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Igor Frederico Canisso, Student

Dr. Barry A. Ball, Major Professor

Dr. Daniel Howe, Director of Graduate Studies

STUDIES ON EQUINE PLACENTITIS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in the College of Agriculture, Food
and Environment at the University of Kentucky

By

Igor Frederico Canisso

Lexington, Kentucky

Director: Dr. Barry A. Ball, Professor of Equine Reproduction

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ABSTRACT OF DISSERTATION

Two types of placentitis were studied: ascending and nocardioform placentitis. Although the first diagnosis of nocardioform placentitis was made three decades ago, little is known about the disease, due to the lack of an experimental model. In attempt to develop a research model, *Crossiella equi* was inoculated through intrauterine, intravenous, intrapharyngeal, and oral routes, but none of the routes resulted in nocardioform placentitis. This may indicate that unidentified factors may play a role in disease pathogenesis and that simple presence of bacteria is not sufficient to induce nocardioform placentitis. The second and major component of this dissertation involved the identification of diagnostic markers for placentitis. Because ascending bacterial placentitis is readily and predictably induced using existing experimental models, this model was used to identify diagnostic markers for placentitis in maternal plasma and fetal fluids. Three potential biomarkers were examined: acute phase inflammatory proteins, steroid hormones produced by the fetoplacental unit, and protein composition of the fetal fluids. Of the three acute phase proteins investigated, serum amyloid A and haptoglobin but not fibrinogen increased in association with experimentally induced ascending placentitis. Androgens and progestins appear to be poor markers for placentitis. Serum estradiol 17 β concentrations were reduced in mares with experimentally induced placentitis and appear to be a good marker for placentitis in mares. Different methods were used to study the protein composition of the fetal fluids. Alpha-fetoprotein was characterized as a major protein present in the equine fetal fluids, and this protein was elevated in plasma of mares with placentitis. In another study, using a high-throughput proteomic technique several new proteins were characterized in the amniotic and allantoic fluids of mares carrying normal pregnancies, and several previously uncharacterized proteins were detected in the allantoic fluid of mares with placentitis. Three secreting proteins were elevated in allantoic fluid of mares with experimentally induced ascending placentitis.

Key-words: ascending placentitis, nocardioform placentitis, pregnancy loss, premature delivery, abortion

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STUDIES ON EQUINE PLACENTITIS

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Et in te!

Credo in somni!

Labor omnia vincit!

Igor Canisso

This dissertation is dedicated to my wife Erotides Capistrano da Silva and to my daughter Pietra Capistrano Canisso. The completion of this work has stolen several valuable hours of their personal time.

In memoriam of the beloved ones that did not have the chance to make through the end of the journey that started 16 years ago:

Alexandre Canisso

Antonio Frederico

Maria M. Canisso

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Chapter 1

Review of the Literature

1.1. Introduction

Late-term pregnancy wastage generates several million dollars of losses to horse owners each year due to the costs of producing a pregnancy (i.e. breeding fees, semen transport, feed, vaccinations, veterinary services, etc.). Additionally, late term pregnancy loss may also cause significant emotional stress to the owner that might have high expectations for the foal from a particular mating. Placentitis, an important cause of late-term pregnancy wastage, is estimated to affect 3 to 5% of pregnancies in Thoroughbred mares (Troedsson & Zent 2004). Therefore, means to prevent pregnancy loss are warranted.

Placental pathology/insufficiency was identified in a large retrospective study as being responsible for greater than 60% of pregnancy losses (i.e. abortions, stillbirths and neonatal deaths up to 24 hours post-delivery) in central Kentucky (Giles *et al.* 1993). Two additional retrospective studies carried several years apart by the same referral diagnostic laboratory supported the finding that placentitis was the most common cause of late pregnancy loss in mares (Hong *et al.* 1993, Williams *et al.* 2004). Outside North America, placentitis has also been indicated as an important cause of pregnancy loss in mares in Brazil (Moreira *et al.* 1998, Marcolongo-Pereira *et al.* 2012), France (Laugier *et al.* 2011), and Australia (Carrick *et al.* 2014). However, in the United Kingdom umbilical cord pathology (e.g. torsion and entrapments) was the leading cause of pregnancy loss in submissions received by a referral diagnostic laboratory (Smith *et al.* 2003). These differences between the United Kingdom and the rest of the world can probably be

accounted for the methodology applied to diagnose placentitis, type of placentas presenting lesions which are submitted for pathologic examination, presence of certain microorganisms (e.g. nocardioform organisms) as well as potential differences in the true incidence of diseases.

1.2. Overview of Etiopathogenesis and Epidemiology of Placentitis

Several infectious agents have been commonly recovered from placentitis cases; bacteria are responsible for the large majority of cases and fungi for the minority (Table 1.1). Bacterial agents commonly associated with the occurrence of placentitis include *Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Leptospira* sp, *Crossiella equi* (*C. equi*) and *Amycolatopsis* species (*Amycolatopsis kentuckyensis*, *Amycolatopsis lexingtonensis* and *Amycolatopsis pretoriensis*) (Hong *et al.* 1993, Giles *et al.* 1993, Moreira *et al.* 1998, Williams *et al.* 2004) (Table 1.1). Fungi associated with equine placentitis primarily include *Aspergillus* ssp and *Candida albicans* (Hong *et al.* 1993).

Leptospiral infection causing abortions are highly variable across the years in central Kentucky (Williams *et al.* 2004, Erol *et al.* 2014). In North America, the serovar Pomona type *Kennewicki* is the most important type associated with Leptospiral abortions in mares (Sheorian *et al.* 2000, Timoney *et al.* 2011, Erol *et al.* 2014); however, in other parts of the world, the serovars Bratislava, Grippytyphosa, Copenhageni, Autumnalis, Hebdomadis, Arborea and Icterohaemorrhagiae are more commonly associated with abortions in mares (for review, Erol *et al.* 2014). Environmental conditions (e.g. precipitation and flooding) are alleged to be important in the dissemination of the bacteria

from wildlife to horses (which are considered incidental hosts). Wild animals (e.g. raccoons, white tailed deer, striped skunks, opossums, and red grey foxes) have been shown to harbor the serovar Pomona and are thought to play a role as source of infection for pregnant mares (Timoney *et al.* 2011).

Table 1.1: Ten most commonly isolated microorganisms in 236 equine placentitis cases in central Kentucky. Adapted from Hong *et al.* (1993).

Microorganisms	Frequency	
	Number of cases	%
<i>Streptococcus equi subspecies zooepidemicus</i>	39	16.5
<i>Leptospira ssp.</i>	37	15.7
<i>Escherichia coli</i>	33	14.0
Fungi	16	6.7
<i>Pseudomonas aeruginosa</i>	11	4.7
<i>Streptococcus equisimilis</i>	11	4.7
<i>Enterobacter agglomerans</i>	5	2.1
<i>Klebsiella pneumonia</i>	5	2.1
α -hemolytic <i>Streptococcus</i>	5	2.1
<i>Staphylococcus aureus</i>	2	0.9

Based upon morphologic lesions and suggested pathogenesis, there are four different types of equine placentitis: ascending, focal mucoid (nocardioform), diffuse (hematogenous), and multifocal (Williams *et al.* 2004) (Figure 1.1). Ascending placentitis, the most frequent type of placentitis, is commonly associated with beta hemolytic streptococci and coliforms (Hong *et al.* 1993) (Table 1.1). Nocardioform placentitis is commonly associated with Gram-positive branching bacilli, *C. equi* and *Amycolatopsis* species (Figure 1.2) (Donahue & Williams 2000). In certain years, nocardioform placentitis is the predominant type of equine placentitis in central Kentucky (Williams *et al.* 2004, Carter *et al.* 2012) (Figure 1.3). A multifocal distribution is a rare type of placentitis and has been sporadically seen in isolated cases in association with fungi or bacteria (Hong *et*

al. 1993, Williams *et al.* 2004). Diffuse placentitis can be diagnosed as isolated cases in association with bacteria or fungi or during outbreaks mainly in association with Leptospirosis (Figure 1.3).

Figure 1.1: Representative photographs of the four morphologic types of equine placentitis.

(A) ascending placentitis (focal extensive lesion in the chorionic surface of the chorioallantois); arrows indicate extensive funisitis, while the asterisk indicates the chorionic surface affected by placentitis, #: indicates aminiotinitis (B) Nocardioform (focal mucoid lesion in the chorionic surface) ** extensive chorionic surface covered by mucus; (C) hematogenous (Diffuse erosive lesions present in the allantoic surface of the chorioallantois); (D) multifocal, in the allantoic surface view, multifoci areas of infection can be observed in this placenta. Courtesy Dr. Neil Williams (photo B) and Dr. Rafaela De Negri (photo C).

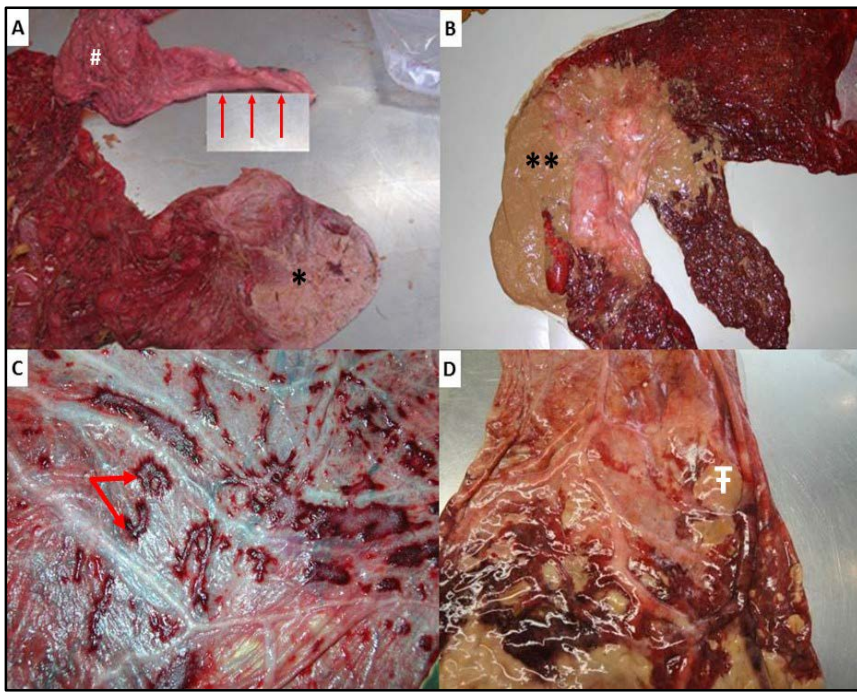


Figure 1.2: Morphologic aspects of nocardioform microorganisms in culture and photomicrographs of Gram-stained microorganisms. (A) *Amycolatopsis* ssp. Three species of *Amycolatopsis* have been identified in association with nocardioform placentitis: *A. kentuckyensis*, *A. lexingtonensis*, and *A. pretoriensis*. (B) Gram staining of *Amycolatopsis* ssp, note the Gram + and branching aspect of the organisms. (C) *Crossiella equi* culture morphology, note the “donut-shape” of each colony. (D) Gram staining of *Crossiella equi*, note the Gram + and branching aspect of the organisms. Courtesy Dr. Erdal Erol.

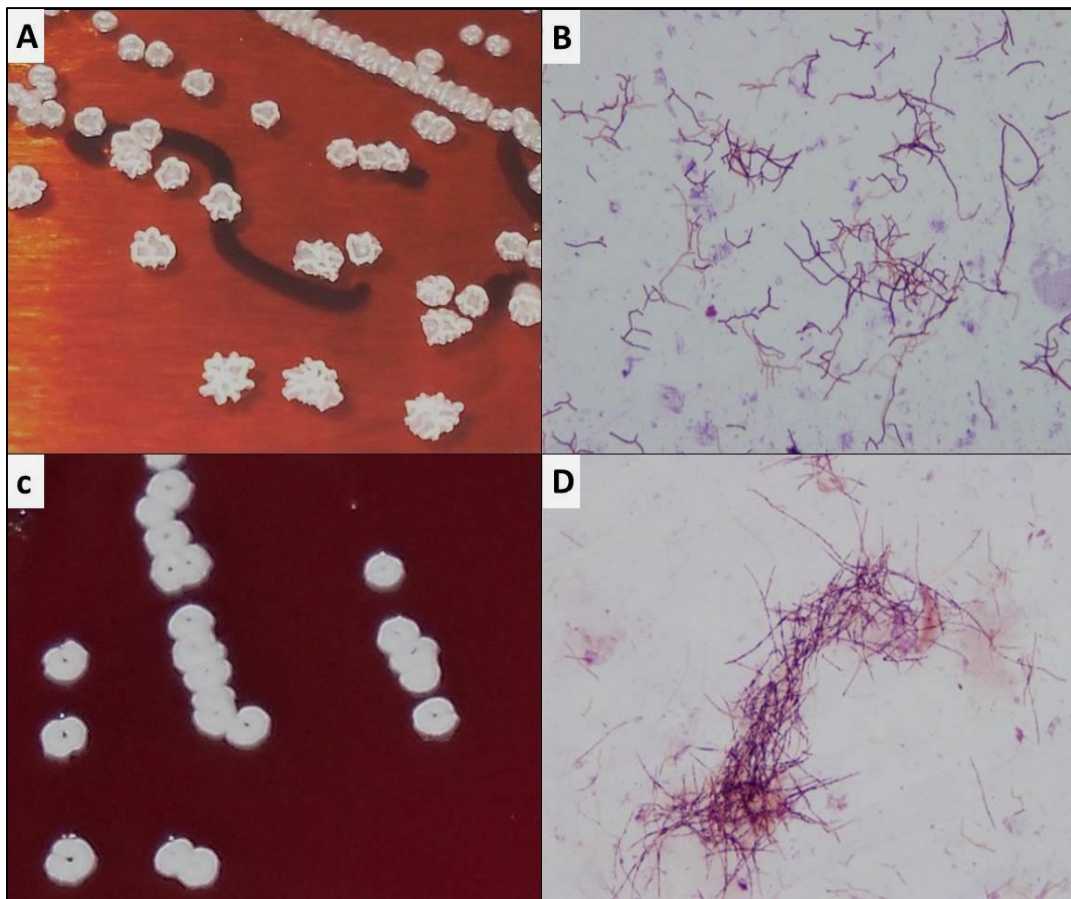
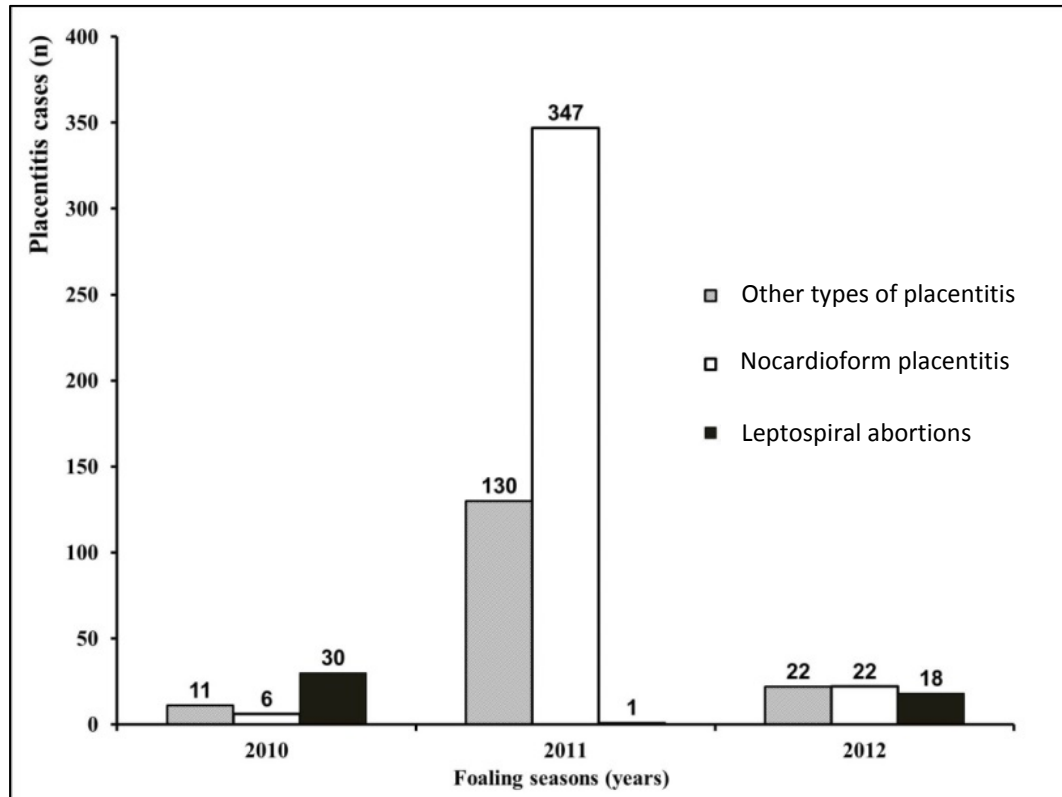


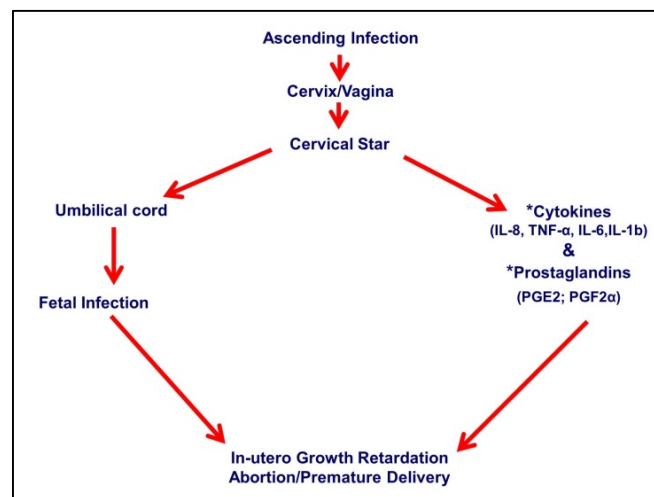
Figure 1.3: Confirmed placentitis cases in cases submitted to the University of Kentucky Veterinary Diagnostic Laboratory during the 2010, 2011 and 2012 foaling seasons. Courtesy Dr. Craig Carter. The graphic below is divided in three groups, other types of placentitis including multifocal, ascending; nocardioform placentitis and leptospiral abortions.



In ascending placentitis, it has been suggested that infectious agents (i.e. bacteria and fungi) enter the uterus via the vagina/cervix and then colonize the caudal pole of the chorioallantois (LeBlanc 2010, Troedsson & Macpherson 2011) (Figure 1.4). Once the infection has been established, clinical signs such premature udder development (\pm streaming of pre-foaling mammary gland secretions), and vulvar discharge may become noticeable. Normal mammary gland development starts about 4 weeks (2 to 6 weeks) from the foaling, with more pronounced development in the week of parturition. Premature

mammary gland development is not a pathognomonic sign of placentitis, but rather a non-specific sign of pending late-term pregnancy loss which can be associated with different conditions, such as twin pregnancy, umbilical cord torsion, and idiopathic imminent abortion (Macpherson 2006, Troedsson & Macpherson 2011). Enlargement of the mammary gland may be observed in mares suffering with hydrops, pre-pubic tendon rupture (mammary gland may be filled with serosanguineous/bloody material and cranially displaced), and normal pre-foaling ventral edema (Lofstedt 2011). In mares with placentitis, premature mammary development might not be present until the placental lesions are well advanced. Vulvar discharge is highly variable, as the discharge can be smeared by and under the mare's tail and easily missed, unless frequent monitoring is applied. If early treatment is not applied, the lesions will progress, and then the infectious agent(s) may gain access to the fetus through the placental vessels and umbilical cord (Figure 1.4).

Figure 1.4: Representative diagram of the suggested pathogenesis of ascending placentitis in mares. Adapted from LeBlanc *et al.* (2012)



Infection of the chorioallantois is associated with inflammation which results in elevation of prostaglandins (i.e. PGE₂ and PGF₂ α) in the fetal fluids and increased expression of pro-inflammatory cytokines (e.g. IL-6 and IL-8) (LeBlanc *et al.* 2002, 2012). Lesions in mares with ascending placentitis are not restricted to the chorioallantois but in certain cases of ascending placentitis, funisitis and amnionitis are also present (Hong *et al.* 1993, Williams *et al.* 2004). Ultimately, prostaglandin release has been suggested to lead to abortion or delivery of a premature foal (LeBlanc *et al.* 2002, 2012) (Figure 1.4). Chronic placentitis leads to placental insufficiency, which may result in growth retardation and delivery of nonviable or weak foals. Foals born alive from mares with placentitis may be born septic and may demand intensive veterinary critical care. In addition, despite treatment pre-partum or and post-delivery there is no guarantee that the foal will be athletically sound. Thus, early diagnosis and subsequent treatment are likely to improve the possibility of delivering a live foal.

Placentitis accelerates the hypothalamic-pituitary adrenal axis (HPA) maturation (Lyle *et al.* 2006, Troedsson & Macpherson, 2011). Mares with placentitis may deliver live and viable foals by 310 days of gestation (Christensen *et al.* 2006, Troedsson & Macpherson 2011), while dead foals often will be delivered, if pregnancies of comparable gestational age of mares without placentitis are interrupted. The mechanisms associated with survival of preterm foals from mares with placentitis are unclear, but it has been suggested that it is related to a premature rise in fetal ACTH and cortisol. Different than other species, final maturation of the equine fetal HPA occurs in the few days preceding normal parturition and continues during the first several weeks after birth (for review, Hart & Barton 2012). Foals born prematurely presented lower cortisol concentrations (<3 μ g/dL)

within 2 hours post-delivery compared to normal full-term foals (12-14 $\mu\text{g}/\text{dl}$) (Rossdale *et al.* 1991). In addition, premature foals showed remarkably higher ACTH concentrations in comparison to normal full-term foals (650 pg/mL vs 300 pg/mL within the first 30 minutes post-delivery). In mares with placentitis, treatment strategies to prevent premature delivery should be applied, thus prolonging fetal time in utero which may allow more fetal maturation and as consequence improve the outcome for neonatal survival.

Nocardioform placentitis was first recognized ~20 years ago in central Kentucky (Giles *et al.* 1986); however, little is known about the pathogenesis of this disease. Lesions in this type of placentitis are located in the uterine body and base of the uterine horns with no association with the cervical star (Hong *et al.* 1993). As a consequence, vulvar discharge is not commonly observed in the mare with nocardioform placentitis unless the mare is about to abort. Based upon submissions to a referral laboratory in central Kentucky, *C. equi* appear to be more pathogenic than *Amycolatopsis* species, as more mares infected with *C. equi* tend to abort, while more mares infected with *Amycolatopsis* ssp tend to delivery live, premature foals. Because nocardioform infection is restricted to the chorioallantois (i.e. amnion, umbilical cord and fetus are not affected) (Hong *et al.* 1993), chorionitis is believed to accelerate fetal maturation and to induce placental insufficiency (i.e. In-utero growth retardation).

Little is known about the pathogenesis of diffuse or multifocal placentitis, it is suggested that the microorganisms reach the uterus hematogenously; however, there has been speculation that multifocal placentitis is the result of a dormant chronic infection of the uterus that became active later in pregnancy. In multifocal placentitis, lesions can be confined to the chorion and may also be present on the allantoic surface (Hong *et al.* 1993,

Williams *et al.* 2004). *Leptospira*, the most important cause of hematogenous (or diffuse) placentitis, is believed to gain access to the uterus through the systemic circulation. Leptospiral lesions include amnionitis, funisitis and acute inflammatory changes in the chorioallantois (Hong *et al.* 1993, Donahue & Williams 2000). Interestingly, leptospiral abortions are associated with high immunoglobulin titers in maternal plasma, different than other types of placentitis (Donahue & Williams 2000, Erdal *et al.* 2014). *Leptospira* is believed to cause abortion directly by infection of the fetoplacental unit, but it appears that systemic inflammation in association with hyperthermia and prostaglandin production may contribute to the pregnancy loss.

1.3. Diagnosis of Placentitis

Currently the diagnosis of placentitis is based on ultrasonography and clinical signs (i.e. premature lactation and/or vulvar discharge) (LeBlanc 2010, Troedsson & Macpherson 2011). Transrectal ultrasonography of the caudal placental pole is commonly used to diagnose ascending placentitis (Renaudin *et al.* 1997, Troedsson & Zent 2004, Bucca *et al.* 2005, Troedsson & Macpherson 2011) (Figure 1.5). While performing this technique, the operator should make a perfect midline alignment of the transducer with the cervix and ventral aspect of the caudal pole, to assure that a branch of uterine artery/cranial vaginal artery is imaged. Three measurements are usually taken of the combined thickness of uterus and placenta (CTUP) and the values can be compared to reported normal ranges (Renaudin *et al.* 1997, Troedsson & Zent 2004, Coutinho da Silva *et al.* 2013). Increased CTUP associated with clinical signs and areas of placental separation are suggestive of

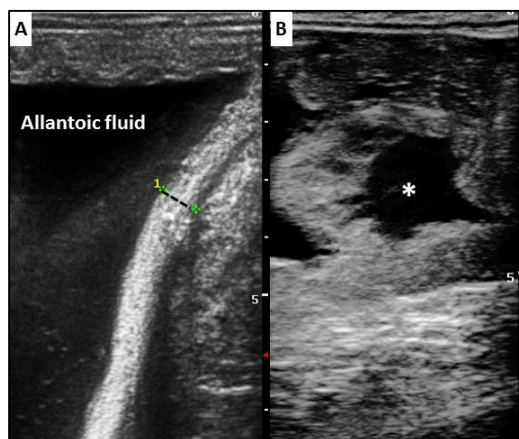
ascending placentitis (Troedsson & Macpherson 2011). Interestingly, there appears to be small variations in normal CTUP between horse breeds (Renaudin *et al.* 1997, Troedsson & Zent 2004, Bucca *et al.* 2005, Hendricks *et al.* 2009, Coutinho da Silva *et al.* 2013). In a Dutch study, parity was reported to have effects on values of CTUP, i.e. nulliparous mares presented higher values compared to multiparous mares (Hendricks *et al.* 2009). Despite the widespread use of transrectal ultrasonography to diagnose ascending placentitis, data on sensitivity and specificity are scant.

Transabdominal ultrasonographic examination of the fetus and placenta has been commonly used as a tool to assess fetal and placental health (Bucca *et al.* 2005, Bucca 2011). This approach is a particularly useful clinical tool in suspected nocardiform placentitis (Wolfsdorf *et al.* 2000, Troedsson & Macpherson 2011). However, since a very limited area of the uterus is accurately visualized by transabdominal ultrasonographic examination, the lack of apparent lesions in the chorioallantois does not exclude the possibility of disease. Areas of placental separation with hyperechoic exudate between the chorioallantois and the uterus are suggestive of nocardiform placentitis (Wolfsdorf *et al.* 2000, Troedsson & Macpherson 2011). Presumptive diagnosis of nocardiform placentitis can be challenging, especially if only a small area is affected. Therefore, the development of an experimental model for this disease would help investigators to better understand its pathogenesis, diagnosis and treatment.

Fetal heart rate monitoring and ultrasonographic character of the fetal fluids can also be evaluated by transrectal and transabdominal ultrasonography (Reef *et al.* 1996, Bucca 2011, Coutinho da Silva *et al.* 2013). Reduced or increased fetal heart rates (i.e. normal heart rates ~80 beats per minute during late pregnancy) are associated with poor

pregnancy outcome (Reef *et al.* 1996, Troedsson & Macpherson 2011). Increased echogenicity of the fetal fluids are associated with imminent abortion (Troedsson & Macpherson 2011). It is worth noting that the echogenicity of the amniotic fluid increases with gestational age (Coutinho da Silva *et al.* 2013), probably related to the vernix being released by the fetus. However, fetal stress (e.g. colic surgery, fetal hypoxia by different reasons) may result in fetal diarrhea, and echogenicity of the amniotic fluid may increase remarkably as consequence of solid particles floating in the amniotic fluid. The normal amnion appears as a thin membrane surrounding the fetus; amnionitis can be observed by ultrasonography as a thickened and irregular amniotic membrane (Troedsson & Macpherson 2011).

Figure 1.5: Transrectal ultrasonography of the caudal pole of the chorioallantois. (A) Normal ultrasonographic appearance of the chorioallantois and combined thickness of uterus and placenta (i.e. <8mm) in a mare at 270 days of gestation (--); (B) Note fluid accumulation and separation of the chorioallantois from the uterus (*), this finding is consistent with early signs of placentitis.

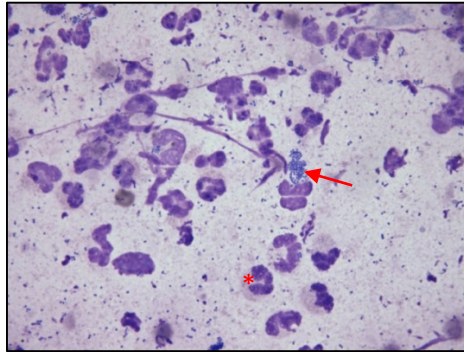


Cytology and culture swabs obtained from the external cervical os in pregnant mares can be beneficial in cases of ascending placentitis, when the cervix is open and

discharge is present (Figure 1.6) (LeBlanc 2010). Samples for culture and cytology can be collected with the use of a double-guarded cytobrush/swab via vaginoscopy or manually with a sterile sleeve. The presence of neutrophils, bacteria, and/or fungal hyphae will aid the presumptive diagnosis and give some clues about the type infection (i.e. Gram positive/negative and shape, or presence of hyphae) while waiting for further laboratorial diagnostic tests.

The definitive diagnosis of placentitis is attained by pathologic and microbiologic examination of the placenta (Hong *et al.* 1993, Giles *et al.* 1993, Donahue & Williams 2000). Ordinarily, the placenta should be laid out in an “F” or “Y” shape, thereafter the chorionic surface of the chorioallantois is examined and sampled (Hong *et al.* 1993, Schlafer 2004). Macroscopic examination of the placenta allows determination of the affected areas, classification of the lesions and sampling for further examinations (i.e. histopathology and microbiologic evaluations). The placenta should be inverted to expose the allantoic surface of the chorioallantois, similarly the placenta should be laid out in an “F” shape, the amnion and umbilical cord should be spread out, examined and sampled for pathologic examination (Schlafer 2004). Affected areas, normal tissues, and areas of transition between the normal and affected tissues should be sampled from the chorioallantois, amnion and umbilical cord (Hong *et al.* 1993, Schlafer 2004).

Figure 1.6: Cervical cytology collected from a mare with ascending placentitis. Note the presence of several segmented neutrophils (example indicated by an asterisk) and several bacterial colonies (*Streptococcus* species indicated by the arrow). Courtesy Dr. Barry Ball.



Swabs collected from affected placental tissues can be used for bacterial culture and PCR testing for abortigenic infectious agents (e.g. *Leptospira*, equine herpesvirus type 1, equine arteritis virus) (Schlafer 2004, Williams *et al.* 2004, Erdal *et al.* 2014). Fetal tissues (e.g. brain, liver, spleen, kidney, heart and lungs) and fetal body fluids (e.g. heart blood, thoracic and abdominal fluids) can be used for diagnostic testing. Fragments of placenta and fetal tissues can be collected, macerated, and be used for culture and PCR testing (Hong *et al.* 1993, Schlafer 2004, Williams *et al.* 2004, Erol *et al.* 2014). A combination of different diagnostic methods will improve the diagnostic accuracy (Hong *et al.* 1993, Schlafer 2004, Erol *et al.* 2012, Erol *et al.* 2014). However, despite extensive testing, in about 20% of abortions submitted to a referral laboratory in central Kentucky, the cause of abortion could not be determined (Williams *et al.* 2004).

Lesions detected upon histopathologic examination of the placenta vary with the infectious agent involved and chronicity of the infection (Hong *et al.* 1993). Acute bacterial placentitis is characterized by neutrophilic infiltration of the inter-villous space and/or necrosis of the chorionic villae (Hong *et al.* 1993, Williams *et al.* 2004). Chronic placentitis is associated with necrosis of the chorionic villi, presence of eosinophilic amorphous

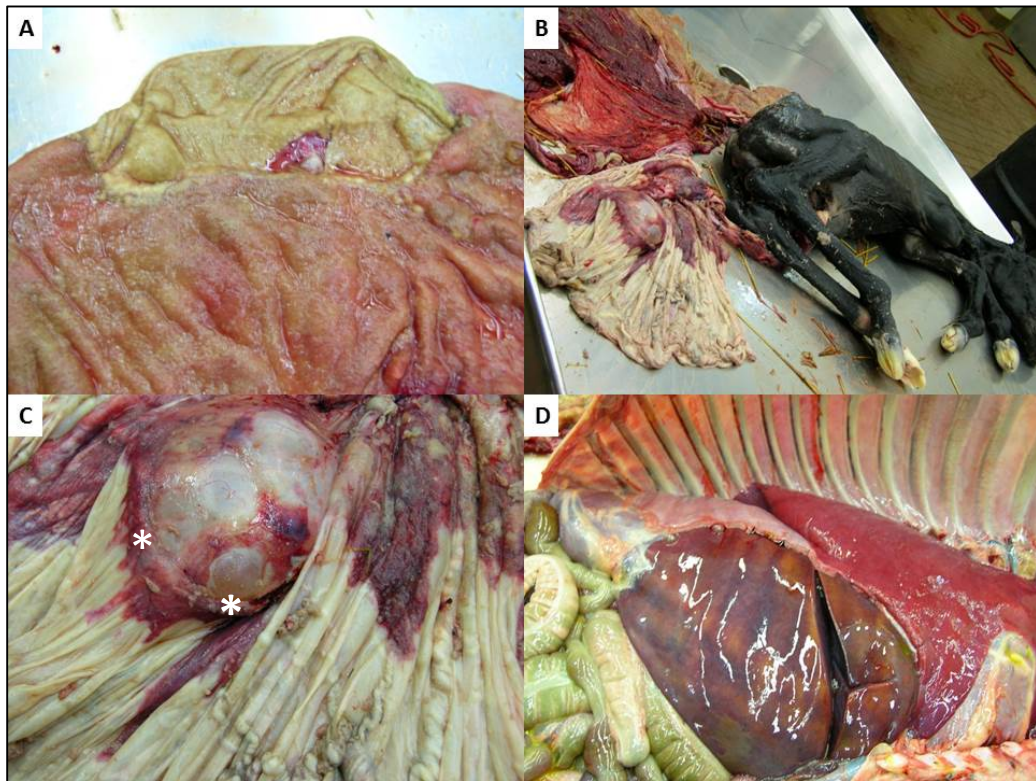
material in the chorion, and/or infiltration of mononuclear cells in the intervillous spaces (Hong *et al.* 1993). Other lesions reported in association with chronic placentitis include chorioangiomas, hyperplasia with or without squamous metaplasia of the chorionic epithelium, and adenomatous hyperplasia of the allantois (Hong *et al.* 1993). Leptospiral placentitis is characterized by edema, placental hemorrhage, brown mucoid material, green discoloration, cystic adenomatous hyperplasia of the allantois, vasculitis, amnionitis and funisitis as well as by the presence of spirochetes (Hong *et al.* 1993, Donahue & Williams, 2000) (Figure 1.7). Severe fetal lesions are also a remarkable feature of leptospiral abortion; the aborted fetus may be emaciated and icteric, present generalized edema and fluid accumulation and severe degenerative inflammatory lesions of lungs, liver, kidneys (Hong *et al.* 1993, Donahue & Williams, 2000, Williams *et al.* 2004).

