standard for analyte detection.

Unlike immunoassays which depend on the quality and development of the antibody used, GC-MS and LC-MS/MS rely on the mass to charge ratio (mass/charge) of the analyte of interest. Pregnanes are quite similar in structure [65] and therefore cross-react easily with antibodies, but they differ in their mass/charge, and this is the basis for detection with mass spectrometry. Short and Holtan demonstrated that P4 was actually low to undetectable in mid to late gestation [2, 3]. Prior to this finding, immunoassays displayed an increase in what was thought to be P4, but what was actually the total pregnanes able to cross-react with the antibody.

Mass spectrometry requires specialized equipment and expertise to analyze the results and is therefore expensive for typical use in diagnosis with pregnancy complications. However, its use in research has been important to understand changes in pregnanes throughout pregnancy as well as in cases of pregnancy complications [3, 9-12]. Mass spectrometry takes advantage of the separation abilities of chromatography and the mass detection of the individual analyte with the mass spectrometer. Whether GC-MS or LC-MS/MS is used, some basic similarities exist for detection and measurement. A purified sample of analyte is needed for method development and serves as a standard reference. Method development includes selection of the mobile phase, selection of the stationary phase, temperature and pressure settings, and analysis of peaks generated from a detector. The selection of the mobile phase and stationary phase takes into consideration the analyte to be measured, possible interactions with the mobile phase (selected gas or solvent), and the affinity for the analyte in the column [77].

Mass spectrometry, along with gas or liquid chromatography, is beneficial as some analytes will have similar retention times (amount of time it takes for the analyte to elute from the column), and therefore simple chromatography wouldn't be adequate for analyte identification. As the analyte enters the mass spectrometer, it encounters an ionization source and the analyte is ionized. The mass of the analyte after ionization will be noted and a mass range is selected. Selection considers mass observed as well as analyte properties and possible solvent interactions, and all masses outside of that range will be ignored. Once the analyte is ionized, it travels in a vacuum through the mass spectrometer and encounters magnetic or electric fields, typically, which will deflect the particles not of interest based on the mass range. In the case of tandem mass

spectrometry, such as LC-MS/MS, a collision cell then fragments the original analyte to daughter ions which can help to further differentiate analytes which are very similar in mass or structure [65]. Detectors at the end of the spectrometer transforms detected ions to interpretable results displayed as peaks of mass to charge ratios. Once the reference standard has been identified, samples can be analyzed and compared to the standard. Like other assay methods, a standard curve is generated to allow the quantification of the analyte in the sample. In addition to the ability to distinguish between similar analytes, GC-MS and LC-MS/MS allow for detection and quantification of multiple analytes within the same sample [9-11], saving time and sample volume.

The complexity of pregnane metabolism and the changes observed through gestation and in cases of pregnancy issues in the late pregnant mare have been brought to light through the use of GC-MS and LC-MS/MS. Though GC-MS and LC-MS/MS have increased detection specificity and sensitivity, as they can detect concentrations down to 0.1 ng/mL [10], they aren't as affordable or easy to use as immunoassays. Much has been learned through their use, but immunoassays will likely continue to dominate as the method of choice for diagnosis with pregnancy issues in the mare. Reference ranges for individual immunoassays and cross-reactivities with the antibody should always be considered, as well as the time in gestation of the mare, to interpret assay results.

Figure 2.1 Pregnane metabolism in the pregnant mare
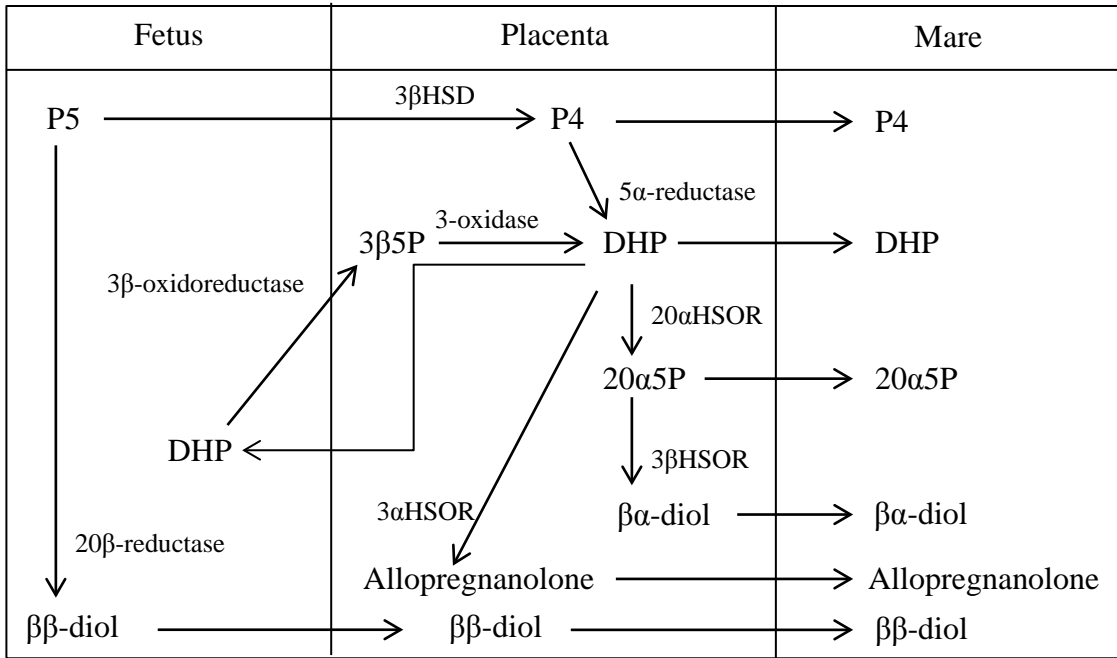
Fetus	Placenta	Mare

P5 —— 3βHSD —→ P4 —————→ P4

P4 —→ 5α-reductase

3β5P —— 3-oxidase —→ DHP —————→ DHP

3β-oxidoreductase

DHP ←———

20αHSOR

20α5P —————→ 20α5P

3βHSOR

3αHSOR

20β-reductase

βα-diol —————→ βα-diol

Allopregnanolone —————→ Allopregnanolone

ββ-diol —————→ ββ-diol —————→ ββ-diol

Pregnenolone (P5), progesterone (P4), 5α-dihydroprogesterone (DHP), 3β-hydroxy-5α-pregnan-20-one (3β5P), 20α-hydroxy-5α-pregnan-3-one (20α5P), 5α-pregnane-3β,20α-diol (βα-diol), and allopregnanolone. Enzymes include 5α-reductase, 3β-oxioreductase, 3-oxidase, 3β-hydroxysteroid dehydrogenase (3βHSD), 3α-hydroxysteroidoxidoreductase (3αHSOR), 20α-hydroxysteroidoxidoreductase (20αHSOR),3β-hydroxysteroidoxidoreductase (3βHSOR). Adapted from Ousey et al., 2003 [4].

Chapter Three: Inhibition of 5α-reductase alters pregnane metabolism during late gestation in the mare

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Abstract

In the latter half of gestation in the mare, progesterone (P4) concentrations decline to near undetectable levels while other pregnanes are elevated. Of these, 5α-dihydroprogesterone (DHP) and allopregnanolone have been reported to have important roles in either pregnancy maintenance or fetal quiescence. During this time, the placenta is necessary for pregnane metabolism, with the enzyme 5α-reductase being required for the conversion of P4 to DHP. The objectives of this study were to assess the effects of a 5α-reductase inhibitor, dutasteride, on pregnane metabolism (pregnenolone (P5), P4, DHP, 20α-hydroxy-5α-pregnan-3-one (20α5P), 5α-pregnane-3β,20α-diol (βα-diol), and allopregnanolone), to determine circulating dutasteride concentrations, and to assess effects of dutasteride treatment on gestational parameters. Pregnant mares (n=5) received dutasteride (0.01 mg/kg/day, IM), and control mares (n=4) received vehicle alone from

300 to 320 days of gestation or until parturition. Concentrations of dutasteride, P5, P4, DHP, 20α5P, βα-diol, and allopregnanolone were evaluated via liquid chromatography tandem mass spectrometry (LC-MS/MS). Samples were analyzed as both days post treatment and as days prepartum, using a random effects mixed model with time, treatment, and time by treatment as fixed effects and mare as random effect. Gestational data and neonatal outcome were analyzed with a Wilcoxon signed-rank test. No significant treatment effects were detected in P5, DHP, 20α5P, βα-diol, or allopregnanolone for either analysis; however, P4 concentrations were increased ($p<0.05$) in dutasteride-treated mares compared to control mares. Dutasteride concentrations increased in the treated mares, with a significant correlation ($p<0.05$) between dutasteride concentrations and P5 or P4 concentrations. Gestational length and neonatal outcomes were not significantly altered in dutasteride-treated mares; however, placentas from treated mares were significantly heavier ($p<0.05$) than the placentas from control mares. Results from this study suggest the ability of dutasteride to inhibit pregnane metabolism as P4 concentrations were altered. Although 5α-reduced metabolites were unchanged, these data suggest an accumulation of P4 precursor with inhibition of 5α-reductase.

Keywords: 5α-reductase, pregnanes, mare, LC-MS/MS

Introduction

Progesterone (P4) is the primary hormone for pregnancy maintenance in most mammalian species [1]. Acting through the progesterone receptor (PR), P4 maintains myometrial quiescence and prevents premature parturition [1, 78]. In contrast to other species, the mare has little detectable P4 in peripheral blood after mid-gestation [2-4, 12].

Although P4 levels rise dramatically during early pregnancy, analysis via mass spectrometry indicates that P4 concentrations begin to decline around 80 days of gestation and are undetectable by day 200 [10, 12]. Several pregnanes are present in high concentrations at this time, including 5α-dihydroprogesterone (DHP), 20α-hydroxy-5α-pregnan-3-one (20α5P), 5α-pregnane-3β,20α-diol (βα-diol), and allopregnanolone [4, 9, 10, 30] (Table 3.1). Specifically, DHP is a direct metabolite of P4 and is known to bind the PR and maintain pregnancy in the absence of P4 [1, 12, 13]. It is believed DHP acts through the PR to block myometrial activity, thereby maintaining pregnancy [30, 79].

The key enzyme responsible for the conversion of P4 to DHP is 5α-reductase (Figure 3.1). Of the two isoforms known to be present in the mare, 5α-reductase type 1 is the predominant isoform in the endometrium and chorioallantois [12]. Once 5α-reductase has metabolized P4 to DHP, DHP can be further metabolized into other pregnanes including allopregnanolone. This hormone is a neuroactive steroid capable of binding $GABA_A$ receptors to enhance inhibitory neurotransmission [80] and is believed to have a sedative-like effect on the fetus [15]. In humans, horses, and sheep, peripheral concentrations of allopregnanolone increase in both the maternal and fetal circulation, with levels peaking near term [9, 10, 81, 82]. Other DHP metabolites include 20α5P and βα-diol. The formation of these pregnanes is dependent upon enzyme metabolism within the uteroplacental tissue, and relative concentrations increase dramatically in mid to late gestation, similar to DHP [10]. These pregnanes are of interest as they have been shown to bind PR *in vitro* [9], and they rely on P4 and DHP as steroidogenic precursors.

To demonstrate the importance of 5α-reductase on pregnane metabolism and pregnancy health, we evaluated the effects of dutasteride, a type-1 5α-reductase inhibitor.

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Specifically, pregnane metabolism was monitored by measuring concentrations of specific pregnanes, and pregnancy outcome was evaluated in mares. The hypothesis of this study was that administration of dutasteride to late pregnant mares would result in a decrease in DHP, 20α5P, βα-diol, and allopregnanolone. With a decrease in these bioactive pregnanes, we further hypothesized a reduced gestational length from mares administered dutasteride when compared to control mares. The objectives for the current study were to assess changes in target pregnanes (P4, P5, DHP, 20α5P, βα-diol, and allopregnanolone) and neonatal outcome in pregnant mares treated with dutasteride compared to controls. Concentrations of dutasteride in the maternal and neonatal circulation, neonatal and placental weight, and neonatal viability were also determined.

Materials and Methods

Animal husbandry

Nine healthy pregnant mares with a mean age of 9.7 yr. ($\pm$ 1.2 yr.) were used in this study. Mares were maintained at the Maine Chance Farm, Department of Veterinary Science, University of Kentucky, Lexington, KY, and all experimental protocols were approved by the Institutional Animal Use and Care committee at the University of Kentucky (Protocol #2012-1067). Gestational age was determined relative to day of ovulation (Day 0). Pregnant mares were maintained on pasture and supplemented with grain and water, hay, salt, and trace minerals ad libitum. When parturition was suspected via determination of milk pH [83], mares were moved to individual box stalls for the night to monitor parturition.

Study design

Pregnant mares were randomly assigned to treatment (n=5) or (n=4) control groups. Beginning on day 298 of gestation, jugular venous blood samples were taken daily in 10-mL heparinized tubes and 10-mL plain tubes (Becton, Dickinson and Company, Franklin Lakes, NJ), and blood sampling continued through two days postpartum. Blood samples were centrifuged at $1,811 \times$ g for ten minutes, and plasma and serum were stored at -20 °C until analysis. Beginning at day 300 of gestation, treated mares received dutasteride (Aurum Pharmaceuticals; Franklin Park, NJ) at 0.01mg/kg body weight, IM, in an ethanol/mygliol (Fisher Scientific, Hampton, NH; Warner Graham Company, Cockeysville, MD) vehicle (60:40, ethanol: mygliol), and control mares received vehicle alone. Dutasteride or vehicle control administration continued daily until day 320 of gestation, or until parturition. Upon mammary development, milk samples were monitored for pH, to assess impending parturition. When milk pH neared 7.0, a FOAL ALERT sensor (Foalalert. Inc., Acworth, GA) was sutured to the vulva, per manufacturer's instructions. Immediately after parturition, blood was collected from the neonate and mare via jugular venipuncture. After foaling, weights of the neonate, mare, and placenta were recorded.

Hormone analysis

Plasma concentrations of P5, P4, DHP, 20α5P, βα-diol, and allopregnanolone were determined via liquid chromatography-tandem mass spectrometry (LC/MS-MS). Samples were analyzed every other day beginning at day 298 of gestation and then daily for nine days prior to parturition. Pregnane concentrations were determined as previously

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reported with an accuracy > 90%, precision < 15%, and average limit of quantification of 0.33 ng/mL [10].

Determination of circulating dutasteride concentrations

Dutasteride concentrations were analyzed at the University of Kentucky Environmental Research Training Laboratory, utilizing a modified protocol [84]. In short, dutasteride was measured from serum with a Varian 410 auto sampler, Varian ProStar 210 pumps, and a Varian 1200L triple quadrupole mass spectrometer (Walnut Creek, CA). This method utilized a Kinetex C18 column (100 x 2.1 mm x 2.6 µm) (Torrance, CA), with mobile phase A consisting of water with 0.1% formic acid and mobile phase B consisting of methanol with 0.1% formic acid. Elution gradient (Table 3.2) and the reporting limit, quality controls (QC), ions, extraction efficiency, accuracy, and precision (Table 3.3) were determined. The internal standard, dutasteride-13C6 (Clearsynth; Mississauga, ON, Canada), was dissolved in methanol at 1mg/mL and diluted to a working concentration of 100 ng/mL. Standards were made by dissolving dutasteride (Aurum Pharmaceuticals; Franklin Park, NJ) in methanol at 1 mg/mL to give an initial concentration of 100,000 ng/mL. Dilutions were made to produce stock standards at 10,000, 1,000, 100, and 10 ng/mL. Charcoal stripped gelding serum was used for the standard curve points, QCs, and blanks. Seven standard points at 80, 50, 25, 10, 5, 0.5, and 0.1 ng/mL were used to generate the standard curve which had an r^2 value of .99. Quality controls were assessed at 35, 15, and 1ng/mL, and triplicates were included of each for the two-batch assays. Each sample, QC, and standard point was analyzed utilizing 1.0 mL of serum.

Statistical analysis

Analysis of endocrine data was conducted using two models. The first model examined endocrine changes between treated and control mares from Day 298 to 318. This model excluded two mares (one control and one dutasteride-treated mare) as they foaled prior to 318 days of gestation, and the biological decline of pregnanes in the three days prior to parturition would have confounded the results [10]. In the second model, endocrine data were analyzed for the nine days prior to parturition with all mares included in the analysis. Endocrine data were analyzed with a random effects mixed model with mare as a random effect and time, treatment, and time-by-treatment interactions as fixed effects. Data were transformed (square root or log transformation) as required and model validity was tested by examination of normal quantile plots of residuals. Gestational data (duration of gestation, neonatal, and placental weights) were analyzed with a Wilcoxon signed-rank test. Dutasteride concentrations were analyzed with regression plots, of drug and individual pregnane concentrations. Analysis was conducted utilizing JMP software (JMP®, Version *12*. SAS Institute Inc., Cary, NC), and significance was set at $p < 0.05$. Data are expressed as mean ± SEM unless otherwise stated.

Results

Foaling outcome

All mares in the study delivered viable foals; however, one of the foals from a control mare had a severe omphalophlebitis and a patent urachus and was humanely euthanized at 5 days of age. Gestational lengths, neonatal weight, and neonatal weight as a proportion of mare weight were not different between dutasteride-treated and control

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mares (Table 3.4). Two of the dutasteride-treated mares retained their placenta beyond three hours postpartum. No control mares retained their placenta. Placental weights were greater in dutasteride-treated mares than in control mares (Table 3.4).

Peripheral pregnane concentrations

For the period between 298 to 318 days of gestation, pregnane concentrations were evaluated in alternate day samples. For P4, there was a significant effect of time and a time by treatment interaction, and P4 was higher in dutasteride-treated mares at Days 314, 316 and 318 of gestation (Figure 3.2b). An effect of time was seen for P5, 20α5P, and βα-diol, but there were no time, treatment or time by treatment effects on concentrations of DHP or allopregnanolone between Days 298-318 (Figure 3.2). For the nine days preceding parturition, there was a significant time effect on P5, DHP, 20α5P, βα-diol, and allopregnanolone with each of these pregnanes decreasing in the three days preceding parturition (Figure 3.3). For P4, there were significant effects of time and a time by treatment interaction such that P4 was greater in dutasteride-treated mares at Days -7, -6, -5, -4, -3 and -2 prior to parturition (Figure 3.3b). Neonate pregnane concentrations were not different between foals from dutasteride-treated or control mares (Table 3.5).

Dutasteride in circulation

To assess circulating dutasteride concentrations, samples from treated mares were analyzed every four days, beginning from day 298 and continuing through parturition (Figure 3.4), along with a single sample from each of the treated foals to determine if dutasteride could cross the placenta. Two samples were selected at random from each

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control mare to include in the analysis. Overall, a time-dependent increase of dutasteride continued for the duration of the treatment. Two mares which foaled after administration of the final treatment exhibited a continual increase in dutasteride concentrations beyond D320. No dutasteride was detected in the control mares. In dutasteride-treated mares, there was a positive correlation between dutasteride concentrations and concentrations of P5 and P4; however, there was no significant correlation between dutasteride concentrations and either DHP or allopregnanolone (Figure 3.5). Dutasteride concentrations were 22.7 ± 6.7 ng/mL for the single sample taken from the foals of treated mares at birth.