In nocardioform placentitis, the macroscopic lesions are focal-extensive at the base of the uterine horn(s) and cranial uterine body on the chorionic surface of the chorioallantois, without involvement of the cervical star (Hong *et al.* 1993; Donahue & Williams 2000). A brown tenacious, mucoid material is found overlying white granular punctuate lesions (Hong *et al.* 1993). Histopathologic lesions are described as chronic-active; severely affected villi are often necrotic and coated with an extensive layer of eosinophilic amorphous material mixed with neutrophils and colonies of long filamentous branching bacteria (Hong *et al.* 1993, Donahue & Williams 2000). In less severely affected areas the same type of exudate is present in the crypts and mononuclear cells are present in the villous stroma and chorionic stroma. Endometritis is a common finding in mares presenting with placentitis. Adenomatous hyperplasia of the allantoic epithelium and hyperplasia with or without squamous metaplasia of the chorionic epithelium is frequently

observed (Hong *et al.* 1993, Donahue & Williams 2000). Bacterial cultures of the affected areas are routinely used as part of the necropsy diagnostic testing in pregnancy loss cases (i.e. abortion cases, premature delivery and suspicious placentitis cases). Methods and procedures for bacterial culture for nocardioform actinomycetes have been recently improved (Labeda *et al.* 2003, Donahue & Williams 2000, Erol *et al.* 2012a). Analyses by PCR analysis for actinomycetes (i.e. *C. equi* and *Amycolatopsis* ssp) has been incorporated as a routine test for the diagnosis of nocardioform placentitis in central Kentucky (Erol *et al.* 2012). The perceived advantage of PCR in comparison to other diagnostic tools includes a rapid turnaround time; however, the specificity and sensitivity for PCR detection have not been reported. At present, as for other abortigenic diseases, the combination of the different diagnostic methods is highly recommended to maximize diagnostic accuracy.

Fungal placentitis is commonly associated with chronic extensive placentitis at the cervical star. Similar to chronic bacterial placentitis, some fungi induce multifocal granulomatous placentitis (Hong *et al.* 1993). Adenomatous hyperplasia of the allantoic epithelium, with or without squamous metaplasia can be commonly observed in chronic placentitis (e.g. fungal placentitis, *E. coli* and nocardioform placentitis) (Hong *et al.* 1993, Shivaprasad *et al.* 1994). Fungal placentitis can be found in association with bacterial placentitis. Anecdotally, treatment for bacterial placentitis may be associated with a secondary fungal infection; however, such associations have not been studied.

Figure 1.7: Leptospiral abortion. (A) Chorionitis, note large area of mottling and extensive placental edema. (B) Severely emaciated fetus aborted in a mare affected with *Leptospira*. (C) Severe amnionitis in a mare aborting due to *Leptospira* infection. Grossly, the amnion was hemorrhagic and necrotic (indicated by asterisks). (D) Icteric fetal carcass (serosa), note the rib impression on the liver as a consequence of the hepatic edema. Courtesy Dr. Rafaela De Negri.



1.3.1 Diagnostic Markers for Equine Placentitis

Several molecules have been measured in blood from pregnant mares to evaluate fetoplacental well-being. Over the last three decades, steroids produced by the fetoplacental unit have received the most attention (Rossdale *et al.* 1991, Ousey *et al.* 2004, Douglas 2004, Morris *et al.* 2007, LeBlanc 2010). Other molecules including relaxin and “equine

feto-protein” have also been investigated in mares with pregnancy loss (Sorensen *et al.* 1990, Ryan *et al.* 2001, Ryan *et al.* 2009). Recently, serum amyloid A (SAA) has been investigated as a prognostic marker for mares with experimentally induced ascending placentitis (Coutinho da Silva *et al.* 2013). Therefore, different diagnostic markers for placental health in mares will be discussed herein.

Fetoplacental steroids

During pregnancy several steroids (i.e. estrogens, progestins and androgens) are produced and metabolized by the fetoplacental unit (Cox *et al.* 1975, Raeside *et al.* 1979, Pashen and Allen 1979, Raeside 2004, Ousey 2011). These steroids achieve extremely high concentrations in the maternal and fetoplacental circulation (Ousey *et al.* 2003, Ousey 2011). At the present, we have very limited understanding of the function of the different steroids during pregnancy, particularly within the fetoplacental unit. Progestins are responsible for maintaining pregnancy (Ginther, 1992, Scholtz *et al.* 2014); however, it is unclear what functions estrogens and androgens have during pregnancy in mares. Removal of fetal gonads did not affect pregnancy maintenance; however, concentrations of PGFM, a metabolite of PGF 2α , was remarkably reduced, and as a consequence, the second stage of labor was delayed (Pashen & Allen 1979, 1982).

Progestins can be subclassified as pregnenes and 5 α pregnanes. Pregnenes include pregnenolone, progesterone and 5-pregnene-3 β ,20 β -diol (P5 $\beta\beta$); 5 α -pregnanes include 5 α hydroxyprogesterone (5 α -DHP), 3 β 5P, 20 α -5P, $\beta\beta$ diol, 5-pregnene-3 β ,20 α -diol (β adiol), however, 5 α DHP, 20 α 5P, and $\beta\alpha$ -diol predominate (Chavatte-Palmer *et al.* 1997, Ousey *et al.* 2003). During the first 100 days of pregnancy, progesterone is the main progestin, with

5 α DHP also being also present in high proportion starting from ovulation (Scholtz *et al.* 2014). It is produced by the primary corpus luteum, and secondary and accessory corpora lutea (Ginther 1992). Starting by 70-100 days of gestation the main source of progestins is the equine placenta (Holtan *et al.* 1978, Hinrichs *et al.* 1987), in fact removal of the maternal ovaries by 100 days of gestation did not affect the pregnancy maintenance (Hinrichs *et al.* 1987), however, removal of maternal ovaries by 70 days of pregnancy was associated with pregnancy loss in 50% of the mares (Holtan *et al.* 1978). It is worth noting that in addition to progesterone, 5 α -DHP is also present in the maternal circulation during early pregnancy and has been recently confirmed as a potent, bioactive progestin (Scholtz *et al.* 2014). Interestingly, from approximately 150 days of pregnancy, progesterone is present in very low concentrations in the maternal plasma (Short 1950), and it appears that other progestins (e.g. 5 α -DHP) are responsible for pregnancy maintenance. During the last 30 days of gestation, maternal concentrations of progesterone rise until immediately prior to parturition (Holtan *et al.* 1975).

Measurement of progestins has been used to monitor placental health in spontaneous and experimental cases of placentitis (Rossdale *et al.* 1991, Douglas 2004, Ousey *et al.* 2007, Morris *et al.* 2007). Chronic placentitis (>7 days post-inoculation) in experimentally induced mares was associated with increased plasma progestin concentrations (Morris *et al.* 2007); however, in the same study, mares that developed acute placentitis (abortion <4 days post inoculation) experienced a rapid decline in plasma progestin concentrations. Spontaneous cases of placentitis have been reported to cause elevation in total progestin concentrations (Douglas 2004, Ousey *et al.* 2007). This increase in concentration of progestins is thought to be linked to the accelerated adrenal gland

maturation in the fetus of pregnant mares experiencing chronic placentitis (Rossdale *et al.* 1991, Lyle *et al.* 2006). The adrenal gland is thought to produce pregnenolone, and this molecule is used as a precursor for progesterone in the equine placenta. It is assumed that placentitis increases the substrate (i.e. pregnenolone) which leads to an increased peripheral progestin concentration in mares with chronic placentitis. It has also been suggested that placentitis affects the ability of the placenta to metabolize progesterone into other progestins, thus progesterone will appear in higher concentration in plasma. Repeated measurements of plasma progestins in mares with placentitis have been proposed as a useful method to identify mares that may abort or deliver prematurely (Douglas 2004, Morris *et al.* 2007, Ousey *et al.* 2007).

During pregnancy, the equine fetal gonads of both male and female fetuses undergo marked hypertrophy and secrete large quantities of androgens, particularly dehydroepiandrosterone (DHEA) and 7-dehydro DHEA (Raeside *et al.* 1973, Pashen *et al.* 1979, Raeside *et al.* 1997). Dehydroepiandrosterone serves as a precursor for classic phenolic estrogens (i.e. estrone, estradiol-17 β , estradiol-17 α , and their sulfoconjugates), and 7-dehydro-DHEA serves as a precursor for ring B unsaturated estrogens (i.e. equilin, equilenin and their hydroxyderivatives 17 α -dihydroequilin, 17 α -dihydroequilenin, 17 β -dihydroequilin, and 17 β -dihydroequilenin) by the equine placenta (Raeside *et al.* 1979, Pashen & Allen 1979, Bhavnani 1988, Raeside *et al.* 1997, Ousey 2011). In addition to DHEA, circulating testosterone is elevated in plasma of pregnant mares (Silberzahn *et al.* 1984, Daels *et al.* 1996). To date, determination of fetoplacental androgens have not been critically assessed in mares experiencing pregnancy losses. Because androgens serve as

precursors of estrogens, it seems logical that placentitis affects androgen concentrations in the maternal circulation.

The equine blastocyst begins to secrete estrogens about 10 days post ovulation (Zavy *et al.* 1984, Ginther 1993); thereafter, estrogens are produced by the corpora lutea starting by 40 days of pregnancy (Daels *et al.* 1991). This ovarian secretion of estrogen is believed to be mediated by eCG and will last until the demise of the corpora lutea around 150 days of gestation (Ginther 1992, Ousey 2011). Concomitant with ovarian secretion of estrogens (i.e. by day 40 of gestation), the fetoplacental unit also starts to secrete estrogens. Concentrations of estrogens in maternal circulation will increase remarkably through the second trimester of pregnancy, and maximal concentrations are achieved by 210-240 days of gestation. From the peak of estrogen concentrations in maternal circulation, there is a progressive reduction in estrogen concentrations through the end of pregnancy with minimal estrogen concentrations at parturition, which is contrary to reports in domestic ruminants (Cox 1975, Ginther, 1992, Raeside 2004, Ousey 2011). The estrogen production parallels the enlargement of the fetal gonads (Schlafer 2011, Ousey 2011). Since estrogens are a final product of the fetoplacental unit, determination of peripheral estrogen concentrations in pregnant mares may provide utility in the diagnosis of equine placentitis.

Determination of estrone sulfate concentrations in maternal circulation has been suggested as a useful marker for fetal wellbeing during early pregnancy (Kashman *et al.* 1988, Jeffcott *et al.* 1987 Stabenfeldt *et al.* 1991); however, studies involving estrone sulfate in advanced pregnancy are scant (Ousey *et al.* 1987, Santschi *et al.* 1991). There are no controlled studies assessing estrone sulfate in mares experiencing placentitis, despite its high popularity among practitioners which frequently use estrone sulfate as a test to

assess fetal-wellbeing and placental health. Field observations carried by a commercial laboratory, reported that aborting mares present significantly lower “total estrogen” (unreported crossreactivity) concentrations than do mares maintaining pregnancy (Douglas 2004). Controlled studies are warranted to confirm field observations concerning the utility of conjugated and unconjugated estrogen concentrations in pregnant mares as markers for placentitis.

Acute phase proteins

Acute phase proteins (APP) are elevated when inflammation is present (Pepsy *et al.* 1989, Peterson *et al.* 2004). This group of proteins is mainly produced by the liver in response to an inflammatory stimulus (i.e. cytokines) (Husebeck *et al.* 1986, Nunokawa *et al.* 1993, Jacobsen & Andersen 2007, Cray 2012). Acute phase proteins are classified as minor, moderate and major; minor and moderate (i.e. constantly present in the plasma and concentration increases 1 to 5 and 5 to 10 fold upon inflammation, respectively) and major (i.e. low or undetectable concentrations in the blood circulation and upon inflammatory stimulus increases >10 and often 100 to 1000 times) (Chrisman *et al.* 2006, Jacobsen & Andersen 2007). The main acute phase protein in the horse is serum amyloid A, whereas the moderate acute phase proteins include haptoglobin (Hp) and fibrinogen (Fb), and minor C-reactive protein (CRP) and α 1-acid glycoprotein (Jacobsen & Andersen 2007, Belgrave *et al.* 2013, Cray & Belgrave 2014). As a moderate APP, Hp is constantly present in plasma of health horses and concentrations are elevated upon inflammation (Jacobsen & Andersen, 2007, Cray, 2012).

In horses, Hp has recently been shown to be a useful marker for a variety of acute-chronic clinical conditions (i.e. >4 days duration) (Cray & Belgrave 2014). Recently,

increases in peripheral blood concentrations of SAA have been reported in mares with experimentally induced bacterial endometritis (Christoffersen *et al.* 2010) and ascending placentitis (Coutinho da Silva *et al.* 2013). In healthy pregnant mares, SAA concentrations remained low throughout the last half of gestation while showing a significant increase beginning approximately 12 hours post-partum. Maternal SAA concentrations then return to baseline within approximately 60 hours postpartum (Coutinho da Silva *et al.* 2013). Interestingly, levels of SAA in peripheral blood have been reported to increase in mares affected by placentitis and decrease again in response to treatment (Coutinho da Silva *et al.* 2013). Fibrinogen, the most widely used APP in the horse, is commonly used in association with white blood cell counts (WBC) as means to assess inflammation in the horse (Chrisman *et al.* 2006, Belgrave *et al.* 2013); however, anecdotally it has been suggested that neither WBC nor Fb change in mares with placentitis. To date SAA or Hp have not been critically evaluated as diagnostic tool for mares with spontaneous and or with experimentally induced ascending placentitis.

Relaxin

Relaxin, a hydrophilic polypeptide hormone, is a member of the insulin-like peptide superfamily. Equine relaxin, which is the smallest relaxin among mammals, is composed of two subunits (i.e. A: alpha 20-residue; and B: beta 28-residue) which are connected by disulfide bonds similar to insulin (Ginther 1992, Hossain *et al.* 2006). It has been suggested that relaxin plays an important role during pregnancy, and the proposed functions include uterine growth, distensibility of uterus, cervix and pelvic ligaments (Ginther 1992). To date the role of relaxin during equine pregnancy is not fully understood.

Relaxin is produced by the equine placenta and can be detected in high concentrations in the peripheral blood from day 80 of gestation until term (Stewart *et al.* 1982, Stewart *et al.* 1992). It has been reported that relaxin is a useful biomarker assess placental health (Ryan *et al.* 1999, 2001). Ryan *et al.* (2009) reported that there was a positive relationship between circulating levels of relaxin and poor outcomes in high risk pregnancies. These investigators suggested that because the placenta is the sole source of relaxin, blood concentrations could be used as a biomarker of placental function (Ryan *et al.* 1999). It is worth noting that relaxin concentrations vary greatly among different breeds (e.g. Thoroughbred vs Standardbreds) and horse types (pony vs standard size horse mares) (Stewart *et al.* 1992, Ryan *et al.* 2001). In addition, the lack of a commercially available test at present precludes the use of relaxin clinically in mares.

1.3.2 Biomarkers for Chorioamnionitis in Humans

In recent years, microRNAs (miRNA), small non-coding ribonucleic acids (~22 nucleotides) have been the focus of human studies as potential biomarkers for diseases (e.g. cancer, inflammatory and cardiovascular pathologies). MicroRNAs are associated with a wide range of biological functions through regulation of gene expression either by degrading or transcriptionally repressing messenger RNA (Ladomery *et al.* 2011). MicroRNAs are relatively stable in the systemic circulation and represent a source of cell-free nucleic acid (Chim *et al.* 2008). In plasma, miRNAs are stable because they are transported within exosomes (non-cellular vesicles), and recent findings have shown that miRNAs present in these vesicles control immune response mediated by T-cells (Mittelbrun *et al.* 2011). A number of studies have characterized pregnancy-associated

miRNAs in women (Ladomery *et al.* 2011 Chim *et al.* 2008, Kotlabova *et al.* 2011) and some have indicated changes in expression of miRNAs associated with abnormal or preterm labor, therefore indicating their potential diagnostic application (Kotlabova *et al.* 2011, Mayor-Lynn *et al.* 2011). To date miRNAs have been received limited attention during equine pregnancy, a recent study demonstrated changes in miRNAs in the dam's circulation during maternal recognition of pregnancy (Cameron 2012). Since miRNA (i.e. miR223 and miR 338) change in the placenta of pregnant women suffering with chorioamnionitis (Montenegro *et al.* 2007), it is reasonable to hypothesize that these or other miRNA may change in mares with placentitis.

Proteomics of the amniotic fluid and fetal circulation has been used in women to identify biomarkers for chorioamnionitis (intra-amniotic infection/inflammation) (Ruetschi *et al.* 2005, Romero *et al.* 2008, Buhimschi *et al.* 2007, Buhimschi *et al.* 2008, Buhimschi & Buhimschi 2010, Buhimschi *et al.* 2011). Ruetschi and collaborators (2005) identified seventeen proteins that were overexpressed in amniotic fluid samples of women with chorioamnionitis. Among these proteins, three proteins were further identified as human neutrophil protein 1-3 as well as calgranulin A and B. Another study identified 39 proteins that are differentially expressed in amniotic fluid samples obtained from patients with intra-amniotic infections (Romero *et al.* 2008). To date proteomics of the equine fetal fluids have not been described; thus characterization of the protein composition of fetal fluids would be a useful to identify potential new markers for equine placentitis. Recently a study involving proteomics of human neonates demonstrated that Hp and haptoglobin-related protein immunoreactivity (Hp-RP) are useful markers for neonatal sepsis

(Buhimschi *et al.* 2011). Proteomics of the fetal fluids and the newborn foal may aid in identifying new diagnostic markers for placentitis and neonatal sepsis.

Macrophage migration inhibitory factor (MIF), an inflammatory marker, is elevated in amniotic fluid in chorioamnionitis (Chaiworapongsa *et al.* 2005). Interestingly, during early pregnancy, non-infectious early pregnancy losses in women were associated with reduced serum levels of MIF (Yamada *et al.* 2003). Macrophage migration inhibitory factor is expressed in high levels by the embryo and endometrium during early pregnancy in horses (Klein & Troedsson 2013). Unfortunately, our preliminary results measuring MIF in plasma of mares with placentitis showed that MIF is highly variable across days and mares, and there were no associations between placentitis and concentrations of MIF (Canisso *et al.* 2012), therefore, measuring MIF does not appear useful as a diagnostic marker for placentitis.

In human medicine, CRP has been the most widely studied APP, as a non-specific biomarker for placental and fetal health (Wu *et al.* 2009, Popowski *et al.* 2011, Lee *et al.* 2012). Changes in concentrations of CRP in the systemic circulation of women suffering with chorioamnionitis have been demonstrated by several authors (Popowski *et al.* 2011, Smith *et al.* 2012, Lee *et al.* 2012). In horses, CRP is a minor APP and has received limited attention (Nunokawa *et al.* 1993, Lavoie-Lamoureux *et al.* 2012), likely due to the lack of remarkable changes in different diseases evaluated to date.

In mammals, infection of the placenta triggers the local innate immune response through activation of Toll-like receptors (TRL), a group of receptors responsible for recognition of pathogen patterns and tissue damage (Kim *et al.* 2004, Romero *et al.* 2007). Activation of TLR (e.g. TRL-2 and TRL-4) initiates a cascade of events of the innate

immune response that results in migration of phagocytic cells to infected/damaged tissues and production of inflammatory mediators (e.g. cytokines, prostaglandins, and many others mediators) (Kim *et al.* 2004; Romero *et al.* 2007). Cytokines are small molecules (peptides, proteins, glycoproteins) that modulate the immune system/response, either by promoting or suppressing inflammation (Oppenheim 2001, Dinarello 2007). Dozens of cytokines are known, and there are several cell-types that produce (e.g. macrophages, monocytes, fibroblasts, dendritic cells, B lymphocytes, NK cells and epithelial cells) and secrete these molecules (Oppenheim 2001, Dinarello 2007). Normal placental tissues of mammals including the mare and women constitutively express pro-inflammatory cytokines (Jana *et al.* 2008; LeBlanc *et al.* 2012, Palm *et al.* 2013, Pitman *et al.* 2013). However, placental infection in women is associated with an increased production of several pro-inflammatory cytokines (e.g. IL-6, IL-8 and TNF- α) not only in placental tissues, but also in the fetal and maternal blood (Vogel *et al.* 2005, Romero *et al.* 2007). Pro-inflammatory cytokines have been shown to be useful markers for placental infection, and valuable prognosticators to determine whether a pregnancy will be carried to term in humans (El-Bastawissi *et al.* 2000, Gervasi *et al.* 2012, Cobo *et al.* 2012, La-Sala *et al.* 2012, Cobo *et al.* 2013). However, there have been few studies evaluating pro and anti-inflammatory cytokines in mares with experimentally induced ascending placentitis (LeBlanc *et al.* 2002, 2012). It is unlikely that measuring cytokines in plasma will be widely used tool in veterinary medicine due to the limited clinical availability of assays for equine cytokines.

1.4.Treatment of Placentitis

Bacterial infection of the equine pregnant uterus in the middle of gestation can result in acute abortions with no lesions in the fetal membranes and minimal to no outward clinical signs (e.g. vulvar discharge, premature mammary gland development) (Hong *et al.* 1993, Giles *et al.* 1993). Therefore, treatment is often not an option to prevent this type of abortion. On the other hand, bacterial infections of the uterus late in pregnancy tend to be associated with chronic placentitis (Hong *et al.* 1993); these mares will usually show the clinical signs as described above, thus if a diagnosis is made early enough, treatment can be applied and pregnancies can be potentially rescued (Troedsson & Zent 2004).

Primarily, treatment for placentitis should be aimed to prolong maintenance of a viable fetus in utero to allow time for fetal development and maturation. To achieve this goal, therapy should: 1) control bacterial infection of placenta and fetus, 2) maintain the myometrial quiescence and 3) block the production of pro-inflammatory cytokines (LeBlanc 2010, Troedsson & Macpherson 2011). To date, there are few evidence-based therapies for mares with placentitis. The limited evidence-based treatment strategies come from mares with experimentally induced placentitis. Unfortunately, as noted above, experimentally induced placentitis does not necessarily mirror spontaneous infections; thus, data generated from experimentally induced placentitis should be interpreted with caution.

Treatment for placentitis is commonly based on a combination of antibiotics, anti-inflammatories, and progestins (LeBlanc 2010, Bailey *et al.* 2010 Troedsson & Macpherson 2011) (Table 1.2). The rationale to use anti-inflammatories is based on the finding that mares with experimentally induced placentitis have increased uterine

contractility (McGlothlin *et al.* 2004) as a consequence of the prostaglandin production (LeBlanc *et al.* 2002) and increased expression of pro-inflammatory cytokines (LeBlanc *et al.* 2012). Similarly, progestin therapy for mares with placentitis is used to block myometrial contractions (Bailey *et al.* 2010). The evidence-based for the use of anti-inflammatories and progestins is based on studies with cloprostenol-induced abortion during early pregnancy. In these studies, all mares receiving cloprostenol but also treated with altrenogest (a progestin) maintained the pregnancy (8/8); however, mares given cloprostenol and also treated with progesterone resulted in 5 of 8 pregnancies maintained, as expected all mares receiving cloprostenol (n=5) but no other treatment aborted (Daels *et al.* 1996). From this study, it has been assumed that mares with placentitis during late pregnancy will behave in a similar manner although this has not been critically evaluated. Antibiotics are included as part of the therapy for the bacterial infection of the fetal membranes and to treat and/or prevent bacteria reaching the fetus (LeBlanc 2010, Troedsson & Macpherson, 2011). To date, few antimicrobials have been shown to cross the placental barrier and to achieve effective minimal inhibitory concentrations (MICs) in the fetal fluids and fetus against common bacteria causing placentitis (LeBlanc 2010, Troedsson & Macpherson, 2011).

Antimicrobials that have been shown to cross the placenta include sulfamethoxazole, trimethoprim, penicillin and gentamicin (Murchie *et al.* 2006, Rebello *et al.* 2006). Ceftiofur crystalline free acid, a drug that has effective in vitro antimicrobial activity against many bacteria causing placentitis was recently tested (Macpherson *et al.* 2013). Unfortunately, administration of ceftiofur crystalline free acid to pony mares did not result in concentrations of desfuroylceftiofur acetamide (i.e. the active metabolite) needed to

effectively treat bacterial placentitis, and it did not improve survival of foals born from mares with experimentally induced ascending placentitis (Macpherson *et al.* 2013). These findings discourage the use of this antibiotic to treat placentitis in mares.

Controversy exists regarding the duration of antimicrobial therapy once a diagnosis of placentitis is made. Anecdotally, short antimicrobial treatment (i.e. 10-15 days) has been advocated to be an effective and a thoughtful approach to treat placentitis as well as an approach to avoid bacterial resistance associated with prolonged antimicrobial therapy (LeBlanc 2010). However, evidence-based findings in mares with experimentally induced placentitis do not support the claim for effectiveness of short-term antimicrobial therapy. In fact, mares with experimentally induced ascending placentitis did not carry foals to term when treatment was discontinued after 2 weeks; however, an apparent increase in survival rates was observed when mares were kept on antimicrobials for a prolonged period of time (Rebello *et al.* 2006, Bailey *et al.* 2010). Another interesting finding about antimicrobial therapy from the same group demonstrated that mares kept on a prolonged treatment with antibiotics, present positive endometrial bacterial culture within 6 hours post abortion or delivery of a viable foal (Diaw *et al.* 2010). These findings suggest that antimicrobials administered to mares with experimentally induced placentitis may suppress bacterial growth, but do not achieve complete bacterial elimination.

Empirically, estrogen supplementation has been advocated to treat mares with placentitis (Douglas 2004). As aforementioned, this author reported that mares with placentitis that subsequently aborted had remarkably lower “total estrogen” concentrations than mares of the same gestational age that maintained pregnancy. As estrogens are produced in high concentrations during the second and third trimesters of pregnancy, it is

unlikely that estrogen supplementation will be able to restore normal estrogen concentrations. For example, it required continuous intravenous infusion of 126-231 mg of estrone sulfate per hour to achieve peripheral pregnancy levels of estrone sulfate (Raeside & Roskopf 1980). Therefore, to date, estrogen therapy has not been critically assessed by controlled studies or prospectively assigned field studies.

Other drugs that have been added to the treatment of equine placentitis include acetylsalicylic acid, pentoxifylline, and dexamethasone (LeBlanc 2010) (Table 1.2). Acetylsalicylic acid has been given to mares with placentitis under the impression that this drug would improve the blood flow to the mare's uterus; however, there is no evidence-basis in the literature to support this practice. In human medicine, low dose of acetylsalicylic acid has been shown to reduce the risks of preeclampsia in women (Henderson *et al.* 2014). Similarly, pentoxifylline has been included as part of the treatment for mares with placentitis with the idea that this drug possesses antiinflammatory and rheolytic properties (Macpherson 2006, Bailey *et al.* 2010) which might improve the oxygenation of the pregnant uterus. Pentoxifylline was found to be present in the fetal fluids of pregnant mares treated with the standard dose (Rebello *et al.* 2006) and has been added to the treatment of mares with experimentally induced placentitis (Bailey *et al.* 2010). However, it is unclear whether this drug has any beneficial effect on the treatment of placentitis. In addition, uterine artery blood flow remained unchanged when pentoxifylline was given to early pregnant mares during a short treatment period (Bailey *et al.* 2012). Dexamethasone has been suggested to be useful for the treatment of placentitis (LeBlanc 2010); however, it is uncertain whether administering this drug improves pregnancy outcome in mares with placentitis. At best, very limited to no evidence-basis

exists to support the practice to include these drugs (i.e. acetylsalicylic acid, pentoxifylline, and dexamethasone) in the treatment of placentitis.

Since the pathogenesis of nocardiform placentitis is unknown, and there are no established experimental models to study this type of placentitis, treatment for nocardiform placentitis has been empirically applied and based on treatments used for other types of equine placentitis. To date there are no evidence-based reports supporting the treatment of nocardiform placentitis in mares. A recent retrospective study demonstrated *in vitro* antimicrobial sensitivity for *Amycolatopsis* *ssp* and *C. equi* (Erol *et al.* 2012b) (Table 1.3). However, it is unclear whether these antimicrobials are effective *in vivo*. It is also unclear whether addition of antiinflammatories is effective or necessary for the treatment of nocardiform placentitis.

Tocolytic agents such as clenbuterol and isoxsuprine have also been used in practice as part of the treatment of mares with placentitis (LeBlanc 2010); however, there is no evidence-basis to support such practice. In fact, in a study carried out with normal pre-foaling mares clenbuterol was not effective in delaying parturition, and treated mares foaled earlier than control mares (Palmer *et al.* 2002). The lack of efficacy of clenbuterol to delay parturition should discourage the practice to give this drug to mares with placentitis.

Table 1.2: Therapeutic agents commonly used to treat placentitis in mares

Therapeutic agent	Dose	Proposed effect in mares with placentitis
Potassium Penicillin G	22,000 IU/kg, IV, QID	Antimicrobial,
Gentamicin	6.6 mg/kg, IV, SID	Antimicrobial
Trimethoprim sulphamethoxazole	15-30mg/kg, PO, BID	Antimicrobial
Altrenogest	0.088 mg/kg, PO, SID	Tocolytic, suggested to prevent prostaglandin mediated abortion
Pentoxifylline	8.5 mg/kg, PO, BID	Anti-pro-inflammatory cytokines, rheolytic
Flunixin meglumine	1.1 mg/kg, PO/IV, SID, or BID	Anti-inflammatory, not detected in the fetal fluids
Acetylsalicylic acid	50 mg/kg, PO, BID	Suggested to improve blood flow to the uterus by inducing thrombocytopenia, this has not been critically tested
Dexamethasone	40, 35, 25 mg, SID 24h, IV for 6 days, decreasing dose every 2 days	This treatment has not been critically tested in mares with placentitis. Clinical experiences and published studies suggested to be useful approach for other complications during pregnancy (e.g. body wall ruptures)

Adapted from: Rebello *et al.* (2006), Murchie *et al.* (2006), LeBlanc (2010), Troedsson & MacPherson (2011).

Table 1.3: In vitro anti-microbial sensitivity for *Amycolatopsis* ssp (n=38) and *Crossiella equi* (n=22) isolated from placentas in mares aborting in central Kentucky. The minimum inhibitory concentrations required to inhibit the growth of 50% and 90% of organisms (MIC50/MIC90) (Adapted from Erol *et al.* 2012b).

Antimicrobials/Bacterial species	Number of isolates with MIC (µg/ml)																MIC ₅₀ /MIC ₉₀					
	0.06	≤0.12	0.12	≤0.25	0.25	0.5	≤1	1	≤2	2	≤4	4	8	>8	16	>16		32	64	>64	128	
Amikacin																						
<i>C. equi</i>																		2	17		3	64/>64
<i>Amycolatopsis</i> spp.							2			13		13	4		1		2	3			4/32	
Amoxicillin-clavulanic acid																						
<i>C. equi</i>									3			14	5								4/8	
<i>Amycolatopsis</i> spp.									7			7	16		7		1				8/16	
Cefepime																						
<i>C. equi</i>							3						2		5		12				32/32	
<i>Amycolatopsis</i> spp.							1			2		2	6		9		18				16/32	
Cefoxitin																						
<i>C. equi</i>																	3	7			12	128/128
<i>Amycolatopsis</i> spp.											1		4		2		7	18			6	64/128
Ceftriaxone																						
<i>C. equi</i>											14			7					1		≤4/8	
<i>Amycolatopsis</i> spp.											15			20		2		1			8/8	
Ciprofloxacin																						
<i>C. equi</i>																						4/4
<i>Amycolatopsis</i> spp.																						2/4
Clarithromycin																						
<i>C. equi</i>																						8/16
<i>Amycolatopsis</i> spp.																						1/16
Doxycycline																						
<i>C. equi</i>																						0.25/0.25
<i>Amycolatopsis</i> spp.																						0.25/8
Imipenem																						
<i>C. equi</i>																						4/8
<i>Amycolatopsis</i> spp.																						4/16
Linezolid																						
<i>C. equi</i>																						≤1/2
<i>Amycolatopsis</i> spp.																						≤1/4
Minocycline																						
<i>C. equi</i>																						≤1/≤1
<i>Amycolatopsis</i> spp.																						≤1/8
Tobramycin																						
<i>C. equi</i>																						16/>16
<i>Amycolatopsis</i> spp.																						16/>16
TMP-SMX																						
<i>C. equi</i>																						0.5/2
<i>Amycolatopsis</i> spp.																						≤1/4

*Concentration ranges tested are shown with gray shading. TMP-SMX = trimethoprim-sulfamethaxazole.

Lately a new practice of antimicrobial therapy has been introduced, without a clinical diagnosis of placentitis; mares are administered antimicrobials for 10 days of the month throughout pregnancy (LeBlanc 2010). The idea of such practice would be that antimicrobial treatment would treat-subclinical/undiagnosed placental infection. This practice not only should be discouraged to the lack of proven efficacy, but also because it

favors bacterial resistant. Thus there is an enormous risk to create super resistant bacteria which may become a major threat not only to the horses but to public health.

1.5 Experimental Models of Bacterial Placental Disease in Large Animals

Experimental models for abortigenic diseases in domestic animals vary according to the animal species, infectious agent, natural route of infection, as well as the ability of the organism to survive in the environment. Different routes of infection have been used to induce disease experimentally; some models involve a vector that is important in the natural infection (e.g. Foot Hill Abortion in cattle), in other models, macerated tissues obtained from aborted materials are directly administered to susceptible animals. In the present review, only ruminant, porcine, and equine models will be briefly reviewed.

Induction of abortigenic diseases with different bacterial species (e.g. *Brucella ovis*, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Chlamydomphila abortus*) in several species of ruminants (i.e. goats, sheep, bison and cattle) and pigs have been achieved by intravenous or subconjunctival inoculation (Meador & Deyoe 1986, Anderson *et al.* 1986, Palmer *et al.* 1996, El Idrissi *et al.* 2001, Jungersen *et al.* 2006, Marques *et al.* 2010, Olsen & Johnson 2011, McCollum *et al.* 2013). Other routes, such as to the nasal and rectal mucus membranes through the inoculation of infected semen with *Brucella ovis* have also been described in sheep (Ridler *et al.* 2014). Oral administration of *Chlamydomphila abortus* (Tsakos *et al.* 2001) have been recently described in sheep. These routes probably mimic the natural infection, while others such as intravenous and subconjunctival by pass the means of natural infection. Once inoculated through either route noted above, bacteria gain access to the pregnant uterus and then cause severe necrotizing placentitis (Meador &

Deyoe 1986, Anderson *et al.* 1986, Palmer *et al.* 1996, El Idrissi *et al.* 2001, Tsakos *et al.* 2001, Jungersen *et al.* 2006, Marques *et al.* 2010, Olsen & Johnson 2011, McCollum *et al.* 2013, Poester *et al.* 2013).

It is likely that *Brucella* species migrate through the systemic circulation in immune cells (i.e. monocytes). Similar mechanisms are thought to occur for other bacterial species. Classically, *Brucella* ssp present tropism for trophoblastic cells, testes and mammary gland (Poester *et al.* 2013). Even though experimental inoculation of *Brucella* ssp through intravenous and subconjunctival routes induces abortion, oral transmission appears to be the most important route, except for *Brucella ovis* and *Brucella suis* where venereal transmission appears to be most relevant. Most species of *Brucella* can infect any mammal, including horses. In horses, “fistulous withers” is the most common clinical presentation in animals exposed to *Brucella abortus*; however, sporadic abortions have also been described (Denny 1973, Robertson *et al.* 1973, Hinton *et al.* 1977). Brucellosis in horses occurs where horses are kept with a herd of cattle where *Brucella abortus* is endemic.

Epizootic bovine abortion (i.e. Foothill abortion) can be induced by administration of macerated products of the aborted fetus (e.g. thymus) subcutaneously or through tick vector (i.e. *Ornithodoros coriaceus*) (Stott *et al.* 2002, Coker *et al.* 2012). From the subcutaneous tissue, the microorganism (an unidentified Deltaproteobacterium member of the order Myxococcales) (King *et al.* 2005), reaches the maternal circulation and the pregnant uterus and fetus. Placental lesions are not commonly present, but severe chronic inflammatory lesions are present in the fetus thymic atrophy, hepatomegaly, ascites, and generalized lymphomegaly (Hall *et al.* 2002).

Models to study Leptospirosis in cattle include inoculation through subconjunctival route, inoculation of bacteria into the central area of a placentome (through laparotomy), and intravenous inoculation (Aycardi *et al.* 1982, Ellis *et al.* 1986, Smith *et al.* 1994). The bacteria enter the systemic circulation and pregnant uterus and then cause placentitis, fetal lesions, abortion, stillbirths and weak neonates (Aycardi *et al.* 1982, Ellis *et al.* 1986, Smith *et al.* 1994, Donahue & Williams 2000). Different serovars of *Leptospira* affect cattle and horses, the subtype Pomona concentrates most of the serovars that affect both species (Schlafer & Palmer 2007).

Experimental induction and challenges with *Campylobacter fetus* subspecies *fetus* in sheep involves oral administration of the bacteria, which is the natural route of infection (Grogono-Thomas & Woodland 1996). Interestingly, a different subspecies of this genera, causes a different disease (i.e. early embryonic death and abortion is less commonly observed) in cattle, thus experimental infection of *Campylobacter fetus* subspecies *venereallis* infection in cows is achieved through intracervical inoculation of the bacteria in non-pregnant cows or intra-preputial inoculation in bulls (Ardila *et al.* 2012).

To date there is only one effective model to induce experimentally bacterial placentitis in mares. The model was initially described by Dr. Michelle LeBlanc's group at the University of Florida (Mays *et al.* 2002, LeBlanc *et al.* 2002). The model involves the inoculation of a pathogenic strain of *Streptococcus equi* subspecies *zooepidemicus* (10^7 to 10^9 cfu), into the cervix of mares during the 3rd trimester of pregnancy. Since the original description of the model, it has been used by other researchers with very predictable outcomes (Christensen *et al.* 2009, Bailey *et al.* 2010, Macpherson *et al.* 2013, Finger *et al.* 2014). One of the limitations of this research model is that a large inoculum is

administered to mares intracervically, with a resulting acute ascending placentitis and rapid abortion. This clinical presentation appears much more rapid than seen in spontaneous placentitis which tends to be insidious and chronic. Therefore, studies to address diagnostic markers and treatment strategies should be interpreted with caution regarding the clinical presentation of spontaneous and experimental placentitis.

1.6. Conclusions

Placental disease is an important cause of pregnancy wastage in broodmares. Bacterial infections are responsible for a large portion of abortion cases. There are four different morphologic types of placentitis: ascending, focal mucoid (nocardioform), diffuse (hematogenous), and multifocal, whereas, ascending is the most frequent type of placentitis, whereby beta-hemolytic streptococci predominate. The diagnosis of equine placentitis is based on clinical signs (i.e. vulvar discharge, premature udder development and lactation) and ultrasonography. While transrectal ultrasonography has been shown to be a valuable clinical tool to diagnose and manage cases of placentitis; the technique is prone to false positive diagnosis and early cases of the disease can be missed. Treatment of placentitis should be aimed on prolonging the permanence of the foal in uterus, to achieve this goal; control of placental and fetal infection, maintaining of the uterus quiescent and by blocking the production of pro-inflammatory cytokines. Few antibiotics (i.e. Penicillin, gentamycin, and sulfa-trimethoprim) have been shown to cross the placenta and achieve satisfactory inhibitory concentrations.

Chapter 2:

Attempts to Induce Nocardioform Placentitis (*C. equi*) Experimentally in Mares

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2.1. Summary:

Reasons for performing study: Nocardioform placentitis in horses is poorly understood, and the development of an experimental horse model would be of help in understanding the pathogenesis of the disease.

Objectives: To investigate whether (1) intrauterine inoculation of *C. equi* during the periovulatory period or (2) i.v., oral or intranasopharyngeal inoculation of *C. equi* during mid-gestation would result in nocardioform placentitis, and (3) before and after mating endometrial swabs present evidence of nocardioform-associated organisms (*C. equi* or *Amycolatopsis* spp).

Methods: Study I, mares (n=20) received an intrauterine inoculation of *C. equi* 24h after artificial insemination. Endometrial swabs were obtained 24h post-inoculation for PCR analysis for *C. equi*. In Study II, pregnant mares (at 180 and 240 days of gestation) were inoculated with *C. equi* by intranasopharyngeal (n=5), oral (n=4) or i.v. (n=4) routes. Sixty

contemporaneous pregnant mares maintained on the same farm served as controls animals. In Study III, privately-owned Thoroughbred mares (n=200) had endometrial swabs collected before and within 24-48h post-mating for detection of nocardioform microorganisms.

Results: In Study I, *C.equi* was identified by PCR in 3/20 mares following intrauterine inoculation. Pregnancy was established in 19 of 20 treated mares. There were 2 embryonic losses and one abortion at 177d of gestation (undetermined cause). Sixteen mares delivered a normal foal and placenta. In Study II, one mare (oral inoculation) aborted at 200 days of gestation (unidentified cause). The remaining mares delivered a normal foal and placenta. In Study III, none of the mares yielded positive endometrial PCR for nocardioform microorganisms.

Conclusions: We were unable to induce nocardioform placentitis, and there was no evidence of nocardioform microorganisms in endometrial swabs of broodmares before or after mating. These findings suggest that nocardioform placentitis is not induced simply via the presence of nocardioform actinomycetes, and that route, insufficient duration of exposure and dose may play a role in the development of disease. Additional predispositions may be involved in the development of nocardioform placentitis.

2.2. Introduction

Placentitis is an important cause of pregnancy wastage in mares. In the USA, placental pathology accounts for the majority of late-term pregnancy losses and neonatal deaths in the first 24h of life (Giles *et al.* 1993, Hong *et al.* 1993, Williams *et al.* 2004). Four different types of equine placentitis have been described according to the morphological lesions and suggested pathogenesis, namely ascending, focal mucoid (nocardioform), diffuse (hematogenous), and multifocal (Williams *et al.* 2004). Overall, ascending placentitis is the most prevalent type of placentitis (Giles *et al.* 1993, Hong *et al.* 1993).

Nocardioform placentitis was first diagnosed in 1985 in central Kentucky by the University of Kentucky Veterinary Diagnostic Laboratory (UKVDL) (Giles *et al.* 1988). Since its initial description, the number of mares suffering from this disease has fluctuated across years in the region (Giles *et al.* 1993, Hong *et al.* 1993, Giles *et al.* 1988, Williams *et al.* 2004, Donahue & Williams 2000, Carter *et al.* 2012, Erol *et al.* 2012a), and nocardioform placentitis has also been diagnosed in other areas of the United States (Christensen *et al.* 2006). Additionally, isolated cases of nocardioform placentitis have been reported in other parts of the world, including South Africa (Volkman *et al.* 2001) and Italy (Cartoli *et al.* 2004). During the 2002 and 2003 foaling seasons, a total of 128 cases of nocardioform placentitis were diagnosed by the UKVDL (Williams *et al.* 2004). Most recently, during the 2011 foaling season, the UKVDL registered 390 confirmed cases of nocardioform placentitis (N.M. Williams, unpublished data).

Microorganisms associated with nocardioform placentitis include *Amycolatopsis* species (*Amycolatopsis kentuckyensis*, *Amycolatopsis lexingtonensis* and *Amycolatopsis pretoriensis*) and *Crossiella equi* (*C. equi*) (Donahue *et al.* 2002, Labeda *et al.* 2003,

Christensen *et al.* 2006, Labeda *et al.* 2009, Erol *et al.* 2012ab). In 2009, two new species of *Streptomyces* (*Streptomyces atriruber* and *Streptomyces silaceus*) were isolated from nocardioform placentitis cases (Labeda *et al.* 2009). Historically, these bacterial agents have been denominated as nocardioform actinomycetes or more recently as gram-positive branching bacilli based on PCR and 16S ribosomal RNA analyses (Erol *et al.* 2012a). In the 2010-2011 foaling season in central Kentucky, *C. equi* and *Amycolatopsis* ssp were responsible for 85% of the cases submitted to the UKVDL. In the remaining cases lesions were present but the bacterial species were not identified (Erol *et al.* 2012a). Based upon observations of cases submitted to the UKVDL, it appears that mares affected with *C. equi* are more prone to abortion, whereas mares infected with other actinomycetes tend to deliver live premature foals (unpublished observations, UKVDL). In addition, it is worth noting that to date the causative organisms have only been cultured from clinical placentitis cases and not from the environment.

A specific distinction between bacteria causing nocardioform placentitis (i.e. *C. equi* and *Amycolatopsis* ssp) and other actinomycetes that affect horses is related to the propensity to infect not only the fetal membranes but also the offspring *in utero*. For example, mares affected by *Cellulosimicrobium cellulans* in Kentucky presented mucoid placentitis associated with fetal lesions (Bolin *et al.* 2004). Similarly, a group of actinomycetes (e.g. *Cellulosimicrobium* sp, *Cellulomonas* sp) and other nonspecific bacterial species have been associated with mucoid placentitis and fetal lesions in a condition known as equine amnionitis and fetal loss (EAFL) that is associated with ingestion of processionary caterpillars in Australia (Todhunter *et al.* 2013). On the other hand, the actinomycetes causing nocardioform placentitis do not infect the fetus in utero as

is commonly seen in other types of placentitis. In other words, the bacterial infection in NPL is limited to the chorionic surface of the placenta (Hong *et al.* 1993, Williams *et al.* 2004, Donahue & Williams 2000).

The definitive diagnosis of nocardioform placentitis is based on the presence of macroscopic and microscopic lesions, bacterial culture and identification of bacterial DNA by polymerase chain reaction (PCR) of samples from the chorioallantois (Hong *et al.* 1993, Donahue & Williams 2000, Erol *et al.* 2012ab). A presumptive clinical diagnosis can be attained through clinical examination and ventral transabdominal ultrasonography (Christensen *et al.* 2006, Wolfsdorf *et al.* 2000). Mares suffering from nocardioform placentitis present areas of placental separation with accumulation of hyperechoic exudate in the ventral uterine body and/or base of the horns (Christensen *et al.* 2006, Wolfsdorf *et al.* 2000, Troedsson & Macpherson 2011). Because the cervical star region is not involved, no vulvar discharge or cervical softening is observed unless the mare is very close to abortion or parturition. Premature mammary gland development, which is the first common sign observed in mares with ascending placentitis, is not always observed in mares during initial stages of nocardioform placentitis. The absence of outward clinical signs (i.e. vulvar discharge and premature mammary development) makes the presumptive diagnosis of nocardioform placentitis challenging for the clinician.

The pathogenesis of nocardioform placentitis is unknown, and a reliable model to reproduce the disease would therefore be useful. The objective of these studies was to develop an experimental model for induction of clinical nocardioform placentitis in mares. Specifically, we investigated (1) whether intrauterine inoculation of *C. equi* during the periovulatory period or (2) intravenous, oral or intranasopharyngeal inoculation of *C. equi*

between 180 and 240d of gestation would result in nocardiform placentitis, and (3) whether endometrial swabs obtained from Thoroughbred mares before and after mating would provide evidence for the presence of *C. equi* or *Amycolatopsis* ssp.

2.3. Material and methods

All the experimental animal work in this research project was carried out at the University of Kentucky, Lexington, KY, USA, from September of 2011 to November of 2012 (Studies I&II) with approval from the Institutional Animal Care and Use Committee (Protocol # 2011-0840). The endometrial swabs (Study III) were collected during the 2012 breeding season at commercial Thoroughbred farms in central Kentucky. In Studies I&II, all the animals were kept in paddocks and supplemented with hay, grain, water, and trace minerals *ad libitum*. In Study II, during more intensive handling procedures and immediately pre-foaling, the mares were kept in individual stalls during the night and turned out on pasture during the day. The *C. equi* strain used in this study was a reference strain, originally isolated from an equine abortion case submitted to the UKVDL (Reference number: LDDC 22291-98^T, NRRLB-24105). This isolate was stored at -80°C and had a very low passage number (passage 3).

Study I: Intrauterine inoculation of *C. equi* in estrous mares 24h after artificial insemination

Twenty mares of different light horse breeds and with a mean age of 10.5 years (range: 5-20 years) were used in this study. Detailed breeding management is described in the appendices. Estrous mares were bred with ≥ 500 million progressively motile sperm in fresh semen pooled from three fertile stallions and diluted using a commercial extender (without antibiotics).

At 24h post breeding, each mare received an intrauterine inoculation of *C. equi* (1×10^4 colony-forming-unit in 1mL of skimmed milk). Bacteria were kept at 37°C in enriched TGY (tryptone-glucose-yeast) broth medium, and then plated onto minimal agar (Czapek's agar) plates for 2-3 days. Bacterial cultures were prepared and preserved in 2 mL cryovials at -80°C until inoculation. Serial dilution and plating were used to determine cfu. Immediately before inoculation, the samples were thawed at room temperature. After inoculation, bacterial culture was performed from each cryovial to confirm viability of the inoculum. At 24h post-inoculation, each mare had an endometrial swab collected for PCR testing for *C. equi* (Sells 1996, Tan *et al.* 2006, Erol *et al.* 2012a).

Pregnancy diagnosis was performed by transrectal ultrasound at 15, 25, 35, 60, 90 and 120 days post-ovulation. From 150 days to term, the mares were examined monthly by transabdominal ultrasonography (transducer 2–6 MHz) to confirm normal pregnancy and to evaluate the fetal membranes (Troedsson & Macpherson 2011).

All the placentas from inoculated mares in Study I were submitted to the UKVDL for pathological evaluation, culture (i.e. for *C. equi* and *Amycolatopsis* ssp) and PCR for both organisms. A swab from each chorioallantois was collected and cultured according to Erol *et al.* (2012ab). PCRs for *C. equi* and *Amycolatopsis* ssp were performed according to established protocols (Sells 1996, Tan *et al.* 2006, Erol *et al.* 2012a) at the UKVDL, an accredited veterinary diagnostic laboratory by American Association of Veterinary Laboratory Diagnosticians. As part of the quality control and assurance cycle, a negative amplification control, a negative extraction control and a positive extraction control (*C. equi* or *Amycolatopsis* reference strains) were performed in each PCR run.

Sixty contemporaneous pregnant mares of various light breeds and ages (mean 10.5yrs; range 4-20yrs) kept at the same farm were used as controls. All placentas were examined by two veterinarians and, if signs of placentitis were observed, the placenta was submitted to the UKVDL for full pathological examination (Hong *et al.* 1993, Williams *et al.* 2004, Donahue & Williams 2000). Unlike mares in the inoculated groups, control mares were not regularly monitored by ultrasonography. Therefore, the experimental end point for the control mares was the presence of nocardioform placentitis lesions after foaling. None of the mares received antibiotics during pregnancy or any type of treatment for placentitis or other clinical problems. Additionally, 10 placentas from control mares were submitted for full pathological examination.

Study II: Intravenous, Intranasopharyngeal and Oral Inoculation of *C. equi* to Pregnant Mares

Thirteen mares of various light breeds and ages (mean 13.2 years; range 8-19 years) were used in this experiment. Mares were bred with fresh extended semen and ovulation was confirmed by ultrasound performed every other day. Before the beginning of the experiment, each mare was examined by transabdominal ultrasonography to confirm normal pregnancy. Mares were randomly allocated into three treatment groups, and inoculated with *C. equi* as follows: nasopharyngeal (n=5), oral (n=4), and intravenous (n=4). The inoculations were performed from 180 to 240d of pregnancy; mares in the intravenous route group received the inoculum once at 180d of gestation, whereas mares in the oral and nasopharyngeal route groups received the bacteria four times at two week intervals (i.e. 180 to 240d of gestation). In this experiment, each inoculum contained 1×10^9

cfu of *C. equi*. Detailed explanation of the bacterial culture is described elsewhere (Erol *et al.* 2012ab) and in the supplemental information.

For all the different routes of inoculation, mares were restrained in stocks and when necessary sedated with xylazine hydrochloride (0.2-0.4 mg/kg intravenously) immediately pre-inoculation. Inoculating doses were prepared at a final concentration of 1×10^9 cfu/mL of *C. equi*, and were transported refrigerated to the farm (<5 min). Immediately before inoculation, the inoculum was thoroughly mixed and diluted 1:4 with sterile Dulbecco's phosphate buffer solution. For respiratory route administration, the inoculum was loaded into a 20 mL sterile syringe and administered deep into the nasopharyngeal cavity using an AI pipette. For oral administrations, the mare's oral cavity was thoroughly flushed with water to remove any residual feedstuff. Thereafter the inoculum (i.e. 5 mL solution) was deposited deep into the mare's mouth. After inoculation through the nasopharyngeal and oral routes the mare's head was held up for 5 min.

Intravenous inoculation was performed through a jugular catheter (14G; 3 inches long). The inoculum was administered slowly (>2 min), and the catheter was then flushed thoroughly with sterile Dulbecco's phosphate buffer solution (100 mL) to avoid bacterial retention on the catheter or in the subcutaneous tissues.

Immediately before inoculation and thereafter for the first 72h, all mares were examined clinically at 12h intervals for evidence of sepsis. At 24h post inoculation, CBC, fibrinogen and serum amyloid A analyses were determined for the mares receiving the bacteria via the i.v. route. Concentrations of serum amyloid A were determined (at 12, 24, 48 and 72h after inoculation for mares receiving the bacteria intravenously) by immunoturbidimetric assay (Jacobsen *et al.* 2006) at the University of Miami.

All mares were thoroughly examined every 2 weeks by transabdominal ultrasonography with a curvilinear transducer 2–6 MHz, from 24h before inoculation until parturition. The control mares described in Study I also served as controls for Study II. All placentas from inoculated mares were submitted for full pathological examination as described for Study I.

Study III: Endometrial PCR for C. equi and Amycolatopsis ssp in Mares

A total of 200 commercial Thoroughbred mares had an endometrial swab collected immediately before and within 24-48h after natural mating. Mares had the perineal area thoroughly prepared in a standard manner. Swabs were submitted to the UKVDL for PCR analysis for *C. equi* and *Amycolatopsis ssp* as described above.

2.4. Results

Study I

The overall pregnancy rate was 95% (19 of 20 mares). One mare was bred during only one cycle and did not become pregnant. This mare went into seasonal reproductive transition after the first estrous cycle and was not rebred. Fifteen mares became pregnant on the first cycle. Three mares became pregnant on the second estrous cycle; one mare became pregnant after 3 cycles. There were 2 embryonic losses (between 25-35d and 35-60d); it is worth noting that both mares had unremarkable ultrasonographic examinations and negative PCR for *C. equi* and bacterial culture upon diagnosis of pregnancy loss. One mare aborted at 177d of gestation. Unfortunately, the placenta and part of the fetus were taken by wild scavengers. Therefore, the cause of abortion could not be determined. Nine

days pre-abortion, transabdominal ultrasonography was unremarkable and the fetus was alive. Fetal material and a nasal swab from the dam were PCR negative for EHV-1. Paired serology taken at three week intervals presented low titers for *Lepstospira* species (data not shown).

Out of 25 inoculations, only three mares had a positive endometrial PCR result for *C. equi* at 24h post inoculation. Two of the mares with positive PCR delivered a normal foal and placenta, and the remaining mare aborted (as noted above). All ultrasonographic examinations were unremarkable during the entire experiment (data not shown). None of the mares presented clinical or pathological (i.e. macroscopic or histopathological lesions) or microbiological (culture and PCR) evidence of nocardioform placentitis.

All mares in the control group delivered normal foals and fetal membranes, although one of these mares had a small (~12cm²) lesion on the chorion that was grossly consistent with nocardioform placentitis. Macroscopically there was loss of villi and increased pallor centrally with raised tan thickenings peripherally. Histopathology confirmed signs consistent with placentitis including necrotic villi with accumulation of eosinophilic debris on the chorionic surface that contained long filamentous bacteria. The villi were infiltrated by moderate numbers of macrophages, neutrophils and lymphocytes and the stroma contained large infiltrates of lymphocytes. The remainder of the placenta was unremarkable. Macroscopic and microscopic findings were consistent with nocardioform placentitis (Donahue & Williams 2000, Williams *et al.* 2004). However, PCR was negative for *C. equi* and *Amycolatopsis* ssp, aerobic culture yielded saprophytic bacteria but no nocardioform organisms were isolated.

Study II

All mares remained healthy after inoculation and physical exam parameters remained within normal ranges (data not shown). Blood PCR at 12 and 24h post IV inoculation were negative for *C. equi* and *Amycolatopsis* ssp. Concentrations of SAA remained baseline (i.e. <20mg/L) from immediately before to 72h after intravenous inoculation (i.e. 12, 24, 48 and 72h, data not shown). Fibrinogen and CBC analyses for the group receiving the bacteria intravenously were within normal ranges by 24h after inoculation.

Twelve mares delivered a normal foal and placenta with no clinical, microbiological or pathological evidence of nocardioform placentitis. One mare aborted at 200d of gestation after two inoculations through the oral route. The cause of abortion was not established but there was no pathological evidence of nocardioform placentitis.

Study III

All the PCR analyses on samples collected shortly before and post-mating were negative for *C. equi* and *Amycolatopsis* ssp.

2.5. Discussion

This is the first report to attempt experimental induction of nocardioform placentitis. None of the experimental approaches in the present study resulted in clinical, pathological or microbiological evidence of nocardioform placentitis. Additionally, none of the uterine swabs recovered from 200 commercial broodmares before or post-mating yielded a positive PCR for actinomycetes nocardioform species.

As there are no established experimental models for nocardioform placentitis, there are no data regarding infective dose, culture conditions or route of inoculation for these

organisms required to induce the disease in horses; therefore the infective dose in this study was determined empirically and general route of inoculations (i.v., intranasopharyngeal and oral routes) were tried in order to address whether the transmission occurs hematogenously. Possible explanations for the inability to induce nocardioform placentitis in the current experimental model include: inadequate infective dose, low pathogenicity of the bacteria, improper route of exposure, insufficient duration of exposure, and absence of host immunosuppression of unknown origin that may make some pregnant mares prone to developing nocardioform placentitis. In Studies I & II the inoculating doses were empirically chosen because there is no basis in the literature for determining the dose of *C. equi* required to establish infection.

Although the source of actinomycetes responsible for nocardioform placentitis remains undetermined, there are some associations which suggest an environmental source for these pathogens. In a retrospective analysis of the weather conditions in central Kentucky, we found a strong negative correlation ($r=-0.7$) between rainfall in August and September and the number of nocardioform placentitis cases during the subsequent winter and spring (unpublished data). In central Kentucky, most Thoroughbred mares will be at 6-8 months of gestation by August-September; therefore in Study II, pregnant mares were inoculated at this stage of gestation in an attempt to mimic supposed natural exposure. Indeed, if the nocardioform actinomycetes are found in the environment (i.e. soil), the oral and nasopharyngeal routes would likely allow prolonged exposure to the environmental source of pathogen, thus, these two routes were evaluated here using multiple inoculations. However, multiple attempts to isolate *C. equi* from the environment (stalls, soil, water, hay, grass) were unsuccessful (Erol E, unpublished observation), either due to low number

of microorganisms in the environment or the presence of these organisms in other areas of the environment. In addition, UKVDL performs thousands of fecal culture from mares yearly; however, these organisms have not been isolated from fecal samples (Erol E, unpublished observation), suggesting that *C. equi* is not shed in feces.

The lesions observed in spontaneously occurring nocardioform placentitis suggest that the bacteria either enter the uterus either hematogenously or during gynecological intrauterine interventions (e.g. uterine lavage, endometrial swabbing, etc) or at mating. Mares in Study I, received the bacteria 24h post-breeding as we considered that this would increase the persistence of the inoculum in the uterus, since mixing the inoculum with the semen would potentially result in rapid drainage of the fluid through the cervix after breeding. It is worth noting that previous attempts, carried out at our facilities in 1998-2000, using intrauterine inoculation (n=5 mares *C. equi*; 10^6 cfu) post-ovulation failed to induce nocardioform placentitis. All mares became pregnant and four mares delivered live normal foals. A normally developed foal from the 5th mare was found suffocated in the placenta. No abnormalities were found in any of the placentas (unpublished data, K.J.McDowell). Additionally, every-other-day intrauterine inoculation (inoculum 10^6 cfu *C. equi*) of five non-bred mares (supplemented with progesterone 150mg/IM) for 10 days post ovulation failed to yield a *C. equi* positive endometrial culture (swabs collected 24h after each of the 5 inoculations) (K.J.McDowell, unpublished data). These results and our findings (Study I) support the concept that *C. equi* grows poorly in the uterine lumen of the non-pregnant mare.

The bacteria appear not to be transmitted during mating, since the 200 mares sampled here had negative PCR for *C. equi* and *Amycolatopsis* ssp (Study III), and mares

receiving the bacteria by the intrauterine route after insemination (Study I) did not develop clinical or pathological evidence of nocardioform placentitis. These experiments do not completely exclude the possibility that nocardioform organisms are acquired during breeding, as the sample size was too small to detect a low rate of infection at mating. If, in fact, nocardioform transmission occurs during mating, using 200 mares with an alpha error of 0.05 and power of 0.8 and based on a one-tailed test, we would have been able to detect an incidence of 2.1% of positive PCR; however, with the estimated incidence of 0.1% nocardioform placentitis in Central Kentucky, >4000 mares would need to be sampled to be sure of detecting infection.

Data presented here support the hypothesis that *C. equi* has low pathogenicity, because bacteria administered intravenously neither induced changes in peripheral leukocyte counts nor resulted in positive blood culture 24h post inoculation or positive PCR at 12, 24, 48 or 72h post inoculation, and intravenous inoculation did not cause elevations in the concentrations of SAA (i.e. 12, 24, 48 and 72h post inoculation), a sensitive marker of inflammation (Jacobsen *et al.* 2006). Absence of clinical signs after intravenous inoculation further corroborates the hypothesis of the lack of pathogenicity of this organism and that the bacteria appear to replicate poorly in the systemic circulation under normal conditions.

In the present Studies (I & II), we hypothesized that different routes of transmission (i.e. intrauterine, oral and intranasopharyngeal) were important in the pathogenesis of nocardioform placentitis, but we were unable to reproduce the disease through any tested route of inoculation. It is possible that the bacteria can be acquired by other routes of transmission not tested here (e.g. ocular, mucosal or dermal); however, this remains

speculative until the route of transmission is established. Another possible explanation for the lack of success in reproducing the disease includes insufficient time or duration of exposure to the actinomycetes. Some of the routes (intrauterine and intravenous) involved a single administration. Perhaps, to become infected a mare needs prolonged exposure to the bacteria (e.g. continual environmental exposure). Anecdotally, it has been suggested that the mechanism leading to uterine nocardioform infections is likely to be related to a deficient immune response of the prospective host (perhaps mediated by progesterone); to date, this hypothesis has not been tested. In a preliminary study, two pregnant mares (240d of gestation) were intracervically inoculated with 1×10^9 cfu of *C. equi*, both mares developed ascending placentitis due to secondary beta hemolytic streptococci (Canisso *et al.* 2013). These findings support the concept that *C. equi* grows poorly in the uterine lumen of normal pregnant mares.

Interestingly, field and experimental cases of EAFL in Australia also present mucoid placentitis (Todhunter *et al.* 2013, Cawell-Smith *et al.* 2013) similar to nocardioform placentitis; however, EAFL is associated with additional placental and fetal lesions. To our knowledge, neither *C. equi* nor *Amycolatopsis* ssp have been associated with EAFL. Researchers believe that intestinal lesions caused by processionary (Todhunter *et al.* 2013, Cawell-Smith *et al.* 2013) and eastern tent (McDowell *et al.* 2010) caterpillars are involved in the pathogenesis of EAFL and mare reproductive loss syndrome (MRLS). However, field and experimental cases of MRLS are not associated with nocardioform placentitis (McDowell *et al.* 2010), instead during the 2001 MRLS outbreak in Kentucky (>3000 pregnancy losses) the UKVDL registered one of lowest incidences of nocardioform

placentitis cases in almost 30 years. Thus at this time there is no evidence linking MRLS and nocardioform placentitis in central Kentucky.

In conclusion, intrauterine inoculation of *C. equi* after ovulation did not result in clinical or pathological evidence of nocardioform placentitis in mares. Intravenous, oral or pharyngeal inoculation of *C. equi* between 180 and 240d of gestation also failed to induce clinical or pathological evidence of nocardioform placentitis in mares. None of the endometrial swabs recovered shortly before or after mating of 200 commercial Thoroughbred broodmares tested positive for *C. equi* or *Amycolatopsis* ssp by PCR. The route of transmission and pathogenesis of equine nocardioform placentitis therefore remains undetermined. Alternative routes of inoculation, using different bacterial isolates and species, doses and intervals should be tested.

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Chapter 3:
**Serum amyloid A and Haptoglobin Concentrations are Increased in Plasma of
Mares with Ascending Placentitis in the Absence of Changes in Peripheral
Leukocyte Counts or Fibrinogen Concentration**

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3.1. Summary

Currently placentitis, an important cause of late pregnancy loss, is diagnosed by clinical signs and ultrasonography. Acute phase proteins (APP) are mainly produced and secreted by the liver in response to acute inflammatory stimuli, and we hypothesized that APP are elevated in mares with placentitis. Concentrations of serum amyloid A (SAA), haptoglobin (Hp), fibrinogen (Fb) and white blood cell counts (WBC) were determined in plasma of mares with experimentally induced placentitis and gestationally aged-matched control mares. Placentitis was induced via intracervical inoculation of *Streptococcus equi* subspecies *zooepidemicus*, a common isolate from clinical cases of bacterial placentitis. Concentrations of SAA and Hp were also determined in 10 days pre-partum in normal

mares. Mares with placentitis aborted within 5-25 days after inoculation. Concentrations of SAA and Hp rapidly increase subsequent to experimental induction of placentitis and remain elevated until abortion. Neither Fb nor WBC appeared to be useful markers for placentitis. Parturition did not trigger an increase in either SAA or Hp in normal foaling mares.

3.2. Introduction

Ascending placentitis is a common cause of late-term abortion and premature delivery in mares (Giles *et al.* 1993, Troedsson & Zent 2004, Williams *et al.* 2004, LeBlanc 2010, Laugier *et al.* 2011); it can be caused by fungal or bacterial agents with bacteria being responsible for about 90% of clinical cases (Hong *et al.* 1993). Bacterial agents commonly associated with the occurrence of ascending placentitis include *Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, of which beta-hemolytic streptococci predominate (Hong *et al.* 1993, Williams *et al.* 2004, LeBlanc 2010). Generally, only one or eventually two species of bacteria are isolated from most clinical cases (Hong *et al.* 1993). The incidence of placentitis in well-managed Thoroughbred farms has been estimated as 3-5% (Troedsson & Zent 2004). The pathogenesis of ascending placentitis in mares is not well established; however, it is believed that bacterial agents enter the uterus via the cervix (LeBlanc 2010). Placental infection/inflammation leads to abortion or delivery of a pre-mature foal (LeBlanc *et al.* 2012). Chronic placentitis may also lead to placental insufficiency, which may result in the delivery of a nonviable foal. Foals born alive from mares suffering placentitis are often septic and demand intensive care and are not necessarily viable athletes (Barr 2005).

Mares with ascending placentitis present premature mammary development and purulent vulvar discharge, and if not treated, mares will delivery prematurely (i.e. abortion, stillbirth, or have septic foals) (LeBlanc 2010, Troedsson & Macpherson 2011). Manifestation of such clinical signs warrant immediate veterinary attention; thus the clinical diagnosis can be confirmed based upon clinical signs and transrectal ultrasonography of the placenta near the cervix (Troedsson & Macpherson 2011). While later stages of placentitis are easily diagnosed via ultrasonography, earlier stages and

subclinical cases of placentitis can be missed. Therefore, improved and earlier diagnostic methods may improve treatment outcomes in some of these cases.

Ascending placentitis is widely associated with focal extensive inflammatory and necrotic changes of the chorioallantois near the cervical star (Hong *et al.* 1993). Mares with experimentally induced ascending placentitis present increased concentration of PGE₂ and PGF_{2α} in the fetal fluids and increased transcripts for IL-6 and IL-8 in the chorioallantois (LeBlanc *et al.* 2002). Unlike placentitis in women (i.e. chorioamnionitis), where the disease can be life-threatening (Fahey 2008, Tita 2010), equine ascending placentitis is thought to be a local inflammatory disease of the pregnant uterus with little or no systemic inflammatory response. Mares with spontaneous or experimentally induced ascending placentitis do not present systemic clinical signs (e.g. sepsis, life threatening complications). A growing body of literature has been dedicated to use acute phase inflammatory proteins (APP) to diagnose placental disease (i.e. chorioamnionitis, funisitis and early-onset of neonatal sepsis) in humans (Wu *et al.* 2009, Popowski *et al.* 2011, Lee *et al.* 2012). A recent study conducted to address the usefulness of serum amyloid A (SAA) determination as prognostic marker for mares with placentitis indicated that mares with experimentally induced ascending placentitis present an increase in SAA concentrations when they did not receive a common treatment (i.e. antibiotics, anti-inflammatories and progestins) for ascending placentitis (Coutinho da Silva *et al.* 2013). In addition to changes associated with placentitis, Coutinho da Silva *et al.* (2013) suggested that parturition was associated with an increase in SAA concentration in normal periparturient mares. Because sample frequency prior to parturition was limited, results from this report were equivocal concerning a pre-partum rise in SAA in the normal mare at parturition.

Haptoglobin (Hp), a moderate APP, is constantly present in plasma of healthy horses, and concentrations are increased upon inflammation (Jacobsen et al. 2007, Cray & Belgrave 2014). Recent studies, involving human neonates, suggested that Hp is a useful marker for neonatal sepsis (Buhimschi *et al.* 2011, Ferrazzi *et al.* 2012). In horses, Hp appeared to be a useful marker to evaluate acute-chronic conditions (i.e. >4-day duration) (Cray & Belgrave 2014). To date, there have been no studies assessing Hp in mares with placentitis.

Fibrinogen (Fb) is the APP used most frequently in the horse to evaluate inflammation; however, to the best of our knowledge, there are no published reports in the literature evaluating this APP in equine placentitis. Total white blood cell counts (WBC) are widely used in human and veterinary medicine as a routine screening test for disease and as a method to monitor treatment response. In women, WBC in peripheral blood has been used as an indicator of chorioamnionitis (Sereepapong *et al.* 2001), although other reports do not support such an assertion (Cray & Belgrave 2014). Other authors found that WBC in fetal fluids along with bacterial culture appears to be superior for the diagnosis of chorioamnionitis compared to peripheral leukocyte counts (Buhimschi *et al.* 2011). To the best of our knowledge there are no controlled studies or randomized trials addressing changes in peripheral WBC counts in equine placentitis.

The objectives of the current study were to: (i) compare plasma concentrations of APP and the WBC in mares with experimentally induced ascending placentitis and gestationally aged-matched control mares carrying normal pregnancies, and (ii) describe SAA (serum amyloid A) and Hp (haptoglobin) concentrations in normal pre-foaling mares.

3.3. Material and Methods

Animal Husbandry

Clinically healthy adult horse mares of different ages were enrolled in these experiments. Mares were maintained at the Maine Chance Farm, Department of Veterinary Science, University of Kentucky, Lexington, KY. All the experimental protocols were approved by the Institutional Animal Use and Care committee at the University of Kentucky (Project #2010-0769). All the animals were kept in paddocks and supplemented with hay, grain and water *ad libitum* along with trace minerals. Immediately pre-abortion or prior to normal parturition; the mares were kept in individual stalls (16 x 16 feet) during the night and turned out in pasture during the day.

Study I: Serum Amyloid A, Haptoglobin, Fibrinogen and WBC in Mares with Ascending Placentitis

Experimental design

Mares carrying normal pregnancies (260-280 days of gestation) were assigned to (i) a control group with (n= 7) or without trans-abdominal ultrasound-guided fetal fluid sampling (FFS; n=10) and (ii) a treatment group with experimentally induced ascending placentitis with (n =7) or without FFS (n=10). Fetal fluid sampling was performed at 0, 5 and 12 days post inoculation (DPI) or until abortion. Similar methodology has been described elsewhere (Canisso *et al.* 2014). Here briefly, the mare was sedated, placed in stocks and fetal fluids were collected with transabdominal ultrasound and an echotip spinal needle (18Gx6"; 30° short bevel, Chiba-Type spinal needle, with stylet, Echo Block PTC

Havel's Cincinnati, OH, USA) (Canisso *et al.* 2014). Fetal fluid sampling was performed in a subset of mares as part of another study.

Experimental Induction of Ascending Placentitis

Ascending placentitis was induced via intracervical inoculation of *Streptococcus equi* ssp. *zooepidemicus*. The experimental model for induction of bacterial placentitis in the mare was modified from that previously described (LeBlanc *et al.* 2002) and has been successfully used by different laboratories as a reliable and predictable model to induce ascending bacterial placentitis in the mare (Coutinho da Silva *et al.* 2013, Morris *et al.* 2007). Sham inoculation of control mares was not performed in the present study as this procedure may induce placentitis in an unpredictable manner; as noted by previous publications. The bacterial inoculum was deposited midway intracervically with the usage of a semi-flexible artificial insemination pipette. The inoculum was contained in a 0.5-mL straw and was deposited with the use of a stylet. The bacterial strain used was isolated from a placenta from a mare diagnosed with spontaneously ascending placentitis by the Veterinary Diagnostic Laboratory at the University of Kentucky. The bacterial isolate was preserved in cryovials containing skim milk at -80°C until use. Prior to inoculation, bacterial cultures were prepared and bacterial counts performed in standard fashion using blood Agar plates (incubated at 37°C), serial dilution and plating were used to determine the number of colony-forming units. The bacterial inoculum, containing 5 million colony forming units (cfu), was suspended in 0.5 mL of phosphate buffered saline. Once inoculation was carried out, a swab was obtained from the leftover in the cryovial and cultured in similar conditions to assure that the bacteria inoculum was still viable.