Discussion

Administration of the 5α-reductase inhibitor, dutasteride, to mares in late gestation did not alter peripheral concentrations of DHP or its downstream metabolites (20α5P, βα-diol, and allopregnanolone). However, administration of dutasteride did increase peripheral concentrations of the precursor, P4, in treated mares. Although metabolism of pregnanes was altered with the administration of dutasteride during late gestation, there was no effect of dutasteride administration on the length of gestation or neonatal outcomes.

In the mare, a normal prepartum rise in circulating pregnanes occurs approximately 30 days before parturition, followed by a rapid decline in the days or hours prior to foaling [9, 10]. In the current study, changes in measured pregnanes in control and treated mares appeared similar to those previously reported [9, 10] with the exception of P4 which increased in mares treated with dutasteride. Ousey et al., reported that administration of

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the 5α-reductase inhibitor, finasteride, beginning at D300 of gestation also increased concentrations of P4 without decreasing concentrations of DHP [57]. In contrast to the observations in the current study, Ousey et al., also reported a reduced gestation length in mares treated with finasteride.

Dutasteride is reported to be 100 times as potent at inhibiting the type 1 isoform of 5α-reductase compared to finasteride in humans [85], therefore dutasteride was expected to have a greater effect in this study when compared to Ousey's study. Based upon *in vitro* observations using equine epididymis as the source of 5α-reductase type 1, both dutasteride and finasteride inhibit the type 1 isoform of this enzyme with equal potency and show a resultant block of conversion of P4 to DHP [56]. This is in contrast to potency differences reported in humans analyzing the enzyme's ability to inhibit testosterone conversion [85]. A limitation of the current study is that animal numbers were small, which may have resulted in our inability to detect treatment effects on gestation length. Further, it is unknown what concentration or duration of administration of dutasteride would be required to elicit significant effects on gestation in the mare.

After intramuscular administration of dutasteride, concentrations of the inhibitor rose slowly and continued to rise in mares after cessation of administration at D320. No data on half-life of dutasteride in the horse is available, but in humans the cited half-life is approximately 5 weeks [86]. Peak concentrations of dutasteride in serum of mares were approximately 86 ng/mL (0.16 μM), well below the published IC50 of dutasteride for equine 5α-reductase activity (1.0 μM) [56]. The slow release of dutasteride from the mygliol vehicle and the low serum concentrations of dutasteride achieved during the treatment period may account for the observed lack of effect of dutasteride on DHP

concentrations in treated mares. Another possible explanation involves the proposed alternate pathway to DHP via oxidation of 3β-hydroxy-5α-pregnan-20-one (3β5P) [24]. However, recent work has refuted this pathway [56], and this explanation remains speculative.

Though dutasteride is typically prescribed for the inhibition of testosterone conversion to 5α-dihydrotestosterone in humans [85], dutasteride significantly reduced conversion of P4 to DHP in both mice (*in vivo*) and humans (*in vitro*) [87, 88]. In human breast tissue, dutasteride resulted in not only a reduction of 5α-reductase activity, but also a significant increase in expression of the enzymes 3α-hydroxysteroid oxidoreductase (3αHSOR) and 20α-hydroxysteroid oxidoreductase (20αHSOR) [88], which are two enzymes necessary for metabolism of DHP to allopregnanolone and 20α5P (Figure 3.1). This suggests dutasteride would decrease the levels of DHP and other downstream metabolites in treated mares, and also potentially increase 20α5P, βα-diol, and allopregnanolone concentrations. However, in the current study, concentrations of these 5α-reduced pregnanes remained unchanged after administration of dutasteride. There was, however, a significant increase in P4 in dutasteride-treated mares that was likely due to a buildup of substrate when the enzyme was inhibited. The significant correlation observed in dutasteride and P5 or P4 concentrations further suggests a buildup of substrate with inhibition. Supporting data for this includes the ability of dutasteride to significantly increase testosterone concentration in treated men when compared to the placebo group [89, 90] when inhibiting 5α-reductase in men with benign prostatic hyperplasia.

Conclusions

Based upon the increase in P4 noted in dutasteride-treated mares during late gestation, dutasteride did inhibit 5α-reductase activity in mares. Although precursor (P4) increased, there were no changes detected in 5α-reduced pregnanes (DHP, 20α5P, βα-diol or allopregnanolone). Results suggest that while a higher dose may have had a greater affect, a possible alternate metabolic pathway may yet be involved in the complex steroid metabolism during late gestation in the mare.

Abbreviation	Chemical name	Trivial name	Alternative abbreviation
P5	pregn-5-ene-3β-ol,20-one (a)	pregnenolone	
	3β-hydroxy-5α-pregnen-20-one (b)		
	3β-hydroxy-5-pregnen-20-one (c,d)		
P4	pregn-4-ene-3,20-dione (a)	progesterone	
	4-pregnene-3,20-dione (b,c,d)		
DHP	5α-pregnan-3,20-dione	(5α)dihydroprogesterone	5αDHP
20αDHP (a)	5α-pregnan-20α-ol-3one (a)	20α-hydroxy DHP (a)	20α5P (b,c)
	20α-hydroxy-5α-pregnan-3-one (b,c,d)		20α5α (d)
3β,20αDHP (a)	5α-pregnan-3β,20α-diol (a)	3β,20α-dihydroxy DHP (a)	βα-diol (b,c,d)
	5α-pregnane-3β,20α-diol (b,c,d)		

Table 3.1: Pregnanes referenced in this paper along with a comparison of pregnane identifications among published articles.
a) Legacki et al., [10] b) Ousey et al., [11] c) Chavatte et al., [21] d) Schutzer et al., [24]

Time	%A	%B	Flow
0:00	50	50	0.2
0:30	50	50	0.2
7:00	5	95	0.2
9:00	5	95	0.2
10:00	50	50	0.2
18:00	50	50	0.2

Table 3.2 Elution gradient, with mobile phase A consisting of water with 0.1% formic acid, and B consisting of methanol with 0.1% formic acid. Flow rate was 0.2 mL/min.

Compound	Rt	Precursor Ion	Daughter Ions	Reporting Limit	QCH %CV/%Acc	QCM %CV/%Acc	QCL %CV/%Acc	%EE
Dutasteride	7.8	529	**461**, 264, 187	0.1	4.8/97.8	5.9/97.6	4/109.7	83.6
Dutasteride 13C6	7.8	535	**467**, 270, 187	0.1	NA	NA	NA	86.7

Table 3.3 Compounds listed with retention time (Rt) in minutes, precursor ions, daughter ions, reporting limit, precision (%CV) and accuracy (%Acc) of quality controls (QCH, QCM, and QCL), and extraction efficiency (%EE). Daughter ions are listed in order of abundance, with the ion of quantification bolded. Reporting limit is listed in ng/mL and was determined by the lowest standard visible with a linear curve. Precision and accuracy for QCH, QCM, and QCL were determined by two runs containing triplicates of each QC. Percent accuracy was assessed by dividing the average QC values by the actual values and multiplying by 100. Extraction efficiency was calculated by dividing the area of the peak with extraction for dutasteride by the area of the peak without extraction for dutasteride and multiplied by 100.

Group	Gestation length (days)	Neonatal weight (kg)	Neonatal/mare weight (%)	Placental weight (kg)
Dutasteride	323 (318, 335)	51 (42, 55)	0.085 (0.073, 0.090)	4.7 (4.5, 5)*
Control	337 (321, 352)	40 (37, 62)	0.071 (0.062, 0.086)	3.1 (2.3, 3.8)*

Table 3.4 Gestational length and neonatal outcome. Data represent the median with the 25th and 75th quartile indicated in parenthesis. Asterisks indicate significant differences between groups (p<0.05)

Group	Neonatal Pregnenolone	Neonatal Progesterone	Neonatal DHP	Neonatal Allopregnanolone	Neonatal Dutasteride
Dutasteride	725.5 (±140.2)	3.3 (±0.6)	21.8 (±2.4)	3.8 (±1.4)	22.7 (±6.7)
Control	834.0 (±308.6)	2.5 (±0.7)	24.6 (±8.7)	4.0 (±1.9)	0

Table 3.5 Concentrations of pregnane and dutasteride concentrations (ng/mL) in neonatal foals born to dutasteride-treated and control mares. No significant differences were observed between groups. Data are mean ± s.e.m.

Figure 3.1 Pregnane metabolism in the pregnant mare
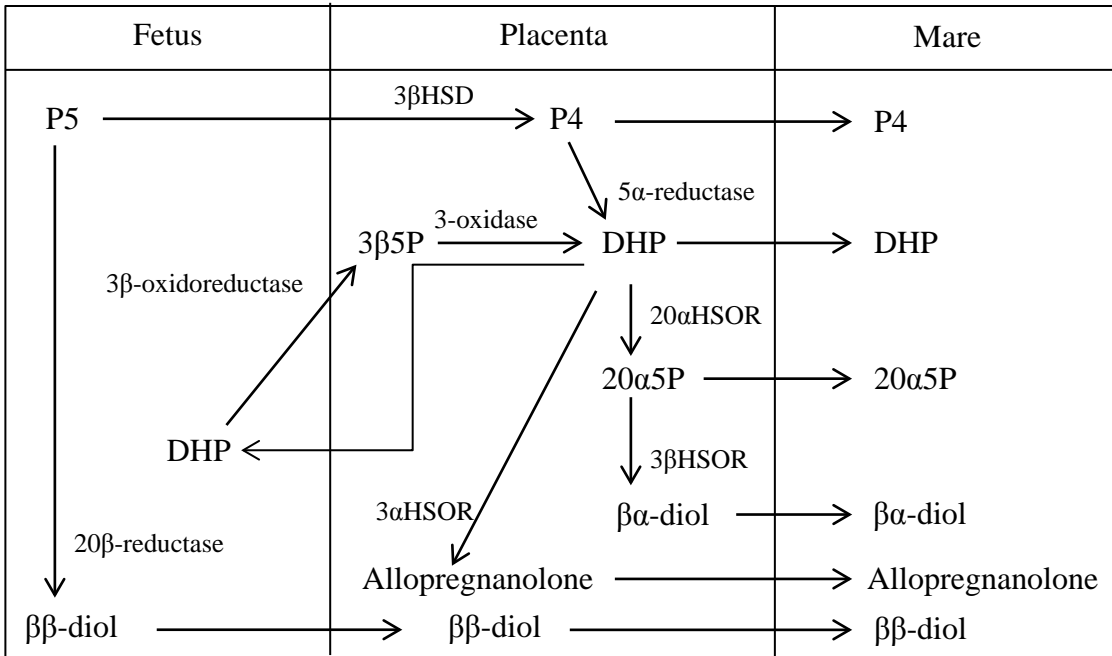
Pregnenolone (P5), progesterone (P4), 5α-dihydroprogesterone (DHP), 3β-hydroxy-5α-pregnan-20-one (3β5P), 20α-hydroxy-5α-pregnan-3-one (20α5P), 5α-pregnane-3β,20α-diol (βα-diol), and allopregnanolone. Enzymes include 5α-reductase, 3β-oxioreductase, 3-oxidase, 3β-hydroxysteroid dehydrogenase (3βHSD), 3α-hydroxysteroidoxidoreductase (3αHSOR), 20α-hydroxysteroidoxidoreductase (20αHSOR), and 3β-hydroxysteroidoxidoreductase (3βHSOR). Adapted from Ousey et al., 2003 [4].

Figure 3.2 Plasma pregnane concentrations in control mares (dashed line) and dutasteride-treated mares (solid line) from 298 – 318 days of gestation.

a)

b)

c)

d)

e)

f)

41
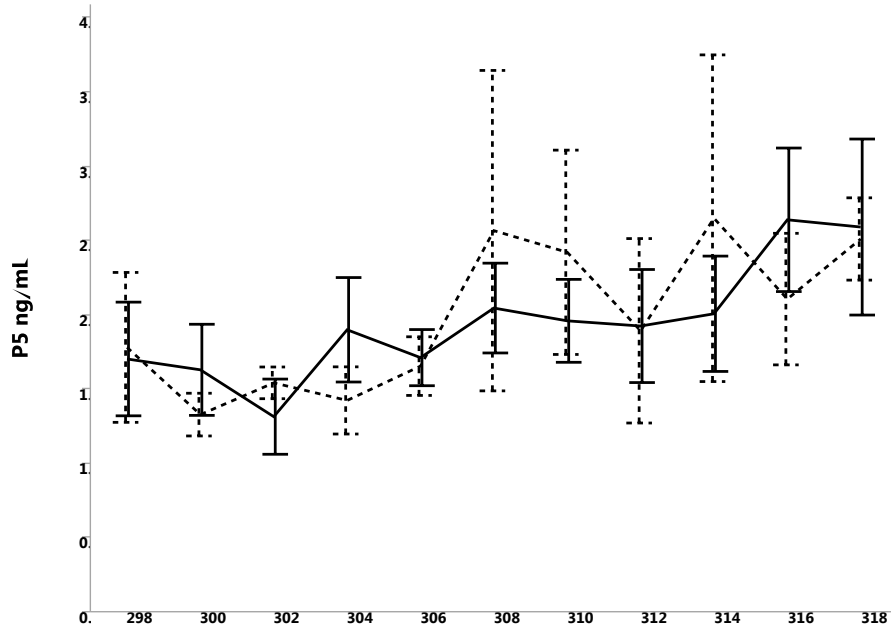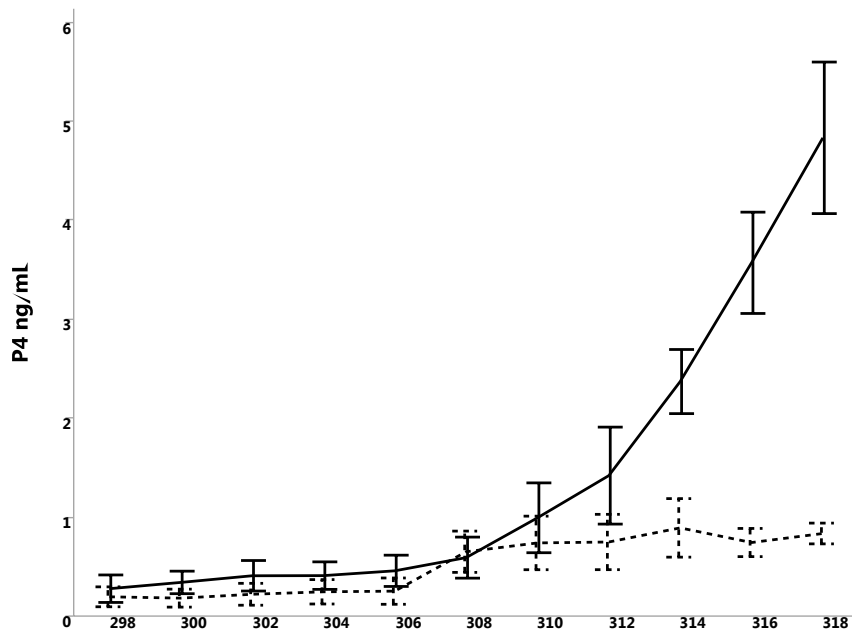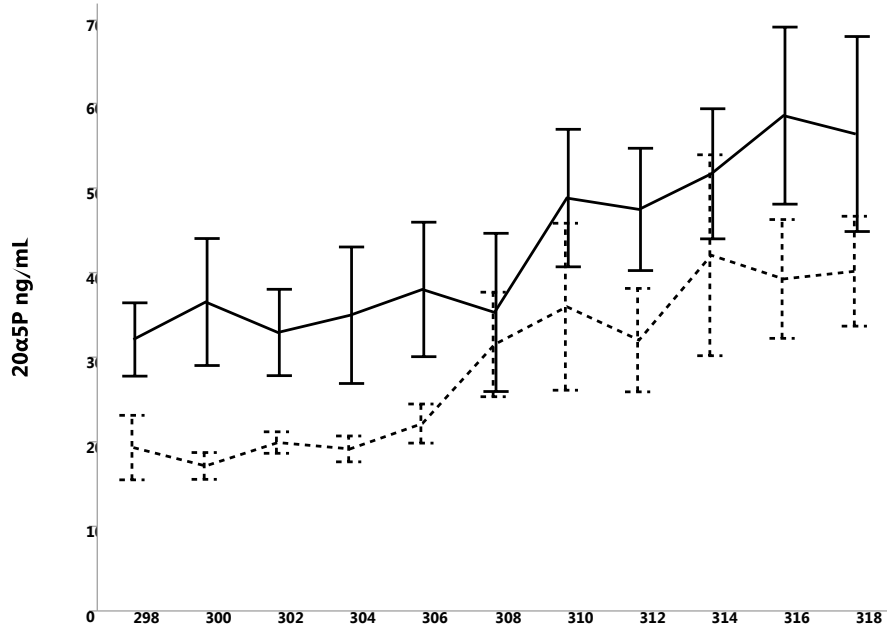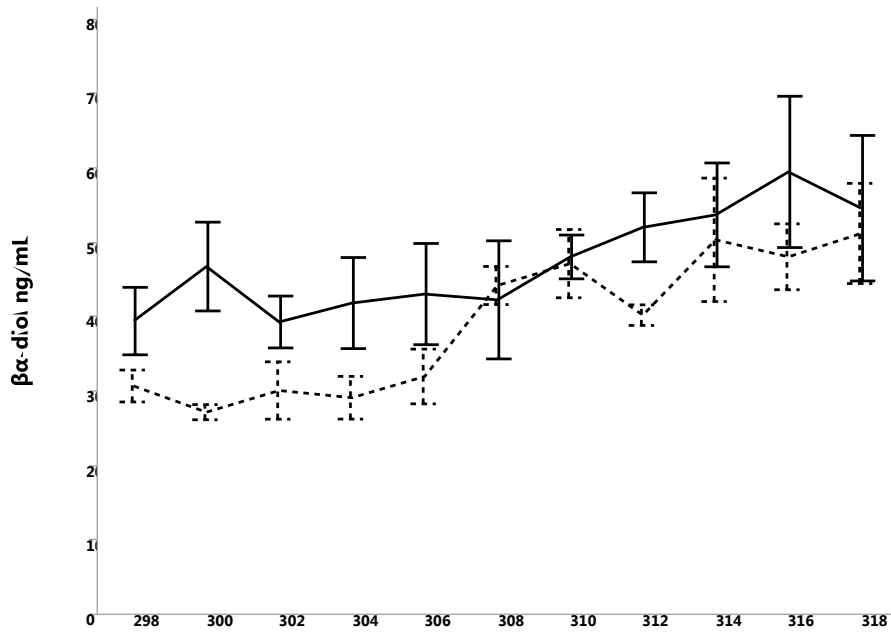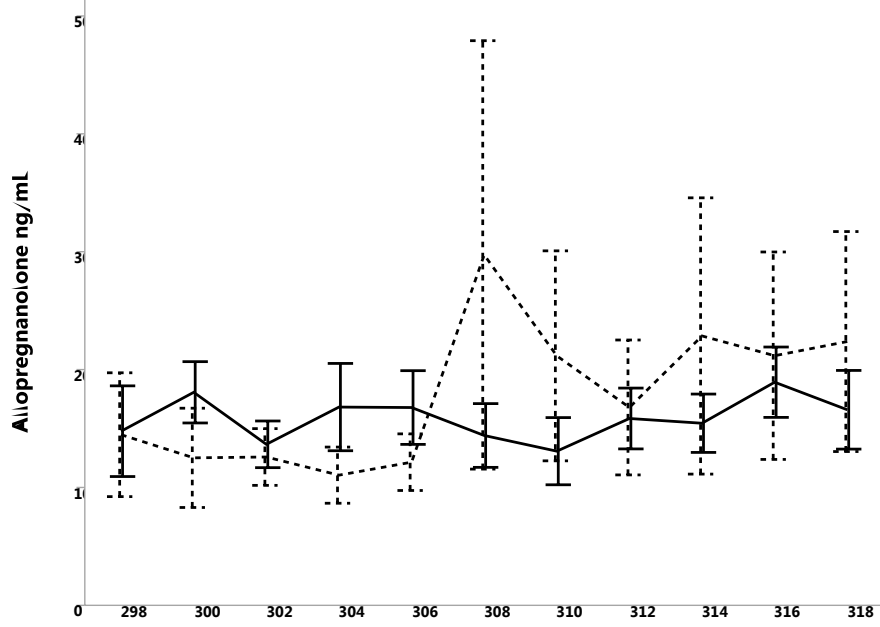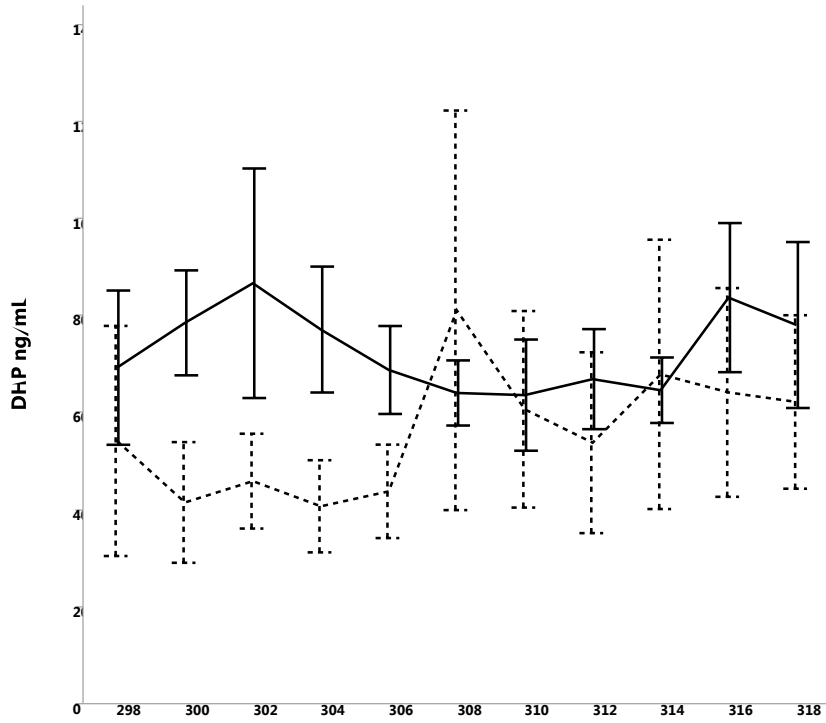
All graphs show days of gestation on the x-axis, with treatment beginning on day 300. Data represent ± 1 SEM.