Transrectal Ultrasonography

Before the beginning of the experiment, mares underwent transrectal ultrasonography to assess the caudal placental pole to assure that all mares were carrying normal pregnancies. Three measurements of the combined thickness of uterus and placenta (CTUP) were taken and compared with previously established normal ranges (Renaudin *et al.* 1997, Coutinho da Silva *et al.* 2013). All mares had CTUP values within previously reported normal ranges and presented normal ultrasonographic appearance of the caudal pole of the placenta (data not shown); therefore all mares were enrolled in this study. Thereafter, all mares had transrectal ultrasonography performed every other day for 12 days or until abortion. Transrectal ultrasonography examinations were performed to follow ultrasonographic changes in inoculated mares and to confirm the lack of changes in mares in the control group. Particularly, we assessed for changes that are highly indicative of placentitis such as areas of placental separation and fluid accumulation between the uterus and chorioallantois.

Blood Sampling and Determination of Serum Amyloid A and Haptoglobin

Blood samples were obtained immediately before inoculation/initial fetal fluid sampling (d=0), and then daily for 12 days or until abortion. Blood was attained by jugular venipuncture into heparinized 10-mL tubes. Immediately after collection, the blood was centrifuged at 600 xg for 10 min at 5° C. Plasma was harvested and preserved at -20° C until further analysis. Concentration of SAA was analyzed by a turbidometric immunoassay. The assay was developed for humans (LZ test SAA, Eiken Chemical® Tokyo, Japan) and has been validated for horses (Jacobsen *et al.* 2006, Belgrave *et al.*

2013). Concentrations of Hp were determined by the use of a commercial colorimetric assay (Phase™, Tri-Delta Diagnostics Inc., Boonton Township, NJ) (Cray & Belgrave 2014). Both SAA and Hp were analyzed at the Acute Phase Proteins Laboratory (Miller School of Medicine, University of Miami, Miami, FL, USA).

Determination of WBC and Fibrinogen

A subset of mares carrying normal pregnancies [(n=9), without (n=3) and with (n=6) FFS] and with experimentally induced placentitis [(n=8), without (n=5) and with (n=3) FFS] were examined to determine leukocyte counts and circulating fibrinogen. Blood was collected daily from inoculation until abortion or for 12 days for control mares. Fibrinogen was determined by comparing total plasma and serum protein. For WBC, an EDTA-anticoagulated blood sample was collected, and analyzed on an automated platform (Genesis, Oxford Science, Oxford CT, USA).

Placental and Fetal Pathology

Placentas from control and inoculated mares, and all aborted fetuses underwent complete pathological (macroscopic and histopathologic examination) and microbiological evaluation (Hong *et al.* 1993). Here briefly, the placenta was laid out and the chorioallantois, amnion, and umbilical cord were examined. Areas with no apparent lesions and areas presenting macroscopic lesions were sampled and preserved in buffered formalin for further histopathologic evaluation. Swabs were collected from the chorioallantois and used to perform aerobic bacterial culture (Hong *et al.* 1993). Fetuses were necropsied in routine fashion and internal organs (liver, spleen, stomach content, lung, heart, and trachea)

were macroscopically examined and sampled for histopathologic evaluations and aerobic bacterial culture (Hong *et al.* 1993).

Study II: Pre-Partum Plasma Concentrations of Serum Amyloid A and Haptoglobin in Mares Carrying Normal Pregnancies

To address the question of whether normal parturition is associated with an increase in plasma concentrations of SAA and Hp, healthy pregnant mares (n=7) were sampled daily beginning between 310 and 320 days of gestations through parturition. Jugular venipunctures were performed daily. Immediately after collection, blood was centrifuged at 600xg for 10 min, and the plasma was harvested and stored at -20° C until further analyses. Concentrations of SAA and Hp in plasma samples were analyzed as aforementioned for days -10, -8, -6, -4, -2, -1, and 0 (the day of parturition). All placentas underwent complete pathologic and microbiologic evaluations as in Study I.

Statistical Analyses

All the data analyses were carried out with JMP 10 (SAS Institute Cary NC). Changes in SAA, Hp and Fb concentrations as well as WBC were analyzed relative to days post-inoculation (DPI, 0 to 7 days) as well as relative to days from abortion (DFA, -7 days to 0) in separate mixed models. Mare was accounted as random effect and treatment; DPI (or DFA) were fixed effects. Fetal fluid sampling was nested within group. As we were interested to evaluate the acute changes in APP and WBC following inoculation, the data were analyzed for changes up to 7 DPI. On the other hand, as the interval from inoculation to abortion was variable, we were interested to examine the changes in the seven days prior to abortion. Two and three way interactions were also included in the different models for

DPI and DFA. Prior to analyses, the data were log transformed. Post-hoc comparisons across days were analyzed by Fisher's Least Significant Difference Test. In study II, changes in SAA and Hp for normal pre-foaling mares were analyzed by mixed model with mare as random effect and days pre-foaling as a fixed effect. Significance was set at $p < 0.05$, statistical tendency was defined as $0.05 \leq p < 0.10$. Data are presented as mean \pm S.E.M.

3.4. Results

Overall, mares with experimentally induced ascending placentitis aborted within 5-25 DPI with a mean of 9.4 ± 1.3 DPI. Only one mare in the control group (subgroup FFS) aborted 8 days after the last sampling; pathological and microbiological examination of the placenta and fetus revealed no lesions and culture yielded saprophytic non-pathogenic bacteria and negative virus isolation (data not shown); thus the cause of abortion was not determined. Three mares (17.6% of the mares that were inoculated) failed to develop placentitis after a single inoculation. One mare with experimentally induced placentitis (subgroup without FFS) did not develop placentitis; she delivered a normal foal and placenta with no pathological or microbiological evidence of placentitis. Two mares in the group with experimentally induced placentitis (one with and another without FFS) did not develop placentitis and were re-inoculated 14 days after the first inoculation. The mare without FFS aborted 20 days after re-inoculation and the other mare with FFS aborted 9 days after re-inoculation. All four of these mares were excluded from the analyses for DPI or DFA for APP or WBC; however, their SAA and Hp concentrations are presented in the supplement (appendices Fig. 3.1 to 3.4). One mare in the control group developed extensive cellulitis at the Viborg's triangle secondary to venipuncture, thus she was excluded from the analysis and her WBC, and APP concentrations are presented separately in the appendices (Fig. 3.5).

Streptococcus equi ssp. *zooepidemicus* was the single bacteria cultured from 12 fetuses and placentas from inoculated mares; however, in other 3 fetuses and placentas from inoculated mares, *Escherichia coli* was also cultured in association with the *Streptococcus equi* ssp. *zooepidemicus*. *Actinobacillus equuli* was isolated in association with *Streptococcus equi* ssp. *zooepidemicus* of one fetus and its placenta. All inoculated mares, but one mare, developed ultrasonographic changes consistent with ascending placentitis (i.e. placental separation, fluid accumulation between uterus and the chorioallantois) (data not shown). All mares in the control group presented normal ultrasonographic findings (data not shown). With the exception of one mare that failed to develop placentitis after experimental induction (noted above) and the control mare with FFS that aborted, all the remaining mares that aborted with experimentally induced ascending placentitis developed gross and histopathologic lesions consistent with ascending placentitis (i.e. neutrophilic infiltration in the intervillous spaces, necrosis of chorionic villi, infiltration of mononuclear inflammatory cells in the intervillous spaces, villous stroma, chorionic stroma, endothelium and allantois) (Hong *et al.* 1993) (data not shown). All mares in the control group delivered macroscopically and microscopically normal placentas. Bacterial culture yielded non-pathogenic saprophytic bacteria (data not shown).

All but two foals from mares with experimentally induced ascending placentitis were dead at delivery. One foal was spontaneously delivered alive and one foal was delivered upon induction of parturition because of sepsis in the mare. Both of these foals had clinical evidence of sepsis and were euthanized immediately post-delivery. *Streptococcus equi* ssp. *zooepidemicus* was isolated in all of their internal organs.

Unexpectedly, one mare with experimentally induced ascending placentitis developed systemic clinical signs of sepsis (hyperthermia (40°C, reference 37-38.8°C); hyperfibrinogenemia (900 mg/dL, reference 200-400 mg/dL); leukocytosis (15.1x10³ WBCs/μL, reference 7-12 x10³ WBCs/μL); dehydration (normal PCV, 42.4%, reference 30-44%, and slightly elevated total proteins 8 g/dL, reference 5.4-7.6 g/dL); neutrophilia (88%, reference 50-70%); lymphopenia (6%, reference 22-40%); and left shift (4%, reference 0-2%)) 14 days post inoculation. The mare received treatment for sepsis including intravenous fluids for approximately 3h and then parturition was induced with oxytocin continuous rate infusion (approximately 1 unit/minute). A live foal was delivered ≤20min of the treatment commence. Intravenous oxytocin treatment was continued and the placenta was delivered in ~30min (Canisso *et al.* 2013). The foal was euthanized immediately and submitted accompanied by its placenta for full pathologic and microbiologic evaluation as aforementioned.

SAA, Hp, Fb and WBC in Mares with Ascending Placentitis

Overall, there were significant effects of time and time x group interaction on concentrations of SAA based upon either DPI (p<0.0001) or DFA (p=0.01) (Fig. 3.1). Concentrations of SAA were significantly elevated from 2 DPI and from -6 DFA until abortion in inoculated mares compared to control mares (Fig. 3.1) (p<0.05). For Hp concentrations, there were significant effects of time and a time x group interaction, based upon DPI (p<0.0001) or DFA (p=0.001) (Fig. 3.2). Concentrations of Hp were elevated from 3 DPI and -3 DFA in inoculated mares compared to control mares (Fig. 3.2) (p<0.05). There was a significant interaction between FFS and group on the concentration of Hp (p<0.0001).

There were effects of DPI ($p=0.03$) and a group by DPI interaction ($P=0.02$) on WBC. However, there were no effects on WBC in the previous 7 DFA ($p=0.26$) and no interaction between group and WBC (DFA, $p=0.98$) (Fig. 3.3). On Fb analyses, there was an effect of time for DPI ($p=0.002$); however, there was no effect on DFA ($p=0.27$). There were no time interactions between group and DPI ($p=0.83$) or DFA ($p=0.61$) (Fig. 3.4) and between group and FFS ($p=0.65$). However, there were significant three way interactions among FFS, group and time ($p=0.02$).

Figure. 3.1 Serum amyloid A concentrations in mares with experimentally induced ascending placentitis and gestationally age-matched control mares carrying normal pregnancies. (a) 0 to 7 DPI; 0 = the day of inoculation or commence of sampling for mares in the control group. (b) -7 to 0 (i.e. day of abortion) DFA. There were no significant differences between mares in the control group and mares with placentitis (DPI $p=0.25$; DFA $p=0.06$). Concentrations of SAA were elevated from 2 DPI and -6 DFA ($p<0.05$). There were significant time and group interactions (DPI $p<0.0001$; and DFA $p=0.01$). *Within day denotes significant statistical difference $p<0.05$.

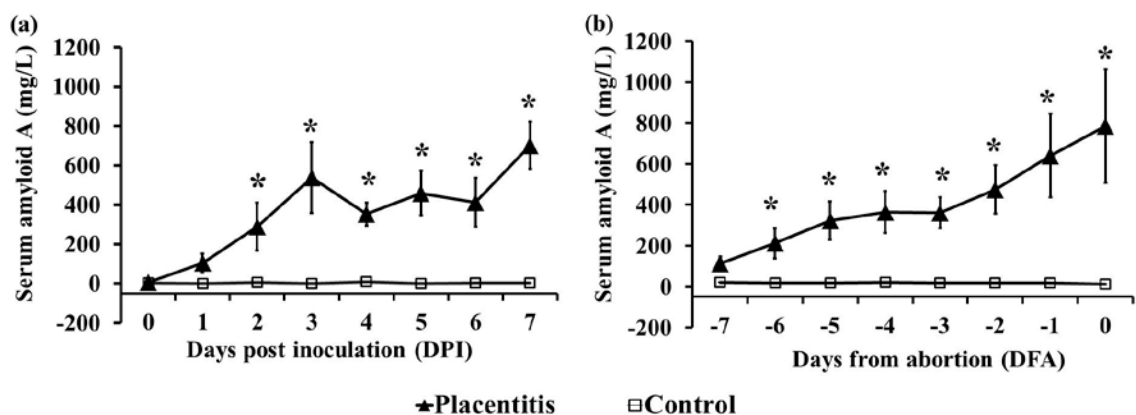


Figure. 3.2 Haptoglobin concentrations in mares with experimentally induced ascending placentitis and gestationally age-matched control mares carrying normal pregnancies. (a) 0 to 7 DPI; 0= the day of inoculation or commence of sampling for mares in the control group). (b) -7 to 0 (i.e. day of abortion) DFA. There were no significant differences between mares in the control group and mares with placentitis (DPI $p=0.61$; DFA $p=0.9$). Concentrations of Hp were elevated by 3 DPI and -3 DFA ($p<0.05$), and there were significant time x group interactions (DPI $p<0.0001$; and DFA $p=0.001$). *Within day denotes significant statistical difference $p<0.05$.

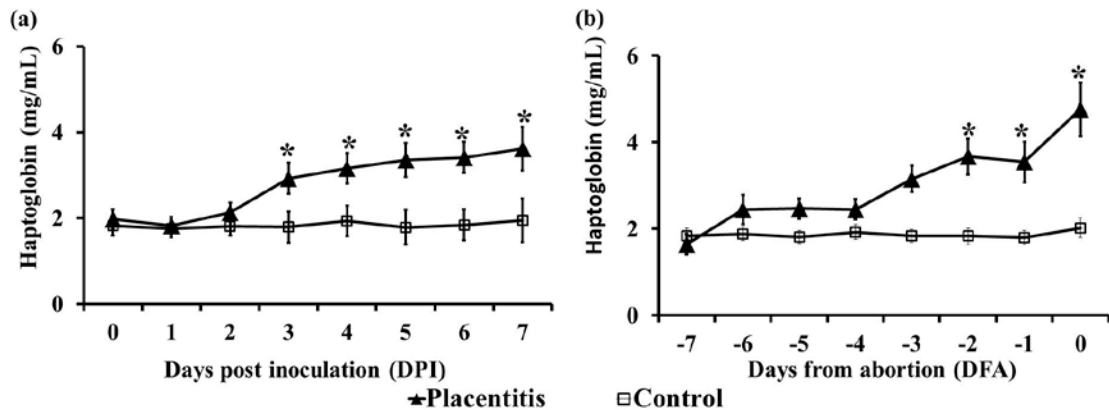


Figure 3.3 White blood cell counts (WBC) in mares with experimentally induced ascending placentitis and gestationally age-matched control mares carrying normal pregnancies. (a) 0 to 7 DPI; 0= the day of inoculation or commence of sampling for mares in the control group. (b) -7 to 0 (i.e. day of abortion) DFA. There were no significant differences between mares in the control group and mares with placentitis (DPI $p=0.76$; DFA 0.70). There were effects of DPI ($p=0.03$) and interaction between group and time (DPI, $p=0.02$). There were no effects on WBC in the previous 7 DFA ($p=0.26$) and no interaction between group and WBC (DFA, $p=0.98$). *Within day denotes significant statistical difference $p<0.05$.

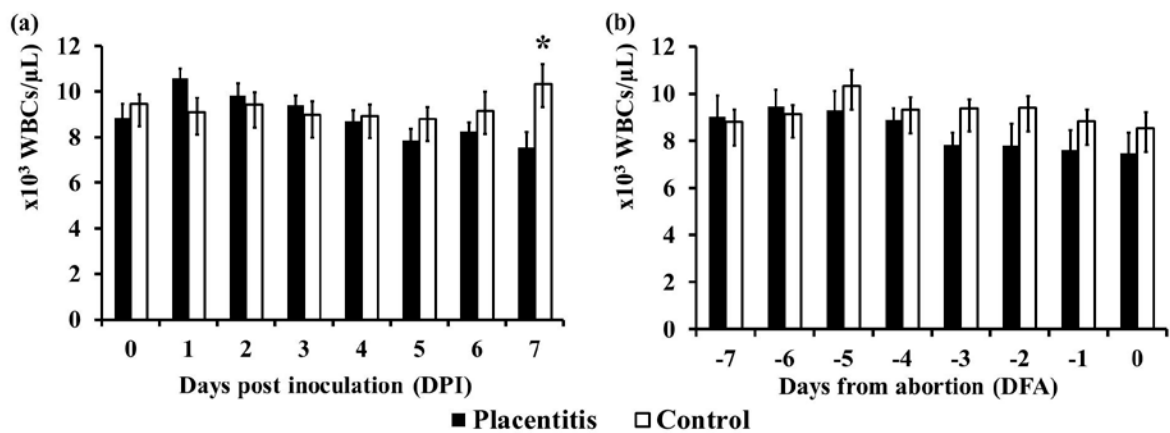
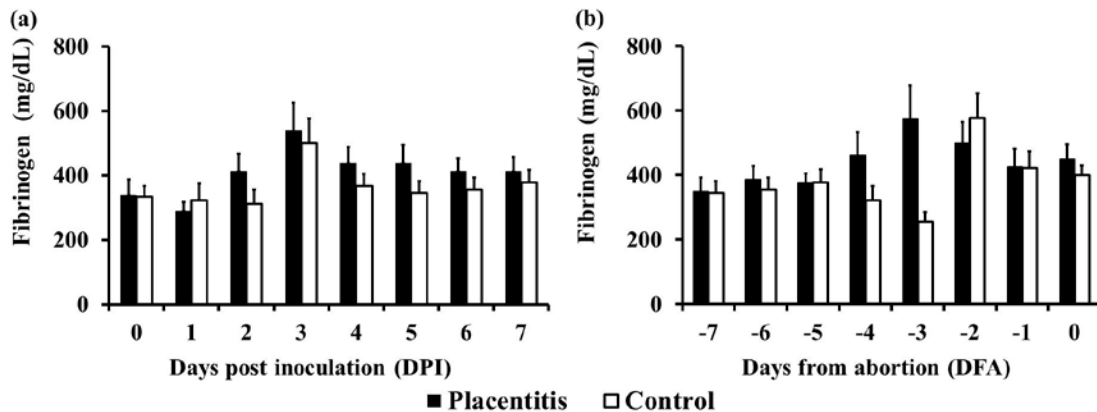


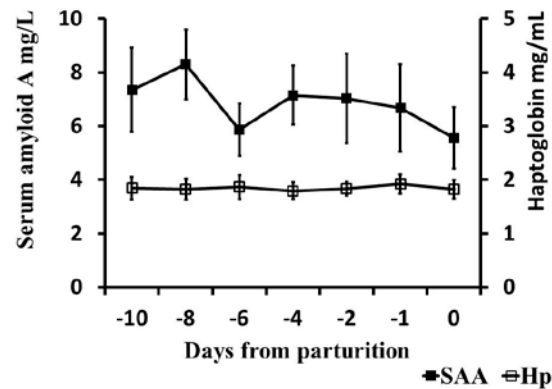
Figure 3.4: Fibrinogen concentrations in mares with experimentally induced ascending placentitis and gestationally age-matched control mares carrying normal pregnancies. (a) 0 to 7 DPI; 0= the day of inoculation or commence of sampling for mares in the control group. (b) -7 to 0 (i.e. day of abortion) DFA. There were no significant differences between mares in the control group and mares with placentitis (DPI, $p=0.87$; DFA= 0.81). There was effect of time for DPI ($p=0.002$); however, there was no effect on DFA ($p=0.27$). There were no time group interactions (DPI $p=0.83$; DFA $p=0.61$) or group and FFS ($p=0.65$). There were significant three way interactions among FFS, group and time ($p=0.02$).



Normal Parturition Does not Cause Elevation in Serum Amyloid A and Hp in Mares

There were no differences in SAA ($p=0.94$) and Hp ($p=0.98$) concentrations in the 10 days prior to parturition (Fig. 3.5).

Figure 3.5: Serum amyloid A (SAA, left axis) and haptoglobin (Hp, right axis) concentrations 10 days prepartum in seven mares carrying normal pregnancies undergoing to uneventful parturition. There were no significant differences across days for SAA ($p=0.94$) or Hp concentrations ($p=0.98$).



3.5. Discussion

After experimental induction of ascending placentitis, concentrations of SAA and Hp changed rapidly and markedly post inoculation. As a major and positive APP, it is expected that SAA will be rapidly elevated after the onset of inflammation (Jacobsen & Andersen 2007). Similar to other research models for diseases in mammals (Hulten *et al.* 2002, 2003, Dabrowski *et al.* 2007) concentrations of SAA were significantly elevated before detectable changes in Hp and several folds of magnitude in comparison to mares in the control group and before experimental induction of placentitis. A new aspect of our study supported by another recent report (Coutinho da Silva *et al.* 2013), is the fact that placentitis induces changes in APP (i.e. SAA and Hp) concentrations at the systemic

circulation. Until very recently, equine placentitis was considered a localized disease of the pregnant uterus with no systemic involvement. However, the finding that placentitis induces changes in APP (i.e. SAA and Hp) concentrations opens a new field of investigation on the inflammatory response in mares with placentitis. In humans, APP (e.g. C-reactive protein; CRP) have been focus of numerous publications involving infection of the placental and fetal tissues (Wu *et al.* 2009, Popowski *et al.* 2011, Lee *et al.* 2012, Ferrazzi *et al.* 2012). Changes in concentrations of CRP in the systemic circulation of women suffering with chorioamnionitis have been demonstrated by several studies (Wu *et al.* 2009, Popowski *et al.* 2011, Lee *et al.* 2012, Ferrazzi *et al.* 2012). In human medicine, CRP is the APP more commonly used to assess risk for premature labor (Smith *et al.* 2012).

Inflammatory APP are produced by the liver in all mammals studied to date (Urieli-Shoval *et al.* 1998, Jacobsen & Andersen 2007, Chrisman *et al.* 2008, Berg *et al.* 2011, Leschi *et al.* 2012). However, transcripts for SAA and Hp were also described in several other normal tissues in cattle and horses (Berg *et al.* 2011) as well as humans (Urieli-Shoval *et al.* 1998). In the horse, the endometrium presented moderate expression (i.e. defined by the authors as >1% of the liver expression) in normal non-pregnant mares (Berg *et al.* 2011). Interestingly, in experimentally induced endometritis using a large inoculum of *E. coli* (1 billion cfu) in non-pregnant mares (Christoffersen *et al.* 2010), the animals presented a systemic inflammatory response (i.e. fever, elevated systemic SAA) and increased endometrial expression of SAA by 3-12h post inoculation. This study suggested upregulation of SAA in the endometrium could result in measurable elevation of systemic SAA in mares. In a more recent study by the same authors (Christoffersen *et al.* 2012), a smaller inoculating dose of *E. coli* (10^5 cfu) was not associated with systemic signs of

endotoxemia or increases in plasma SAA concentrations; however endometrial expression of SAA was 10-100 fold higher compared to pre-inoculation expression of SAA. Therefore, our findings along with those of Coutinho da Silva *et al.* (2013) raise a question on the origin of SAA and perhaps Hp in mares with placental inflammation. It appears likely that the liver may not be the sole source of APP in these mares, but that other tissues such as endometrium may contribute to the systemic elevation of SAA and Hp in mares with experimentally induced placentitis.

On a comparative basis, it is worth noting that the epitheliochorial placenta of equids allows limited exchange of large molecules between maternal and fetal circulation in comparison to the hemochorial placenta of humans. Thus the structural and morphofunctional differences between fetal membranes of mares and women may explain why infection of the maternal-fetal unit can be life threatening in women (Fahey 2008, Tita 2010) but is rarely so in mares where the disease is restricted to the gravid uterus.

As observed here and supported in other studies using this experimental model, only a single bacterial specie or less commonly two species of bacteria are isolated in clinical cases or experimental cases of ascending placentitis (Hong *et al.* 1993). In the present report, *Streptococcus equi* ssp. *zooepidemicus* was the single isolate in 80% of the experimental mares; however, in the remaining mares, a second bacterial specie was also isolated (i.e. *Escherichia coli* and *Actinobacillus equuli*). However, in the human clinical condition known as chorioamnionitis/funisitis, mixed infection with enteric bacteria appears to be a more common clinical presentation (Hillier *et al.* 1991, Sherman *et al.* 1997).

In the study by Coutinho da Silva and collaborators (2013), concentration of SAA was elevated by four days post experimental induction in all five mares that were experimentally induced with ascending placentitis and did not receive treatment after onset of clinical signs. This is two days later than the present report in which SAA was already significantly elevated from 2 DPI and remained elevated until abortion. Coutinho da Silva *et al.* (2013) used a different immunoassay method to determine the concentrations of SAA, used a different bacterial isolate of *Streptococcus equi* ssp *zooepidemicus* and their inoculating dose was remarkably higher than the one used in our study (10 million cfu versus 5 million). Perhaps a higher inoculating dose may explain why mares in the group with experimentally induced ascending placentitis that did not receive treatment for placentitis aborted ~2 days (Coutinho da Silva *et al.* 2013) earlier than the average in the present report (~9DPI).

Both Hp and Fb are classified as moderate APP in the horse (i.e. constantly present in the blood, and upon inflammation present only a moderate elevation of 1 to 10 times baseline), thus it is expected that upon inflammatory insult both molecules will be elevated from two to five days post insult in the horse (Jacobsen & Andersen 2006, Cray & Belgrave 2014). However in the present study Fb was not elevated in mares with experimentally induced ascending placentitis but there was an effect of time. Interesting, total WBC was higher in control mares at 7 DPI. While we cannot explain the origin of this higher concentration of WBC, upon normal foaling no placentae from mares in the control group presented evidence of inflammation or other changes that suggest placental lesion. In addition, the total WBC in control mares remained within normal ranges (reference 7-12 x10³ WBCs/ μ L). However, from the sampling period (~280 days of gestation) to normal

parturition (~340 days of gestation) in the present report corresponded approximately to a 2-months period, so changes induced by the sampling could have disappeared. Additionally, the origin of the inflammation could have been the body wall or uterine wall. Interesting that SAA, a very sensitive marker for inflammation, was not elevated in the control group. This is interesting because FFS had no effect on SAA concentrations, and this APP is known to be more sensitive marker for inflammation than Fb and WBC in the horse (Belgrave *et al.* 2013). We did not expect Fb or WBC to be elevated in mares with experimentally induced ascending placentitis, based on our experiences with spontaneously occurring cases. However, we believe that a controlled study was warranted to confirm clinical observations.

Parturition did not trigger an elevation in SAA or Hp in mares carrying normal pregnancies and delivering normal foals. A prior report (Coutinho *et al.* 2013) suggested that parturition induced changes in serum concentrations of SAA in mares carrying normal pregnancies and delivering normal foals. These authors (Coutinho *et al.* 2013) used a different assay to measure SAA and the mares were sampled in weekly intervals than the more frequent protocol used here. Our findings support the hypothesis that increases in SAA and Hp are not associated with parturition, but with placentitis as evidenced by the lack of effect of normal parturition.

In conclusion, SAA and Hp rapidly increase in mares subsequent to experimental induction of ascending placentitis and remain elevated until abortion with SAA increasing earlier than Hp. Neither Fb nor WBC change in mares with placentitis, therefore are not markers for placentitis. There were no detectable changes in the concentrations of SAA and Hp in normal mares in the immediate prepartum period. The findings of the present

study open a new field of investigation. Elevations of SAA and Hp in mares with experimentally induced ascending placentitis suggest that these two APP might be useful, but nonspecific, biomarkers for mares with spontaneously occurring placentitis, thus this should be tested in prospective controlled designed studies with field cases. It appears likely that SAA and Hp will be useful markers to diagnose field cases of placentitis in mares; however, these two APP should be tested prospectively to confirm whether these APP are useful biomarkers in spontaneous clinical cases.

3.6. Acknowledgments

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Chapter 4
Experimentally-Induced Ascending Placentitis Does not Change Peripheral
Androgen Concentrations in Mares

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4.1. Summary

Reasons for performing study: Currently, the diagnosis of ascending placentitis is based on clinical signs and transrectal ultrasonography; while advanced stages of placentitis can be easily diagnosed, early stages can be missed. Thus additional tools to diagnose placentitis are necessary.

Objectives: To characterize peripheral dehydroepiandrosterone sulphate (DHEA-S) and testosterone concentrations in mares carrying normal pregnancies (Study I) and to compare plasma concentrations of DHEA-S and testosterone in mares with placentitis and gestationally age-matched control mares (Study II).

Methods: In study I, mares (n=18) had serum samples collected every two weeks from 100d of gestation to term. In study II, pregnant mares (260-280d of gestation) were assigned to a control group (n=17) and to a group with experimentally induced placentitis (n=17). Within each group, a subset (n=7) of mares had fetal fluid sampling (FFS) performed at 0, 5, 12 days post inoculation (DPI) or until abortion. Placentitis was induced via intracervical inoculation of *Streptococcus equi* ssp *zooepidemicus*. Blood was collected

at inoculation/commence for control mares (day=0) and daily for 12 DPI or until abortion. Concentrations of DHEA-S and testosterone were determined by ELISA.

Results: In study I, androgen concentrations increased during pregnancy with concentrations of DHEA-S peaking by 180 days of gestation. Testosterone concentrations were progressively elevated from days 100 to 180 with a plateau until ~240d and a progressive decline until 290d of gestation. In study II, mares with experimentally induced placentitis aborted within 5-25 DPI (mean 9.4 ± 1.3 days). Concentrations of DHEA-S and testosterone were not significantly different between groups, except DHEA-S was significantly reduced on the day of abortion for mares in the placentitis group (subset having FFS).

Conclusions: Concentrations of DHEA-S and testosterone were elevated and varied through pregnancy; however, neither testosterone nor DHEA-S concentrations in the pregnant mares were predictive of impending abortion in mares with experimentally induced ascending placentitis.

Keywords: pregnancy, testosterone, dehydroepiandrosterone, placentitis, abortion, equine

4.2. Introduction

Placentitis has been identified as the major cause of late-term pregnancy loss (i.e. abortions, stillbirths and neonatal deaths) in broodmares in central Kentucky (Giles *et al.* 1993, Hong *et al.* 1993, Williams *et al.* 2004, Troedsson & Zent 2004). Outside North America, placentitis has also been reported to be an important cause of pregnancy wastage in France (Laugier *et al.* 2011) and Australia (Carrick *et al.* 2012). However, in the United Kingdom umbilical cord pathology (e.g. torsion and entrapments) was recognized as the leading cause of pregnancy loss in submissions received by a referral laboratory (Smith *et al.* 2003). Differences in incidences across countries can be explained by surveillance systems, methodology to perform the pathologic examination, and true incidence of infectious agents.

Ascending placentitis is commonly associated with bacteria and less commonly with fungi; infrequently, mixed bacterial fungal infections have also been observed (Hong *et al.* 1993). Common bacterial isolates associated with the occurrence of ascending placentitis are similar to ordinary isolates in endometritis and include *Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli*, *Streptococcus equisimilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, whereby beta-hemolytic streptococci predominate (Giles *et al.* 1993, Hong *et al.* 1993, Williams *et al.* 2004).

Currently, ascending placentitis is diagnosed based on clinical signs (i.e. vulvar discharge, premature udder development and lactation) and transrectal ultrasonography (Renaudin *et al.* 1997, LeBlanc 2010, Troedsson & Macpherson 2011). While transrectal ultrasound of the caudal placental pole has been shown to be a useful tool to diagnose and manage clinical cases of ascending placentitis (Troedsson & Zent 2004) the technique is prone to false positive diagnoses and early stages of the disease can be missed. Additional

diagnostic tools for placentitis have the potential to improve the identification of early stages of the diseases and may therefore improve the likelihood to deliver a viable foal as well as help to avoid unnecessary treatment of normal pregnant mares.

During pregnancy several estrogens, progestins and androgens are produced in high concentrations by the equine fetoplacental unit and are released into the maternal circulation (Cox 1975, Raeside *et al.* 1979, Pashen & Allen 1979, Raeside 2004, Ousey 2011) In particular, the hypertrophied fetal gonads of both male and female fetuses secrete large quantities of androgens, particularly dehydroepiandrosterone (DHEA, 3 β -hydroxyandrost-5-en-17-one also known as 5-androsten-3 β -ol-17-one) and 7-dehydro DHEA (3 β -hydroxy-5,7-androstadien-17-one) (Raeside *et al.* 1997).

Dehydroepiandrosterone serves as a precursor for classic phenolic estrogens (i.e. estrone, estradiol-17 β estradiol-17 α , and their sulfoconjugates) and 7-dehydro-DHEA serves as a precursor for ring B unsaturated estrogens (i.e. equilin, equilenin and their hydroxyderivatives 17 α -dihydroequilin, 17 α -dihydroequilenin, 17 β -dihydroequilin, and 17 β -dihydroequilenin) by the equine placenta (Raeside *et al.* 1979, Pashen & Allen, 1979, Bhavnani 1988, Raeside 2004, Ousey 2011). Normally, both precursors are rapidly aromatized by the placenta into estrogens; however, some DHEA, particularly the sulfoconjugate (DHEA-S) is present in high concentrations in maternal circulation during pregnancy in mares (Meinecke & Gips 1990). In addition to DHEA-S, circulating testosterone is elevated in plasma of pregnant mares (Silberzahn *et al.* 1984, Daels *et al.* 1996). During early pregnancy, the corpora lutea have been shown to be an important source of testosterone (Ginther 1992); however, late in pregnancy this androgen is probable

synthesized from DHEA (through androstenedione as an intermediate) and released into the maternal circulation (Bhavnani 1988, Raeside *et al.* 1997, Ousey 2011).

The fetal gonads produce DHEA and 7-dehydro DHEA from cholesterol and from farnesyl pyrophosphate, respectively (Bhavnani 1988, Tait *et al.* 1985, Raeside 2004). The sulfoconjugated form of DHEA predominates in the blood of pregnant mares blood (6:1), (Meinecke & Gips 1990) however, in the newborn foal the non-conjugated form predominates (i.e. DHEA) (Raeside *et al.* 1997) Based on immunolabeling and labelled precursors studies, DHEA is converted into androstenedione by 3 β -hydroxysteroid dehydrogenase, and then androstenedione can be converted into estrone by aromatase or into testosterone by 17- β hydroxysteroid dehydrogenase (17- β -HSD) (Bhavnani 1988, Henderson *et al.* 1984, Chavatte *et al.* 1995, Han *et al.* 1995, Ousey 2011). Estrone can be converted into estradiol 17- β by 17- β HSD in the chorioallantois (Ousey 2011). In the mare, it is thought that estrone sulfate can be converted into estradiol 17 β sulfate by 17- β HSD in the endometrium (Ousey 2011).

Controlled studies and field investigations have reported that estrogens change in mares experiencing pregnancy loss (Kashamn *et al.* 1988, Douglas 2004). However, there are no reports on changes in DHEA-S or testosterone in mares suffering placental disease. Since mares experiencing placentitis, both spontaneous (Douglas 2004) and experimentally induced (unpublished observations), present remarkable reduction in estrogens concentrations, and androgens are products of the fetoplacental unit used as substrate to produce estrogens, we hypothesize that placentitis would result in increased concentrations of androgens in the maternal plasma. Therefore, the objectives of this study were: (i) to describe DHEA-S and testosterone concentrations in mares carrying normal pregnancies;

(ii) to compare plasma concentrations of DHEA-S and testosterone in mares with experimentally induced placentitis to those in control mares.

4.3. Material and Methods

Mares were maintained at the Maine Chance Farm University of Kentucky. All the experimental protocols were approved by the Institutional Animal Care and Use committee at the University of Kentucky (Project #2010-0769). All the animals were kept in paddocks and supplemented with hay, grain and water *ad libitum* along with trace minerals. In study I, mares were kept in pastures during the entire experiment and foaling was not attended. In study II, immediately pre-abortion or prior to normal parturition; the mares were kept in individual stalls (16 x 16 feet) during the night and turned out in pasture during the day.

Study I: Concentrations of DHEA-S and testosterone in mares carrying normal pregnancies

Animals

Eighteen light-breed mares were enrolled in this study. The mares were bred with fresh extended semen during the breeding season of the northern hemisphere. Ovulation was determined by transrectal ultrasonography. Postpartum placentas and foals were examined to assure normality.

Blood Sampling and Immunoassays

Blood samples were collected every two weeks from 100 days of gestation to term. This gestational age was chosen because that is the suggested time that the fetal-placental unit starts to be the predominant steroidogenic organ (Ousey 2011). Testosterone is elevated starting at 40 to 50 days of pregnancy, and this elevation is mainly associated with

the ovarian production of testosterone (Ginther 1992, Daels *et al.* 1996). Blood was collected by jugular venipuncture, allowed to clot, centrifuged at 600 xg for 10 min and then serum was harvested and preserved at -20 °C. Determination of DHEA-S and testosterone were achieved with specific equine immunoassays.

Analysis of testosterone was performed in triplicate using a testosterone antibody (R156/7 UC-Davis) and testosterone conjugated with horseradish peroxidase and testosterone. The standard curve ranged from 0.02 ng/mL to 10 ng/mL, with a limit of detection of 0.021 ng/mL (CV's; intrassay 9.7% and interassay 17%). The testosterone antisera has a high cross-reactivity with only one testosterone metabolite 5 α dihydrotestosterone (57%) and low cross reactivity with androstenedione (0.3%) (Thompson *et al.* 2008). The testosterone immunoassay has been previously validated against a radio immunoassay (Coat-a-count total testosterone radioimmunoassay; PITKTT-8), and chemiluminescence immunoassay (Immulite total testosterone; PILKTTW-12) (unpublished observations).

Determinations of DHEA-S concentrations were performed in duplicate using a DHEA-S antibody (Schwarzenberger *et al.* 1993) and DHEA-S conjugated with 3-horseradish peroxidase. The standard curve ranged from 0.05 ng/mL to 20 ng/ml, with a limit of detection of 0.0315 ng/ml (CV's; intra-assay 6.5% and inter-assay 10.3%). Cross-reactivity for the DHEA-S antisera has been previous reported (Schwarzenberger *et al.* 1993). Here briefly, DHEA 100%, 5-androsten-3 β -ol-17-one 38.2%, 5-androstene-3 β , 17 β -diol 0.14%, 5-androstene -3,17-dione 6.6%, 4-androstene-3, 17-dione 0.3%, testosterone <0.1% β , epiandrosterone 8.8%, androsterone 3.2%, 5 α -diol 1.6%, estrone <0.1%. The DHEA-S antiserum was raised in sheep against 3- β -hydroxy-5-androsten-17-

one-3 hemisuccinate/BSA. The antiserum was kindly provided by Dr James Raeside and has been previously reported for immunoassay of DHEA-S (Raeside *et al.* 1997, Schwarzenberger *et al.* 1993). The dilution of antiserum was prepared at 1:9000 in sodium bicarbonate solution (0.05 M, pH 9.6). Standard solutions were prepared with DHEA-S at the concentrations 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 ng/ml. Plates were incubated for two hours at room temperature, before being washed with an automated plate washer. Substrate solution containing citric acid (0.05 M), ABTS (40mM; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), and H₂O₂ (0.5 mM) was added to the plates; thereafter, plates were incubated at room temperature for two hours. Stop solution (i.e. 0.15 M hydrofluoric acid, 0.006 M NaOH, 1.0 M EDTA) was added at the end of the incubation, and reading was carried out by the use of an ELISA microplate reader.

Study II: Concentrations of DHEA-S and testosterone in mares with experimentally induced placentitis

Experimental Design

Mares of light-breed types carrying normal pregnancies (260-280 days of gestation) were assigned to (1) a control group with (n= 17) and (2) a group with experimentally induced ascending placentitis (n =17). As part of another study, within each group, 7 mares had transabdominal ultrasound guided fetal fluid sampling at 0, 5 and 12 days post inoculation (DPI) or until abortion. Similar methodology has been published elsewhere (Canisso *et al.* 2014). Here briefly, the mare was sedated, placed in stocks and fetal fluids samples were collected with transabdominal ultrasound and an echotip spinal needle (18Gx6"; 30° short bevel, Chiba-Type spinal needle, couple with a stylet).

Experimental Induction of Ascending Placentitis

Ascending placentitis was induced via intracervical inoculation of *Streptococcus equi* ssp. *zooepidemicus*. The experimental model for induction of bacterial placentitis in the mare was modified from that previously described (LeBlanc *et al.* 2002). This protocol has been successfully used by different laboratories as a reliable and predictable model to induce ascending bacterial placentitis in the mare (Morris *et al.* 2007, Christensen *et al.* 2010, Coutinho da Silva *et al.* 2013). Sham inoculation of control mares was not performed in the present study as this procedure may induce placentitis in an unpredictable manner; as noted by previous publications (Christensen *et al.* 2010, Macpherson *et al.* 2013, Coutinho da Silva *et al.* 2013) The bacterial inoculum was deposited midway in the cervical lumen with a semi-flexible artificial insemination pipette. The inoculum was contained in a 0.5-mL straw and was deposited with the use of a stylet. The bacterial strain used was isolated from the placenta from a mare diagnosed with spontaneously ascending placentitis by the University of Kentucky Veterinary Diagnostic Laboratory. The bacterial isolate was preserved in cryovials containing skim milk at -80°C until use. Prior to inoculation, bacterial cultures were prepared and bacterial counts performed in standard fashion using blood Agar plates (incubated at 37°C), serial dilution and plating were used to determine the number of colony-forming units. The bacterial inoculum (5 million cfu) was suspended in 0.5 ml of phosphate-buffered saline. Once inoculation was carried out, a swab was obtained from the leftover in the cryovial and cultured in similar conditions to assure that the bacteria inoculum was still viable.

Blood Sampling and Immunoassays

Blood samples were obtained immediately before inoculation/initial fetal fluid sampling (d=0), and then daily for 12 days DPI or until abortion. Blood was attained by jugular venipuncture into heparinized 10-mL tubes. Immediately after collection, the blood was centrifuged at 600 xg for 10 min at 5° C. Plasma was harvested and preserved at -20° C until further analysis for DHEA-S and testosterone as described in study I.

Placental and Fetal Pathology

All aborted fetuses and placentas from inoculated mares and control underwent pathologic and microbiological examination as previously described (Hong *et al.* 1993) at the University of Kentucky Veterinary Diagnostic Laboratory. Here briefly, the placenta was laid out and the chorioallantois, amnion, and umbilical cord were examined. Areas with no apparent lesions and areas presenting macroscopic lesions were sampled and preserved in buffered formalin for further histopathological evaluation. Swabs were collected from the chorioallantois and used to perform aerobic bacterial culture (Hong *et al.* 1993). Fetuses were necropsied in routine fashion and internal organs (liver, spleen, stomach content, lung, heart, and trachea) were macroscopically examined and sampled for histopathologic evaluations and aerobic bacterial culture (Hong *et al.* 1993).

Statistical Analyses

All analyses were performed using JMP software. In study I, data were log-transformed and analysed with a mixed model (mare as random effect and time as a fixed effect). Post hoc comparisons were made by Fisher's protected least significant difference (day effect) test. In study II, data were log-transformed and analysed with a mixed model.

Because time of abortion varied from 5 to 25 days, changes in DHEA-S and testosterone concentrations were analysed relative to days from abortion (DFA, -8 days to 0). Mare was accounted as random effect and group and time (i.e. DFA) were fixed effects. Fetal fluid sampling was nested within group. Two way interactions were also included in the different models. Significance was set at $p < 0.05$. Data are presented as mean \pm S.E.M.

4.4. Results

Study I

All mares had normal gestation lengths (mean 348 ± 2.8 days; range: 328-362 days) and delivered normal placentas. Sixteen mares delivered live healthy foals; two mares had dystocias and delivered upon obstetrical interventions; one at 328 and another at 339 days of gestation. Pathological examination of the fetal membranes and foals concluded that dystocia was the cause of the intra-partum death. There was significant day-effect for both androgens ($p < 0.0001$) (Fig. 4.1 & 4.2). Concentrations of DHEA-S peaked by six months of gestation ($p < 0.05$) (Fig. 4.1). However, testosterone was progressively elevated from days 100 to 180, and then remained elevated until ~ 240 days of gestation; thereafter a progressive decline was observed towards 290 days of gestation. Concentrations of testosterone remained constant between ~ 290 days and term ($p > 0.05$) (Fig. 4.2).

Figure. 4.1: Dehydroepiandrosterone sulfate (DHEA-S) concentrations in eighteen light-breed mares carrying normal pregnancies from ~100 days of gestation to term. There were significant differences across sampling days ($p < 0.0001$), with a peak between 175 and 189 days of gestation ($p < 0.05$).

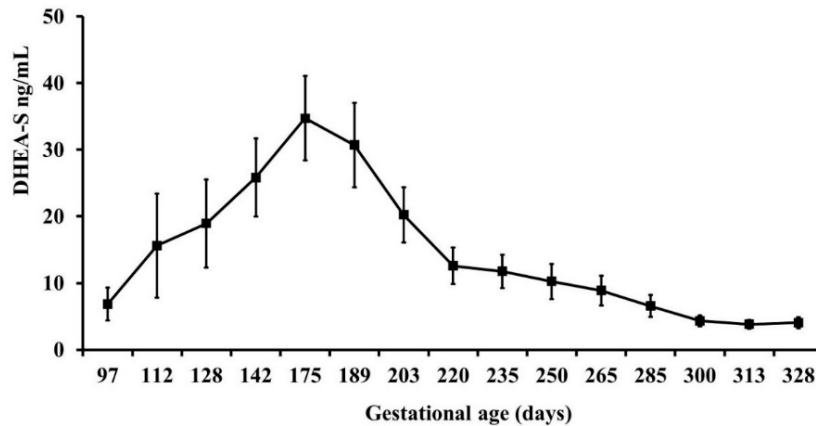
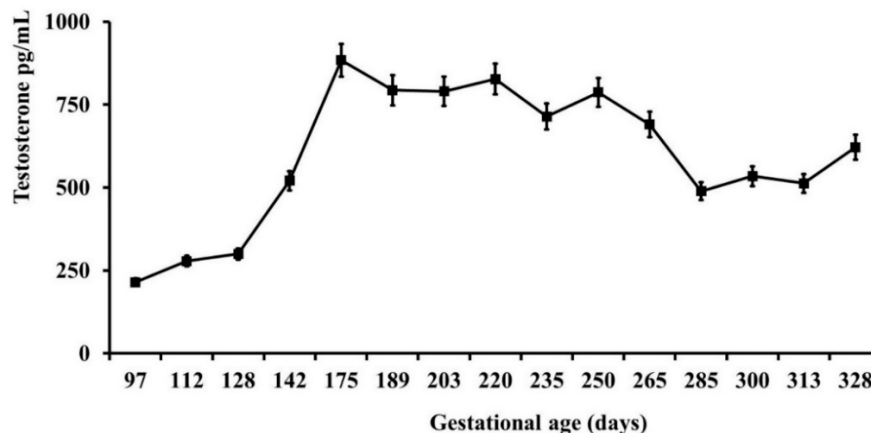


Figure 4.2: Testosterone concentrations in eighteen light-breed mares carrying normal pregnancies from 100 days of gestation to term. There were significant differences across sampling days ($p < 0.0001$). Under the conditions of the present study, testosterone did not present a distinct peak. However, testosterone was progressively elevated from days 100 to 180, and then remained plateaued until ~240 days of gestation; thereafter a progressive decline was observed towards 290 days of gestation.



Study II

The mean time from inoculation to abortion was 9.4 ± 1.3 days (range: 5-25 DPI) for mares with experimentally induced ascending placentitis. One control mare in subgroup FFS aborted 8 days after the last sampling; pathological and microbiological examination of the placenta and fetus revealed no lesions and culture yielded saprophytic non-pathogenic bacteria (Table 4.1) and negative virus isolation (data not shown); thus the cause of abortion was not determined. One mare in the placentitis group (subgroup without FFS) failed to develop placentitis after experimental inoculation; she delivered a normal foal and placenta with no microbiological (Table 4.1) or pathological evidence of placentitis. These two mares were excluded from the analyses. Two mares in the group with experimentally induced placentitis (one with and another without FFS) did not develop placentitis and were re-inoculated 14 days after the first inoculation. Results from 14 days following the first inoculation were excluded from the analyses for DHEA-S or testosterone. The re-inoculated mare without FFS aborted at 20 DPI and the other re-inoculated mare with FFS aborted 9 DPI.

As expected, *Streptococcus equi* ssp. *zooepidemicus* was the most prevalent isolate from placentas and fetuses from inoculated mares, however, in other fetuses and placentas from inoculated mares, *Escherichia coli* (n=3) and *Actinobacillus equuli* (n=1) were also cultured in association with the *Streptococcus equi* ssp. *Zooepidemicus* (Table 4.1). Except for one mare that failed to develop placentitis after experimental inoculation (noted above) and the control mare with FFS that aborted, all the remaining mares that aborted with experimentally induced ascending placentitis developed gross and histopathologic lesions consistent with ascending placentitis (i.e. neutrophilic infiltration in the intervillous spaces,

necrosis of chorionic villi, infiltration of mononuclear inflammatory cells in the intervillous spaces, villous stroma, chorionic stroma, endothelium and allantois) (Hong *et al.* 1993) (data not shown). All mares in the control group delivered macroscopically and microscopically normal placentas. Bacterial culture yielded non-pathogenic saprophytic bacteria (Table 4.1). All but two fetuses from the experimentally induced ascending placentitis group were dead at delivery. These two foals delivered alive (one spontaneously and one upon induced parturition), had clinical evidence of sepsis and were euthanized immediately post-delivery. *Streptococcus equi* ssp. *zooepidemicus* was isolated in all of their internal organs.

Analyses with the full mixed model for the concentrations of DHEA-S showed no differences between groups ($p=0.88$), or effects of time (DFA $p<0.0001$), but there were interactions between groups and DFA ($p<0.0001$) and between FFS and DFA ($p=0.0001$). Therefore, the data were re-analyzed in separated mixed models (with mare as random effect, group and DFA as fixed effects), for mares having FFS and mares without FFS. Comparisons between groups were performed as aforementioned. Concentrations of DHEA-S in mares without FFS, revealed no differences between groups ($p=0.52$), or effects of time (DFA $p=0.1$) or interactions between group and time (Group*DFA $p=0.17$) (Figure 4.3). However, for the subset of mares having FFS, there were no differences between groups ($p=0.57$), but there were effects of time (DFA $p<0.0001$), and time by group interaction (DFA*group $p<0.001$). Post hoc comparisons showed that the day of abortion was significantly different in mares with experimentally induced ascending placentitis ($p<0.05$) (Figure 4.3).

For the testosterone concentrations, there were no significant differences between mares in the control group and mares with placentitis (DFA $p=0.15$) (Fig. 4). There were no effects on time (DFA $p=0.07$) or interactions between group and time (DFA $p=0.43$) (Fig. 4.4).

Figure 4.3 Dehydroepiandrosterone sulfate (DHEA-S) concentrations in mares with experimentally induced ascending placentitis (n=17) and gestationally age-matched control mares carrying normal pregnancies (n=17). Days from abortion (DFA), -8 to 0 (i.e. day of abortion) DFA. Within groups, a subset of 7 mares had fetal fluid sampling (FFS) performed at 0, 5 and 12 days post inoculation or until abortion. (A) Represents mares from both groups without FFS. There were no groups differences ($p=0.52$), effects of (DFA $p=0.10$) or interactions between group and time (group*DFA $p=0.17$). (B) Represents the subset of mares from both groups having FFS. There were no group differences ($p=0.57$). However, there were effects of time (DFA $p<0.0001$), and interaction between time and group (group*DFA $p<0.001$). As denoted by the letters on the figure above, the day of abortion was significantly different than the remaining sampling days ($p<0.05$).

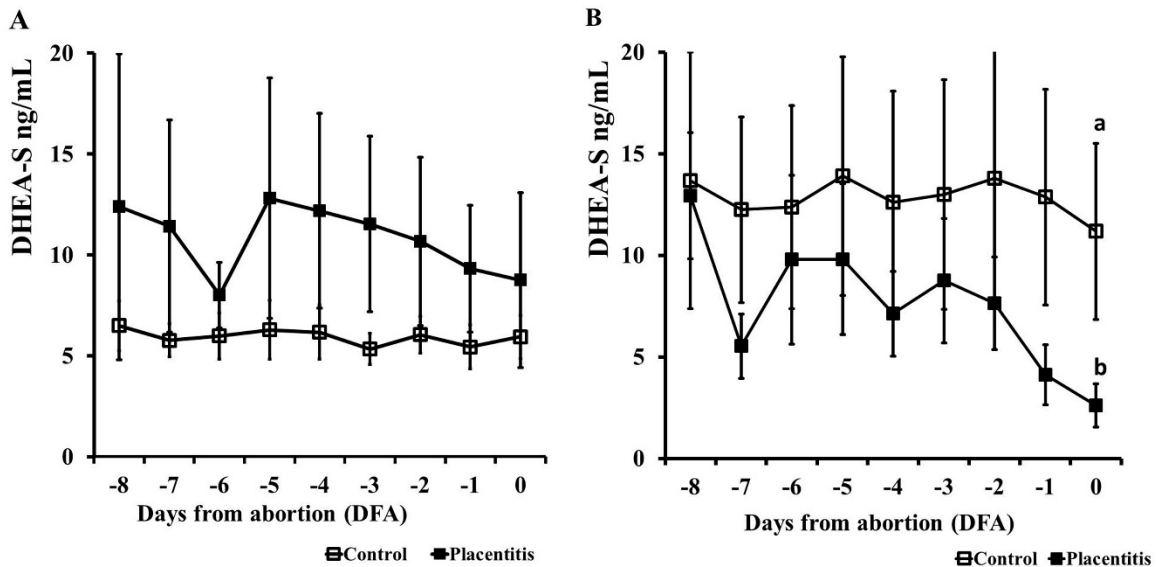


Figure. 4.4 Testosterone concentrations in mares with experimentally induced ascending placentitis (n=17) and gestationally age-matched control mares carrying normal pregnancies (n=17). Days from abortion (DFA), -8 to 0 (i.e. day of abortion) DFA. There were no significant differences between mares in the control group and mares with placentitis (DFA p=0.15). There were no effects of fetal fluid sampling (FFS p=0.64), or effects time (DFA p=0.07) or interactions between group and time (DFA p=0.43) and group and time and FFS (p<0.99).

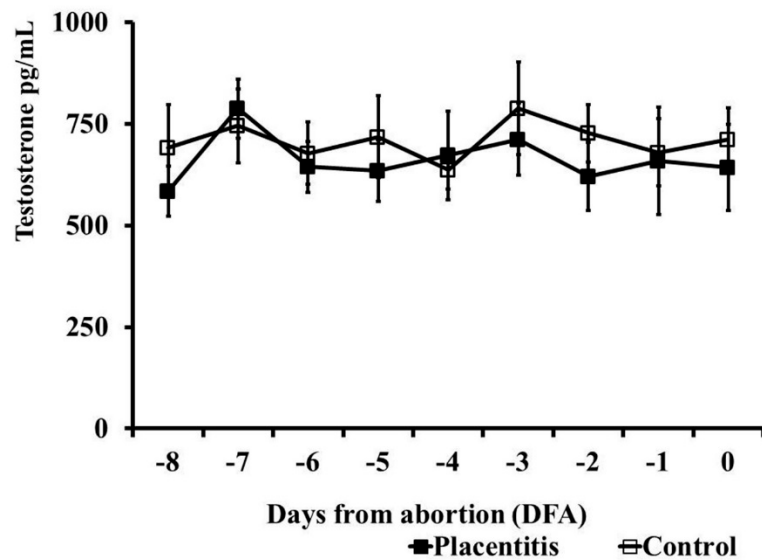


Table 4.1: Bacterial culture results from aborted fetuses and placentas from mares in the control group and mares with experimentally induced ascending placentitis.

Organism	Number
<i>S. equi</i> ssp <i>zooepidemicus</i>	12
<i>S. equi</i> ssp <i>zooepidemicus</i> & <i>E.coli</i>	3
<i>S. equi</i> ssp <i>zooepidemicus</i> & <i>Actinobacillus equuli</i>	1
<i>Non-pathogenic bacteria</i> *	18

*This represents all mares in the control group and one mare that did not develop placentitis after inoculation and that was not re-inoculated.

4.5. Discussion

Because both DHEA-S and testosterone are products of the fetal placental unit, we hypothesized that disruption of placental function resulting from experimentally induced ascending placentitis would affect the ability of these tissues to metabolize the precursor (DHEA) to estrogens and testosterone. However, contrary to our hypothesis, concentrations of both androgens examined herein were not different between groups after experimental induction of ascending placentitis in mares. Therefore, our findings in mares with experimentally induced ascending placentitis suggest that neither androgen can be used as a diagnostic marker for spontaneous placentitis; however this has not been critically tested.

The experimental model used to induce ascending placentitis differs from spontaneous clinical cases of ascending placentitis, as spontaneous cases tend to be more insidious and chronic in comparison with the experimental model, which induces a more

acute placentitis. However, we used a smaller inoculating dose (5 million vs. ~10million cfu) compared to previous studies (Leblanc *et al.* 2002, Christensen *et al.* 2010, Bailey *et al.* 2010, Macpherson *et al.* 2013, Coutinho da Silva *et al.* 2013) and mares here aborted from 5-25 days after inoculation with an average of ~9.5 days. Furthermore, we did not observe acute abortions (i.e. 2-3 days post inoculation) as reported elsewhere. (Coutinho da Silva *et al.* 2013). Nonetheless, concentrations of both androgens did not vary remarkably throughout the experimental days, except DHEA-S which was significantly reduced on the day of abortion for mares (subgroup with FFS) with experimentally induced ascending placentitis.

Recent findings from our laboratory appeared to support our hypothesis that placentitis affects peripheral androgen concentrations in mares with placentitis; the study demonstrated that inhibition of estrogen synthesis using letrozole (a non-steroidal aromatase inhibitor) resulted in elevation of DHEA-S and testosterone in plasma of late-term pregnant mares (unpublished data). We considered that the placental insult prompted by placentitis would cause similar effects for both androgens. Unpublished observations from our laboratory had shown that mares with experimentally induced ascending placentitis present a reduction in estrogens concentrations post-inoculation, therefore it was plausible to hypothesize that concentrations of androgens could be elevated in mares with placentitis. Since ascending placentitis only causes partial compromise of the chorioallantois (based on pathologic lesions), the remaining unaffected tissues may compensate for the affected portion. This could explain why we failed to observe differences for both androgens.

Mares experiencing pregnancy loss present lower estrogen concentrations in comparison to normal mares for the same gestational age (Douglas 2004). It is believed that the placental damage would compromise the ability of the placenta to produce estrogens, as a consequence we hypothesized that the precursor (DHEA) would accumulate in the maternal plasma and as consequence DHEA-S would be also elevated as a consequence of increased substrate. Perhaps the mechanism leading to the reduction of estrogens in mares with placentitis is related to other unknown mechanisms. Here we reported the first controlled study attempting to use androgens, particularly DHEA-S and testosterone, as diagnostic markers for mares with placentitis. Previously, a clinical descriptive case report had measured DHEA-S and DHEA in eight high risk pregnant mares; however, the authors found that neither DHEA-S nor DHEA appeared to be particularly useful markers in these mares (Meinecke & Gips 1990). Our findings appear to support the clinical observations by Meinecke & Gips (1990).

In normal pregnant mares, concentrations of both DHEA-S and testosterone varied greatly within mares across 100 days of gestation to term. Concentrations of DHEA-S peaked by ~180 days of gestation which is similar to a previous report involving five pregnant mares (Rance *et al.* 1978) and slightly before previously reported peak concentrations for estrogens (Ginther 1992). The earlier rise in DHEA suggests that the equine fetal gonads are synthesizing the precursor (i.e. DHEA) and that the placenta is not fully converting DHEA into estrogens. Interestingly, contrary to an earlier report,^[19] testosterone did not peak, but rather plateaued from approximately 180 -290 days of gestation. The absolute concentrations of testosterone in the current study were about three-fold higher than those reported previously (Silberzahan *et al.* 1984). Potential

reasons for the disparity in testosterone concentrations in these studies includes differences on the assays (ELISA vs RIA) and antibody crossreactivity. The testosterone antibody used in this study has a high cross-reactivity with dihydrotestosterone; however, at this point it is unknown whether this metabolite of testosterone is present in significant concentrations in plasma of mares in the second and third trimester of pregnancy.

In conclusion, DHEA-S and testosterone were present in high concentrations in pregnant mares and varied through day 100 to term. There were no significant differences for concentrations of DHEA-S and testosterone between mares with experimentally induced ascending placentitis and gestationally age-matched control mares. Based on our experimental results we suggest that neither androgen here evaluated in mares with experimentally induced ascending placentitis appear to be a useful marker for spontaneous placentitis.

4.6. Acknowledgments

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Chapter 5

Estradiol 17 β , Estrone Sulfate and Progestins in Mares with Experimentally Induced Ascending Placentitis

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5.1. Summary

Reasons for performing study: Placental steroids have been suggested as useful diagnostic markers for pregnancy loss in mares; however, there are few controlled studies that have examined changes in steroids in mares with placentitis.

Objectives: To compare plasma concentrations of estradiol 17 β , estrone sulfate and progestins in mares with placentitis and with gestationally age-matched control mares.

Methods: Pregnant mares (260-280d of gestation) were allocated to a control group and to a group with experimentally induced placentitis. Induction of placentitis was achieved via intracervical inoculation of *Streptococcus equi* ssp *zooepidemicus*. Plasma was collected at inoculation for mares in the placentitis group or commence for control mares (day=0), and then daily until abortion or for 12 days post inoculation (DPI). Concentrations of estrone sulfate and progestins were determined by ELISA, whereas estradiol 17 β was

determined by a chemiluminescence immunoassay. Data were analyzed by mixed models, for DPI and for days from abortion (DFA) for the different steroids.

Results: Mares with experimentally induced placentitis aborted within 5-25 DPI. There were significant effects of time (DPI, $p < 0.0001$; DFA $p = 0.0008$) and time by group interactions (DPI or DFA $p < 0.001$) for the concentrations of estradiol 17- β . Concentrations of estradiol 17- β were significantly reduced at 4 DPI and at - 2 DFA ($p < 0.05$). Analyses of estrone sulfate revealed effects of time (DPI $p < 0.003$; DFA $p = 0.01$) and time by group interactions (DPI $p = 0.002$; DFA $p < 0.0001$). Estrone sulfate concentrations were significantly reduced 6 and 7 DPI and on the day of abortion (0 DFA) for mares with experimentally induced placentitis. There were no effects of time (DPI $p = 0.72$; DFA $p = 0.76$) or time by group interactions (DPI*group $p = 0.99$; DFA*group $p = 0.54$) on progesterin concentrations.

Conclusions: Concentrations of estrogens but not progestins changed significantly in mares with experimentally induced ascending placentitis. Prospectively controlled studies are warranted to evaluate whether estrogens are useful markers for spontaneous placentitis.

5.2. Introduction

Placentitis is an important cause of pregnancy wastage in broodmares (Giles *et al.* 1993, Williams *et al.* 2004). Currently, diagnosis of placentitis is based upon ultrasonography and clinical signs (i.e. premature lactation and/or vulvar discharge) (Troedsson & Macpherson 2011). Transrectal ultrasonography of the cervical pole of the placenta (Renaudin *et al.* 1997) has been shown to be a useful tool to confirm and to manage clinical ascending placentitis (Troedsson & Zent 2004). However, data on sensitivity and specificity are scant. Clinical signs vary and may only be detected in advanced stages of placentitis (Troedsson & Macpherson 2011); thus, development of new diagnostic tests for placentitis will potentially enhance veterinary care for pregnant mares.

The endocrine fetoplacental unit synthesizes and metabolizes steroids (i.e. progestins, androgens, and estrogens) (Cox 1975, Pashen & Allen 1979, Raeside 2004), which are responsible for pregnancy maintenance and for preparation of the mare for parturition (Cox 1975, Pashen & Allen 1979, Raeside 2004, Ousey 2011). The fetal adrenal gland plays a role in endocrine support of pregnancy as an important source of substrate (i.e. pregnenolone) for the placental production of progestins (Ousey 2011). The fetal adrenal gland expresses P450 side chain cleavage (P450scc) that converts cholesterol to pregnenolone (Chavattee *et al.* 1995, Han *et al.* 1995, Ousey 2011, Weng *et al.* 2007). Pregnenolone is then metabolized to progesterone by the action of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in the equine placenta (Chavattee *et al.* 1995, Han *et al.* 1995, Ousey 2011). Under, normal conditions, progesterone is rapidly converted into 5 α -dihydroprogesterone (5 α -DHP; 5 α -pregnane,3,20-dione) by the action of a 5 α reductase present in the equine placenta and endometrium (Ousey *et al.* 2003, Ousey 2011), and 5 α DHP may be further converted into 20 α -hydroxy-5 α -pregnan-3-one (20 α 5P) by 20 α -

reductase expressed in the placenta (Ousey *et al.* 2003, Ousey 2011). Progestins can be sub-classified as pregnenes and 5 α pregnanes. Pregnenes include pregnenolone, progesterone and 5-pregnene-3 β ,20 β -diol (P5 $\beta\beta$) (Ousey *et al.* 2003); 5 α -pregnanes include 5 α -DHP, 3 β 5P, 20 α -5P, $\beta\beta$ diol, 5-pregnene-3 β ,20 α -diol (β adiol) (Ousey *et al.* 2003); however, 5 α DHP, 20 α 5P, and $\beta\alpha$ -diol predominate (Chavatte *et al.* 1997).

During pregnancy, the hypertrophied fetal gonads of both male and female fetuses produce large quantities of androgens, particularly dehydroepiandrosterone (DHEA) and 7-dehydro DHEA from cholesterol and from farnesyl pyrophosphate, respectively [12-14,17]. Classic phenolic estrogens (i.e. estrone, estradiol-17 β estradiol-17 α , and their sulfoconjugates) are synthesized from DHEA (Raeside 2004). . Ring B unsaturated estrogens (i.e. equilin, equilenin and their hydroxyderivatives 17 α -dihydroequilin, 17 α -dihydroequilenin, 17 β -dihydroequilin, and 17 β -dihydroequilenin) are produced from 7-dehydro-DHEA by the equine placenta (Cox 1975, Raeside *et al.* 1979, Pashen & Allen 1979, Raeside, Ousey 2011). Immunolabeling studies of the chorioallantois and labelled precursor studies suggested that DHEA is converted into androstenedione by 3 β -HSD and that androstenedione can be converted into estrone by aromatase or into testosterone by 17- β hydroxysteroid dehydrogenase (17- β -HSD) (Tait *et al.* 1985, Bhavnani 1988, Chavattee *et al.* 1995, Raeside 2004, Ousey 2011). In turn, estrone can be converted into estradiol 17- β by 17- β HSD in the chorioallantois (Raeside 2004, Ousey 2011). It is thought that estrone sulfate can be converted into estradiol 17 β sulfate by 17- β HSD in the endometrium (Ousey 2011). The key role of the placenta in steroidogenesis during equine pregnancy suggests that pathologic conditions of the fetoplacental unit will compromise the ability of different steroids to be metabolized and released in the maternal circulation.

Abnormalities of pregnancy may alter the normal profile of placental steroids and serial determinations of peripheral steroids in the pregnant mare have been suggested as useful diagnostic markers for placentitis (Kashman *et al.* 1988, Rossdale *et al.* 1991, Douglas 2004, Ousey *et al.* 2005). Mares with placental pathology may have increased plasma concentrations of progestins as a result of fetal stress (Douglas 2004, Ousey *et al.* 2005, Ousey 2011). Estrone sulfate has been suggested to be a useful marker for fetal well-being during early pregnancy (Kashman *et al.* 1988); however, studies involving estrone sulfate in advanced pregnancy are scant (Kashman *et al.* 1988, Ousey *et al.* 1987). To our knowledge, there are no controlled studies assessing estrone sulfate in mares experiencing placentitis, despite its frequent use to assess fetal well-being. Field studies carried by a commercial laboratory reported that mares at risk for abortion had significantly lower “total estrogen” (unreported cross-reactivity) concentrations than mares that maintained pregnancy (Douglas 2004). Controlled studies are warranted to confirm field observations about estrogens and progestins and to assess whether estrone sulfate is a useful marker for placentitis. Therefore, we hypothesized that experimentally induced placentitis will be associated with the reduction of estrogens and increase in progestin concentrations. The objective of the current study was to compare plasma concentrations of progestins, estradiol 17 β , and estrone sulfate in mares with experimentally induced ascending placentitis and gestationally aged-matched control mares carrying normal pregnancies.

5.3. Material and Methods

Animal Husbandry

Horse mares enrolled in these experiments were maintained at the Maine Chance Farm. All the experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. All mares were kept in paddocks and supplemented with hay, grain and water *ad libitum* along with trace minerals. Upon imminent abortion or normal parturition, mares were kept in individual stalls (16 x 16 feet) during the night and turned out in pasture throughout the day.

Experimental Design & Induction of Ascending Placentitis

Mares carrying normal pregnancies (260-280 days of gestation) were assigned to (1) a control group and to (2) a group with experimentally induced ascending placentitis. As part of another study, within each group, a subset of mares had fetal fluid sampling (FFS) performed at 0, 5 and 12 days post inoculation (DPI) or until abortion. Similar methodology has been described elsewhere (Paccamonti *et al.* 1995) and has been slightly modified (Canisso *et al.* 2014). Briefly, the mare was sedated, placed in stocks and fetal fluids were collected with transabdominal ultrasound and an echotip spinal needle (18G×6"; 30° short bevel, Chiba-Type spinal needle, with stylet, Echo Block PTC Havel's Cincinnati, OH, USA) (Canisso *et al.* 2014).

Ascending placentitis was induced via intracervical inoculation of *Streptococcus equi* ssp. *zooepidemicus*. The experimental model for induction of bacterial placentitis in the mare was modified from that previously described (LeBlanc *et al.* 2002) and has been successfully used by different laboratories as a reliable and predictable model to induce

ascending bacterial placentitis in the mare (Coutinho da Silva *et al.* 2013, Morris *et al.* 2007). Sham inoculation of control mares was not performed in the present study as this procedure may induce placentitis in an unpredictable manner; as noted by previous publications. The bacterial inoculum was deposited midway intracervically with the usage of a semi-flexible artificial insemination pipette. The inoculum was contained in a 0.5-mL straw and was deposited with the use of a stylet. The bacterial strain used was isolated from a placenta from a mare diagnosed with spontaneously ascending placentitis by the Veterinary Diagnostic Laboratory at the University of Kentucky. The bacterial isolate was preserved in cryovials containing skim milk at -80°C until use. Prior to inoculation, bacterial cultures were prepared and bacterial counts performed in standard fashion using blood Agar plates (incubated at 37°C), serial dilution and plating were used to determine the number of colony-forming units. The bacterial inoculum, containing 5 million colony forming units (cfu), was suspended in 0.5 ml of phosphate buffered saline. Once inoculation was carried out, a swab was obtained from the leftover in the cryovial and cultured in similar conditions to assure that the bacteria inoculum was still viable.

Transrectal Ultrasonography

Before the beginning of the experiment, mares underwent transrectal ultrasonography to assess the caudal placental pole to assure that all mares were carrying normal pregnancies. Three measurements of the combined thickness of uterus and placenta (CTUP) were taken and compared with previously established normal ranges (Renaudin *et al.* 1997). All mares had CTUP measures within previously reported normal ranges and presented normal ultrasonography appearance of the caudal pole of the placenta (data not shown); therefore all mares were enrolled in this study. Thereafter, all mares had

transrectal ultrasonography performed every other day for 12 days or until abortion to follow ultrasonographic changes in inoculated mares and to confirm the lack of changes in mares in the control group. Particularly, we assessed changes that are highly indicative of placentitis including placental separation and fluid accumulation between the uterus and chorioallantois. It was out of the scope of this study to compare or associate ultrasonographic findings with steroid concentrations. A previous publication using a different progesterin assay had addressed these associations (Morris *et al.* 2007).