Fig. a) Mean pregnenolone (P5) concentrations. No difference noted between groups.

Fig. b) Mean progesterone (P4) concentrations. Significant differences, indicated with asterisks ($p<0.05$), noted between groups on days 314, 316, and 318.

Fig. c) Mean 5α-dihydroprogesterone (DHP) concentrations. No differences noted between groups.

Fig. d) Mean 20α-hydroxy-5α-pregnan-3-one (20α5P) concentrations. No difference noted between groups.

Fig. e) Mean 5α-pregnane-3β,20α-diol (βα-diol) concentrations. No differences noted between groups.

Fig. f) Mean allopregnanolone concentrations. No difference noted between groups.

Figures 3.3 Plasma pregnane concentrations in control mares (grey) and dutasteride-treated mares (black) for the last nine days of gestation.

a)

b)

c)

d)

e)

f)

45
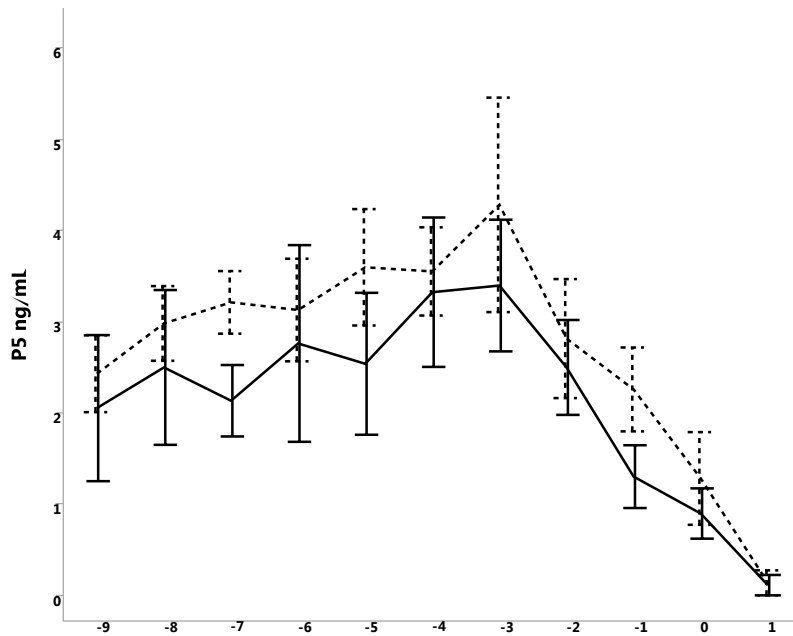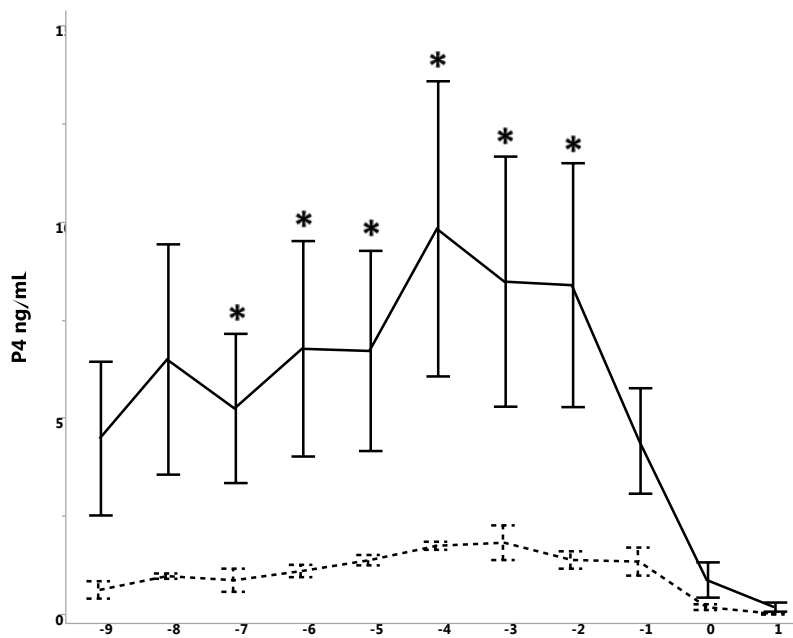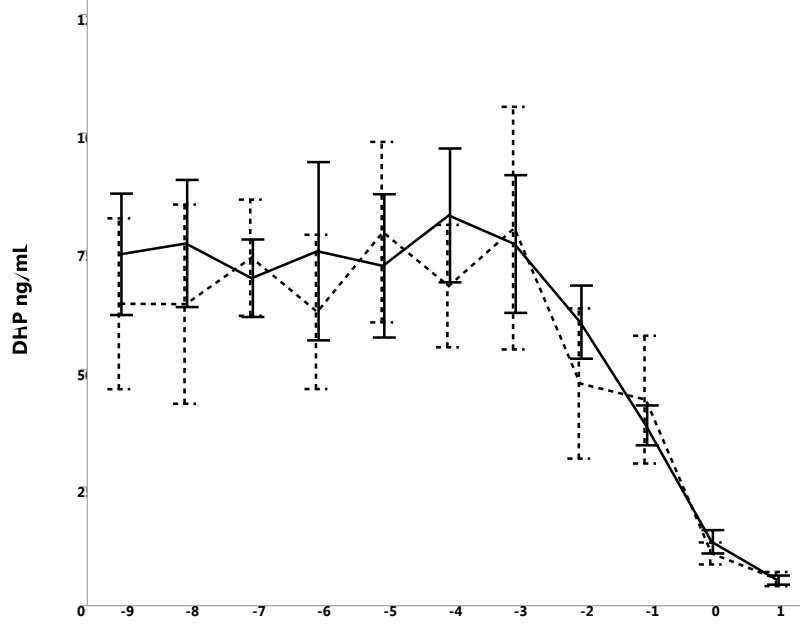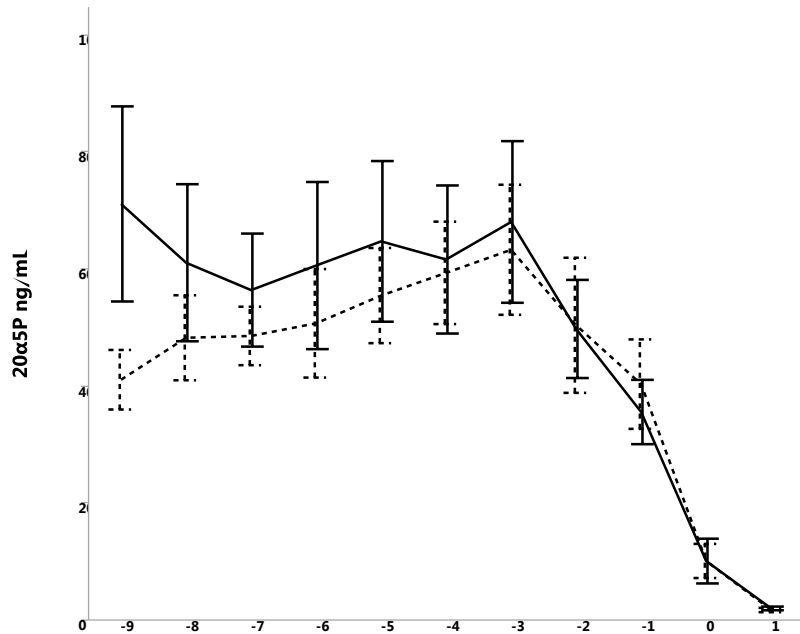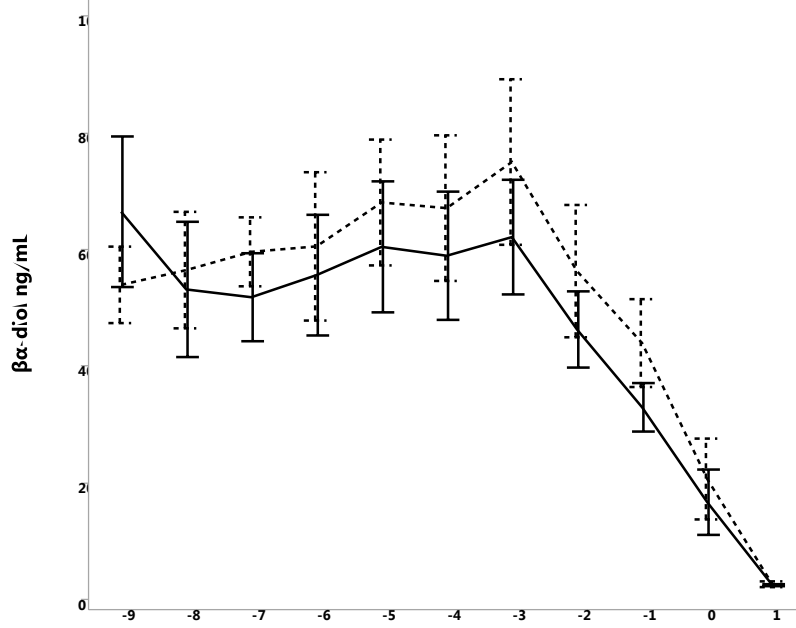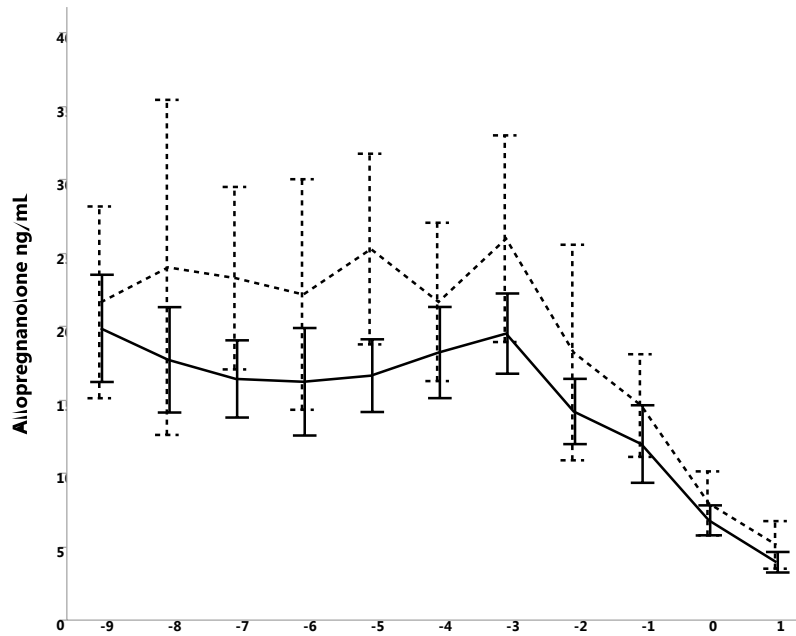
All graphs show days preceding parturition on the x-axis. Data represent ± 1 SEM.

Fig. a) Mean pregnenolone (P5) concentrations. No differences noted between groups.

Fig. b) Mean progesterone (P4) concentrations. Significant differences, indicated with asterisks (p<0.05), noted between groups for days -7, -6, -5, -4, -3, and -2 preceding parturition.

Fig. c) Mean 5α-dihydroprogesterone (DHP) concentrations. No differences noted between groups.

Fig. d) Mean 20α-hydroxy-5α-pregnan-3-one (20α5P) concentrations. No differences noted between groups.

Fig. e) Mean 5α-pregnane-3β,20α-diol (βα-diol) concentrations. No differences noted between groups.

Fig. f) Mean allopregnanolone concentrations. No differences noted between groups

Figure 3.4 Dutasteride concentrations in treated mares, showing the increase in dutasteride concentration during and post treatment. Days of gestation are indicated on the x-axis with the last day of treatment on day 320, and concentrations of dutasteride are shown along the y-axis in ng/mL. Individual mare identification is listed above the graphs. Mares A8 and C4 foaled prior to the end of treatment.
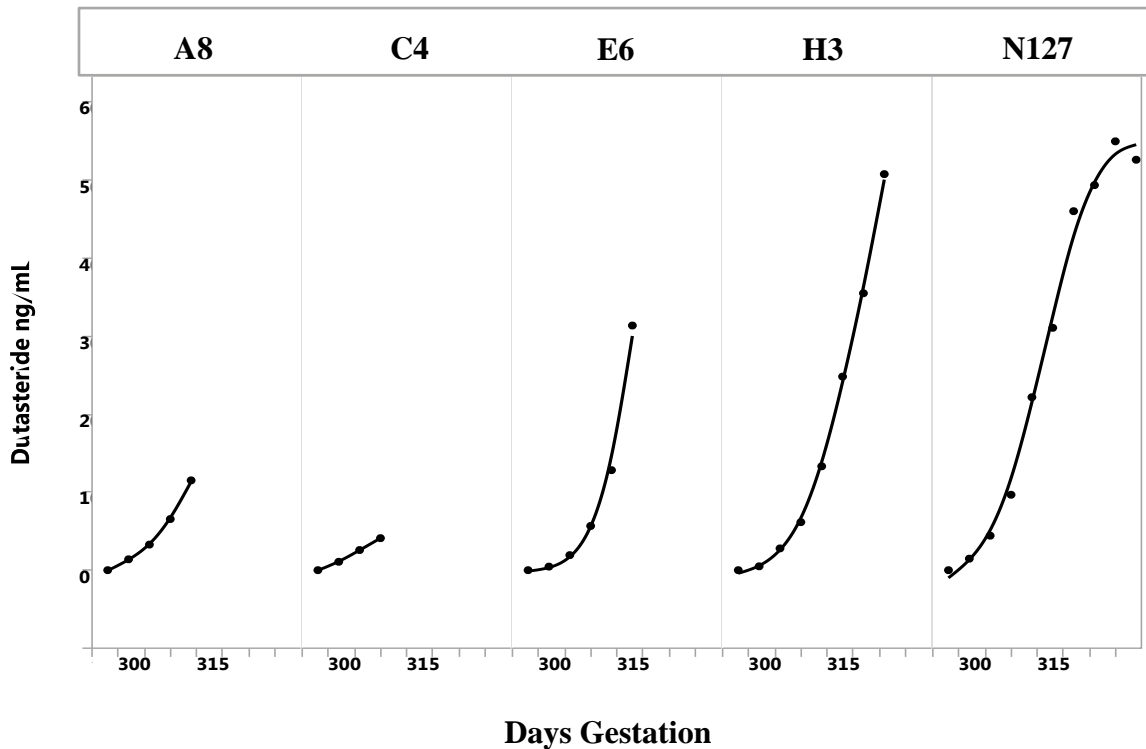
Figures 3.5 Regression plots for pregnanes and dutasteride concentrations in mares treated with dutasteride from 298-320 days of gestation.

a)

b)

c)

Y-intercept: 67.14
Slope: 0.23
R2: 0.03

d)