Determination of Estradiol 17 β , Estrone sulfate and Progestins Concentrations

Blood samples were obtained immediately before inoculation/initial FFS (d=0), and then daily for 12 days or until abortion. Blood was attained by jugular venipuncture into heparinized 10-mL tubes. Immediately after collection, the blood was centrifuged at 600 xg for 10 min at 5° C. Plasma was harvested and preserved at -20° C until further analysis.

Estradiol 17 β concentrations were determined in 17 mares in the control group and 14 mares in the group with experimentally induced ascending placentitis. As part of another study, FFS was performed in a subset of 7 mares in the control group and 5 mares in the placentitis group. Concentrations of estradiol 17 β were determined by chemiluminescent immunoassay (Estradiol, Immulite 1000, Siemens; intra-assay CVs were 1.2-1.4%; interassay CVs were 2-3.15%). The detection range for this assay varies from 20 to 2,000 pg/mL with an analytical sensitivity of 15 pg/mL. According to the manufacturer, the cross reactivity for this assay is non-significant for most adrenal and reproductive steroids (the reader is referred to kit's manual, for more information), thus the highest reported cross reactivity was 2.09% with estrone.

Concentrations of estrone sulfate were measured in 28 mares, with 14 in each group. As part of another study, FFS was performed in a subset of 7 mares in the control group and 6 mares in the placentitis group. Estrone sulfate was determined (in triplicate) using a competitive immunoassay previously validated for horses (Stabenfeldt *et al.* 1991, Carneiro *et al.* 1998). The standard curve ranged from 0.05 ng/mL to 20 ng/mL with a limit of detection of 0.05 ng/well (intra-assays CV of 10.2% and inter-assay CV of 16.6%). The reported cross-reactivity of the polyclonal antibody (R583) used for this assay are estrone-3-sulphate (100%), estrone-3-glucuronide (70%), estrone (269%), and estradiol-17 β (9.8%) (Stabenfeldt *et al.* 1991).

Progesterin concentrations were determined in 14 mares in the control group and 14 mares in the group with experimentally induced ascending placentitis. As part of another study, FFS was performed in a subset of 6 mares in the control group and 7 mares in the placentitis group. Progesterins concentrations were determined (in duplicate) by the use of a competitive immunoassay (Munro & Stabenfeldt 1984). Cross-reactivity for this antisera was reported in the initial description as 11 α -hydroxyprogesterone 21.4%, 17 α -hydroxyprogesterone 0.25%, 20 α -hydroxyprogesterone 0.32%, 20 β -hydroxyprogesterone 2.38%, 5 α -DHP 29.5%, estradiol 17 β 0.02%, cortisol 0.02%, androstenedione 0.07%, and testosterone 0.03% (Munro & Stabenfeldt 1984). The standard curve ranged from 0.05 ng/mL to 20 ng/mL, with a limit of detection of 0.25 ng/well (intra-assays CV of 15.4% and inter-assay CV of 18.5%).

Placental and Fetal Pathology

Placentas from control and inoculated mares, and all aborted fetuses underwent complete pathological (macroscopic and histopathologic examination) and microbiological evaluation (Hong *et al.* 1993). Here briefly, the placenta was laid out and the chorioallantois, amnion, and umbilical cord were examined. Areas with no apparent lesions and areas presenting macroscopic lesions were sampled and preserved in buffered formalin for further histopathologic evaluation. Swabs were collected from the chorioallantois and used to perform aerobic bacterial culture (Hong *et al.* 1993). Fetuses were necropsied in routine fashion and internal organs (liver, spleen, stomach content, lung, heart, and trachea) were macroscopically examined and sampled for histopathologic evaluations and aerobic bacterial culture (Hong *et al.* 1993).

Statistical Analyses

All the data analyses were carried out with JMP 10 (SAS Institute, Cary NC, USA). Concentrations of estradiol 17 β , estrone sulfate and progestins were analyzed relative to days post-inoculation (DPI, 0 to 7 days) as well as relative to days from abortion (DFA, -8 days to 0) in separate mixed models. Mare was accounted as random effect, and treatment and time (DPI or DFA) were fixed effects. Fetal fluid sampling was nested within group. As we were interested in evaluating the acute changes in steroids concentrations following inoculation, the data were analyzed for changes up to 7 DPI. On the other hand, as the interval from inoculation to abortion was variable, we examined the changes in steroids in the eight days prior to abortion. Two and three way interactions were also included in the different models for DPI and DFA. Since there were no effects of FFS, data were combined

within groups for further analyses. Prior to analyses, the data were log transformed as necessary. Post-hoc comparisons across days were analyzed by Fisher's Least Significant Difference Test. Data are presented as mean \pm S.E.M. Correlations between estradiol 17 β and estrone sulfate were determined by pairwise comparisons. □

5.3. Results

Mares with experimentally induced placentitis aborted between 5 and 25 DPI. Three mares that were inoculated failed to develop placentitis after a single inoculation. One mare with experimentally induced placentitis (subgroup without FFS) did not develop placentitis; she delivered a normal foal and a placenta with no pathological or microbiological evidence of placentitis, this mare was excluded from the analyses. Two mares in the group with experimentally induced placentitis (one with and another without FFS) did not develop placentitis and were re-inoculated 14 days after the first inoculation. The mare without FFS aborted 20 days after re-inoculation and the other mare with FFS aborted 9 days after re-inoculation. The first 14 days following the first inoculation in these two re-inoculated mares were excluded from the analyses.

As expected and noted in a parallel publication, *Streptococcus equi* ssp. *zooepidemicus* was the predominant bacterial isolate, however *E. coli* was also isolated in association with *Streptococcus equi* ssp. *zooepidemicus*. All inoculated mares, but one mare, developed ultrasonographic changes consistent with ascending placentitis (i.e. placental separation, fluid accumulation between uterus and the chorioallantois). All mares in the control group presented normal ultrasonographic findings (data not shown). With the exception of one mare that failed to develop placentitis after experimental induction (noted above) and the control mare with FFS that aborted, all the remaining mares that aborted

with experimentally induced ascending placentitis developed gross and histopathologic lesions consistent with ascending placentitis (Hong *et al.* 1993) (data not shown). All mares in the control group delivered macroscopically and microscopically normal placentas. Bacterial culture yielded non-pathogenic saprophytic bacteria (data not shown).

There were significant effects of time (DPI, $p < 0.0001$; DFA $p = 0.0008$) and time by group interactions (DPI or DFA $p < 0.001$) for the concentrations of estradiol 17- β (Figure 1). Post hoc comparisons revealed that concentrations of estradiol 17- β was significantly reduced 4 DPI and - 2 DFA ($p < 0.05$). Analyses of estrone sulfate revealed effects of time (DPI $p < 0.003$; DFA $p = 0.01$) and time by group interactions (DPI $p = 0.002$; DFA $p < 0.0001$). Estrone sulfate concentrations were significantly reduced 6 and 7 DPI and in the day of abortion (0 DFA) for mares with experimentally induced ascending placentitis (Figure 2). For the progestin concentrations, there were no effects of time (DPI $p = 0.72$; DFA $p = 0.76$) or time by group interactions (DPI*group $p = 0.99$; DFA*group $p = 0.54$) (Figure 3). Estradiol 17 and estrone sulfate concentrations were moderately correlated ($r = 0.34$, $p = 0.001$).

Figure 5.1. Estradiol 17- β concentrations in mares with experimentally induced ascending placentitis (n=14) and gestationally age-matched control mares carrying normal pregnancies (n=17). The average time from inoculation to abortion was 7.8 days (range 5-14 days). (A) Represents 0 (i.e. day of inoculation/commence of sampling for control mares) to 7 days post inoculation (DPI). (B) Represents samples for days from abortion (DFA) -8 to 0 (i.e. day of abortion). There were significant effects of time (DPI, $p < 0.0001$; DFA $p = 0.0008$) and time by group interactions (DPI or DFA $p < 0.001$). Post hoc comparisons revealed that concentrations of estradiol 17- β was significantly reduced 4 DPI and -2 DFA ($p < 0.05$). *Within day denotes significant difference ($p < 0.05$) between groups.

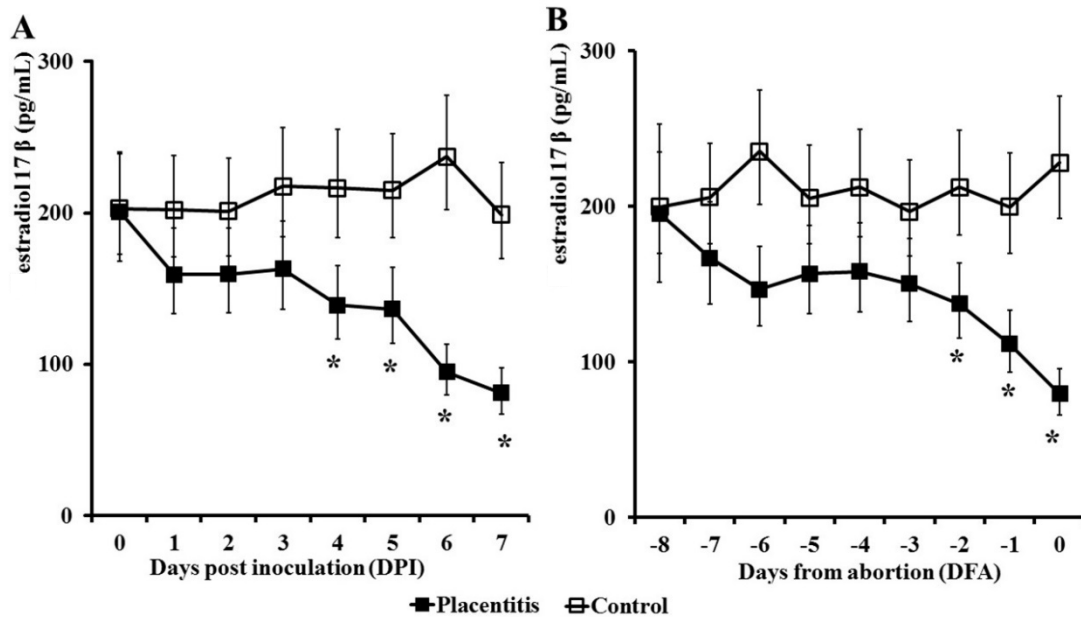


Figure 5.2: Estrone sulfate concentrations in mares with experimentally induced ascending placentitis (n=14) and gestationally age-matched control mares carrying normal pregnancies (n=14). The average time from inoculation to abortion was 9.4 days (5-25 days). (A) Represents 0 (i.e. day of inoculation/commence of sampling for control mares) to 7 days post inoculation (DPI). B) Represents samples for days from abortion (DFA) -8 to 0 (i.e. day of abortion). There were effects of time (DPI $p < 0.003$; DFA $p = 0.01$) and time by group interactions (DPI $p = 0.002$; DFA $p < 0.0001$). Estrone sulfate concentrations were significantly reduced 6 and 7 DPI and on the day of abortion (0 DFA) ($p < 0.05$). *Within day denotes significant statistical difference $p < 0.05$.

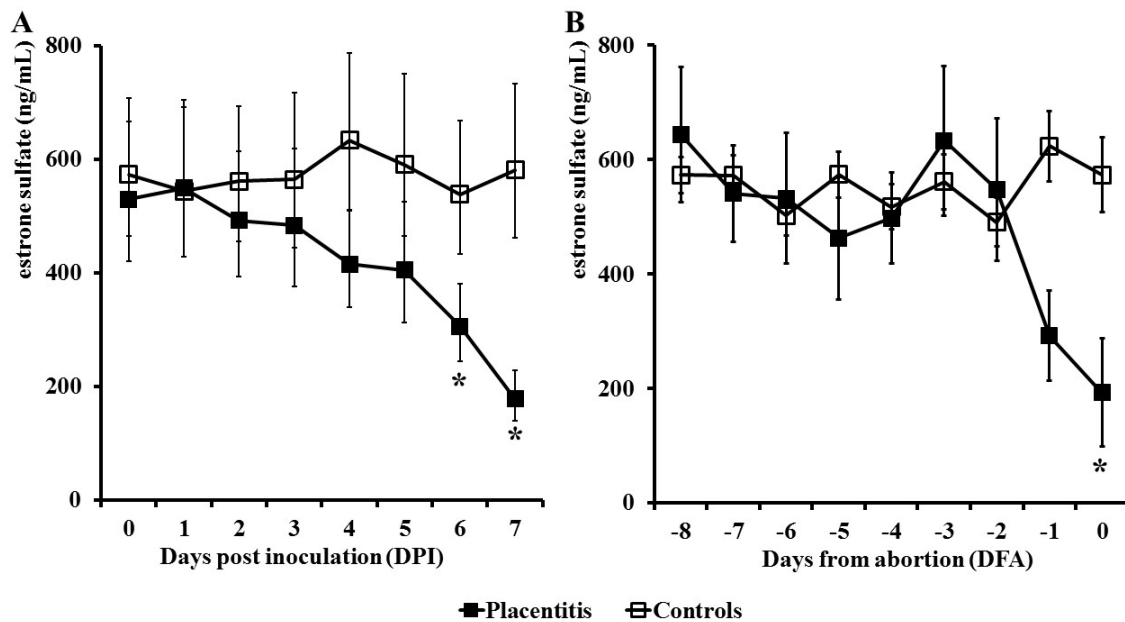
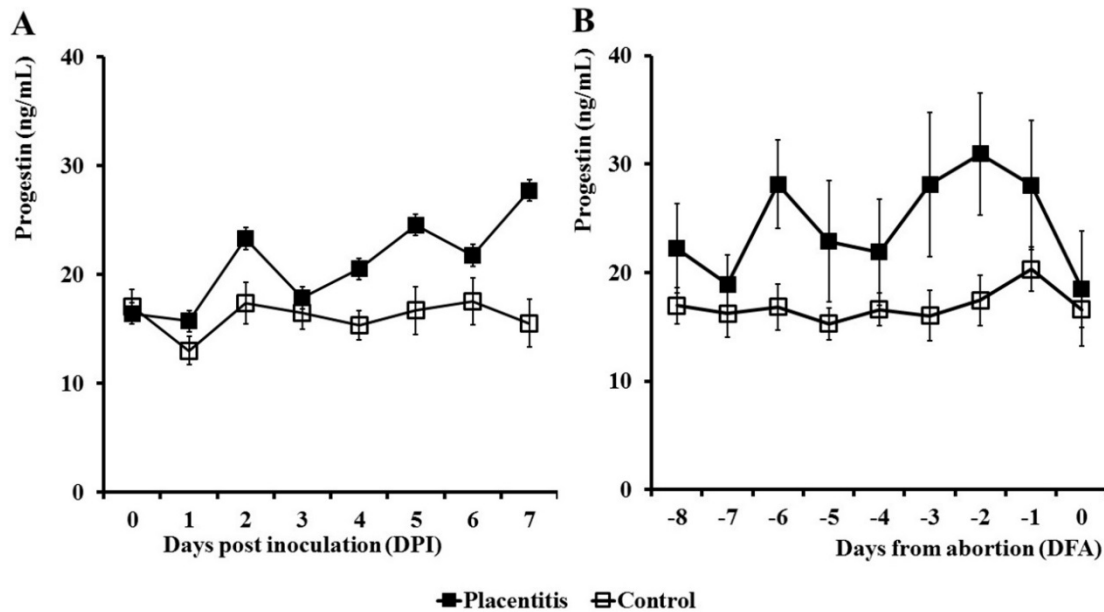


Figure 5.3: Progesterone concentrations in mares with experimentally induced ascending placentitis (n=14) and gestationally age-matched control mares carrying normal pregnancies (n=14). The average time from inoculation to abortion was 9.4 days (range 5-25 days) (A) Represents 0 (i.e. day of inoculation/commence of sampling for control mares) to 7 days post inoculation (DPI). B) Represents samples for days from abortion (DFA) -8 to 0 (i.e. day of abortion). There were no effects of time (DPI $p=0.72$; DFA $p=0.76$) or time by group interactions (DPI*group $p=0.99$; DFA*group $p=0.54$) for the concentrations of progesterone.



5.5 Discussion

Although peripheral estrogens in the mare have been suggested as a possible marker for fetoplacental well-being for many years (Kashman *et al.* 1988), this is the first report to demonstrate time-dependent changes in estrogen concentrations subsequent to experimentally induced, ascending placentitis in mares. Approximately, 99% of the

estradiol 17 β in pregnant mares is present as a sulfoconjugate; however, the remaining 1% is present in the maternal circulation as estradiol 17 β (Ginther 1992). Here, we demonstrated that free estradiol 17 β was significantly reduced following experimental induction of placentitis, therefore, this steroid may be a useful diagnostic marker for placentitis in mares with spontaneous disease. Interestingly, estrone sulfate, a commonly used hormone to monitor the fetal wellbeing, was only reduced several days after inoculation/or close to abortion. This suggest that this marker only changes in advanced stages of placentitis, but also may reflect the long half-life of this hormone in maternal plasma which may limit its clinical applications. Concentrations of estradiol 17 β and estrone sulfate presented low correlations, this lack of associations can be explained by the different half-lives, as sulfoconjugated steroids are thought to have longer half-lives.

Prior studies have reported changes in estrone sulfate concentrations relative to early fetal loss in the mare; however, the experimental induction of pregnancy loss in these mares involved acute induction of fetal death or luteolysis which have very different effects on placental function than the induction of ascending placentitis in the current study (Kashman *et al.* 1988, Stabenfeldt *et al.* 1991). Santschi *et al.* (1991) examined changes in estrone sulfate concentrations in pregnant mares with a variety of medical and surgical conditions and concluded that estrone sulfate concentrations in late gestation frequently overlapped with those of normal pregnancy. In contrast, Douglas (2004) reported significantly lower “total serum estrogen” concentrations in mares that aborted with placentitis than mares that delivered live foals during the interval between 150 and 280 days of gestation. In Douglas’ study (2004), estrogens were determined using an assay for 17 α estradiol, thus limited direct comparisons can be drawn.

It is surprising that progesterin concentrations did not change in mares with experimentally induced placentitis in the current study. In a previous study, significant differences in progesterin concentrations were reported following placentitis (Morris *et al.* 2007). In mares with experimental placentitis, it was found that chronic placentitis (defined by the authors as mares aborting >7 days after inoculation) was associated with increased plasma progesterin concentrations (Morris *et al.* 2007). Conversely, mares that developed acute placentitis and abortion soon after infection experienced a rapid drop in plasma progesterin concentrations (Morris *et al.* 2007). The authors suggested that measurement of repeated samples of plasma progesterin concentrations in mares with placentitis might be a useful method to identify mares that may abort or deliver prematurely, especially if combined with transrectal ultrasonography. In the present study, we did not analyze changes in progesterin concentrations based upon interval to abortion, but rather examined changes in concentrations for progesterins for all mares based upon days from abortion. In the current study, there were no significant changes in plasma progesterin concentrations in mares aborting with experimentally induced placentitis. In another report with seven spontaneous cases of placentitis, gas chromatography-mass spectrophotometry analyses revealed changes in different progesterins which appeared to be elevated (i.e. P4, P5, 3 β 5P, P5 $\beta\beta$, $\beta\beta$ -diol, $\beta\alpha$ -diol, 5 α -DHP, 20 α 5P, 20 β -OH) (Ousey *et al.* 2007). Since the mares in that report were not sampled at regular and similar intervals, additional studies should be carried with mares sampled at regular intervals and gestationally age-matched control mares should be also sampled. It is also worth noting that the antibody used in our study for determination of progesterin concentrations was different than the antibody used by Morris *et al.* (2007). As noted before, the antibody used in the present study (Munro &

Stabenfeldt 1984) cross-reacts with 5 α -DHP in ~30%; however, the assay used by Morris *et al.* (200), the authors reported that their assay cross-reacted with 5 α -DHP 10%. It is possible that differences in cross-reactivity can explain the discrepant results between Morris *et al.* (2007) and our findings.

In conclusion, estrogens but not progestins changed following experimental induction of placentitis in mares. Estradiol 17 β concentrations changed rapidly in plasma of mares with experimentally induced ascending placentitis, contrary to estrone sulfate which changed only close to abortion. Concentrations of estradiol 17 β and estrone sulfate are only moderately correlated. Our observations with experimental cases of ascending placentitis should be expanded to prospectively controlled field studies to confirm the usefulness of estradiol 17 β as a diagnostic maker for placental disease in the mare.

5.6 Acknowledgments

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Chapter 6
Alpha-fetoprotein is Present in the Equine Fetal Fluids and is Increased in Plasma
of Mares with Experimentally Induced Ascending Placentitis

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6.1. Summary

The objectives of this study were to: (i) determine alpha-fetoprotein (AFP) concentrations in fetal fluids (FF), (ii) compare plasma concentrations of AFP in mares with placentitis (n=17) and gestationally age-matched control mares (n=17), and (iii) use cytology of the FF to determine its origin. Fetal fluid sampling (FFS, n=7/group) was performed at 0, 5 and 12 days post inoculation (DPI) or until abortion. Plasma was harvested daily for 12 days or until abortion. Placentitis was induced via intracervical inoculation of *Streptococcus equi* ssp. *zooepidemicus*. Proteins present in the FF were resolved by 1D-SDS-PAGE. Immunoblotting was used to detect the presence of AFP in fetal fluids. Concentrations of AFP in FF and plasma were determined with a chemiluminescence immunoassay. Mixed models for DPI, and for days from abortion (DFA) were used to

analyze plasma concentrations of AFP. A protein band ~68 kDa consistent with the AFP size was present in all samples. Immunoblotting for AFP revealed a single protein (~68 kDa) in all samples. Concentrations of AFP in FF appeared higher than those in maternal plasma. There were effects of time (DPI $p < 0.0001$; DFA $p = 0.0002$) and time-by-group interactions (DPI*Group $p < 0.06$; Group*DFA $p < 0.001$). Amniotic fluid was found to be rich in epithelial cells; however, allantoic fluid was acellular. This study confirmed that AFP is present in the FF of mares during the third trimester of pregnancy. Cytology can be used to identify the origin of the FF. Experimentally induced placentitis was associated with an elevation in plasma concentrations of AFP.

6.2. Introduction

Ascending placentitis, an importance cause of pregnancy wastage, is the most prevalent type of placentitis in mares (Hong *et al.* 1993); three other less common morphologic forms have been recognized (i.e. focal mucoid, hematogenous, and multifocal) (Williams *et al.* 2004). Ascending placentitis can be caused by fungal or bacterial agents with bacteria being responsible for about 90% of clinical cases (Hong *et al.* 1993). Bacterial agents commonly associated with the occurrence of ascending placentitis include *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*), *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, although beta-hemolytic streptococci predominate (Hong *et al.* 1993, Giles *et al.* 1993, Williams *et al.* 2004).

Currently the diagnosis of ascending placentitis is based on clinical signs (i.e. premature mammary gland development \pm streaming milk, and vulvar discharge) and transrectal ultrasonography of the caudal pole of the placenta (Troedsson & Macpherson, 2011). Although ultrasonography of the placenta has been shown to be a useful tool for the clinical diagnosis of ascending placentitis (Troedsson & Zent 2004), information on the sensitivity and specificity of the technique is limited. Furthermore, early stages of placentitis may not be detected with transrectal ultrasonography. Therefore, new diagnostic markers are needed for early diagnosis and to avoid unnecessary treatment of normal pregnant mares.

To date several markers have been investigated in mares experiencing pregnancy losses. Among these markers, steroids produced by the fetoplacental unit have been the focus of the majority of the reports (Rossdale *et al.* 1991, Douglas 2004, Ousey *et al.* 2005,

Morris *et al.* 2007). Relaxin, a placental specific hormone has also been investigated in high risk pregnant mares (Ryan *et al.* 2009); however, the lack of a commercially available equine assay precludes the use of relaxin. Lately, inflammatory acute phase proteins, particularly serum amyloid A and haptoglobin have also been investigated (Canisso *et al.* 2014a); however, it is unclear whether these inflammatory proteins are useful markers for spontaneous cases of placentitis.

Different diagnostic markers have been identified in amniotic fluid of women with chorioamnionitis. Some of these markers are also present in blood and have been used as diagnostic tools for pathologic conditions of the placenta and fetus (Gravett *et al.* 2004, Ruetschi *et al.* 2005, Romero *et al.* 2008, Buhimschi & Buhimschi 2012). However, in comparison to humans, the physiology and pathology of the fetal fluids in domestic mammals, particularly in the horse, is poorly understood. In horses, studies have been conducted primarily to examine the presence of certain enzymes also present in other body systems as well as quantification of electrolyte composition (Schmidt *et al.* 1991, Williams *et al.* 1993, Holdstock *et al.* 1995, Paccamonti *et al.* 1995, Lyle *et al.* 2006, Zanella *et al.* 2013, Finger *et al.* 2014). A more detailed study of protein composition of equine fetal fluids would potentially allow the identification of proteins that are highly expressed in the fetal fluids, and possibly also present in blood.

Data from other domestic mammals suggested that alpha-fetoprotein (AFP) is a major protein present in the allantoic and amniotic fluids (Okano *et al.* 1977, Smith *et al.* 1979, Luft *et al.* 1984, Deutsch 1991); Alpha-fetoprotein is a member of the albuminoid superfamily, the other protein members of this superfamily are vitamin D binding protein, alpha-albumin, and afamin (Lichenstein *et al.* 1994, Mizejewski 1995). In mammalian

fetuses, among other functions, AFP is associated with estrogen-binding (allowing development of GnRH surge center in females), anti-oxidative activities (through binding of heavy metals), and immunoregulation (Peck *et al.* 1978, Mizejewski 1995, Gabant *et al.* 2002, De Mees *et al.* 2006). In horses, AFP is highly expressed during early pregnancy by the conceptus (Simpson *et al.* 2000). Although there are no reports of AFP concentrations associated with equine fetal fluids later in gestation, it seems likely that AFP continues to be a major protein present during the later stages of equine gestation. Cytologic preparations of the equine fetal fluids have been suggested to be a useful method to identify the type of fetal fluid (i.e. allantoic or amniotic) (Lyle *et al.* 2006), however, we were unable to find any report describing the cytology appearance of these fluids. Therefore, we were interested to confirm whether AFP is present in the fetal fluids of mares during the third trimester of pregnancy and whether this protein can be measured in plasma of the pregnant mare, and whether concentrations of this molecule change followed experimental induction of placentitis. The objectives of this study were to: (i) describe AFP concentration in the equine fetal fluids, (ii) compare the concentrations of alpha-fetoprotein in plasma of mares with experimentally induced placentitis and gestationally age-matched control mares, and (iii) use cytological preparations of the fetal fluids to determine its origin.

6.3. Material and methods

Animal Husbandry

Mares were maintained at the Maine Chance Farm, Department of Veterinary Science, University of Kentucky, Lexington, KY. The Institutional Animal Care and Use Committee at the University of Kentucky approved all the experimental protocols (Project #2010-0769). All the mares were kept in paddocks and supplemented with hay, grain and

water *ad libitum* along with trace minerals. Immediately prior to normal parturition or pre-abortion; the mares were kept in individual stalls (16 × 16 feet) during the night and turned out on pasture during the day.

Study Design

Mares carrying normal pregnancies (260-280 days of gestation) were assigned to (1) a control group with (n=7) or without (n=10) trans-abdominal ultrasound-guided fetal fluid sampling (FFS) and (2) a treatment group with experimentally induced ascending placentitis with (n =7) or without FFS (n=10). Fetal fluid sampling was performed at 0, 5 and 12 days post inoculation (DPI) or until abortion. Similar methodology has been previously described elsewhere (Paccamonti et al., 1995) and slightly modified by us (Canisso et al., 2014b). Briefly, the mare was sedated, placed in stocks and fetal fluids were collected with transabdominal ultrasound and an echotip spinal needle (18G×6”; 30° short bevel, Chiba-Type spinal needle, with stylet, Echo Block PTC Havel’s Cincinnati, OH, USA) (Canisso *et al.* 2014b). Blood samples were obtained immediately before inoculation/initial FFS (d=0) and then daily for 12 days or until abortion.

Immediately after collection, aliquots of fetal fluids were frozen in liquid nitrogen and preserved at -80°C until further analyses. To confirm the origin of each sample, an aliquot was submitted to the Veterinary Diagnostic Laboratory at the University of Kentucky (UKVDL) for determination of creatinine, calcium, sodium and chloride as previously described (Holdstock *et al.* 1995; Paccamonti *et al.* 1995). Amniotic fluid has low creatinine concentrations and relatively high chloride concentrations, whereas allantoic fluid has high creatinine concentrations and low calcium concentrations (Paccamonti *et al.* 1995). Data for creatinine and electrolyte concentrations are presented

in the supporting information. In addition, cytologic examination of all fetal fluid samples was performed. The slides were prepared by standard cytopspin technique and stained by Diff-Quick. All fetal fluid samples were submitted for routine aerobic bacterial culture at the UKVDL.

Blood was collected daily from inoculation until 12 DPI or abortion. Blood was attained by jugular venipuncture into heparinized 10-mL tubes. Immediately after collection, the blood was centrifuged at 600×g for 10 min at 5° C. Plasma was harvested and preserved at -20° C until further analysis.

Experimental Induction of Ascending Placentitis

Placentitis was induced via intracervical inoculation of *S. zooepidemicus*. The experimental model for induction of bacterial placentitis in the mare was modified (Canisso *et al.* 2014a) from that previously described (Mays *et al.* 2002; LeBlanc *et al.* 2002). The bacterial inoculum was deposited midway intracervically with a semi-flexible artificial insemination pipette. The inoculum was contained in a 0.5-mL straw was deposited with the use of a stylet (Canisso *et al.* 2014a). The bacterial strain used was isolated from the placenta of a mare diagnosed with spontaneously ascending placentitis by the UKVDL. The bacterial isolate was preserved in cryovials containing skim milk at -80°C until use. Prior to inoculation, bacterial cultures were prepared and bacterial counts performed in standard fashion using blood-agar plates (incubated at 37°C) with serial dilution and plating to determine the number of colony-forming units. The bacterial inoculum was suspended in 0.5 ml of phosphate-buffered saline. Once inoculation was carried out, a swab was obtained from the remaining sample in the cryovial and cultured in similar conditions to assure that the bacteria in the inoculum remained viable.

Placental and Fetal Pathology

Placentas from control and inoculated mares, and all aborted fetuses underwent complete pathological (macroscopic and histopathologic examination) and microbiological evaluation (Hong *et al.* 1993). Briefly, the placenta was laid out and the chorioallantois, amnion, and umbilical cord were examined. Areas with no apparent lesions and areas presenting macroscopic lesions were sampled and preserved in buffered formalin for further histopathologic evaluation. Swabs were collected from the chorioallantois and used to perform aerobic bacterial culture (Hong *et al.* 1993). Fetuses were necropsied in routine fashion and internal organs (liver, spleen, stomach content, lung, heart, and trachea) were macroscopically examined and sampled for histopathologic evaluations and aerobic bacterial culture (Hong *et al.* 1993).

Electrophoresis

Total protein concentration of fetal fluids was determined with the Coomassie Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Samples (10 µg total protein) were diluted (1:1 v/v) with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and incubated at 95°C for 4 min followed by electrophoresis on 4-20% SDS polyacrylamide gels (Mini-PROTEAN TGX™ Precast Polyacrylamide Gel, Bio-Rad Laboratories, Hercules, CA, USA). The gels were run with a running buffer (Tris-Glycine 0.1% SDS) at 200V for 45 min. All gels were stained with Coomassie blue (0.05%, w/v) and subsequently de-stained with methanol (20%) and glacial acetic acid (10%). Images were obtained using an automatic reader (Cell & Biosciences, San Andreas, CA, USA).

Immunoblotting Alpha-fetoprotein

To confirm the specificity of the AFP antibody, western blotting was performed using fetal fluid samples. Samples (10 µg total protein) were diluted with Laemmli sample buffer (BioRad, Hercules, CA, USA) and incubated at 95°C for 4 min followed by electrophoresis on 10% SDS polyacrylamide gels. After electrophoresis, proteins were transferred to 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) using a wet electroblotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated for 1 h in 1×PBS with 0.05% Tween (1×PBS-T) with 5% non-fat dry milk and 3% BSA to block non-specific binding followed by incubation with goat anti-human AFP polyclonal antibody (1:1,000, Santa Cruz Biotechnology Inc., Dallas, TX, USA) diluted in 1×PBS-T with 0.3% BSA for 2 h at room temperature. Following several wash steps with 1×PBS-T, binding of primary antibody was detected using horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (1:5,000, R&D Systems, Minneapolis, MN, USA) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA). Images were visualized using an automatic analyzer (Cell & Biosciences, San Andreas, CA, USA). Dilutional parallelism (10 µg-1 µg) was confirmed with fetal fluid samples and equine fetal sera samples (data not shown). Plasma samples of pregnant mares (n=3), non-pregnant mares (n=3), and geldings (n=4) were immunoblotted, and AFP was not detected in plasma of these animals (data not shown).

Determination of Alpha-fetoprotein Concentrations

Concentrations of AFP in fetal fluids and plasma were measured with a heterologous commercial immunoassay (AFP Siemens Healthcare Diagnostics Tarrytown,

NY, USA) on a chemiluminescence platform (Immulite® 1000 platform; Siemens Healthcare Diagnostics Tarrytown, NY, USA). This assay has a range of 0.2 to 300 units of AFP, samples above the upper detection limit were diluted automatically by entering the dilution ratio in the platform with the diluent that accompanies the commercial kit. Since AFP is a glycoprotein of the albuminoid superfamily (i.e. closely related to albumin) (Deutsch 1991), we examined whether the AFP immunoassay would recognize equine albumin (i.e. the major plasma protein in horses). Plasma samples of geldings (n=4), equine fetuses (n=3; 300 d gestational age, thought to have high concentrations of AFP), pregnant mares in the second and third trimester (n=5) and non-pregnant mares (n=3) were assayed for AFP. According to the manufacturer, conversion of units/mL to ng/mL of human AFP is accomplished with a correction factor of 1.21.

Statistical Analyses

All the data analyses were carried out with JMP 10 (SAS Institute, Cary, NC, USA). Plasma concentrations of AFP were analyzed using a mixed model for days from abortion (DFA, -7 to 0), with time being treated as a continuous variable, mare as a random effect, and group as a fixed effect. Plasma concentrations of AFP were also analyzed using a mixed model for days post inoculation (DPI, days 0, 5, 6 and 7). Two way interactions were examined between fixed and continuous effects in both models. In both models, FFS did not significantly affect ($P > 0.05$) plasma AFP concentrations, and data from both subsets (i.e. mares having FFS) were combined within groups for further analyses. Post hoc comparisons for DPI were performed with Tukey's HSD test.

6.4. Results

Mares with experimentally induced ascending placentitis aborted on average 9.4 ± 1.3 DPI (range 5 to 25 days). One mare in the control group (subgroup FFS) aborted 8 days after the last FFS; placental and fetal examination revealed no pathologic lesions and culture yielded saprophytic non-pathogenic bacteria and negative virus isolation (data not shown). Therefore, the cause of abortion in this mare was not determined. Three mares (17.6% of the mares that were inoculated) failed to develop placentitis after a single inoculation. One of these mares (subgroup without FFS) was not re-inoculated, a normal foal was delivered, and there was no evidence of placentitis. The two other mares (one with and another without FFS) that failed to develop placentitis were re-inoculated 14 days after the first inoculation. One mare (subgroup without FFS) aborted 20 days after re-inoculation, whereas the other mare (subgroup with FFS) aborted 9 days after re-inoculation. The mare that was not re-inoculated (treatment group) and the control mare that aborted were excluded from the analyses. Similarly, the first 14 days of samples for the two re-inoculated mares were excluded from the analyses.

Twelve fetuses and placentas from inoculated mares had *S. zooepidemicus* as the sole isolate; *E. coli* was cultured in association with *S. zooepidemicus* in 3 fetuses and placentas from inoculated mares. One fetus and its accompanying placenta had *Actinobacillus equuli* isolated in association with *S. zooepidemicus*. With the exception of the mare that failed to develop placentitis after experimental induction (above) and the control mare with FFS that aborted, all mares that aborted developed gross and histopathologic lesions consistent with ascending placentitis (Hong *et al.* 1993) (data not shown). All mares in the control group delivered macroscopically, histologically and

microbiologically (i.e. non-pathogenic saprophytic bacteria) normal placentas (data not shown).

Fourteen fetuses from aborting mares in the group with experimentally induced placentitis were nonviable at delivery. Two foals were delivered alive, one foal was delivered alive after spontaneous delivery, and one foal was delivered upon induction of parturition due to sepsis in its dam. Both of these foals had clinical evidence of sepsis and were euthanized immediately post-delivery.

Except one mare that had positive bacterial culture at 5 DPI, none of the mares in the control group or the group with experimentally induced ascending placentitis presented positive bacterial culture of the fetal fluids. As expected, *S. zooepidemicus* was isolated in the amniotic fluid of this mare.

Several protein bands of different molecular weights were present in both allantoic and amniotic fluids (Fig.6.1). A protein band consistent (~68 kDa) with alpha-fetoprotein (AFP) was present in all samples. Two other protein bands were present in most samples (i.e. ~250 kDa, 43/44 samples; ~200 kDa, 39/44 samples). To confirm the identity of the 68-kDa protein, immunoblotting for AFP was performed in all samples (Fig. 6.2). A single band consistent with AFP was present in all samples of both allantoic and amniotic fluid.

As expected, concentrations of AFP were below the limits of detection for the immunoassay (0.2 units/ml) for geldings and non-pregnant mares, there appear no apparent crossreactivity with albumin. As predicted, equine fetuses presented high concentrations of AFP in plasma (ranging from 600 to >1200 units/mL). The manufacturer states that the assay is not accurate for samples with concentrations above 600 units/mL; despite possible dilution, samples obtained from equine fetuses can be interpreted as having very high

concentrations of AFP. As part of the validation of this assay, we examined the concentrations of AFP (units/mL) during the last five months of gestation, the results were: 7th (0.8 ±0.1), 8th (0.5 ±0.05), 9th (0.4±0.03), 10th (0.3 ±0.02) and 11th (0.6 ± 0.2) months of gestation (n = 5 mares). Based upon this biological validation, it appears that the heterologous human AFP recognized this protein in plasma of pregnant mares, fetal fluids, and in equine fetal plasma. The AFP immunoassay confirmed the presence of AFP in all fetal fluid samples from mares with experimentally induced ascending placentitis and gestationally age-matched control mares (Table 6.1). Analyses of maternal plasma concentrations of AFP relative to DPI demonstrated no effect of group (p=0.73); however, there were effects of time (DPI, p<0.0001) and a time-by-group interaction (DPI*Group p<0.06) (Fig.3). Post hoc comparisons across days and groups showed that AFP concentrations at 7 DPI were significantly higher in mares with experimentally induced placentitis when compared to controls (p<0.05) (Fig 6.3). For the analyses of maternal plasma AFP relative to DFA, there were effects of group (p<0.0001), time (DFA p=0.0002) and a group by time interaction (Group*DFA p<0.001) (Fig 6.4).

Upon cytological examination of the amniotic fluid, we observed that this fluid is rich in epithelial cells, and that scant debris is present surrounding these cells, contrarily, the allantoic fluid is acellular and remarkable amorphous material was observed (Fig 6.5). There were no apparent differences between mares in the control group and mares with experimentally induced ascending placentitis, except for one mare that presented numerous bacterial colonies surrounding the epithelial cells in the amniotic fluid (Fig 6.5).

Figure.6.1: Representative one-dimensional SDS-PAGE of allantoic and amniotic fluids, in mares with experimentally induced ascending placentitis and gestationally age-matched control mares at 0 (D0), 5 (D5) and 12 (D12) days post-inoculation. Day 0 was defined as the day of inoculation, or commencement of sampling for mares in the control group. L: represents the molecular weight markers. Mare 1: 0-12 represent allantoic samples; Mare 2: 0-5 amniotic samples; Mare 3 & 4: represent allantoic samples. Several proteins were present in these samples; however, one protein band (68 kDa) was consistently present in all samples.

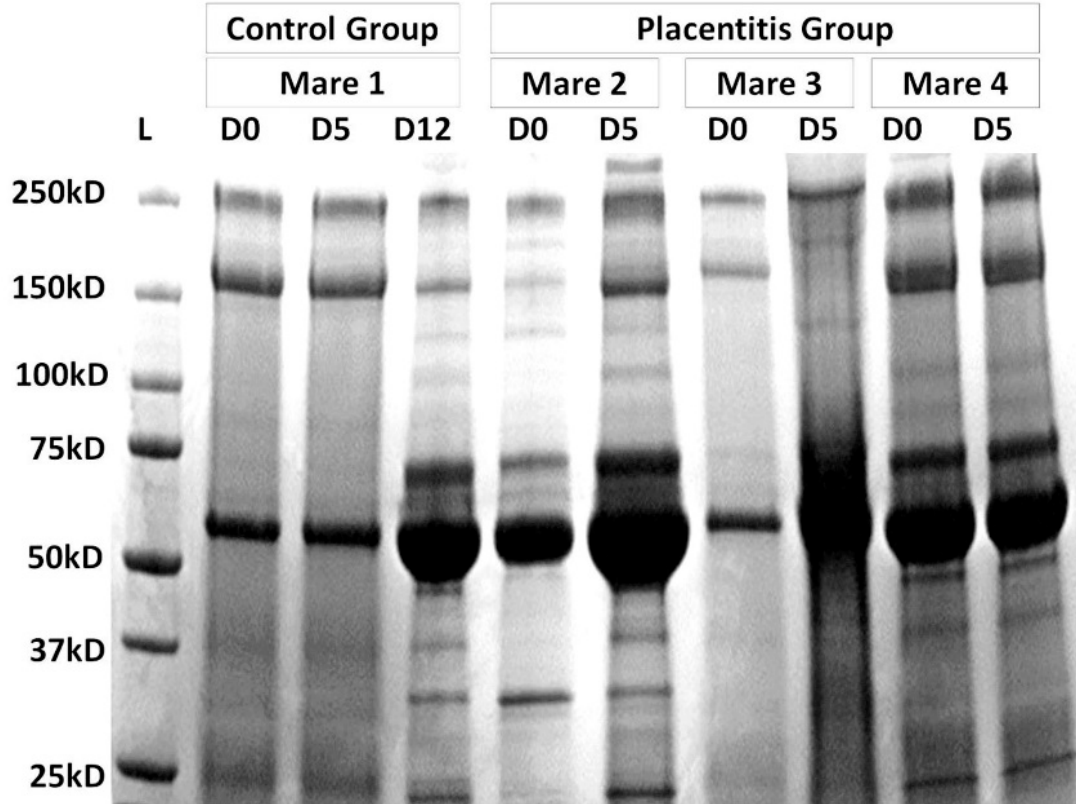


Figure 6.2: Immunoblotting for alpha-fetoprotein (AFP) in allantoic and amniotic fluids of mares with experimentally induced ascending placentitis and gestationally age-matched control mares. Fetal fluid samples were collected at days 0, 5 and 12. Day 0 was defined as the inoculation day for mares in the placentitis group and commencement of sampling for mares in the control group. A single immunoreactive protein (~68kDa) was detected with AFP in all samples of both types of fetal fluids. All the bands in the figure above represent serial sampling from 3 mares.

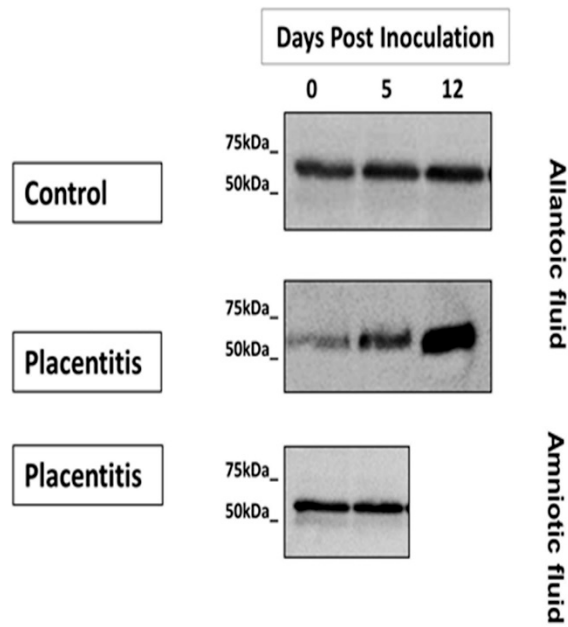


Figure. 6.3. Alpha fetoprotein (AFP) concentrations in plasma of mares carrying normal pregnancies and mares with experimentally induced ascending placentitis for 0, 5, 6 and 7 days post inoculation (DPI). There were no group differences ($p=0.73$). There were effects of time (DPI, $p<0.0001$) and a time-by-group interaction (DPI*Group; $p<0.06$). Post hoc comparisons across days and groups showed that AFP concentrations at 7 DPI were significantly higher in mares with experimentally induced placentitis compared to controls ($p<0.05$). To convert units/mL to ng/mL, multiply units/ml by a correction factor of 1.21. *Within days different letters denotes significant statistical differences ($p<0.05$).

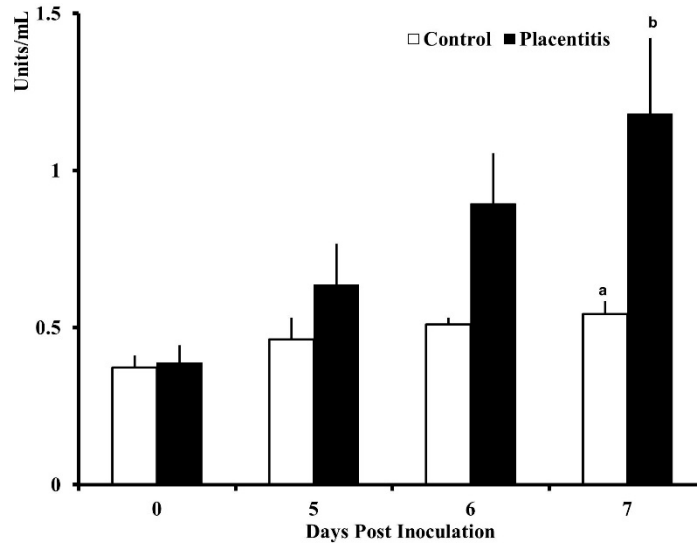


Figure 6.4: Alpha fetoprotein (AFP) concentrations in plasma of mares carrying normal pregnancies and mares with experimentally induced ascending placentitis for days from abortion (DFA, -7 to 0). There were effects of group ($p < 0.0001$), time (DFA $p = 0.0002$) and a group-by-time interaction (Group*DFA $p < 0.001$).

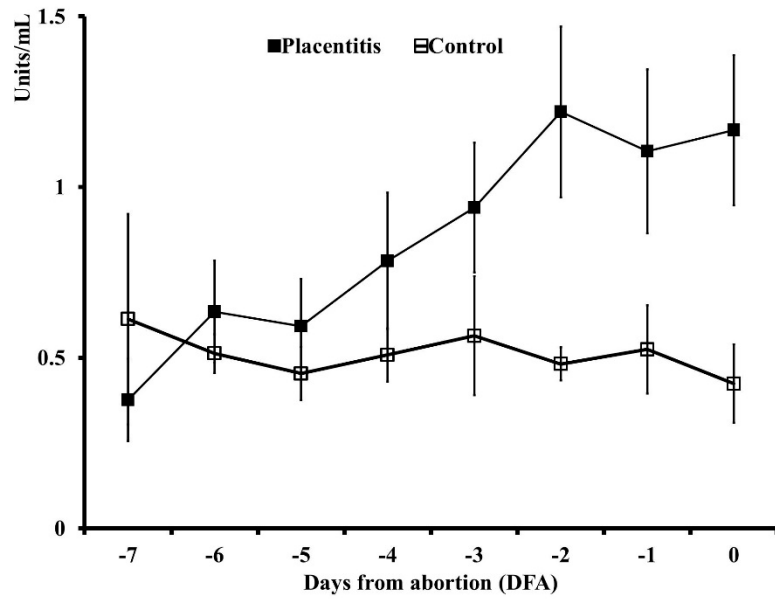


Figure 6.5. Representative cytological preparations of the fetal fluids in mares with experimentally induced ascending placentitis and gestationally age-matched control mares. These slides were prepared by cytospin and stained by Diff-Quick. A & B represents the allantoic fluid of a control mare at 0 and 5 DPI; C & D represents the amniotic fluid at 0 and 5 DPI; E & F Represents the allantoic fluid at 0 and 5 DPI in mares with experimentally induced placentitis; G & H represents the amniotic fluid at 0 and 5 DPI. The amniotic fluid was rich in epithelial type cells and presented relative scant debris among cells; allantoic fluid was acellular, and remarkable amorphous material was observed. There were no apparent differences between mares in the control group and mares with experimentally induced ascending placentitis, except for one mare (represented in G & H above), note numerous bacterial colonies surrounding the epithelial cells (H), *Streptococcus equi* subspecies *zooepidemicus* was cultured from this mare (contained in the circle). (Scale bars = 25 μ m).

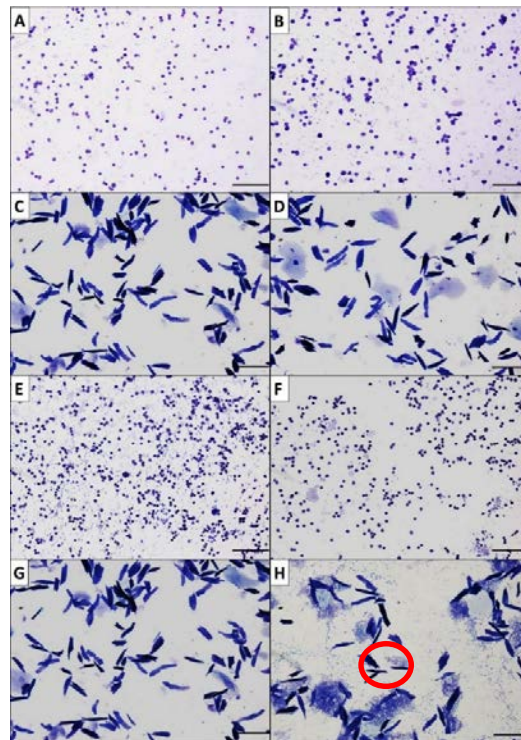


Table 6.1: Concentration of alpha-fetoprotein (AFP) (units/mL) in the allantoic and amniotic fluids of mares with experimentally induced ascending placentitis and gestationally age-matched control mares at days 0, 5 and 12 post-inoculation (DPI). Day 0 was defined as the day of inoculation or the commencement of sampling for mares in the control group. Numbers in parentheses represent number of samples assayed. Data are expressed as Mean \pm SEM.

Fluid type	Day 0		Day 5		Day 12	
	Control	Placentitis	Control	Placentitis	Control	Placentitis
Allantoic	120.6 \pm 14.3(5)	107.6 \pm 18.6(5)	100 \pm 12(6)	168 \pm 39.2(5)	110 \pm 14.5(4)	166 \pm 73.8(2)
Amniotic	111 \pm 5.6(2)	196(1)	65.5(1)	385 \pm 148(3)	260 \pm 201(3)	—

To convert units/mL to ng/mL, multiply units/mL by a correction factor of 1.21.

6.5. Discussion

To our knowledge, this is the first study to confirm the presence of AFP in the allantoic and amniotic fluids of mares during the third trimester of pregnancy. However, this finding was not surprising, as studies in other mammals had long shown that this protein is present in the fetal fluids throughout pregnancy (Okano *et al.* 1977, Smith *et al.* 1979, Luft *et al.* 1984, Deutsch 1991). Previously, in the horse, indications that AFP was present in the fetal fluids late in pregnancy came from a study conducted with slaughter specimens (Zanella 2008), whereby the author sampled mares across pregnancy and based on fetal measurements, the gestational age was estimated. Upon electrophoresis, using 1D-SDS-PAGE, the author observed a protein band consistent with AFP, in all fetal fluids samples (Zanella 2008).

In the present study, AFP was elevated in plasma of mares with experimentally induced ascending placentitis. This increase may be due to up regulation and/or an increased vascular permeability across the placenta. For either mechanism, this finding provides a new area of investigation, as we confirmed that AFP, a protein produced by the fetus (and present in the fetal fluids), can be detected in the maternal circulation and that disease (i.e. placentitis) can increase the concentration of AFP in the maternal circulation. It is highly suggestive, that similar to AFP, other fetal proteins might cross the placenta upon normal and pathological conditions.

Despite the extensive use of AFP in human medicine as a marker for placental and fetal health for several years, apparently, this is the first study to report AFP in mares with experimentally induced placentitis. Recent studies in human medicine have shown the importance of AFP as a prognosticator of neonatal infection (for review, Buhimschi &

Buhimschi 2012). Although AFP is elevated in plasma of mares with experimentally induced placentitis, it remains to be determined if similar elevations of AFP concentrations in plasma occur in mares with spontaneous placentitis. It is worth to note, that AFP was elevated in the later course of the disease, perhaps this protein may have limited application as early marker for placentitis, but it may have clinical utility as later marker for placentitis (i.e. chronic stage of placentitis), thus, probable it may be a useful prognosticator for placentitis since it changed late stages of disease.

Contrary to our results, a study in pregnant cows experimentally infected with *Aspergillus fumigatus*, *Sarcocystis cruzi* or *Campylobacter fetus* failed to demonstrate elevation of AFP in association with placentitis induced by these infectious agents (Baetz *et al.* 1981). Biological differences between species and pathogenesises of these diseases in cattle and horses, may explain the contrasting findings. It is worth-noting, that cows may take several days to expel a dead fetus; however, in mares this is not usually common. Differences between the assays with regards to sensitivity may also explain the contrasting results.

In a clinical trial involving a large group of commercial mares, Sorensen and collaborators (1990) developed an assay for a molecule termed “equine fetoprotein”. From that report, it is unclear whether this protein is AFP or if it is some other molecule derived from the fetoplacental unit. If “equine fetoprotein” is similar to AFP, their findings concur with ours that placentitis induces changes in peripheral concentrations of AFP. Unfortunately, the authors did not report the absolute concentrations derived in the study, under the allegation that the assay was under revision for patent protection.

Therefore, we cannot compare absolute concentrations if indeed, they measured the same molecule.

As previously suggested, but not shown by the authors (Lyle *et al.* 2006), the cytological appearance of these fluids are distinct enough that amniotic and allantoic fluid can be easily differentiated from one another, thus creatinine and electrolytes measures can be replaced by the cytological examination. An interesting aspect of cytology of the fetal fluids is the fact that only one mare had detectable bacteria, none of the remaining mares had evidence of bacterial colonies; aerobic bacterial culture confirmed the presence of the bacteria. However, it is worth-noting that amniotic fluid represented the minority of samples in the present study; it is possible that more mares would have presented positive bacterial cultures of the fetal fluids if more amniotic samples were harvested in the present study.

Since we performed several bacterial cultures in the allantoic fluid, and none of the samples yielded positive bacterial cultures, despite severe placentitis, we suggest that during spontaneous infection (i.e. the bacteria infecting the cervical pole of the placenta), bacteria likely gain access to the fetus through the umbilical cord. It seems very logical to us that if bacteria would gain access to the fetus through the allantoic fluid, we would have seen evidence of this infection (i.e. positive bacterial culture of the allantoic fluid). On the other hand, positive culture of the amniotic fluid may suggest that the fetus was first infected, and then respiratory and gastrointestinal secretions of bacteria into the amniotic fluid may happen as a secondary event to the fetal infection, rather than a primary event. Therefore, we suggest that infection of allantoic fluid may not play an important role in the mechanism leading to the fetal infection.

In conclusion, AFP is present in all fetal fluid samples and is increased in plasma of mares with experimentally induced ascending placentitis, either due to upregulation and/or increased vascular permeability across the placenta. Cytological preparations of the fetal fluids can be used to identify its origin. It appears that infection of the allantoic fluid may not play an important role in the mechanism leading to the fetal infection. Although, experimentally induced placentitis was associated with an elevation in plasma concentrations of AFP.

6.6. Acknowledgments

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Chapter 7
Proteomics of the Fetal Fluids in Mares Carrying Normal Pregnancies and in Mares
with Experimentally Induced Ascending Placentitis

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7.1. Summary

Little is known about the protein composition of equine fetal fluids. The objectives of this study were to (i) describe the normal protein composition of the amniotic and allantoic fluids of mares at 300d of gestation, and (ii) compare the protein composition of the allantoic fluid before and after experimental induction of ascending placentitis. In study I, three mares carrying normal pregnancies were euthanized at 300 d of gestation and fetal fluids were collected and preserved at -80°C until further analyses. In study II, three control mares and three mares with experimentally induced ascending placentitis had allantoic fluid collected by transabdominal, ultrasound-guided puncture. Fetal fluids were analyzed with a high-throughput proteomic technique (LC-MS/MS) to characterize the protein composition of fetal fluids in late-term mares carrying normal pregnancies and allantoic fluids from mares with experimentally induced placentitis. Approximately 100 proteins were present in both allantoic and amniotic fluids. Three proteins (i.e. lactoferrin, serum

transferrin, alpha-1-proteinase were present in allantoic samples and were elevated at least two fold in mares with experimentally induced ascending placentitis. It remains to be determined how these changes in fetal fluid proteins are related to ascending placentitis and whether they have any diagnostic application for placental disease in the mare.

7.2. Introduction

Ascending placentitis is an important cause of abortion in mares, its diagnosis is based on clinical signs (vulvar discharge and premature mammary gland development \pm milk streaming) and transrectal ultrasonography (Troedsson & Macpherson 2011). The clinical signs and associated ultrasonographic changes (e.g. placental separation) are variable and may only be present in advanced stages of disease; therefore, early diagnosis would potentially improve the likelihood of a delivery of a viable foal. Recently, our laboratory has been focused on developing new diagnostic tests for equine placentitis. Several markers have been investigated in experimental cases and some appear to be useful tools to detect experimentally induced placentitis. Among these markers, acute phase proteins, particularly serum amyloid A, appear to be a sensitive but nonspecific marker for placentitis (Canisso *et al.* 2012, Canisso *et al.* 2014).

In the horse, little is known about the protein composition of fetal fluids (Williams *et al.* 1993, Paccamonti *et al.* 1995, Zanella 2008, Zanella *et al.* 2013). However, in human medicine, amniotic fluid has been the focus of numerous reports (Ruetschi *et al.* 2005, Buhimschi *et al.* 2008, Romero *et al.* 2008 Buhimschi &Buhimschi 2012). Two dimensional gels and LC/MS have been used to identify proteins in the amniotic fluid of humans (Romero *et al.* 2008 Buhimschi &Buhimschi 2012, Tambor *et al.* 2012, Tambor *et al.* 2013) .To date, studies conducted with horses were carried out to examine the electrolyte composition or to investigate the presence of specific proteins present in other body systems (Paccamonti *et al.* 1995, Holdstock *et al.* 1995, Zanella *et al.* 2013, Canisso *et al.* 2014). The protein composition of the equine fetal fluids has received limited attention either during normal pregnancy (Zanella 2008) or during pathologic conditions (Finger *et al.* 2014). Proteomic analyses applied to study the protein composition of the

equine fetal fluids in earlier studies (Zanella 2008, Finger *et al.* 2014) provided only limited identity of protein composition; therefore a high-throughput proteomic technique would allow more definitive identification of proteins in equine fetal fluids.

In the recent years, high-throughput proteomics techniques have been used to identify new diagnostic markers in the amniotic fluid of women experiencing chorioamnionitis or to identify fetal defects (for review, Klein *et al.* 2014). The application of a high-throughput proteomic technique such as liquid chromatographic mass spectrometry (LC-MS) associated with bioinformatics tools provides an opportunity to identify proteins that have not yet been described in equine fetal fluids . We hypothesize that the protein composition of the amniotic fluid differs from that of allantoic fluid and that ascending placentitis alters the protein composition of the allantoic fluid. The objectives of this study were to (i) describe and compare the protein composition of amniotic and allantoic fluids of mares carrying normal pregnancies at 300d gestation (ii), and to compare the protein composition of allantoic fluid before and after experimental induction of ascending placentitis in normal and control mares.

7.3. Material and methods

Clinically healthy adult horse mares were enrolled in these experiments. Mares were maintained at the Maine Chance Farm, Department of Veterinary Science, University of Kentucky, Lexington, KY, USA. All the experimental protocols were approved by the Institutional Animal Use and Care committee at the University of Kentucky (Project #2010-0769). All the animals were kept in paddocks and supplemented with hay, grain and water *ad libitum* along with trace minerals. Immediately pre-abortion or prior to normal parturition the mares were kept in individual stalls (16 x 16 feet) during the night and turned out in pasture during the day.

Study I: Protein composition of amniotic and allantoic fluids in mares carrying normal pregnancies in the third trimester of pregnancy

Allantoic and amniotic fluids from mares (n=3) carrying normal singleton pregnancies, were collected after euthanasia at 300 days of gestation. Within approximately 20 minutes postmortem, samples were harvested through direct access of both fetal compartments (i.e. allantoic and amniotic) and frozen immediately. A syringe was used to collect aliquots of the fetal fluids, aliquots of 2 mL were loaded into cryovials and samples were immersed in liquid nitrogen. From liquid nitrogen, samples were stored at -80°C until further analyses.

Immediately before analyses, samples were slowly thawed on ice. A fixed volume of 10 µl of fetal fluid was submitted to vacuum centrifugation for 3 minutes at 38°C to concentrate the final volume of protein. Samples (5 µL) were diluted (1:1 v/v) with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and incubated at 95°C for 4 min followed by electrophoresis on 10% SDS polyacrylamide gels (Mini-PROTEAN

TGX™ Precast Polyacrylamide Gel, Bio-Rad Laboratories, Hercules, CA, USA). The gels were run with Tris-Glycine 0.1% SDS at 200V for 20 min. All gels were briefly stained with Coomassie blue (0.05%, w/v) and subsequently de-stained with methanol (20%) and glacial acetic acid (10%). Images were obtained using an automatic reader (Cell & Biosciences, San Andreas, CA, USA).

After destaining, proteins were analyzed at the laboratory of Protein Analyses (Department of Molecular and Cellular Biochemistry, University of Kentucky) where proteins were excised from the gel and digested with trypsin. Peptides resultant from this digestion were analyzed by tandem LC-MS. Peptides obtained with from the LC-MS analyses were analyzed by an ion trap mass analyzer (Orbitrap®) (Zubarev & Makarov 2013). Resulting spectra were submitted for a MASCOT search sequence for similarity against other mammalian proteins present in the SwissProt database (excluding humans, rats and mice). Proteins present were identified by uniprot entries (www.uniprot).

Study II: Protein composition of the allantoic fluid in mares carrying normal pregnancies and mares with experimentally induced ascending placentitis

Allantoic fluid samples from six mares carrying normal pregnancies (265-280 days of gestation) were used in this study. Three mares were non-inoculated controls and three mares had experimentally induced ascending placentitis. As noted in a parallel publication, ascending placentitis was induced via intra-cervical inoculation of *Streptococcus equi* subspecies *zooepidemicus* (Canisso *et al.* 2014a). Allantoic fluid was obtained by transabdominal ultrasound guided puncture at 0 and 5 days post inoculation/commence of the study for control mares (Canisso *et al.* 2014b).

To assure that control mares had normal pregnancies and to confirm that experimentally inoculated mares developed placentitis, all placentas were submitted for routine histopathologic examination (Hong *et al.* 1993). All mares with experimentally induced ascending placentitis had inflammatory necrotic changes consistent with bacterial placentitis and *Streptococcus equi* subspecies *zooepidemicus* was the single isolate in the placenta and aborted fetuses. All mares in the control group delivered normal foals and placentas.

Only descriptive analyses were used to characterize proteins present in the amniotic and allantoic fluids of normal mares. In study II, paired t-tests were used to compare abundance of proteins present in allantoic fluid samples from mares at Days 0 and 5, and unpaired t-tests were used to compare abundance of proteins across groups. Protein abundance was based upon spectral count (Zhu *et al.* 2010). Significance was set at $p < 0.05$

7.4. Results

Based upon non-redundant sequences, a total of 37 proteins were identified in the amniotic fluid (Table 7.1) and 59 proteins were identified in the allantoic fluid of mares carrying normal pregnancies at 300 days of gestation (Table 7.2). Most of the proteins described are secretory proteins, structural proteins. The list of all proteins including ion scores are listed in the appendices (D). For study II, a total of eight proteins were present in all allantoic fluid samples (Table 7.2), and among these eight proteins, lactotransferrin, serotransferrin and alpha-1-antiproteinase were increased at least two-fold from 0 to 5 days post inoculation in allantoic fluid from mares with experimentally induced ascending placentitis ($p < 0.05$) and remained unchanged in control samples. Furthermore, lactotransferrin, serotransferrin and alpha-1-antiproteinase were significantly increased in

allantoic fluid at day 5 post inoculation in mares with experimentally induced placentitis compared to control ($p < 0.05$) (Table 7.3).

Table 7.1: Proteins identified in amniotic fluid samples from normal mares at 300d gestation based upon LC-MS-MS.

Proteins	Proteins
Serum albumin	Alpha-1B-glycoprotein
Fibronectin	Glycoprotein hormones alpha chain
Apolipoprotein	Actin-related protein 3
Gelsolin	Stress-70 protein, mitochondrial
Alpha-fetoprotein	Angiotensinogen
Keratin, type II cytoskeletal 1	Apolipoprotein D
Keratin, type II cytoskeletal 5	Angiotensin-converting enzyme 2
Keratin, type II cytoskeletal 7	Decorin
Keratin, type I cytoskeletal 10	Apolipoprotein E
Keratin, type II cytoskeletal 75	Surfeit locus protein 6
Alpha-2-macroglobulin	Lactase-phlorizin hydrolase
Lactotransferrin	Proteasome subunit beta type-8
Lumican	Type III iodothyronine deiodinase
Coatomer subunit beta	Alpha-1-antiproteinase 2
Hemoglobin subunit beta	Clusterin
Alpha-1-antiproteinase	Melanotransferrin
Major allergen Equine chromosome 1	Uteroglobin
Serotransferrin	Transmembrane protein 198
Phosphatidylinositol 3-kinase regulatory subunit gamma	

Table 7.2: Proteins identified in allantoic fluid samples from normal mares at 300d gestation based upon LC-MS-MS.

Proteins	
Serum albumin	Peptidase inhibitor 16
Fibronectin	Cationic trypsin
Hemoglobin	Actin, alpha skeletal muscle
Alpha-fetoprotein	Inter-alpha-trypsin inhibitor
Serotransferrin	Cadherin-2
Alpha-1-antiproteinase	Regulator of G-protein signaling
Annexin A1	Thrombospondin-1
Keratin, type II cytoskeletal 5	G-protein coupled receptor
Keratin, type II cytoskeletal 75	Ubiquitin-40S ribosomal protein S27a
Keratin, type II cytoskeletal 7	Polyubiquitin-B
Calsequestrin-2	Secretogranin-1
Keratin, type II cytoskeletal 79	Phytanoyl-CoA dioxygenase, peroxisomal
LXR motif-containing protein 7	Glycerate kinase
Amyloid beta A4 protein	Hemoglobin fetal subunit beta
CD44 antigen O	Tenascin
Insulin-like growth factor-binding protein	Peptidase inhibitor
Alpha-1B-glycoprotein	Alpha-1-antitrypsin
Keratin, type I cytoskeletal 10	Cationic trypsin
Ezrin	Calsequestrin-2
Moesin	SPARC
Radixin	Inter-alpha-trypsin inhibitor
SPARC	LXR motif-containing protein 7
G-protein coupled receptor	G-protein coupled receptor
14-3-3 protein zeta/delta	Uroplakin-1b
Coatomer subunit beta'	14-3-3 protein zeta/delta
Actin-related protein	Surfeit locus protein 6
Keratin, type II cytoskeletal 1	Metalloproteinase inhibitor 2
Metalloproteinase inhibitor 2	SCO-spondin
Collagen alpha-2 (IV) chain	Major allergen Equ chromosome 1

Table 7.3: List of proteins present in the allantoic fluid of mares carrying normal pregnancies and mares with experimentally induced placentitis based upon LC-MS-MS.

Proteins
Lactotransferrin
Hemoglobin
Inter-alphatrypsin inhibitor
Serotransferrin
Serum albumin
Alpha-1-antiproteinase
Alpha-fetoprotein
Fibronectin

7.5. Discussion

To the authors' knowledge, this is the first report using high-throughput protein sequencing to study the protein composition of fetal fluids in mares with normal pregnancies and in mares with experimentally induced placentitis. Several proteins were newly identified both in the allantoic and amniotic fluids. Our findings may guide future research aiming to investigate the physiology of the fetal fluids and potential role of proteins in the fetal maturation. Since several proteins were newly known to be present in equine fetal fluids, investigators can use other techniques to quantify specific proteins described by us.

Interestingly, most of the proteins detected in equine fetal fluids are similar to the proteins present in the maternal plasma. It should be noted that the type of placentation in the horse limits exchange of large molecules, therefore, proteins present in the fetal fluids are likely originated in the fetus, and for instance some proteins like keratin could originate from fetal skin. It is worthnoting that some molecules present in the fetal fluids are released into the maternal circulation (e.g. alpha-fetoprotein) and that placentitis increases its

concentration in maternal plasma, probably related to increased permeability (Chapter 6). In the present study, three secretory proteins (i.e. serum transferrin, lactotransferrin and alpha-antiproteinase) were increased at least two-fold in the allantoic fluid of mares with experimentally induced placentitis. It is uncertain whether the increase in these proteins observed here can be used to assess fetal well-being. The increase in these three plasma proteins in the fetal fluids suggest that the increased permeability may have occurred. Interestingly, lactoferrin has anti-bacteriostatic capabilities (Kolm *et al.* 2006). Since lactoferrin is produced and secreted by the endometrium and is increased in endometritis (Kolm *et al.* 2006), increase secretion of this enzyme in the fetal fluids may reflect the presence of endometritis-associated with placentitis. It is unknown whether placentitis is associated with increased secretion of lactotransferrin in the fetal fluids, although this hypothesis should be tested. Serum transferrin is responsible for ion transport, in human medicine genetic defects have been associated with excessive and absent congenital serum transferrin (Bartnikas 2012), in equine medicine, the role of serum transferrin in equine placentitis is unknown.

Proteomic studies in humans demonstrated that amniotic fluid presented more than 800 types of proteins (Cho *et al.* 2007). However, in our study about 100 proteins were found to be present. Reasons for that discrepancy can be associated with biological variations, different methodology and the current data base available for proteins of equine origin. In the present study, we use 1-dimensional electrophoresis to separate the electrolytes known to be present in the fetal fluids (Paccamonti *et al.* 1995). It is possible that some proteins that were overabundant in the samples precluded identification of less abundant proteins. Alternative methods to isolate proteins, such as protein precipitation

could have been used to remove electrolytes and then concentrate proteins for LC/MS. Application of different methods would likely result in different outcomes; however, it was outside the scope of this study to compare different methods.

Alpha-1-antitrypsin is an acute phase protein. It which acts as an anti-inflammatory protein, which is increased several-fold upon inflammation (Kolarich *et al.* 2006, Ahsan *et al.* 2014). It is believe that this protein has protective effects against several lytic proteins secreted by neutrophils. In human medicine, the levels of alpha-1-antitrypsin in plasma have been used as a diagnostic and prognostic tool for keratoconjunctivitis (Ahsan *et al.* 2014). In horses, this protein has been described but its role in placentitis is unknown. Because this protein was elevated in fetal fluids in mares with experimentally induced placentitis, and because it is an acute phase protein, alpha-1-antitrypsin inhibitor should be evaluated as a possible biomarker for equine placentitis.

Protein composition of the fetal fluids has been the focus of numerous publications in human medicine, however, these fetal fluids have received limited attention in equine reproductive medicine, particularly in mares with placentitis. In the present study, protein composition of the fetal fluids, determined by high-throughput proteomic techniques (i.e. LC-MS-MS) identified several proteins in the amniotic and allantoic fluids of mares carrying normal pregnancies. In addition, we demonstrated several proteins that were previously undescribed in the allantoic fluids of mares with placentitis. Further ongoing evaluations may determine whether the any of the three proteins found to be elevated in the allantoic fluid are also elevated in plasma. It remains to be determined whether the increase is related to protein extravasation or to increased production of these proteins or whether these proteins are of fetal or maternal origin. For either mechanism, our results in

both studies open a new field of investigation regarding diagnostic markers present in the fetal fluids that may be increased or reduced in maternal plasma.