Y-intercept: 16.4
Slope: 0.003
R2: 0.00

48
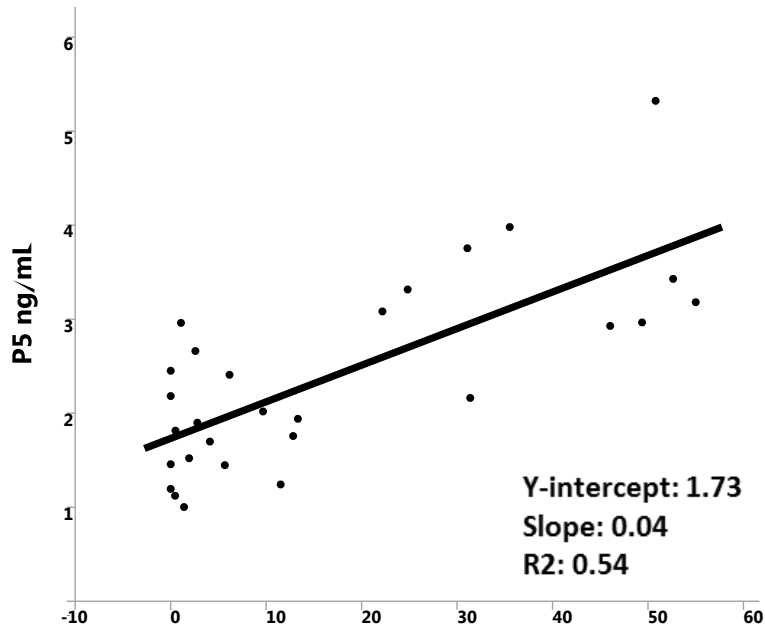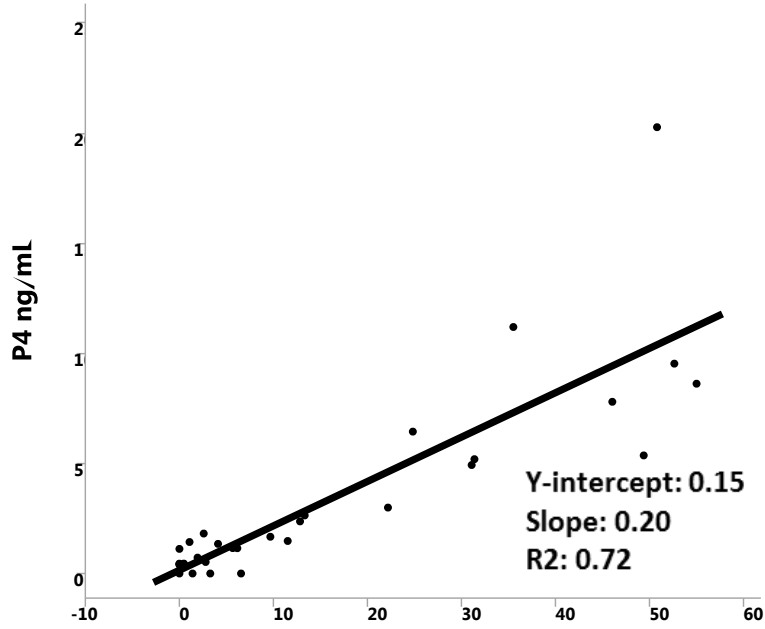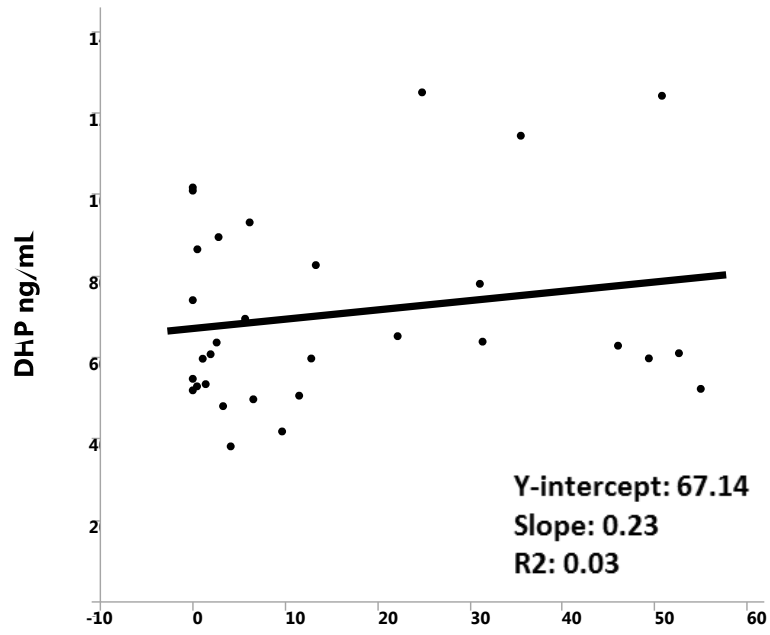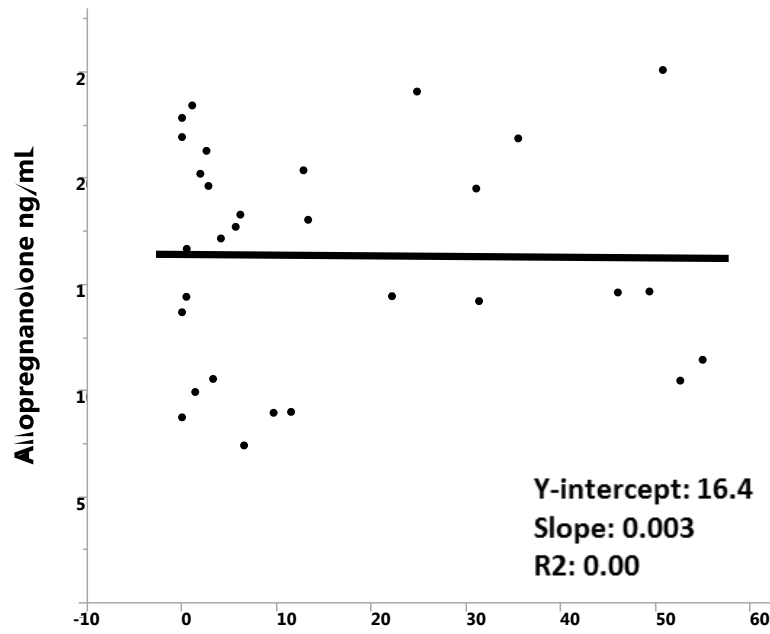
For each graph, pregnane concentration is indicated along the y-axis in ng/mL and dutasteride concentration along the x-axis in ng/mL. Slope, intercept, and coefficient of determination (R^2) can be seen in the upper left corner of each graph.

Fig. a) Pregnenolone (P5) concentrations were significantly ($p<0.05$) and positively associated with dutasteride concentrations.

Fig. b) Progesterone (P4) concentrations were significantly ($p<0.05$) and positively associated with dutasteride concentrations.

Fig. c) 5α-dihydroprogesterone (DHP) concentrations were not significantly associated with dutasteride concentrations.

Fig. d) Allopregnanolone concentrations were not significantly associated with dutasteride concentrations.

Chapter Four: Changes in maternal pregnane concentrations in mares with experimentally induced ascending placentitis

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Abstract

Endocrine biomarkers, such as pregnanes, are commonly measured to help diagnose compromised pregnancies in mares in late gestation. Interpretation of pregnane concentrations are complicated during late gestation as multiple pregnanes are found in high concentrations and may variably cross-react in immunoassays. Therefore, immunoassay results are assay dependent when attempting to quantify pregnanes in late gestation. Several pregnanes are thought to be important in late gestation, based on either their concentration or bioactivity, but cannot be specifically measured by immunoassay. For this reason, quantification of progesterone and other pregnanes has been conducted with gas chromatography mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) in order to better identify changes in pregnanes of interest. This study's objective was to measure via LC-MS/MS specific pregnanes in mares with experimentally induced ascending placentitis (n = 7) and to observe changes in these target hormones when compared to a group of gestationally age matched control

mares (n = 8). Target analytes were measured for days -8, -6, -4, -3, -2, -1, and 0 days preceding abortion in the treated mares, and for the matched days of gestation for the control mares by LC-MS/MS and by immunoassay. Measured analytes included pregnenolone (P5), progesterone (P4), 5α-dihydroprogesterone (DHP), allopregnanolone, 3β-hydroxy-5α-pregnan-20-one (3β5P), 20α-hydroxy-5α-pregnan-3-one (20α5P), 5α-pregnan-3β,20α-diol (βα-diol), and 5α-pregnan-3β,20β-diol (ββ-diol). Data was analyzed with a random effects mixed model with time, treatment, and time × treatment interaction as fixed effects and mare as random effect. For total pregnanes by immunoassay and P4, 20α5P, and βα-diol by LC-MS/MS, a significant effect ($p < 0.05$) of time, treatment, and time × treatment interaction was detected. A time and time × treatment interaction was observed for DHP, allopregnanolone, and 3β5P, but no significant effects were detected for P5 or ββ-diol. Inoculated mares demonstrated a significant increase in measured pregnanes when compared to the control mares. This premature rise in pregnanes in the inoculated mares mirrors that of the normal change in pregnanes seen in healthy pregnancies just prior to parturition.

Keywords: equine, pregnancy, LC-MS/MS, pregnanes, placentitis

Introduction

The endocrine profile of pregnanes in the mare in late gestation is complex and unique. Unlike many other mammals [1], progesterone (P4) concentrations in pregnant mares decline around mid-gestation and remain low or undetectable (<1 ng/mL) [2, 3, 10, 12] throughout late gestation until the time of parturition, when a slight rise in P4 concentrations has been identified [1, 10]. As P4 is considered critical for pregnancy

maintenance, these low concentrations of P4 in mid to late gestation represent a somewhat unusual aspect of reproductive biology in the horse. However, the mare is known to produce a number of other pregnanes and progestogens during this time which may be bioactive and thereby serve important functions in pregnancy maintenance [10, 12].

The metabolism of these pregnanes is complex, requiring contributions from the fetus, placenta and endometrium of the pregnant mare [4, 16] (Figure 4.1). Several pregnanes have gained attention for their abundance or possible bioactivity in late gestation. These include: 5α-dihydroprogesterone (DHP), allopregnanolone, 3β-hydroxy-5α-pregnan-20-one (3β5P), 20α-hydroxy-5α-pregnan-3-one (20α5P), 5α-pregnane-3β,20α-diol (βα-diol), and 5α-pregnane-3β,20β-diol (ββ-diol) [3, 10]. Of these, 20α5P and βα-diol are the most quantitatively important, reaching concentrations of >300ng/mL in late gestation [3, 11]. Others, (DHP, 3β5P, and ββ-diol), have been shown to increase to concentrations of >30 ng/mL during late gestation [4, 10]. While the bioactivity of certain pregnanes have been evaluated in-vivo [9, 12], the bioactivity of many pregnanes in the mare remains unknown, despite their high concentrations in late gestation [3, 9, 10]. Of the pregnanes evaluated for possible bioactivity, P5, P4, DHP, and allopregnanolone are considered important for their bioactive role or as necessary precursors for bioactive progestogens [4, 12, 14, 15].

The role of P4 during pregnancy is well documented, having the ability to bind progesterone receptors (PR), maintain myometrial relaxation, and serve as a precursor for further metabolites [1, 4, 10]. Progesterone is a metabolite of pregnenolone (P5) which may originate from the fetal adrenal cortex [4], and possibly the fetal gonads [24, 28],

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and P5 is metabolized in the placental tissue by 3β-hydroxysteroid-dehydrogenase (3βHSD; Figure 4.1) [91]. Progesterone is then converted to DHP via 5α-reductase in the placenta [4]. 5α-dihydroprogesterone can be metabolized to other hormones or serve its proposed function in binding PRs and maintaining myometrial quiescence [4, 12, 24]. Allopregnanolone is an important metabolite of DHP and a potent neurosteroid, known for binding $GABA_A$ receptors [80]. Allopregnanolone helps maintain fetal sleep and fetal quiescence in utero [14, 15].

Immunoassays, specifically P4 immunoassays, have been traditionally used to evaluate changes in pregnane concentrations which could be indicative of diseased states or abnormal pregnancy in mares. Chronic disease involving the placenta, such as placentitis, show a gradual increase in pregnanes, while acute disease, such as fetal demise, result in an abrupt decline in pregnanes [5, 6, 50]. Attempts to study pregnane concentrations as a predictive tool have been reported with pregnane measurements by immunoassay prior to 308 days of gestation, indicating placental dysfunction in mares with differing chronicity of placentitis [50]. Because these observations were made in mares with spontaneous clinical placentitis, gestational ages, sample number, causative organism, and pregnancy outcomes varied.

Unlike immunoassays, which report all pregnanes able to cross-react with the antibody, analysis by mass spectrometry is able to report concentrations of specific pregnanes in late gestation. Ousey et al., used gas chromatography mass spectrometry (GC-MS) to describe notable changes in profiles of specific pregnanes in mares with abnormal pregnancies compared to a group of mares with healthy pregnancies. Within the cases reported, mares with placentitis had serum concentrations of 20α5P, βα-diol, and ββ-diol

which were markedly increased when compared to the control mares (above 99.8% confidence limits). Concentrations of DHP and 3β5P increased, but to different degrees with different causes of placentitis (placentitis, fungal placentitis, focal placentitis). Changes in P5 ranged from highly decreased to highly increased among the differing cases of placentitis [11]. While these changes gave important information about the measured precursor or specific metabolites and how they related to fetal or placental function, the samples were generated from clinical cases where gestational ages, sample number, causative organism, and pregnancy outcomes varied. Therefore, analysis by mass spectrometry from a controlled study with serial samples from mares with a known diagnosis may better define target pregnane profiles. The purpose of this study was to investigate the effects of experimentally induced ascending placentitis on a set of target pregnanes (P5, P4, DHP, allopregnanolone, 3β5P, 20α5P, βα-diol, and ββ-diol), measured by LC-MS/MS, compared to gestationally age matched control mares.

Materials and Method

All plasma samples used in this study were generated from previous experiments in which placentitis was induced via intracervical inoculation of *Streptococcus equi* spp. *zooepidemicus* in mares between 260-280 days of gestation [53, 92]. Samples from inoculated mares (n = 7) in which the interval from inoculation to abortion was ≥ 8 days were selected for analysis as a previous report indicated that this interval was associated with elevations in plasma progestins [50]. Plasma samples from gestationally age matched mares (n = 8) were used as controls. In inoculated mares, the interval from inoculation to abortion was 13.4 ± 6.4 days (mean ± sd). Blood samples were drawn daily via jugular venipuncture into 10-mL heparinized tubes (Becton, Dickinson and

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Company, Franklin Lakes, NJ). Immediately following collection, blood was centrifuged at $600 \times g$ for ten min. Plasma was then frozen at -20°C until time of analysis. Samples were analyzed for days -8, -6, -4, -3, -2,   -1, and 0 days preceding abortion for the treated mares and for the matched days of gestation for the control mares.

LC-MS/MS

Standards and solutions

Standards for the hormones (Table 4.1.) examined in this study were purchased from Steraloids (Newport, RI, USA): P5, P4, DHP, allopregnanolone, 3β5P, 20α5P, βα-diol, ββ-diol, and d9-P4.   A master stock solution of each analyte in methanol (Fisher Scientific, Hampton, NH) at an initial concentration of 100,000 ng/mL was used to produce working solutions at concentrations of 10,000, 1,000, 100, and 10 ng/mL.   An internal standard of d9-P4 (IS) at an initial concentration of 100,000 ng/mL in methanol was diluted to a working concentration of 100 ng/mL.

Sample preparation and extraction

One milliliter of serum was added to a 13x100 mm glass screw-top tube and 100 μL of working IS was added and briefly vortexed. Standard curve points (ranging from 0.01-1000 ng/mL) and quality control samples (high (QCH): 300 ng/mL, and low (QCL): 30 ng/mL) were simultaneously prepared by the addition of 100 μL of working IS and the corresponding volume of working solutions to one milliliter of charcoal stripped gelding serum and briefly vortexed. Five milliliters of diethyl ether, (Fisher Scientific, Hampton, NH) was added to each sample and mixed for 15 min on a rotating rack. Samples were centrifuged at 3000 x g for five minutes. Tubes were held in liquid nitrogen for 45 sec,

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until the serum was frozen, and the supernatant was decanted into a 12x75 mm glass tube. Samples were dried under a steady stream of nitrogen gas using a drying rack (Pierce Reacti-Therm Heating Module) until ~0.5 mL remained, at which time a glass pipette was used to rinse the sides of the tube with 0.5 mL diethyl ether, and the samples were allowed to dry completely under nitrogen gas. Samples were reconstituted with 150 µL of methanol and vortexed for 15 sec. Standard curve points were generated at the beginning of each assay, with a repeat analysis of the 100 ng/mL standard at the end of the assay to assess possible assay drift. Quality control samples were included in every assay. The IS was selected based on its close resemblance to the analytes of interest.

LC-MS/MS analysis

LC-MS/MS analysis of samples was achieved with the use of a Varian 410 auto sampler, Varian ProStar 210 pumps, and a Varian 1200L triple quadrupole mass spectrometer (Walnut Creek, CA) with argon as the collision gas. This method utilized a C18 column (100 x 2.1 mm x 2.6 µm) (Kinetex; Torrance, CA), with mobile phase A consisting of water with 0.1% formic acid and mobile phase B consisting of methanol with 0.1% formic acid. An elution gradient for this method can be seen in Table 4.2. Injection volume was 25 µL with a flow rate of 0.20 mL per minute. Ionization was achieved with an atmospheric-pressure chemical ionization (APCI) box in positive mode. Extraction efficiency was assessed by comparing recovery peaks of each analyte at equal amounts of working solution from non-extracted and extracted samples. Efficiencies were found to be >87% for each analyte, with an average of 92%. Accuracy was found by dividing the QC results from seven runs by the predicted value and multiplying by 100. Average accuracy was 98.1%. Precision was evaluated with coefficients of variation (CV) using

the QCs across seven assays, with an average CV of 9.4%. The reporting limit for each analyte was found by assessing the lowest linear standard point on the curve which could be clearly identified. Extraction efficiencies, details of analyte identification, and QC data can be seen in Table 4.3.

Sample preparation, extraction, and analysis by immunoassay

Sample analysis by immunoassay was conducted with P4 antibody R4859 (Clinical Endocrinology Laboratory, University of California, Davis, CA) utilizing a modified protocol [93]. Ten microliters of serum was mixed with 0.5 mL of petroleum ether (Fisher Scientific, Hampton, NH) in a glass tube and vortexed for 45 seconds. Serum was frozen by holding the tube in liquid nitrogen, and the ether was decanted into a clean glass tube and dried overnight under a fume hood. Samples were reconstituted with 100 µL of phosphate buffered saline with 0.1% bovine serum albumen (Sigma, St. Louis, MO) and HRP conjugated with P4 (Clinical Endocrinology Laboratory, University of California, Davis, CA) at 1:45,000. Progesterone standard was purchased from Steraloids (Newport, RI). Progesterone dissolved in methanol (Fisher Scientific, Hampton, NH) was used to generate a standard curve with nine standard points, ranging from 2.5 - 2500 ng/mL. Standard points were made by pipetting appropriate amounts of solution into glass tubes and allowing to dry, then reconstituting with 2 mL of HRP-conjugated buffer.

For the standard plate and separate sample plates, 50 µL of HRP-conjugated buffer was pipetted across a 96-well plate, previously coated with antibody. The standard curve was generated by adding 50 µL of each standard in replicates of eight across the plate.

Curves were linear with correlation coefficients of >0.99. Samples were pipetted on separate plates in duplicate at 50 µL. High (300 ng/mL) and low (9ng/mL) controls were included in duplicate on each plate. After two hours of incubation, the plate was washed three times and substrate (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in citric acid (Sigma, St. Louis, MO)) was pipetted across the plate at 100 µL/well. After a second incubation of 1.5 hours, 100 µL of stop solution (EDTA in hydrofluoric acid (Sigma, St. Louis, MO)) was added to each well and the plates were read with an Epoch microplate spectrometer (BioTek, Winooski, VT). All samples were analyzed in a single assay with an intra-assay CV of 8.4%.

Statistical analysis

Pregnane concentrations were analyzed with a random effects mixed model with mare as a random effect and time, treatment, and time $\times$ treatment interactions as fixed effects. Data were log transformed, and model validity was assessed by examination of normal quantile plots of residuals for the mixed model. For P4 and P5, censored data (below the LOD of the assay) were substituted using LOD/sqrt 2 as the estimate for values below LOD [94]. Pairwise comparisons were made using preplanned comparisons in the test-slice function of JMP. Analysis was conducted utilizing JMP software (JMP®, Version *12*. SAS Institute Inc., Cary, NC, 1989-2007.), and significance was set at p<0.05. Data are expressed as the mean ± SEM.

Results

There were significant time, treatment, and time $\times$ treatment interactions for P4, 20α5P, and βα-diol by LC-MS/MS, and total pregnanes by immunoassay. Likewise, there were

significant time and time × treatment interactions for DHP, allopregnanolone and 3β5P. There were no significant time, treatment or time × treatment interactions for P5 or ββ-diol. Significant increases in target pregnanes (excluding P5 or ββ-diol) were detected in the eight days leading to abortion in the mares with experimental placentitis when compared to the matched controls (Figure 4.2). In contrast, there were no significant changes in measured pregnane concentrations over the eight days sampled in control mares.

Discussion

To the authors' knowledge, this is the first report to examine changes in maternal concentrations of pregnanes measured by LC-MS/MS in a controlled study of mares with experimentally induced ascending placentitis. Control mares with normal pregnancies maintained concentrations of target and total pregnanes comparable to results previously established for their gestational age, and these concentrations did not vary significantly during the period under study [9, 10]. Changes in the pregnanes measured in the inoculated mares were as expected for the disease state, which affects the function of the placenta and presumably the fetal adrenal gland [5]. This study demonstrated that profiles of pregnanes in mares with experimentally induced ascending placentitis mirror those of normal pregnancies, just prior to parturition.

In healthy mares, pregnane profiles have been well established utilizing immunoassays [3, 7], with variations expected with the P4 antibody used. However, research utilizing LC-MS/MS or GC-MS has allowed the measurement of individual pregnanes in late gestation, which would not be possible with immunoassays. Control mares from this

study ranged in gestational age from 260-280 days, and displayed total pregnane profiles by immunoassay typical for the given time in gestation. Target pregnanes measured in the control mares by LC-MS/MS in this study are similar to those previously reported by LC-MS/MS or GC-MS [4, 9, 10].

In contrast to normal pregnant mares, total pregnanes determined by immunoassay were significantly elevated in the eight days preceding abortion in mares with experimentally induced ascending placentitis. This is consistent with reports of elevated pregnanes by immunoassay in cases of chronic placentitis, defined by an interval of 8 or more days from inoculation to abortion in experimental cases [6]. Profiles of specific pregnanes by mass spectrometry in mares with late pregnancy complications have not been well defined. However, the inoculated mares in this study had significantly higher concentrations of most of the target pregnanes. Maximum fold changes were highest for 20α5P (4.6x) and βα-diol (3.6x), with 3β5P, allopregnanolone, and DHP each increasing approximately two-fold. Concentrations of P4 were also significantly increased with a maximum fold change of 3.2 after adjusting for concentrations below the LOD, although P4 concentrations remained relatively low (<2 ng/ml) even in mares with placentitis. Concentrations of P5 and ββ-diol did not demonstrate significant changes in mares with placentitis.

Of the target pregnanes measured by LC-MS/MS in this study, all pregnanes which were significantly increased in the induced mares are pregnanes metabolized in the utero-placental tissue. A healthy feto-placental unit metabolizes almost all precursor (P5) coming from the fetus to produce the pregnanes measurable in the periphery of the late pregnant mare [4]. Previous reports indicate an increase in pregnane concentrations at

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the end of gestation, just prior to a marked decrease which occurs approximately three days prior to parturition [9, 10]. The increase is thought to be the result of activation of the fetal hypothalamic-pituitary-adrenal axis (HPA) axis [18] and the fetal adrenal, not yet able to produce cortisol [26], is stimulated and produces more P5. Evidence of this can be seen when maternal pregnanes increase in response to fetal administration of ACTH [41]. The decrease is thought to occur when the enzyme necessary for cortisol production, $P450_{C17}$, is expressed in the fetal adrenal gland [26]. With a shift in synthesis to cortisol, pregnane concentrations decline.

Similarly, cases of placental compromise or other pregnancy insults can lead to increases in P5 concentrations, and therefore, elevated concentrations of metabolized pregnanes [11]. This excess in production of P5 is thought to be a response from the fetus to stress from the disease [44]. Concentrations of P4, 20α5P, βα-diol, DHP, allopregnanolone, 3β5P, P4 and immunoreactive pregnanes appeared to decline in mares with placentitis in the two days preceding abortion (Figure 4.2), mirroring what would be seen in a healthy pregnancy just prior to parturition.

Of the target pregnanes in this study, DHP, 20α5P, and βα-diol are the ones most commonly discussed in previous reports as they are found in the highest concentrations in maternal serum during late gestation. The significant increase in concentrations of these pregnanes with disease was not surprising, as this has been previously observed and reported [11]. While not commonly discussed in many other studies, 3β5P showed a significant increase in this study. Ousey et al. reported changes in concentrations of 3β5P from compromised pregnancies in clinical cases, with differences in changes

corresponding to the diagnosis [11]. However, this is the first study to report increases in this pregnane in a controlled experiment of mares with ascending placentitis.

Interestingly, measured concentrations of P5 and ββ-diol, which are directly derived from the fetus [24, 26], were not different in inoculated mares compared to the control group. Knowing that P5 is the precursor to the downstream pregnanes measured in this study, it was curious that P5 was not significantly increased in the inoculated mares, when placental pregnanes were significantly elevated. It is possible that P5 metabolism was such that, if excess precursor was generated by the fetal adrenal, it was quickly metabolized before any excess was available for maternal circulation. 5α-pregnane-3β,20β-diol (ββ-diol) is a pregnane thought to be metabolized in the fetal liver from P5 or a P5 metabolite (DHP or 3β5P) [24]. Despite the possibility of metabolism of ββ-diol via DHP or 3β5P, a lack of increased P5 may have also resulted in a lack of increase in ββ-diol for this study. It may also be possible that a lack of increase in P5 or ββ-diol is associated with ascending placentitis, specifically. However, further work would be necessary to confirm this.

A comparison of mass spectrometry results from this study and Ousey's study show similarities in dramatic increases in total pregnanes, 20α5P, and βα-diol from mares with placentitis when compared to a control group [11]. Ousey also reported significant increases for P4, DHP, and 3β5P, as did the current study. However, Ousey reported changes from multiple placentitis diagnosis, and the increases in these three pregnanes were not consistent across the diagnosis. Perhaps the most interesting comparison between these two studies is the results for P5 and ββ-diol. In contrast to Ousey's results, this study found no increase in P5 or ββ-diol. The complication in making this

comparison between studies potentially lies in the variations of sampling. Ousey's study utilized samples from mares with various diagnoses, times in gestation, and sample numbers. Additionally, as these were mares in a clinical setting, results may have been confounded by any treatments the mares were receiving. Conversely, parameters in this current study were well controlled, and the induced mares received no treatments.

Conclusion

Results from this study demonstrate a significant increase in total pregnanes by immunoassay as well as pregnanes (determined by LC-MS/MS) that are metabolized in the placenta (P4, DHP, allopregnanolone, 3β5P, 20α5P, and βα-diol) in mares with induced ascending placentitis. This study demonstrates that in a controlled experiment, mares induced with ascending placentitis had a similar pregnane profile preceding abortion to that of mares with normal pregnancies preceding normal parturition. However, no significant increase by LC-MS/MS was noted for the two pregnanes coming directly from the fetus (P5 and ββ-diol). The similarities noted between pregnane profiles from induced mares in this study and those of healthy mares just prior to parturition suggest a common mechanism which leads to these changes.

Steroid Analyzed	Chemical Name	Abbreviation	CAS#
Pregnenolone	5-Pregnen-3β-ol-20-one	P5	145-13-1
Progesterone	4-Pregnen-3,20-dione	P4	57-83-0
5α-dihydroprogesterone	5α-Pregnan-3,20-dione	DHP	566-65-4
	3β-hydroxy-5α-pregnan-20-one	3β5P	516-55-2
	20α-hydroxy-5α-pregnan-3-one	20α5P	516-59-6
	5α-pregnan-3β,20α-diol	βα-diol	566-56-3
	5α-pregnan-3β,20β-diol	ββ-diol	516-53-0
Allopregnanolone	5α-Pregnan-3α-ol-20-one		516-54-1
Deuterated Progesterone	4-Pregnen-3,20-dione-2,2,4,6,6,17α,21,21,21-d9	dp-P4	57-83-0

Table 4.1 Steroids analyzed with common name (if available), chemical name, common abbreviation used in the text, and CAS number.

Time	%A	%B	Flow
0:00	50	50	0.2
0:30	50	50	0.2
1:30	40	60	0.2
21:00	15	85	0.2
23:00	15	85	0.2
24:00	50	50	0.2
32:00	50	50	0.2

Table 4.2 Elution gradient, with mobile phase A consisting of water with 0.1% formic acid, and B consisting of methanol with 0.1% formic acid. Flow rate was 0.2 mL/min.

Compound	Rt	Precursor ion	Daughter ions	Reporting limit	QCH %CV/%Acc	QCL %CV/%Acc	%EE
P5	11.2	299	**281**, 159, 131	1	8.5 / 92.9	9.1 / 99.1	91.1
P4	8.7	315	**97**, 109, 297	0.5	8.9 / 96.4	10.7 / 104.5	94.6
DHP	11.7	317	**281**, 189, 241	1	10.9 / 89.2	14.5 / 97.9	92.9
3β5P	12.5	301	**283**, 189, 135	1	11.6 / 89.7	7.8 / 98.1	93.8
20α5P	11.5	319	**283**, 161, 175	1	7.9 / 94.6	9.6 / 107.2	88.2
βα-diol	11.3	285	**135**, 175, 189	2.5	8.2 / 95.3	10.1 / 106.9	92.4
ββ-diol	14.2	285	**135**, 175, 189	1	8.1 / 93.9	12.5 / 104.3	96.8
Allopregnanolone	14.1	301	**283**, 189, 135	0.5	10.1 / 92.3	8.2 / 98.6	92.7
d9-P4	8.5	324	**100**, 113, 306	NA	NA	NA	NA

Table 4.3 Compounds listed with retention time (Rt) in minutes, precursor ions, daughter ions, reporting limit, precision (%CV) and accuracy (%Acc) of QCH and QCL, and extraction efficiency (%EE). Daughter ions are listed in order of abundance, with the ion of quantification bolded. Reporting limit is listed in ng/mL. Precision and accuracy for QCH and QCL were determined by seven runs, one for each batch of samples. Percent accuracy was assessed by dividing the average QC values by the predicted values and multiplying by 100. Extraction efficiency was calculated by dividing the area of the peak with extraction for each analyte by the area of the peak without extraction for each analyte and multiplied by 100.

Figure 4.1 Pathway of hormones of interest, including proposed alternate pathways.
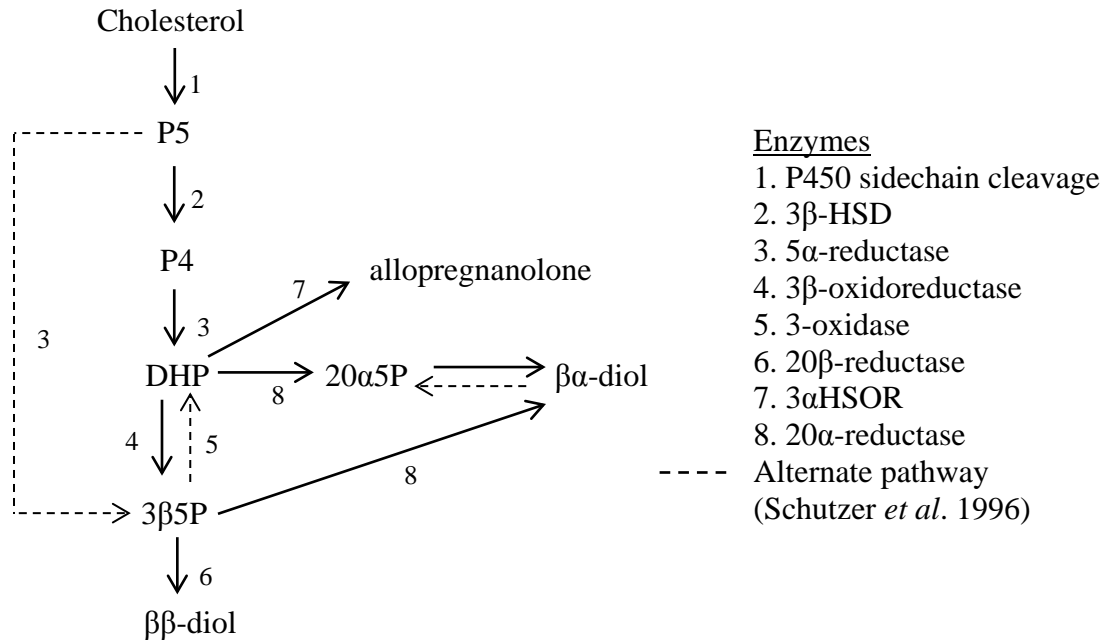
Hormones of interest: pregnenolone (P5), 4-Pregnen-3,20-dione (P4), 5α-dihydroprogesterone (DHP), 3β-hydroxy-5α-pregnan-20-one (3β5P), 5α-pregnan-3β,20β-diol (ββ-diol), allopregnanolone, 20α-hydroxy-5α-pregnan-3-one (20α5P), 5α-pregnan-3β,20α-diol (βα-diol). Adapted from Ousey et al., 2004 [95].

Figure 4.2 Measured pregnanes by LC-MS/MS

a)

b)

c)

d)

e)

f)

g)

h)

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i)
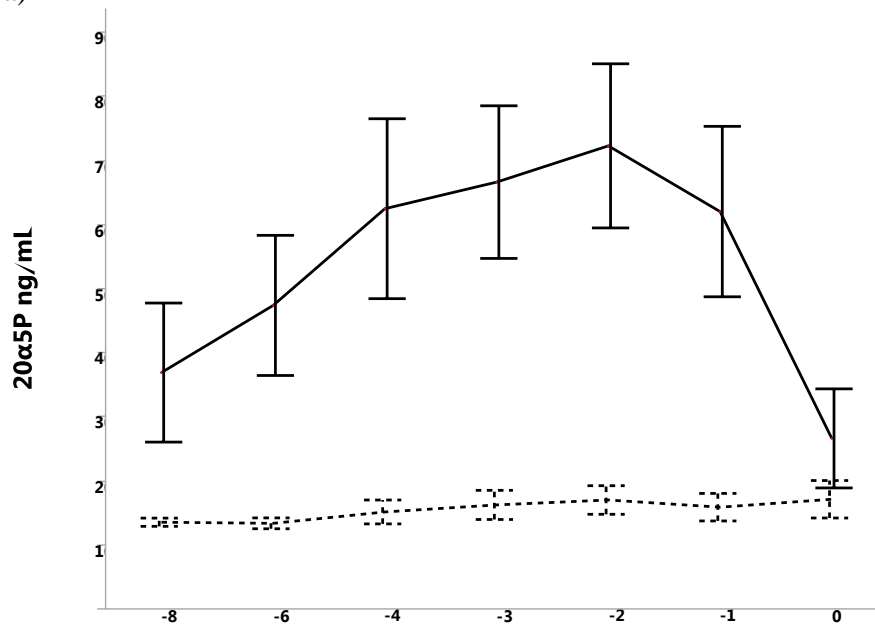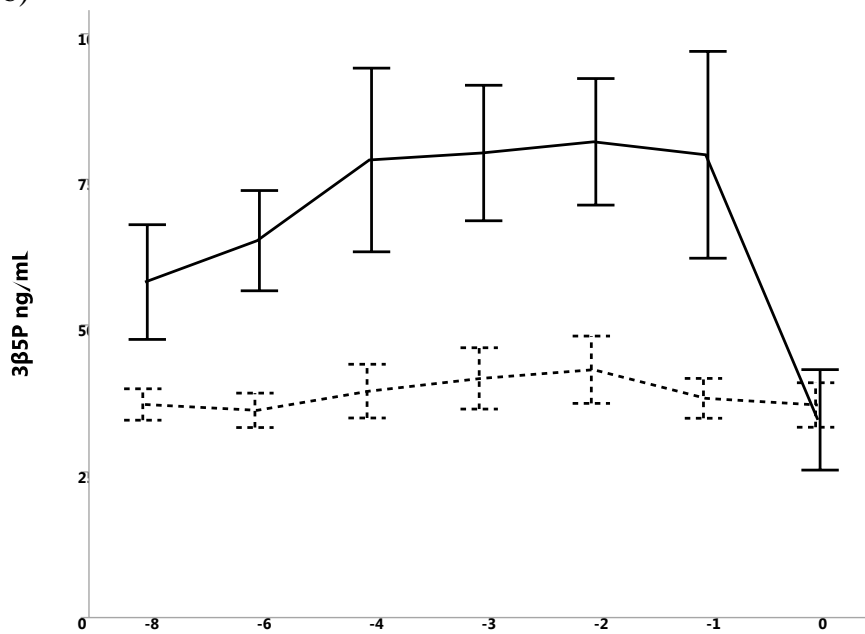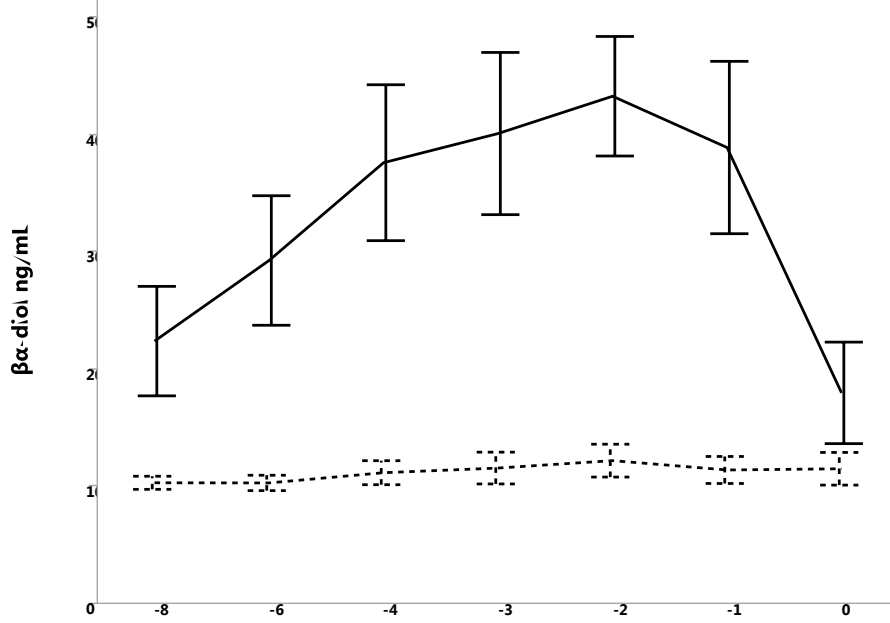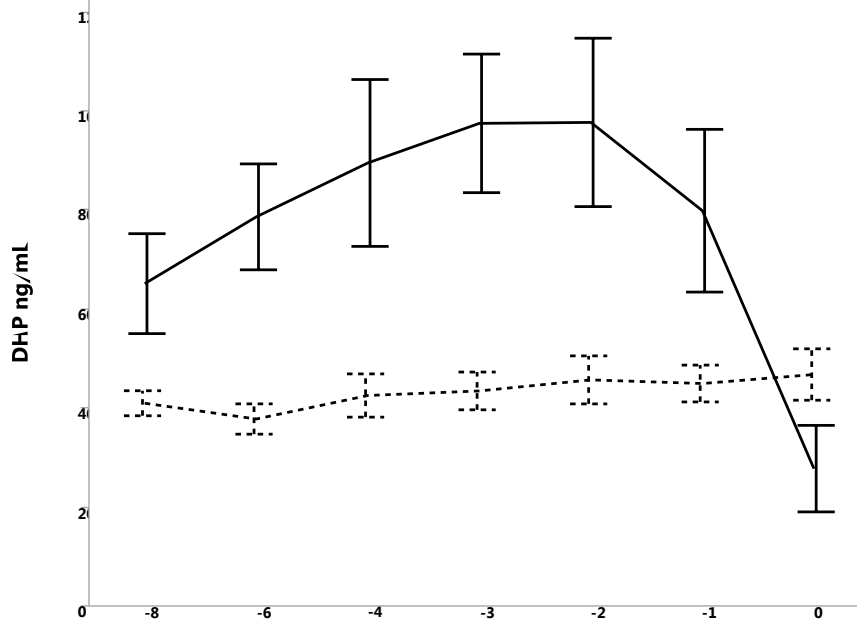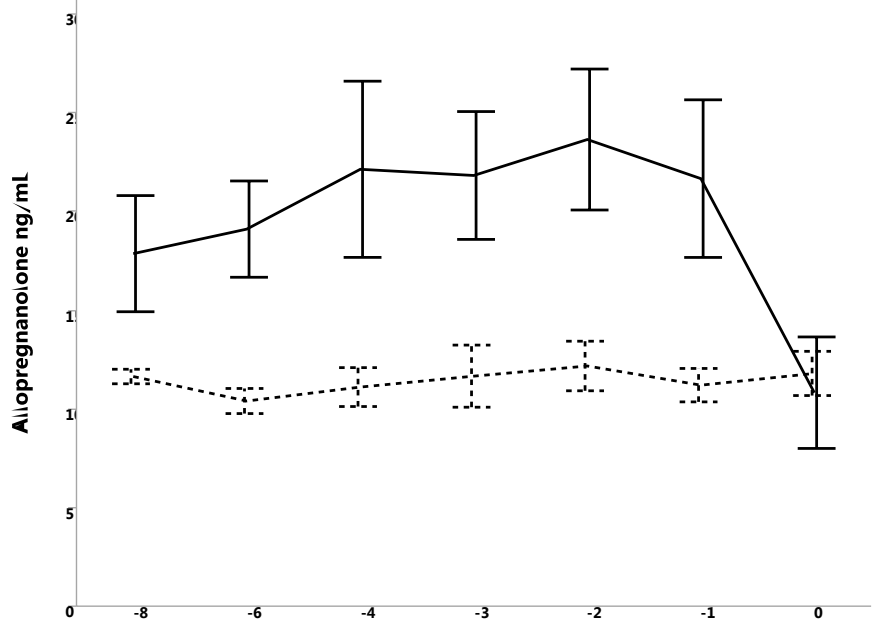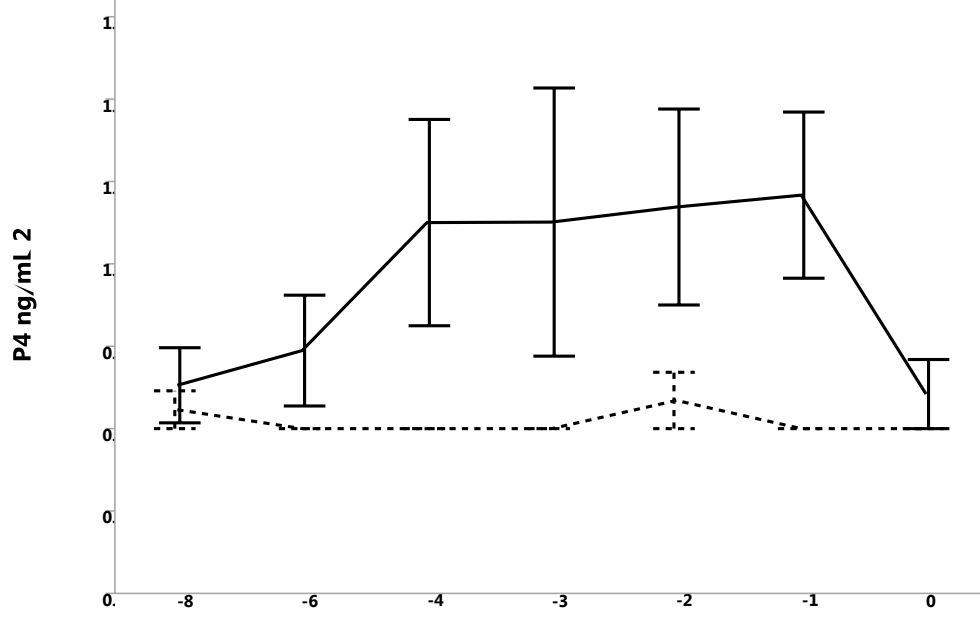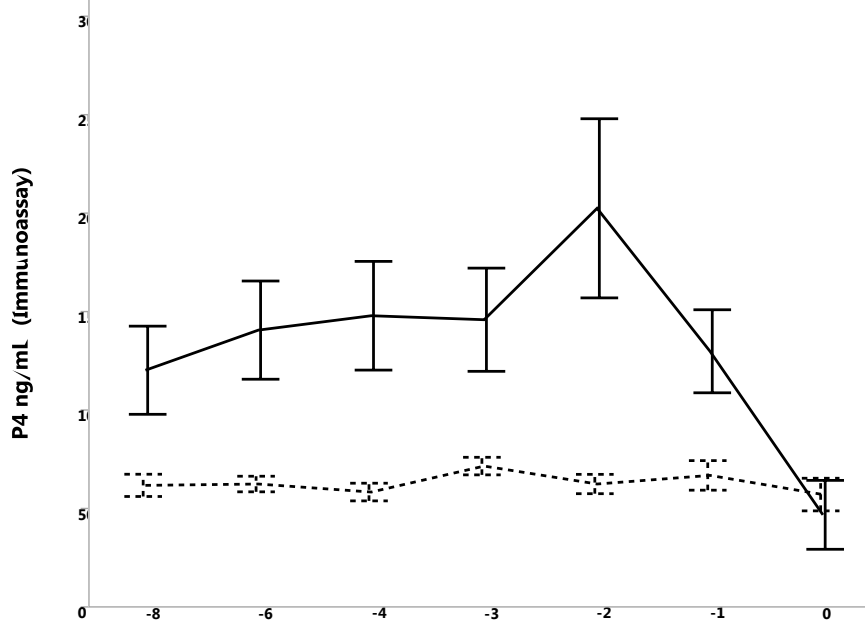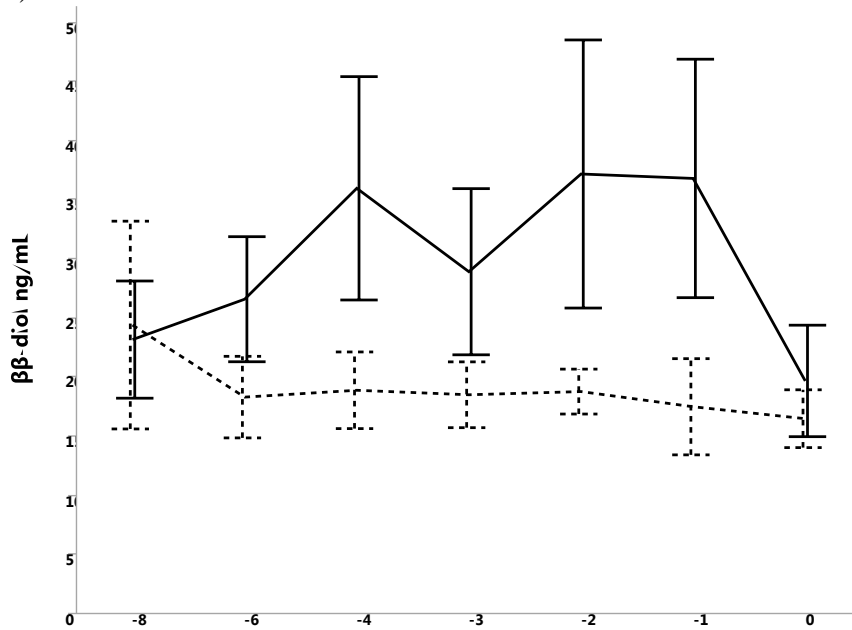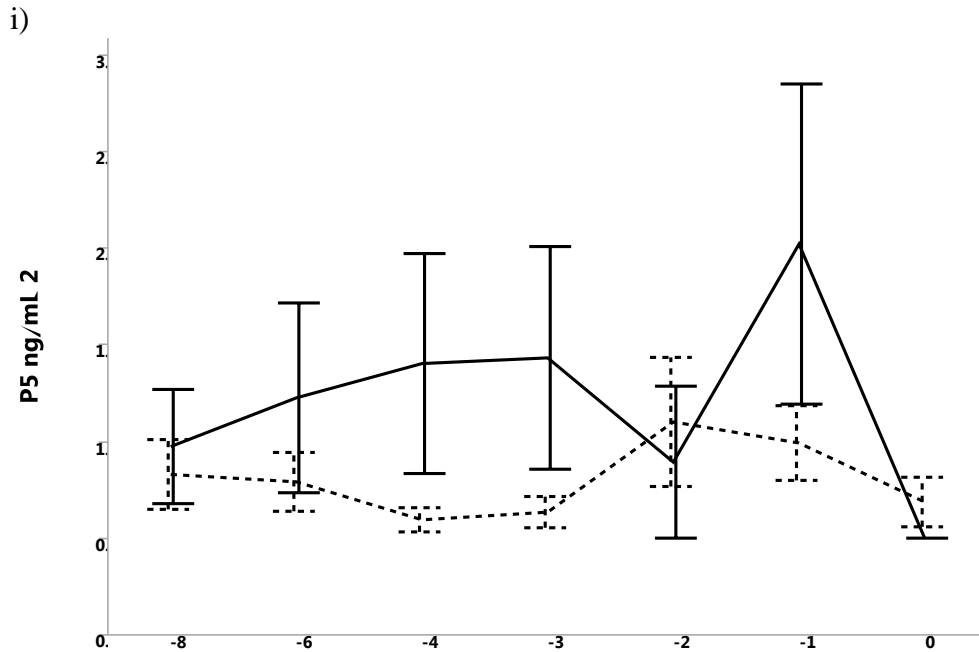
Each graph shows results for treated mares (black) and control mares (grey). Concentrations in ng/mL are reported along the y-axis for each graph and the days preceding abortion for the treated mares shown along the x-axis. Control mares were matched based upon relative days of gestation. Each graph includes error bars representing ± 1 SEM. a) 20α-hydroxy-5α-pregnan-3-one (20α5P). b) 3β-hydroxy-5α-pregnan-20-one (3β5P). c) 5α-pregnan-3β,20α-diol (βα-diol). d) 5α-dihydroprogesterone (DHP). e) Allopregnanolone. f) Progesterone (P4). g) Progesterone (P4) immunoassay (total pregnanes). h) 5α-pregnan-3β,20β-diol (ββ-diol). i) Pregnenolone (P5). For all analysis excluding results for ββ-diol and P5, a significant difference was observed between groups. Asterisks indicate significant difference between groups ($p < 0.05$).

Chapter Five: A comparison of progesterone assays for determination of peripheral
pregnane concentrations in the late pregnant mare

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Abstract

During the latter half of gestation in mares, there is a complex milieu of pregnanes
detectable in peripheral circulation. Pregnane concentrations are often measured during
late gestation to assess pregnancy well-being by immunoassay. However, interpretation
of these results is complicated by the numerous pregnanes present in high concentrations
during late gestation in the mare and by the cross-reactivity of immunoassays with these
pregnanes. Further, many mares are supplemented with an exogenous progestin,
altrenogest, which may also cross-react with existing assays. Therefore, the first
objective of this study was to compare assay results from four immunoassays (mini
VIDAS®, Immulite 1000, and two in-house ELISAs; antibody R4859 and CL425) to P4
and DHP concentrations measured with liquid chromatography tandem mass

spectrometry (LC-MS/MS). The second objective was to assess the cross-reactivity of pregnenolone, P4, DHP, allopregnanolone, and altrenogest in these four immunoassays, as these pregnanes are important in late gestation. Blood samples from four healthy mares in late gestation were evaluated by immunoassays and LC-MS/MS, with the data analyzed by regression and Bland-Altman analyses. Results indicate actual concentrations of P4 (via LC-MS/MS) correlated best to results by Immulite 1000, while DHP concentrations correlated best to mini VIDAS® results. Cross-reactivity findings resulted in no cross-reaction of altrenogest with any immunoassay, but varying degrees of cross-reactivity with other pregnanes analyzed. Results from Bland-Altman analysis indicate considerable variations in method results, with all methods significantly different than the P4 concentrations found with LC-MS/MS. These data confirm pregnane determination by immunoassay during late gestation varies depending upon the assay used and the cross-reactivity to other pregnanes present in late gestation, although the synthetic progestin altrenogest did not affect the results of any immunoassay tested. Though high correlation is observed in results from the immunoassays tested and P4 by LC-MS/MS, each assay reported poor agreement in measurement methods when compared to LC-MS/MS.

Keywords: Immunoassay, cross-reactivity, pregnanes, LC-MS/MS, equine, pregnancy

Introduction

Maintenance of pregnancy is largely attributed to progesterone (P4); however, other progestogenic hormones are quantitatively more important in pregnancy maintenance

during the latter half of gestation in the mare. For the first 80 days of gestation, P4 is considered the primary progestogen (steroid hormone that is able to bind the progesterone receptor) and is produced by the primary and secondary corpora lutea [3, 10, 96]. However, a luteo-placental shift occurs in the latter half of gestation, at which time P4 concentrations drop to low or undetectable levels (< 1ng/mL) and the primary site for pregnane (steroid hormones in the progesterone family, not necessarily bioactive) production shifts from the ovaries to the feto-placental unit [2-4, 10]. Specifically, the feto-placental unit initiates production of a number of pregnanes, originating from the precursor pregnenolone (P5), synthesized by the fetus [4, 23, 24]. These pregnanes are believed to play a role in maintenance of pregnancy in the latter half of gestation, and are present in concentrations ranging from 10 to >400 ng/mL [3, 9, 10, 24, 30]. Though the bioactivity of most of these pregnanes remains unknown, 5α-dihydroprogesterone (DHP) is bioactive in the mare and is able to bind the equine progesterone receptor with equal affinity as P4 [12, 13]. Additionally, DHP is able to maintain pregnancy in the absence of P4 [12]. A derivative of DHP, allopregnanolone, is also active in late gestation. Working through $GABA_A$ receptors, allopregnanolone elicits a suppressive response on the nervous system [34], and may play a role in maintaining fetal quiescence in late gestation [15]. While the biological function of other pregnanes is currently unclear, they are present in high concentrations in late gestation [9, 10, 95] and frequently cross-react with antibodies used in P4 immunoassays [7].

Determination of progesterone concentrations by immunoassay in late gestation is commonly carried out to help diagnose issues with the pregnancy such as placentitis [5, 6, 54]. In placentitis, acute disease typically results in a rapid decrease in total pregnanes

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whereas chronic disease is associated with increase in total pregnane concentrations [5, 6]. In both situations, interpretation of peripheral progesterone concentrations is complicated by the variable cross-reactivity inherent with immunoassays [7], as well as the high concentrations of pregnanes present in maternal circulation during late gestation which are structurally similar (Figure 5.1). Further, comparisons of results from lab to lab can be difficult as assays will report different concentrations, depending upon the antibody used. Due to these complications, a gold standard in detection has become liquid chromatography tandem mass spectrometry (LC-MS/MS). The use of LC-MS/MS has resulted in the ability to specifically identify and quantify individual pregnanes in late gestation. By being able to measure specific pregnanes, researchers have been better able to understand how certain pregnancy complications in late gestation can affect the feto-placental unit, and unique patterns of target pregnanes are associated with certain pathologies [11]. However, limitations exist due to the high cost and expertise required for LC-MS/MS analysis. Conversely, immunoassays are far more affordable and more readily available. Therefore, immunoassays continue to be the preferred method for pregnane detection in late gestation.

Another potentially confounding variable is the widespread supplementation of pregnant broodmares with the synthetic progestin, altrenogest. Beyond its typical prophylactic use, altrenogest is also supplemented in suspected cases of placental dysfunction, such as placentitis, to counteract the release of PGF2α [97] and to minimize myometrial contractility. Due to the similarities in structure between altrenogest and pregnanes (Figure 5.1), the possibility of cross-reaction in immunoassays exists, potentially leading to confounding results. Currently, the preferred method to specifically measure

altrenogest concentrations is liquid chromatography-tandem mass spectrometry (LC-MS/MS) [98-100].

Differences in results between immunoassays as well as differences in results from mass spectrometry when compared to immunoassays have been reported [11, 72, 73, 75]. This demonstrates the difficulty of trying to make comparisons across immunoassays or to quantify a targeted hormone in late gestation in the mare. The objectives of this study were: 1) to compare differences in pregnane concentrations determined with four immunoassays compared to LC-MS/MS and 2) to assess cross-reactivity observed with the same immunoassays, specifically considering P4, P5, DHP, allopregnanolone, and altrenogest.

Materials and Methods

Animal husbandry

Four healthy pregnant mares used in this study were maintained at the Maine Chance Farm, Department of Veterinary Science, University of Kentucky, Lexington, Ky. All mares were maintained on pasture and supplemented with grain and water, hay, salt, and trace minerals ad libitum. Experimental protocols were approved by the Institutional Animal Use and Care committee at the University of Kentucky (Project #2012-1067).

Study design

Beginning on day 298 of gestation (Day 0 = ovulation), jugular venous blood samples were taken daily (10-mL plain VACUTAINER), with sampling continued through

parturition. Following collection, blood was allowed to clot, samples were centrifuged at

$1,811 \times g$ for ten minutes, and serum was stored at -20°C until analysis.

Hormone Analysis

To allow for consistent sampling intervals, due to variations in gestation length, samples

were analyzed for 17, 13, 9, 5, 3, and 1 day preceding parturition and also for the day of

parturition. Pregnane concentrations were determined by two in-house ELISAs, with P4

antibodies R4859 and CL425 (Clinical Endocrinology Laboratory, University of

California, Davis, CA) and by two commercial, automated immunoassay platforms: mini-

VIDAS® (mini-VIDAS®; Biomérieux, Boston, MA) and Immulite 1000 (Siemens

Healthcare Diagnostic Products, Ltd., Malvern, PA). Each assay was performed

according to the manufacturer's recommended protocol, or as described below. Aliquots

of the same samples were sent to the University of California, Davis and analyzed via

LC-MS/MS following an established protocol [10] (see below) to determine P4 and DHP

concentrations, specifically. Details for each assay can be seen in Table 5.1. Intra- and

inter-assay coefficients of variation (%CVs) for each assay can be seen in Table 5.2.

Analysis by antibodies R4859 and CL425

Ten microliters of serum was mixed with 0.5 mL of petroleum ether (Fisher Scientific,

Hampton, NH) in a glass tube and vortexed for 45 s. Serum was frozen by holding the

tube in liquid nitrogen, and the ether was decanted into a clean glass tube and dried

overnight under a fume hood. Samples were reconstituted with 100 µL of phosphate

buffered saline with 0.1% bovine serum albumin (Sigma, St. Louis, MO) and HRP

conjugated with P4 (Clinical Endocrinology Laboratory, University of California, Davis,

CA) at 1:45,000. Progesterone standard was purchased from Steraloids (Newport, RI). Progesterone dissolved in methanol (Fisher Scientific, Hampton, NH) was used to generate a standard curve with nine standard points, ranging from 2.5 - 2500 ng/mL. Standard points were made by pipetting appropriate amounts of solution into glass tubes and allowed to dry, then reconstituted with 2 mL of HRP-conjugated buffer.

For the standard plate and separate sample plates, 50 µL of HRP-conjugated buffer was pipetted across a 96-well plate, previously coated with antibody. The standard curve was generated by adding 50 µL of each standard in replicates of eight across the plate. Curves were linear with correlation coefficients of >0.99. Samples were pipetted on separate plates in duplicate at 50 µL. High (300 ng/mL) and low (9ng/mL) controls were included in duplicate on each plate. After two hours of incubation, the plate was washed three times and substrate (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) in citric acid (Sigma, St. Louis, MO)) was pipetted across the plate at 100 µL/well. After a second incubation of 1.5 hours, 100 µL of stop solution (EDTA in hydrofluoric acid (Sigma, St. Louis, MO)) was added to each well and the plates were read with an Epoch microplate spectrometer (BioTek, Winooski, VT).

Analysis by LC–MS/MS

Standards were purchased from Steraloids: DHP, P4, and d9-progesterone. A master mix of all reference standards was prepared and diluted in methanol (10, 1, 0.1 and 0.01 ng/mL). Methanol and water were of HPLC grade and obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid and methyl tert-butyl ether were of ACS grade and obtained from EMD (Gibbstown, NJ, USA).

Samples were extracted according to the method developed and described by Legacki et al., [10]. Inter- and intra-accuracy and precision were assessed at four QC concentrations (0.6, 1.5, 20, and 80 ng/mL) for P4 and DHP using six replicates. The analytes were measured with ≤15% deviation from the expected concentrations for the three highest QC concentrations and %CV of ≤15%. The analytes had a percent accuracy >90% and a precision <15%. Curves were linear with correlation coefficients of >0.99.

Cross-reactivity: Standards and solutions

Standards were prepared to determine possible cross-reactions across the immunoassays for a subset of pregnanes present during equine pregnancy. The following steroids were obtained from Steraloids: P4, DHP, P5, allopregnanolone, and altrenogest. All steroids were purchased as crystalline powder and were dissolved in methanol at an initial concentration of 100,000 ng/mL. Phosphate buffered saline with 0.1% bovine serum albumen served as the matrix for the standards. Control buffer was tested for matrix effects on each assay prior to any sample analysis, with no effect noted.

Sample preparation and evaluation

Serial dilutions of each steroid were made at concentrations of 50, 20, 10, 2, 1, 0.5, 0.2, and 0.1 ng/mL by first drying the stock solution in methanol under air, then reconstituting with buffer. These concentrations constituted the standard curve for each steroid, and were used in each immunoassay. Results were transformed using B/B0, with B representing treatment, and B0 representing the buffer control. Curves were generated utilizing Gen 5 software (BioTek Instruments, Inc.), with cross-reactions calculated using the fifty percent binding found for each steroid. (Brown et al., 2003). Specifically, we

identified the x-value (concentration) where the curve for each pregnane or altrenogest crossed 50% binding. The value for P4 was then divided by the value for each steroid and multiplied by 100 to obtain cross-reactivity estimates.

Statistics

To compare assay results, data were analyzed by linear regression analysis using data from LC-MS/MS as the x-value and the tested immunoassay as the y-value in the regression. Data were also analyzed using a Bland-Altman plot (pregnane concentration measured by LC-MS/MS minus concentration by immunoassay vs. pregnane concentration measured by LC-MS/MS plus concentration measured by immunoassay / 2). Data were analyzed utilizing JMP software (JMP®, Version *12*. SAS Institute Inc., Cary, NC, 1989-2007.). Significance was set at p<0.05.

<div align="center">Results</div>

Pregnane analysis via LC-MS/MS

All mares in this study had normal pregnancies and delivered live foals with a mean gestational age of 329 ± 5.2 d. Average P4 and DHP concentrations peaked at three days prior to parturition with P4 concentrations of 1.8 ng/mL and DHP concentrations of 79.8 ng/mL (Figure 5.2).

Pregnane analysis via immunoassay

Based upon Bland-Altman analysis, all four immunoassays evaluated gave higher (P < 0.0001) measured concentrations of progesterone than did LC-MS/MS (Figure 5.3). Mean differences between measurements of pregnanes between LC-MS/MS and

immunoassay methods were lowest for the Immulite 1000 platform and greatest for the immunoassay based upon monoclonal antibody CL425 (Table 5.3). Similarly, regression slopes for pregnane concentrations for immunoassays were positive (P<0.001) with the Immulite 1000 assay having the lowest slope and the immunoassay based upon monoclonal antibody CL425 having the greatest slope (Table 5.3). For each immunoassay, measured pregnane concentrations were moderately to strongly correlated (P < 0.001) with measured P4 concentrations determined by LC-MS/MS (Table 5.3). Linear regression analysis for concentrations of DHP measured by LC-MS/MS versus pregnane concentrations determined by immunoassay revealed significant positive slopes for all immunoasays and y-intercepts were greater than zero for both the Immulite 1000 and mini VIDAS® (Table 5.3).

Cross-reactivity

Estimates for cross-reactivity (50% binding (B/B$_o$) of each measured steroid compared to progesterone) varied across target pregnanes and immunoassay (Table 5.4 and Figure 5.4). In general, reported and measured cross reactivities against DHP for antibodies R4859 and CL425 appeared much greater than the automated commercial progesterone assays (mini VIDAS® and Immulite 1000). None of the assays in this study cross-reacted with altrenogest.

Discussion

Results from this study demonstrate the complications of using immunoassays in late gestation in the mare. While identical samples were analyzed, variations in cross-reactivity with the antibody used in each assay resulted in large differences in reported

concentrations. Immunoassay results from each of the four assays tested did show a positive and linear correlation with P4 and DHP by LC-MS/MS. However, when comparing methods, results indicate poor agreement of each immunoassay to P4 concentrations by LC-MS/MS. Each assay cross-reacted with the tested hormones to a different degree than reported, indicating an additional complication in interpreting assay result in late gestation. The synthetic progestin, altrenogest, had not been previously tested for cross-reactions on any of the four assays and reported no cross-reactions in this study.

High concentrations of circulating pregnanes in late gestation in the mare can create difficulties in interpretation of immunoassay results due to the various immunoassays available and the cross-reactions that can occur with the antibodies. Previous work with mass spectrometry has analyzed healthy and compromised equine pregnancies in late gestation [9-11], with unique profiles of specific pregnanes observed in clinical diagnosis from mares with compromised pregnancies [11]. While this method of analysis is the superior choice, demand has not yet warranted this technology in most labs. Until then, immunoassays will remain the dominate method of hormone quantification. As this study has demonstrated, an emphasis should be placed in understanding a particular P4 assay when analyzing blood samples from mares in late gestation. Being familiar with the reported cross-reactions and the gestational stage when the sample was taken can greatly assist the clinician in understanding assay results. However, the clinician should be aware that differential cross-reactions are possible, even if not reported. An additional complication in comparing reported cross-reactivities of antibodies is the lack of standardization in nomenclature (Table 5.5).

Although it is widely believed that monoclonal antibodies are more specific than polyclonal antibodies, this is still dependent on the individual antibody. Within this study, we utilized a monoclonal antibody with very high levels of cross-reactivity (P4 antibody CL425), as well as a polyclonal antibody with minimal cross-reactivity (Immulite 1000) (Tables 5.1 and 5.5). All assays in this study demonstrated a positive, linear correlation to P4 and DHP concentrations, despite the variations in reported concentrations. This was expected, as pregnanes in the mare increase with time leading up to parturition [3, 9-11]. Further, all assays resulted in better correlation to DHP results than P4. Again, this is expected as P4 concentrations are negligible in late gestation, while DHP can reach concentrations of approximately 30-100 ng/mL [9, 10].

When considering each assay individually, the cross-reactivities (Table 5.4 and Table 5.5) help to explain the correlation results (Table 5.3). The Immulite 1000 had the greatest correlation to P4 by LC-MS/MS. The reported cross-reactivities are very low, and this platform is the most specific for P4 of the immunoassays tested. The study results indicated no cross-reactivity with the target pregnanes; however, this did not agree with the reported values. We speculate that lengthening the curve with additional standard points at higher concentrations would have resulted in cross-reactions for P5, DHP, and allopregnanolone (Figure 5.4).

The mini VIDAS® had the best correlation with DHP by LC-MS/MS. Again, when considering the cross-reactivity rates, this assay is reported to cross-react with DHP but little else. Study results for cross-reactions indicate possible cross-reactions with DHP and P5, which was not reported to cross-react, but not to allopregnanolone or altrenogest.

Similar to the Immulite 1000, it appears as though additional standard points in higher concentrations would have resulted in a cross-reaction for allopregnanolone (Figure 5.4).

Antibodies R4859 and CL425 exhibited the highest cross-reactivity in terms of number of pregnanes/hormones and percent of possible cross-reactions (Table 5.4 and 5.5). Therefore, it is no surprise that they had the poorest correlations of the assays tested with P4 and DHP concentrations by LC-MS/MS. Results from cross-reactions tested indicate differential degrees of cross-reactivity, and an additional cross-reaction not previously reported (allopregnanolone with antibody R4859). Similar to the previous assays, altrenogest did not cross-react with either antibody, and in contrast to the pregnanes, it does not appear that additional standard points in higher concentrations would have affected the results for any of the assays tested (Figure 5.4). Reported cross-reactions and tested cross-reactions (besides those for P4) were different in each assay. Possible differences in reagents, lab techniques, and human error may explain these variances.

Analysis with Bland-Altman plots were included to showcase the vast differences in results that can be seen in differing P4 assays during late gestation in the mare. Several P4 immunoassays are available, but the reported concentrations will be different from assay to assay, and certainly different from concentrations found using mass spectrometry. Previous work has shown that while results from different methods may strongly correlate, poor agreement can exist when comparing methods [101]. In this study, each P4 immunoassay significantly correlated to P4 concentrations by LC-MS/MS as discussed previously; however, poor agreement was observed in each immunoassay when compared to LC-MS/MS as immunoassays report results from all possible cross-reactions and actual P4 is negligible at this time in gestation.

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As stated previously, earlier studies have demonstrated variations in immunoassay results. However, to the author's knowledge, possible cross-reactions with altrenogest have not been evaluated with current immunoassays. A previous report showed no cross-reaction with altrenogest by radioimmunoassay [102], but this updated analyses may be useful in cases when a mare is supplemented and the owner or veterinarian may be concerned with possible cross-reactions. If altrenogest did cross-react with the chosen immunoassay, it could lead to confusion with the diagnosis or a missed diagnosis. However, the assays tested in this study indicated that altrenogest did not alter immunoassay results.

Conclusion

High concentrations of circulating pregnanes in late gestation in the mare create difficulties in measuring concentrations of specific pregnanes. Adding to this, large differences between immunoassays are present due to the variable levels of cross-reactivity within individual antibodies. Immunoassay results consistently correlated more strongly with DHP values than P4 in the late pregnant mare, as measured by LC-MS/MS; however, all immunoassays showed poor agreement to concentrations of P4 by LC-MS/MS. When cross-reactivity levels were analyzed, significant levels of cross-reactivity were seen with targeted pregnanes, but not with altrenogest. Overall, this study demonstrates the importance of gaining familiarity with your selected immunoassay when analyzing pregnane concentrations in the late pregnant mare.

	Immulite 1000	mini VIDAS®	R4859	CL 425
Antibody Description	Polyclonal	Monoclonal	Polyclonal	Monoclonal
Sensitivity/Limit of Detection	0.20 ng/mL	0.25 ng/mL	0.02 ng/mL	0.04 ng/mL
Reportable Range:	0.20-40 ng/mL	0.25-80.0 ng/mL	0.02-200 ng/ml	0.04-200 ng/mL

Table 5.1 Assay details for each of the immunoassays used. Table indicates the type of antibody used, the sensitivity or limit of detection, the reportable range, and reported cross-reactivity for each assay.

	R4859	CL425	mini VIDAS®	Immulite 1000
Intra-assay %CV	8.4	11.7	6.3	7.4
Inter-assay %CV	NA	NA	8.2	8

Table 5.2 Average coefficients of variation (%CV) for each assay. Coefficients of variation for immunoassays R4859 and CL425 were calculated by assaying all samples and controls in duplicate. No inter-assay CV was calculated for either of these assays as all samples were ran in one assay. For the mini VIDAS®, a control and calibration standards are included in each new kit and assayed prior to samples, with the %CVs reported from the manufacturer. Similarly, the %CVs for the Immulite 1000 are reported from the manufacturer and high and low controls are assayed in two week intervals.

	r	Slope	y-intercept	Bland-Altman mean difference	95% CI
P4 by LC-MS/MS					
CL425	0.69	182.6*	59	211.4**	171-297.8
R4859	0.68	73.3*	42.2*	99.8**	85.4-136.3
mini VIDAS®	0.72	20.5*	22.1*	38.4**	35-48.4
Immulite 1000	0.76	3.4*	2.5*	4.5**	4-5.9
DHP by LC-MS/MS					
CL425	0.88	4.1*	-12.1		
R4859	0.9	1.7*	11.5		
mini VIDAS®	0.88	0.4*	15.3*		
Immulite 1000	0.86	0.1*	1.8*		

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Table 5.3 Results from regression analysis showing correlation coefficients (r), slopes of the curves, and y-intercepts. Also showing mean difference by Bland-Altman and 95% confidence intervals (CI) by for each immunoassay platform when comparing results of P4 or DHP by LC-MS/MS.
*Values for slope and intercept differ from zero (p<0.05).
**Mean difference in Bland-Altman differs from zero (P < 0.01)

Steroid	R4859		CL425		mini VIDAS®		Immulite 1000	
	Reported %	Study results %	Reported %	Study results %	Reported %	Study results %	Reported %	Study results %
Progesterone	100	100	100	100	100	100	100	100
DHP	12.2	29.3	55	30.6	13	8.6	1	0
P5	0.1	14.5	12.5	18.8	not tested	2.7	0.6	0
Allopregnanolone	0	10.9	64	10	not tested	0	not tested	0
Altrenogest	not tested	0	not tested	0	not tested	0	not tested	0

Table 5.4 Reported cross-reactivities and cross-reactivities found from this study for each compound, across each assay tested

Immulite 1000		mini VIDAS®		R4859		CL 425	
Steroid	%	Steroid	%	Steroid	%	Steroid	%
Progesterone	100	Progesterone	100	Progesterone	100	4-Pregnen-3α-ol-3.20-dione	188
5β-Dihydro-progesterone	6.6	5β-dihydroxyprogesterone	17.39	11α-OH-Progesterone	40	4-Pregnen-3β-ol-20-one	172
Corticosterone	1.9	5α-dihydroxyprogesterone	12.95	5α-Pregnane-3,20-dione	12.19	4-Pregnen-11α-ol-3,20-dione	147
20α-Dihydro-progesterone	1.0	17α-hydroxyprogesterone	1.18	17α-OH-Progesterone	0.38	Progesterone	100
11-Deoxy-corticosterone	0.8	Deoxycorticosterone	1.15	20α-OH-Progesterone	0.13	5α-Pregnan-3β-ol-20-one	94
Pregnenolone	0.6	6β-hydroxyprogesterone	0.29	20β-OH-Progesterone	0.13	Allopregnanolone	64
17α-Hydroxy-progesterone	0.6	16α-hydroxyprogesterone	0.2	Pregnenolone	0.12	5α-Pregnan-3,20-dione	55
Testosterone	0.1	Corticosterone	0.09	Cortisol	<0.04	5β-Pregnan-3b-ol-20-one	12.5
		20α-hydroxyprogesterone	0.03	Pregnanediol	<0.01	5β-Pregnan-3,20-dione	8
		Testosterone	0.01	Estradiol 17β	<0.01	4-Pregnen-11β-ol-3,20-dione	2.7
		Estrone	0.01	Estrone	<0.01	5β-Pregnan-3α-ol-20-one	2.5
		Estradiol	<0.01	Testosterone	<0.01	5α-Pregnan-3α,20β-diol	<0.1
						Pregnanediol	<0.1
						Androstenedione	<0.1
						Corticosterone	<0.1

Table 5.5 Reported cross-reactivities from each assay

Figure 5.1 Structures of progesterone (P4), pregnenolone (P5), 5α-dihydroprogesterone DHP), allopregnanolone, and altrenogest.

P4

P5

DHP

Allopregnanolone

Altrenogest
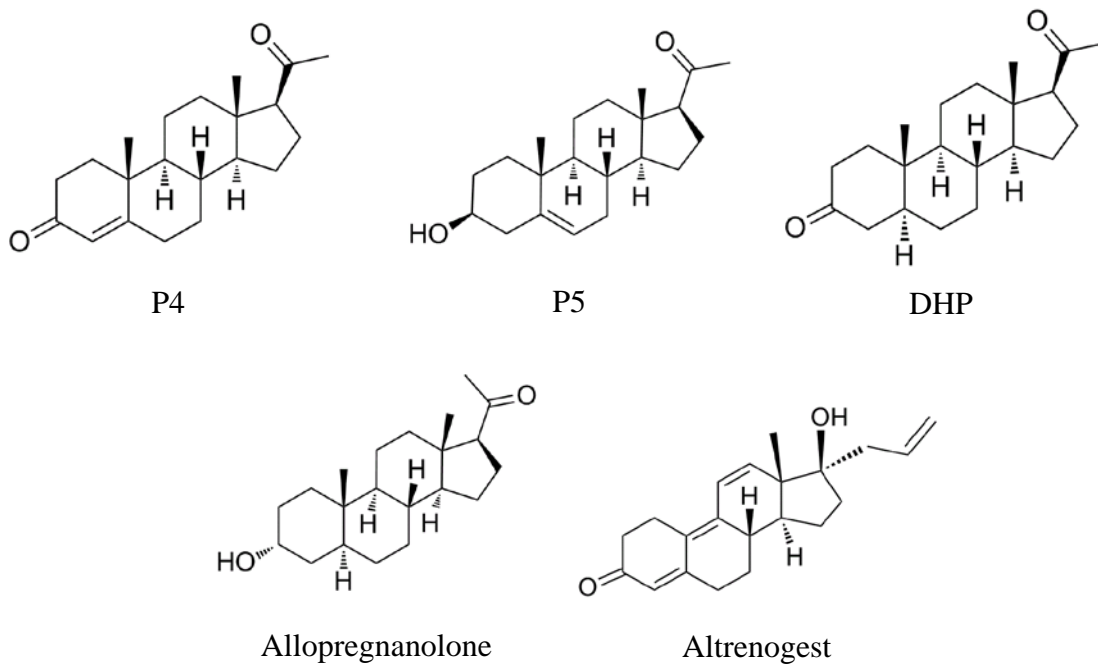
Figure 5.2 Average pregnane concentrations by four immunoassays and LC-MS/MS

Comparison of Immunoassays to P4 and DHP
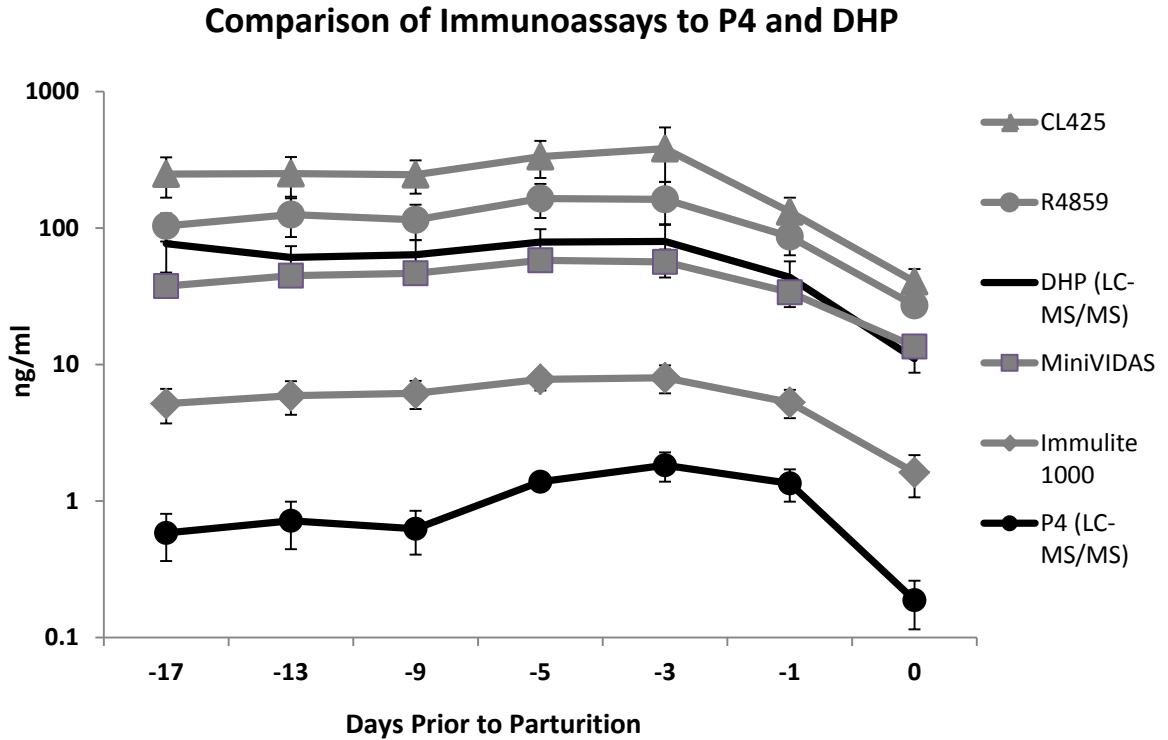
Average pregnane concentrations (ng/mL) measured in pregnant mare serum (n=4) in days prior to parturition from antibody CL425 (grey triangles), antibody R4859 (grey circles), mini VIDAS (grey squares), and the Immulite 1000 (grey diamond). Concentrations of P4 (black circles) and DHP (black line) measured with LC-MS/MS. The y-axis was log-transformed to separate the curves. Date represent mean ± S.E.M.

Figure 5.3 Bland-Altman plots
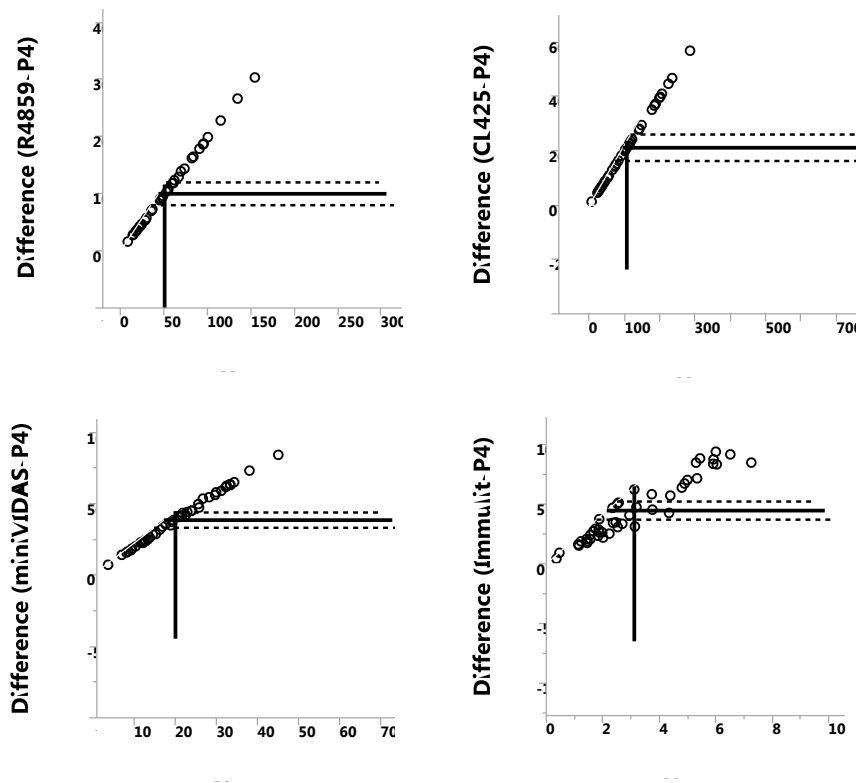
Bland Altman plots showing differences in results for each immunoassay compared to progesterone values found with LC-MS/MS. The difference between a paired set of measures is plotted on the y-axis, with the mean of the two plotted along the x-axis. Solid lines indicate mean difference, with dotted lines indicating the upper and lower 95% confidence levels.

Figure 5.4 Cross reactivity graphs

R4859

Legend:
- ✳ Altrenogest r=0.87
- ✕ Allopregnanolone r=0.99
- ▲ P5 r=1
- ■ DHP r=0.99
- ◆ P4 r=1

CL425

Legend:
- ✳ Altrenogest r=0.36
- ✕ Allopregnanolone r=1
- ▲ P5 r=1
- ■ DHP r=0.99
- ◆ P4 r=1
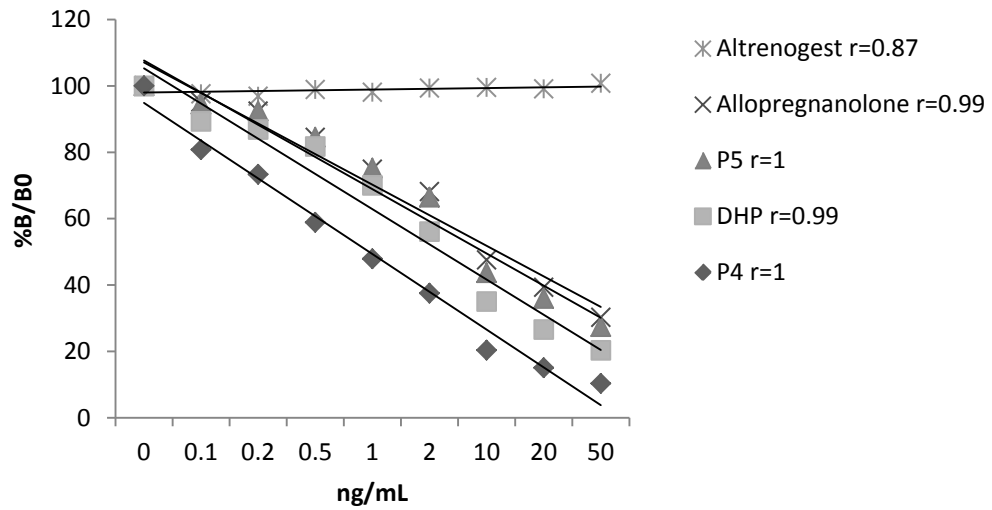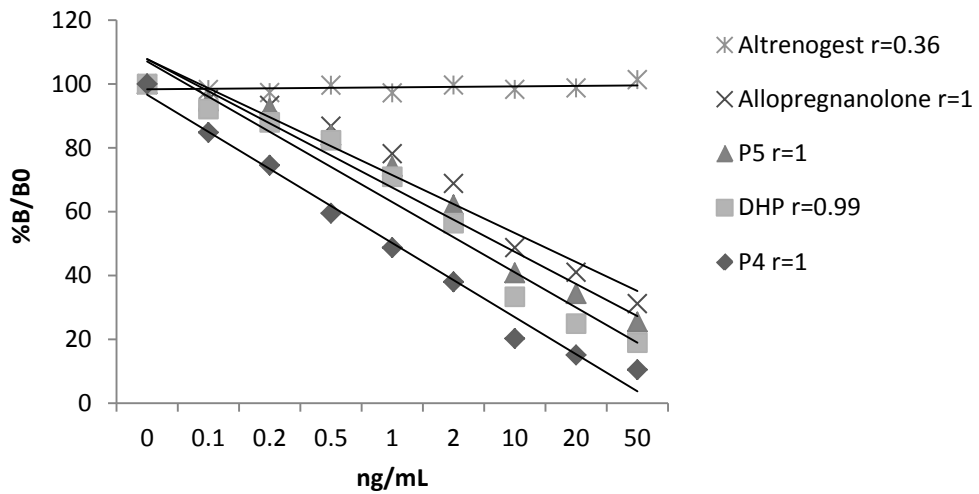
miniVIDAS

Immulite

Cross-reactivity graphs. The y-axis for each graph shows %B/B0 values (defined as signal from a sample divided by the maximum intensity of signal), and the x-axis is shown as pregnane concentration (ng/mL). Correlation coefficients (r) are given for each compound on each assay platform. Cross-reactivities were determined at 50% B/B0.

Chapter six: Summary and conclusions

Pregnane profiles are complex in the late pregnant mare, as the relationship between the fetus and placenta is essential in pregnane metabolism. Of the numerous pregnanes that have been identified, only P4, DHP, allopregnanolone, and potentially 20α5P and βα-diol have demonstrated bioactivity. While the function of the remaining pregnanes is currently not understood, we can speculate that they hold some importance due to their elevated concentrations at this time in gestation. Measurement of specific pregnanes with compromised pregnancies has demonstrated significant changes in pregnane profiles. However, these key changes cannot be detected by immunoassay due to high inc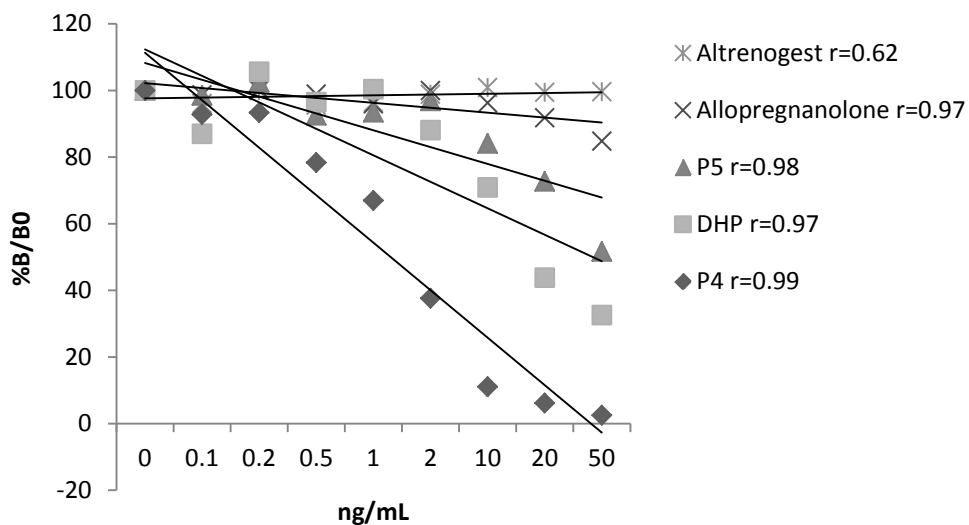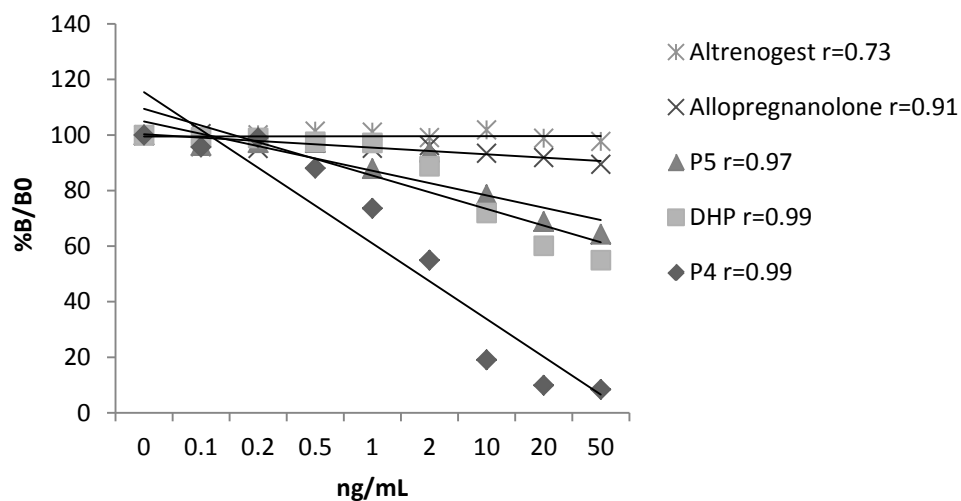idences of cross-reactions. Future work utilizing LC-MS/MS or GC-MS will allow a better understanding of changes in pregnanes as they relate to healthy or compromised pregnancies.

The work in this thesis has demonstrated that attempts to significantly alter pregnancy outcome by enzyme inhibition is not an easy task. Inhibition of 5α-reductase did not have a significant effect on gestation length or concentrations of DHP. It did significantly increase P4 concentrations in the treated mares, indicating its activity by producing an increase in a precursor to 5α-reductase. Although concentrations of dutasteride were seen to increase even beyond the cessation of treatment, the overall lack of response suggests that a higher dose or longer treatment period is necessary to elicit a reliable and significant change in pregnancy outcome or DHP concentrations.

Changes in pregnane concentrations as they relate to pregnancy complications in the mare have been previously studied. Although immunoassays offer a cost-effective means

of measurement, the high incidences of cross-reactions make interpretation of assay results difficult. For this reason, we utilized LC-MS/MS to measure changes in P5, P4, DHP, allopregnanolone, 20α5P, 3β5P, ββ-diol, and βα-diol, specifically, in a group of mares with induced ascending placentitis. Concentrations of pregnanes metabolized in the placenta (P4, DHP, allopregnanolone, 20α5P, 3β5P, and βα-diol) were significantly increased in the inoculated mares. Changes in pregnane profiles of inoculated mares just prior to abortion closely mirrored those observed in healthy pregnancies just prior to parturition, indicating a common mechanism at the end of gestation.

As stated previously, immunoassay results are challenging to interpret in late gestation. To demonstrate this difficulty, a comparison of four P4 immunoassays was made against P4 and DHP concentrations by LC-MS/MS. Significant correlations were observed with all immunoassay results when compared to actual P4 or DHP concentrations, as would be expected with the natural rise in pregnanes in late gestation. However, making comparisons across assays is nearly impossible in late gestation with the variations in antibodies resulting in significant differences in assay results.

In conclusion, the measurement and interpretation of pregnane concentrations is currently taking place at a tipping point in technology. Although immunoassays provide a cost-effective means of measurement, mass spectrometry is currently the only way to measure changes in specific pregnanes in late gestation. Alterations to important enzymes through either naturally occurring disease in pregnancy or in controlled studies will result in certain changes in pregnanes. These changes, measured by mass spectrometry, will lead to a better understanding of fetal and placental health and function in late gestation in the mare, ultimately resulting in improved diagnostic abilities for compromised pregnancies.

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Abstracts

M.A.A. Wynn, E.L. Legacki, A.J. Conley, S.A. Loux, A. Esteller-Vico, S.D. Stanley, E.L. Squires, M.H.T. Troedsson, and others. Determination of peripheral progestin concentrations in the late pregnant mare based upon immunoassays and liquid chromatography-tandem mass spectrometry. Journal of Equine Veterinary Science, Vol. 35, Issue 5, p441–442

M. Wynn*, E. Legacki, A. Conley, S. Loux, A. Esteller-Vico, S. Stanley, E. Squires, M. Troedsson, and B. Ball Inhibition of 5α-reductase during late gestation in the mare Reproduction, Fertility and Development 28(2) 163-163 https://doi.org/10.1071/RDv28n2Ab67 Published: 3 December 2015

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<u>Publications</u>

Reproduction. 2016 Oct;152(4):323-31. doi: 10.1530/REP-16-0227. Progestin withdrawal at parturition in the mare. Legacki EL1, Corbin CJ1, Ball BA2, Wynn M2, Loux S2, Stanley SD3, Conley AJ4.

<u>Presentations</u>

Determination of peripheral progestin concentrations in the late pregnant mare based upon immunoassay and liquid chromatography - tandem mass spectrometry
Conference of the Center for Clinical and Translational Science, 34th Annual Symposium in Women's Health and Reproductive Science. April 2015, Lexington, KY.

Determination of peripheral progestin concentrations in the late pregnant mare based upon immunoassays and liquid chromatography-tandem mass spectrometry
M.A.A. Wynn, E.L. Legacki, A.J. Conley, S.A. Loux, A. Esteller-Vico, S.D. Stanley, E.L. Squires, M.H.T. Troedsson, and others
Equine Science Symposium. May 2015, St. Pete Beach, FL

A prospective case-control study of biomarkers for feto-placental well-being in the mare
Conference of the Center for Clinical and Translational Science, 35th Annual Symposium in Women's Health and Reproductive Science. April 2016, Lexington, KY.

Inhibition of 5α-reductase during late gestation in the mare
M. Wynn*, E. Legacki, A. Conley, S. Loux, A. Esteller-Vico, S. Stanley, E. Squires, M. Troedsson, and B. Ball (Abstract 67)
International Embryo Transfer Society. January 2016, Louisville, KY

<u>Publications in Preparation</u>

Michelle Wynn, Barry A. Ball, Erin Legacki, Alan Conley, Shavahn Loux, John May, Alejandro Esteller-Vico, Scott Stanley, Kirsten Scoggin, Edward Squires, Mats Troedsson. Inhibition of 5α-reductase alters pregnane metabolism during late gestation in the mare.

Michelle Wynn, Barry A. Ball, John May, Alejandro Esteller-Vico, Igor Canisso, Edward Squires, Mats Troedsson. Changes in maternal pregnane concentrations in mares with experimentally induced ascending placentitis.

Michelle A. Wynn, Erin L. Legacki, Alan J. Conley, Shavahn C. Loux, Alejandro Esteller-Vico, Scott D. Stanley, Thomas E. Curry, Jr., Edward L. Squires, Matts H. Troedsson, Barry A. Ball. A comparison of progesterone assays for determination of peripheral pregnane concentrations in the late pregnant mare