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Chapter 8

General Conclusion and Future Directions

Bacterial placentitis is an important cause of pregnancy loss in mares. Two types of placentitis were examined in this dissertation: ascending and nocardioform placentitis. Both types of placentitis occur late in pregnancy, and acute-chronic inflammatory lesions are present upon delivery/abortion. Acute infections of the gravid equine uterus during mid-gestation are commonly associated with fulminant bacterial infection of the fetus with minimal or no lesions present in the placenta, this type of infection is unnoticed and invariably result in abortion. On the other hand chronic infections of the placenta occurring from the mid to late term are associated with acute-chronic lesions present in the placenta. Ascending placentitis and nocardioform placentitis manifest as chronic insidious infections.

Despite the fact that the first diagnosis of nocardioform was made three decades ago, little is known about the pathogenesis of nocardioform placentitis. Until now, all our knowledge about nocardioform placentitis in mares results from epidemiological observations carried out by the University of Kentucky Veterinary Diagnostic Laboratory. Lack of a research model to experimentally induce nocardioform placentitis has limited our understanding of the disease. Since nocardioform lesions are located at the base of the uterine horn and body, it has been speculated that the bacteria gain access to uterus during breeding or other gynecological interventions; however, inoculation of mares with *C. equi* after artificial insemination did not result in nocardioform placentitis. In addition, endometrial swabs collected from commercial Thoroughbred mares immediately before or after mating did not provide evidence or suggest that nocardioform placentitis is

transmitted during mating. Furthermore, oral, respiratory, and intravenous inoculation of *C. equi* did not result in clinical nocardioform placentitis. These findings demonstrated that the simple presence of *C. equi* was not sufficient to induce disease. This may indicate that other unidentified factors may play a role in disease pathogenesis. Alternative routes of inoculation (e.g. ocular), using different bacterial isolates and species of microorganisms, different doses and intervals of inoculation should be tested.

The second and major component of this dissertation involved the identification of diagnostic markers for placentitis. Because ascending bacterial placentitis is readily and predictably induced using existing experimental models, this type of placentitis was used to identify diagnostic markers for placentitis in maternal plasma and fetal fluids. Currently, the diagnosis of ascending placentitis is based on transrectal ultrasonography and clinical signs. Often clinical signs and associated ultrasonographic changes are only present in well-advanced stages of placentitis, thus there is a real need to investigate new markers for placentitis. In the present dissertation, three main fields were explored to identify new markers for bacterial placentitis as follows: acute phase inflammatory proteins, steroid hormones produced by the fetal placental unit, and protein composition of the fetal fluids.

Of the three acute phase proteins investigated, serum amyloid A and haptoglobin but not fibrinogen increased remarkably in association with experimentally induced ascending placentitis. Until very recently, placentitis was thought to be a disease of the equine pregnant uterus without detectable inflammatory changes in maternal plasma. The anecdotal suggested lack of changes in WBC cells and fibrinogen in maternal plasma, as these two are the most widely used means to evaluate inflammation in the horse. Indeed, our experimental findings here confirmed that placentitis was not associated with changes

in WBC and fibrinogen. The findings that serum amyloid A and haptoglobin are elevated in maternal plasma raise the question of the origin of this elevation. It is likely that this detectable increase in maternal plasma has some involvement of the endometrium, since experimentally induced endometritis using a high inoculating dose was associated with systemic elevation in serum amyloid A. It is likely that other inflammatory markers change in mares with placentitis, thus further investigation in this particular field may enhance our knowledge about placentitis and may allow us to identify new markers for placentitis.

Steroids produced by the fetoplacental unit including androgens, estrogens and progestins were investigated in the present dissertation. The use of fetoplacental unit steroids as markers for placental health are not necessary a novel idea, however, we investigated steroids (i.e. testosterone, estrone sulfate, DHEA-S, and estradiol 17 β) that have not been investigated yet in mares with placentitis. Androgens appear to be poor marker for experimentally induced ascending placentitis. On the other hand, concentration of estradiol 17 β and estrone sulfate were reduced in mares with experimentally induced placentitis. Estrone sulfate has been suggested for years to be a good marker to assess fetal-wellbeing; however, until now it has not been evaluated in mares with placentitis. Mares with experimentally induced placentitis presented reduced concentrations of estrone sulfate close to abortion, and this probably limits the use of this molecule as a biomarker for placentitis in mares. On the other hand, estradiol 17 β was reduced in mares with experimentally induced placentitis, and this molecule appears to be a good potential marker for placentitis in mares.

Protein composition of the fetal fluids has been the focus of numerous publications in human medicine; however, analysis of fetal fluids has received limited attention in

equine reproductive medicine. In the present dissertation, different methods were used to study the protein composition of the fetal fluids. In the first study, alpha-fetoprotein was characterized as a major protein present in the equine fetal fluids, and this protein was elevated in plasma of mares with placentitis. In a second study, conducted to evaluate the protein composition of the fetal fluids, using a high-throughput proteomic technique (i.e. LC-MS-MS) several new proteins were discovered in the amniotic and allantoic fluids of mares carrying normal pregnancies. In addition, for the first time using a LC-MS-MS, we demonstrated several proteins that were unknown to be present in the allantoic fluids of mares with placentitis. Further ongoing evaluations, may determine whether some of these newly discovered proteins are elevated in allantoic fluid. In the first study involving protein composition of the fetal fluids, we demonstrated that alpha-fetoprotein, a protein originated in the fetoplacental unit is released in the maternal circulation, and that placentitis increases its concentrations. It remains to be determined whether the increase is related to protein extravasation or to increased production of this molecule. For either mechanism, our results in both studies open a new field of investigation regarding diagnostic markers present in the fetal fluids that may be increased or reduced in maternal plasma.

In the present dissertation, an experimental model to induce ascending bacterial placentitis was used to identify new diagnostic markers for this disease; however, further studies should be carried to investigate whether the diagnostic markers for experimentally induced placentitis are useful markers for spontaneous placentitis.

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Appendices

Appendix A:

The information listed in the present manuscript has been included as supplementary material in the manuscripts generated out of this dissertation.

Study I: Breeding Management

The mares were examined 3 to 4 times per week by transrectal palpation and ultrasonography (coupled with a linear transducer 5 MHz Titan[®] Sonosite Bothell, WA, USA) until a preovulatory follicle (i.e. ≥ 35 mm, presence of endometrial edema) was detected. If needed, mares were given prostaglandin F2 α (7.5 mg, IM, Pfizer Animal Health, USA) to induce estrus. The mares were bred with fresh extended semen using a commercial extender (Equi-Pro[®], Minitube of America, Verona, WI, USA) without antibiotics.'

Once mares had a preovulatory follicle, they were bred with ≥ 500 million progressive motile sperm with pooled semen from three fertile stallions. A single dose of human chorionic gonadotropin (i.e. 2,500 units, Intervet/Schering-Plough Animal Health New York, NY USA) was given intravenously to induce ovulation. If the mare did not ovulate by 48 h after the first breeding, she was re-bred every 48 h until an ovulation was detected.

At 24 h post breeding, each mare received an intrauterine inoculation of *C. equi* (1×10^4 cfu in 1mL of skimmed milk). The inoculum was deposited into the uterine body through the use of a commercial (Minitube of America, Verona, WI, USA) semi-flexible AI pipette containing a stylet for deposition. Each inoculum was cultured to confirm

viability of the bacterial agent. At 24h post inoculation, each mare had an endometrial swab collected for PCR testing for *C. equi* [Reference 7]. The mares were re-checked by transrectal palpation and ultrasonography every 48 h until ovulation was detected. The mares accumulating intrauterine fluid received oxytocin injections twice daily (20 iu; IM, Bimeda Animal Health, Oakbrook Terrace, IL, USA) until fluid accumulation was not detectable with ultrasound.

Study II

Bacterial culture

The culture was prepared on blood agar plates, and incubated at 37°C for at least 3 days in aerobic culture. Pure culture of *C. equi* was confirmed using the characteristic pungent/soil odor, hard colonies, dark field examinations, gram staining and PCR analysis [References:7,14]. Briefly, 20-30 colonies were picked, suspended in 2 ml of Mueller Hinton Broth (with cation adjusted) in Magna lyser Green Beads Tubes (Roche Diagnostics, Indianapolis). To break hard *C. equi* colonies, suspension was homogenized using a commercial bead-beating system (Roche Diagnostic, Indianapolis) at the speed of 7,000 rpm for 30 sec. The homogenate was centrifuged at 12000 rpm for 3 min, and then the cell pellet was washed 3 times with PBS. The final pellet was resuspended in PBS and kept on refrigerated. This final suspension was then 10 fold-serial diluted and plated on agar plates. Cell numbers were counted, cfu was calculated and inoculation was performed next day. Since approximately 30% of the bacterial cells become dead by next day by this procedure (unpublished data, Erol), inoculum (cfu) was adjusted accordingly to make 10⁹ viable *Crossiella. equi*.

Appendix B

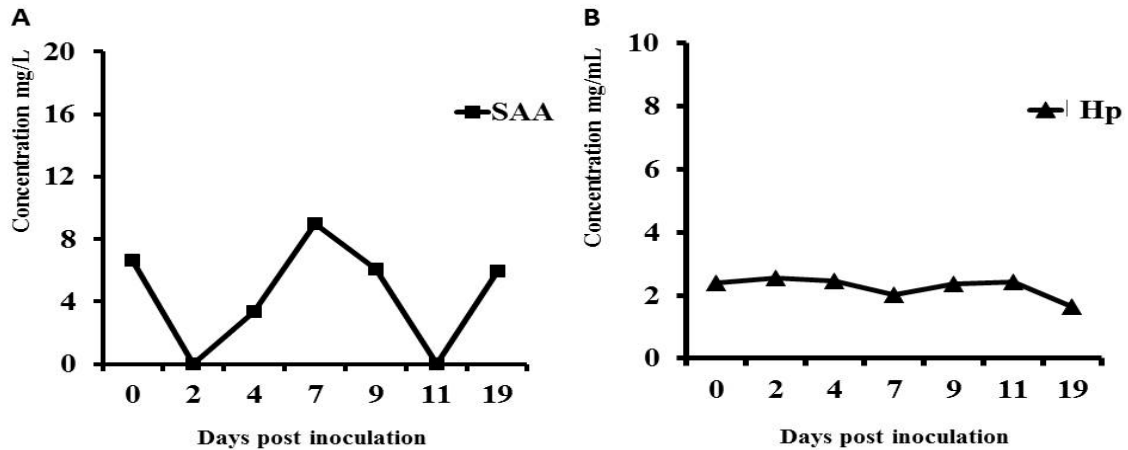


Figure 3.1 (A & B): Serum amyloid A (SAA) and haptoglobin (Hp) concentrations in a control mare excluded from the statistical analyses. During the entire sampling period the concentrations of SAA (<20mg/L) and Hp (<2.7 mg/mL) remained within normal ranges. Transabdominal guided fetal fluid sampling was performed at days 0, 5 and 12. She aborted 8 days after the last fetal fluid sampling. Pathological and microbiological examination of the placenta and fetus revealed no lesions and culture yielded saprophytic non-pathogenic bacteria and negative virus isolation; thus the cause of abortion was not determined.

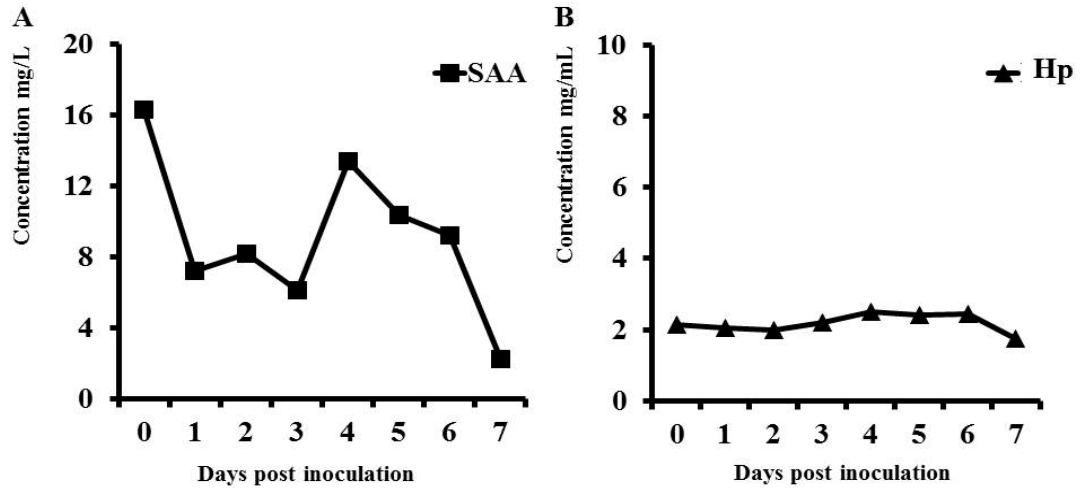


Figure 3.2 (A &B): Serum amyloid A (SAA) and haptoglobin (Hp) concentrations in a mare that failed to develop placentitis after experimental induction of placentitis. This mare was excluded from the statistical analyses. During the entire sampling period the concentrations of SAA (<20mg/L) and Hp (<2.7 mg/mL) remained within normal ranges. This mare was not re-inoculated. At parturition, there was no evidence of placentitis. This mare belonged to the subgroup without fetal fluid sampling. The mare delivered a normal foal and placenta with no pathological or microbiological evidence of placentitis.

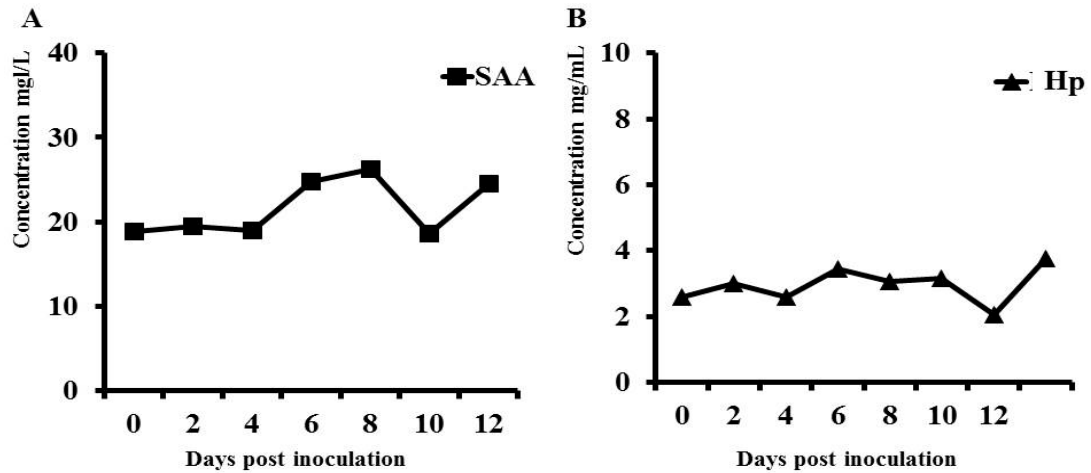


Figure 3 (A & B): Serum amyloid A (SAA) and haptoglobin (Hp) concentrations in a mare that failed to develop placentitis after experimental induction of ascending placentitis. During the first two weeks follow to inoculation concentrations of of SAA (<20mg/L) and Hp (<2.7 mg/mL) remained close to normal ranges. Transabdominal guided fetal fluid sampling was not performed at any time on this mare. There were no signs of placentitis upon ultrasonographic examination. This mare was re-inoculated two weeks after the first attempt. Data for SAA and Hp concentrations in the first two weeks were excluded from statistical analyses. After re-inoculation this mare developed signs of placentitis and aborted 20 days after re-inoculation.

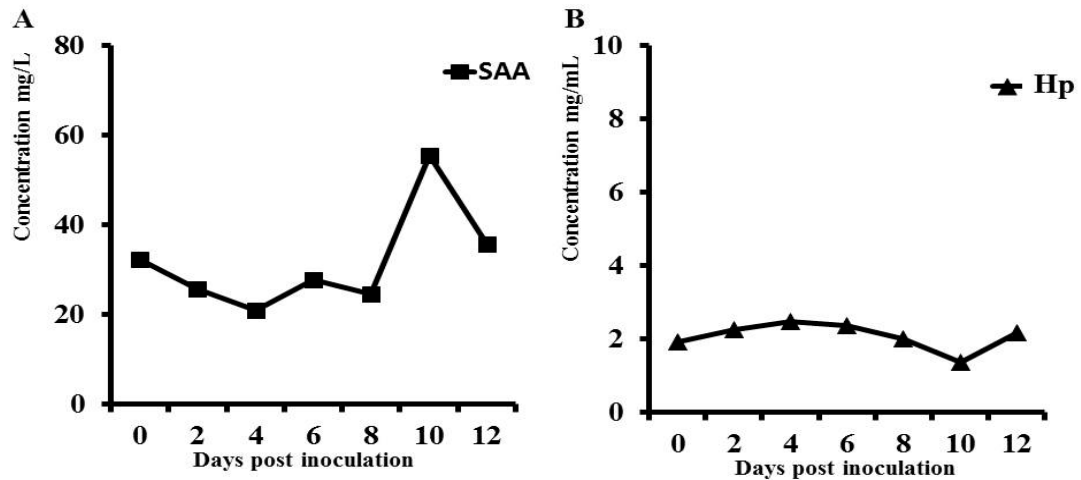


Figure 4 (A & B): Serum amyloid A (SAA) and haptoglobin (Hp) concentrations in a mare after experimental induction of ascending placentitis. Transabdominal guided fetal fluid sampling was performed at days 0, 5 and 12. SAA concentrations remained slightly elevated normal ranges up to across all days (i.e. <20mg/L). Concentrations of Hp remained within normal ranges across all days (i.e. < 2.7 mg/mL). This mare failed to develop placentitis; there were no signs of placentitis upon ultrasonographic examination. Data for SAA and Hp concentrations in the first two weeks post the inoculation were excluded from statistical analyses. The mare was re-inoculated 14 days post inoculation, developed signs of placentitis and aborted 9 days post-re-inoculation.

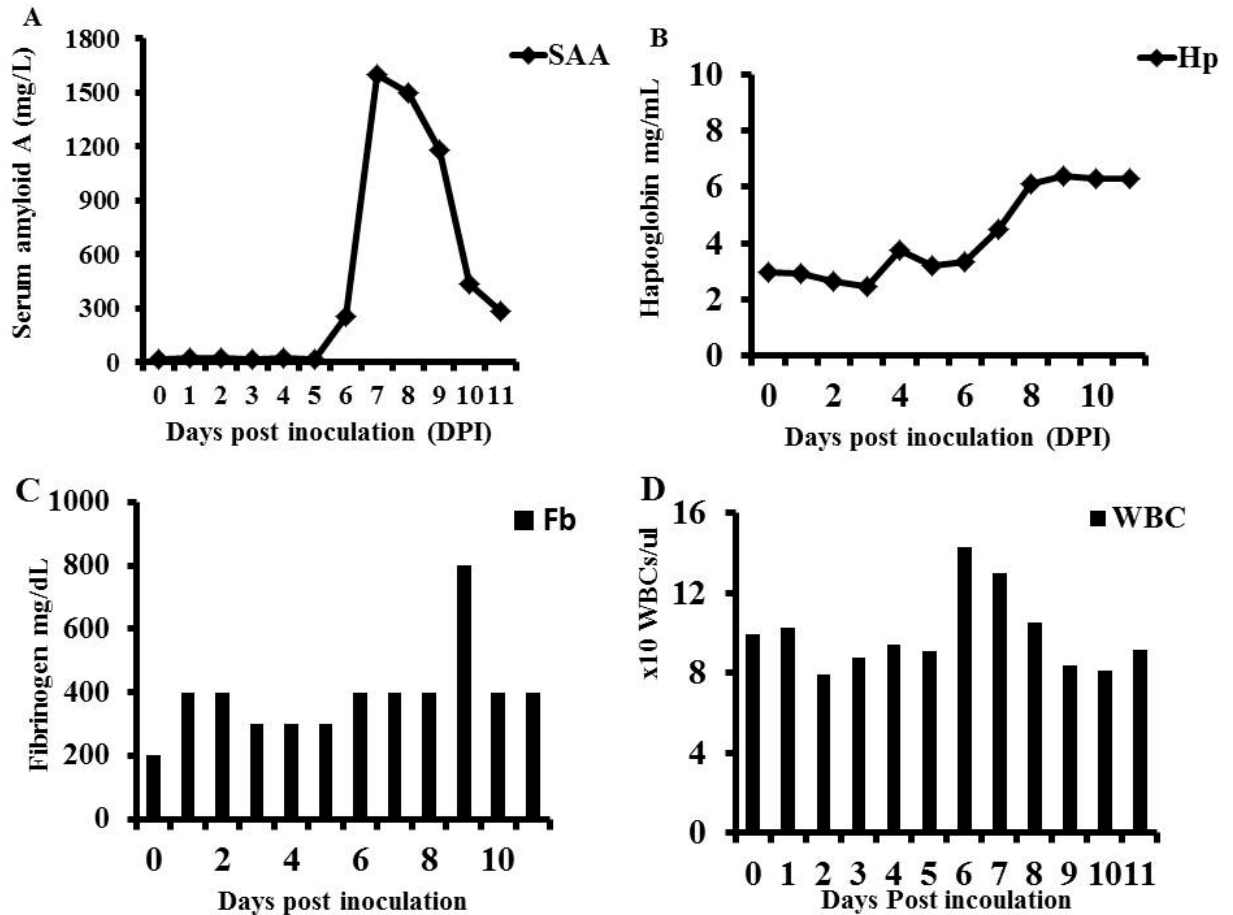


Figure 3.5: Serum amyloid (SAA), haptoglobin (Hp), fibrinogen (Fb) concentrations and white blood cell counts (WBC) in a control mare subgroup with fetal fluid sampling. This mare developed extensive cellulitis at the Viborg’s triangle secondary to venipuncture, thus she was excluded from the analysis. Concentrations of SAA, Hp, Fb and WBC were elevated once the mare developed the condition. The mare delivered a health foal and normal placenta at 330 days of gestation.

Table 3.1: Separation of the chorioallantois from the endometrium near the cervical star in mares (n = 14) with experimentally induced ascending placentitis. The placenta was examined by transrectal ultrasonography performed every other day from inoculation until abortion.

Interval between inoculation and initial detection of placental separation (days)	Number of mares with detected placental changes / total number of mares	Interval from detected placental separation to abortion for each mare (days)
0-2	3/14	0,0, 6
3-4	4/14	2,2,3, 4
5-6	5/14	3,4,4,8,8
7-8	1/14	4
9-10	1/14	14

As noted in the manuscript, three mares did not develop placentitis (data not included in the table above). During the first 14 days post inoculation none of these three mares developed areas of placental separation. Two of the mares were re-inoculated 14 days following the first inoculation; both mares were detected to have areas placental of separation by 5-6 days post inoculation, one mare aborted 3 days later, another mare aborted 14 days.

Appendice C

Table 1: Proteins present in the amniotic fluid in 1 or 2 samples. The entries on the left column represent the accession number on uniprot.

Protein identification	Comon name (Species)
P49822	Serum albumin (<i>Canis familiaris</i>)
P02062	Hemoglobin subunit beta (<i>Equus caballus</i>)
P38029	Alpha-1-antiproteinase (<i>Equus caballus</i>)
Q95182	Major allergen Equ c 1 (<i>Equus caballus</i>)
P24627	Lactotransferrin (<i>Bos taurus</i>)
P09571	Serotransferrin (<i>Sus scrofa</i>)
P34955	Alpha-1-antiproteinase (<i>Bos taurus</i>)
P12725	Alpha-1-antiproteinase (<i>Ovis aries</i>)
P23035	Alpha-1-antiproteinase (<i>Oryctolagus cuniculus</i>)
O46404	Phosphatidylinositol 3-kinase regulatory subunit gamma (<i>Bos taurus</i>)
Q2KJF1	Alpha-1B-glycoprotein (<i>Bos taurus</i>)
P35605	Coatomer subunit beta' (<i>Bos taurus</i>)
P01219	Glycoprotein hormones alpha chain (<i>Sus scrofa</i>)
P61157	Actin-related protein 3 (<i>Bos taurus</i>)
Q6E1Y9	Keratin, type II cytoskeletal 1 (<i>Canis familiaris</i>)
P85295	Serum albumin (Fragments) (<i>Capra hircus</i>)
Q3ZCH0	Stress-70 protein, mitochondrial (<i>Bos taurus</i>)
Q95182	Major allergen Equ c 1 (<i>Equus caballus</i>)
P20757	Angiotensinogen (<i>Ovis aries</i>)
Q29477	Lactotransferrin (<i>Capra hircus</i>)
Q32KY0	Apolipoprotein D (<i>Bos taurus</i>)
P37153	Apolipoprotein D (<i>Oryctolagus cuniculus</i>)
Q56H28	Angiotensin-converting enzyme 2 (<i>Felis catus</i>)
Q58DD0	Angiotensin-converting enzyme 2 (<i>Bos taurus</i>)
Q56NL1	Angiotensin-converting enzyme 2 (<i>Paguma larvata</i>)
Q29443	Serotransferrin (<i>Bos taurus</i>)
Q28888	Decorin (<i>Oryctolagus cuniculus</i>)
Q7M2U7	Apolipoprotein E (<i>Zalophus californianus</i>)
P18649	Apolipoprotein E (<i>Canis familiaris</i>)
Q03247	Apolipoprotein E (<i>Bos taurus</i>)
P18287	Apolipoprotein E (<i>Oryctolagus cuniculus</i>)
Q0VCY3	Surfeit locus protein 6 (<i>Bos taurus</i>)
P09849	Lactase-phlorizin hydrolase (<i>Oryctolagus cuniculus</i>)
Q3T112	Proteasome subunit beta type-8 (<i>Bos taurus</i>)

Q5W416	Proteasome subunit beta type-8 (<i>Canis familiaris</i>)
Q29576	Proteasome subunit beta type-8 (Fragment) (<i>Sus scrofa</i>)
Q5I3B1	Type III iodothyronine deiodinase (<i>Bos taurus</i>)
Q6QN11	Type III iodothyronine deiodinase (<i>Sus scrofa</i>)
Q6DN07	Type III iodothyronine deiodinase (<i>Ovis aries</i>)
P38029	Alpha-1-antiproteinase 2 (<i>Equus caballus</i>)
	Fibronectin (<i>Bos taurus</i>)
P14632	Lactotransferrin (<i>Bos taurus</i>)
Q29482	Clusterin (<i>Equus caballus</i>)
P14632	Lactotransferrin (<i>Sus scrofa</i>)
O97490	Melanotransferrin (<i>Oryctolagus cuniculus</i>)
Q6EIZ0	Keratin, type I cytoskeletal 10 (<i>Canis familiaris</i>)
Q8MKG2	Uteroglobin (<i>Equus caballus</i>)
	Serotransferrin (<i>Sus scrofa</i>)
	Keratin, type II cytoskeletal 1 (<i>Canis familiaris</i>)
	Alpha-1B-glycoprotein (<i>Bos taurus</i>)
Q08E36	Transmembrane protein 198 (<i>Bos taurus</i>)
P18649	Apolipoprotein E (<i>Canis familiaris</i>)
Q03247	Apolipoprotein E (<i>Bos taurus</i>)
Q3SZ57	Alpha-fetoprotein (<i>Bos taurus</i>)

References

- Ahsan A, Salman KA, Alam S, Siddiqui AH, Naeem SS, Ahmad A & Khan IM** 2014 Alpha-1-antitrypsin, a diagnostic and prognostic marker of vernal keratoconjunctivitis. *J Clin Diagn Res* 8 CC08-10.
- Anderson TD, Cheville NF, Meador VP** 1986 Pathogenesis of placentitis in the goat inoculated with *Brucella abortus*. II. Ultrastructural studies. *Vet Path* 23 227-39.
- Aycardi E, Rivera B, Torres B & De Bohorquez V** 1982 Experimental infection with a *Leptospira hardjo* strain isolated from cattle of the eastern plains of Colombia. *Vet Microbiol* 7 545-550.
- Ardila AA, Indjein L, Koya A, Lew-Tabor A, Venus, B, de Weit SC, Power J, Greer RM, Boe-Hansen G & McGowan M** 2012 Experimental infections with *Campylobacter fetus* subspecies *venereallis* in cattle. Proceedings 6th International Veterinary Vaccines & Diagnostics Conference, Cairns, Australia.
- Baetz AL, Crandell SE, Schmer MJ, Barnett D, & Bryner JH** 1981. Plasma alpha-fetoprotein concentrations in pregnant cows exposed to *Sarcocystis cruzi*, *Campylobacter fetus*, or *Aspergillus fumigatus*. *Am J Vet Res* 42 2146-21468.
- Bailey CS, Sper RB, Schewmaker JL, Buchanan CN, Beacher TM, Pozor MA & Whitacre MD** 2012. Uterine artery blood flow remains unchanged in pregnant mares in response to short-term administration of pentoxifylline. *Theriogenology* 77 430-6.
- Bailey CS, Macpherson ML, Pozor MA, Troedsson MHT, Benson S, Giguere S, Sanchez LC, LeBlanc MM, Vickory TW** 2010 Treatment efficacy of trimethoprim sulfamethoxazole, pentoxifylline and altrenogest in experimentally induced equine placentitis. *Theriogenology* 74 402-412.
- Barr BS**: The outcome of foals born to mares treated for placentitis. In Uterine Infection in Mares and Women: a Comparative Study II, MM LeBlanc, JF Wade, L Foster (eds). South Carolina, USA: R& W communications, 2005, p 45.
- Bartnikas TB** 2012 Known and potential roles of transferrin in iron biology *Biometals* 25 677-686.
- Beasley JA, Goldbeck JM, Bright BC, Morton DJ, Whitby PW, Stull TL**. Haptoglobin concentrations in preterm and term newborns. *J Perinatol* 2011, 7:500-3.
- Berg LC, Thomsen PD, Andersen PH, Jensen HE Jacobsen S** 2011 Serum amyloid A is expressed in histologically normal tissues from horses and cattle. *Vet Immunol Immunopathol* 144 155-9.
- Belgrave RL, Dickey MM, Arheart KL, Cray C**. 2013 Assessment of serum amyloid A testing for horses and its clinical application in a specialized equine practice. *J Am Vet Med Assoc* 243 113-9.
- Bhavnani BR** 1988 The saga of the ring B unsaturated equine estrogens. *Endocr Rev* 9 396-416.
- Bolin DC, Donahue JM, Vickers ML, Giles RC, Harrison L, Jackson C, Poonacha KB, Roberts JE, Sebastian MM, Sells SE, Tramontin R, & Williams NM** 2004 Equine abortion and premature birth associated with *Cellulosimicrobium cellulans* infection. *J Vet Diagn. Invest.* 16, 333-6.
- Buhimschi IA & Buhimschi CS** 2012. Proteomics/diagnosis of chorioamnionitis and of relationship with the fetal exposure. *Sem Fetal Neonatal Med* 17 36-45.

- Buhimschi CS, Bhandari V, Dulay AT, Nayeri UA, Abdel-Razeq SS, Pettker CM, Thung S, Zhao G, Han YW, Bizarro M & Buhimschi IA** 2011 Proteomics mapping of cord blood identifies haptoglobin “switch-on” pattern as biomarker of early-onset neonatal sepsis in preterm newborns. *PLoS One* 6:e.2611.doi:10.1371.
- Buhimschi IA, Zhao G, Rosenberg VA, Abdel-Razeq S, Thung S, Buhimschi CS** 2008. Multidimensional proteomics analysis of amniotic fluid to provide insight into the mechanisms of idiopathic preterm birth. *PLoS One* 3 e2049. doi: 10. 137 /journal .pone. 00 02049.
- Buhimschi CS, Bhandari V, Hamar BD, Bahtiyar MO, Zhao G, Sfakianaki AK, Pettker CM, Magloire L, Funai E, Norwitz ER, Paidas M, Copel JA, Weiner CP, Lockwood CJ, Buhimschi IA** 2007 Proteomic profiling of the amniotic fluid to detect inflammation, infection, and neonatal sepsis. *PLoS Med* 4:e18.
- Buhimschi CS & Buhimschi IA** 2010 The role of proteomics in the diagnosis of chorioamionitis and early-onset neonatal sepsis. *Clin Perinatol* 37 355-74.
- Buhimschi CS, Bhandari V, Dulay AT, Nayeri UA, Abdel-Razeq SS, Pettker CM, Thung S, Zhao G, Han YW, Bizarro M & Buhimschi IA** 2011. Proteomics mapping of cord blood identifies haptoglobin “switch-on” pattern as biomarker of early-onset neonatal sepsis in preterm newborns. *PLoS One* 6 e.2611.doi:10.1371.
- Canisso IF, Ball BA, Squires EL & Troedsson MH** 2014. How to perform transabdominal ultrasound-guided fetal fluid sampling in mares. *J Equine Vet Sci* in press.
- Canisso IF, Rodriguez JS, Sanz MG & Coutinho da Silva MA** 2013. A clinical approach to the diagnosis and treatment of retained fetal membranes with an emphasis placed on the critically ill mare. *J Equine Vet Sci* 33 570-579.
- Canisso IF, Ball BA, Troedsson MHT, Claes A, Scoggin KE, Erol E, Williams NM & Squires EL** 2013. Experimental induction of nocardioform placentitis in mares. In Proceedings Annual Meeting Society for Theriogenology, August 2013 (abstract) p65.
- Carneiro GF, Liu IKM, Illera JC & Munro CJ** 1998 Enzyme immunoassay for the measurement of estrone sulfate in cryptorchids, stallions, and donkeys. *Proceedings Am. Ass. Equine Practns* 44, 3-5.
- Carrick JB, Perkins NR & Zalucki MP** 2014 Causes of abortion in Australia (2005-2012) – proportion of cases due to Equine Amnionitis and Foetal Loss (EAFL). *J Equine Vet Sci* 34 212-214.
- Carter CN, Cassady JC, Cohen N, Kennedy LA, Erol E, Malm T, Donahue M, Sells S, Williams NM, Smith J & Dwyer R** 2012 Equine Nocardioform Placentitis and Abortion Outbreak and Farm-Based Risk Factor Study, 2010-2011. (2012) *In Proceedings European Association of Veterinary Laboratory Diagnosticians*. Kazimierz-Dolny, Poland S3-O-04.
- Cattoli G, Vascellari M, Corro M, Capua F, Mutinelli F, Sells SF & Donahue JM** 2004 First case of equine nocardioform placentitis caused by *Crossiella equi* in Europe. *Vet Record* 154,730-731.
- Cawdell-Smith AJ, Todhunter KH, Perkins NR & Bryden WL** 2013 Exposure of mares to processionary caterpillars (*Ochrogaster lunifer*) in early pregnancy: an additional dimension to equine amnionitis and fetal loss. *Equine Vet J* 45 755-60.
- Chaiworapongsa T, Romero R, Espinoza J, Kim YM, Edwin S, Bujold E, Gomez R & Kuivaniemi H** 2005. Macrophage migration inhibitory factor in patients with preterm

- parturition and microbial invasion of the amniotic cavity. *J Matern Fetal Neonatal Med* **18** 405-16.
- Chavatte PM, Rossdale PD & Tait AD.** 1995 Modulation of 3 beta-hydroxysteroid dehydrogenase (3-beta HSD) activity in the equine placenta by pregnenolone and progesterone metabolites. *Equine Vet J* **27** 342-47.
- Chavatte P, Holtan D, Ousey JC & Rossdale PD** 1997 Biosynthesis and possible biological roles of progestagens during equine pregnancy and in the newborn foal. *Equine Vet J Suppl.* **24** 89-95.
- Chavatte PM, Rossdale PD & Tait AD** 1995 Modulation of 3 beta-hydroxysteroid dehydrogenase (3-beta HSD) activity in the equine placenta by pregnenolone and progesterone metabolites. *Equine Vet J* **27**, 342-47.
- Chim SSC, Shing TKF, Hung ECW, Leung TY, Lau TK, Chiu RWK & Lo YMD.** 2008 Detection and characterization of placental micrnas in maternal plasma. *Clin Chem* **54** 482-490.
- Chiu RWK & Lo YMD.** 2010 Pregnancy-associated micrnas in maternal plasma: A channel for fetal-maternal communication? *Clin Chem* **56**1656-1657.
- Cho CK, Shan SJ, Winsor EJ & Diamandis EP** 2007. Proteomics analysis of human amniotic fluid. *Mol Cell Proteomics* doi. 10.1074/mcp. M700090-MCP200.
- Christensen BW, Roberts JF, Pozor MA, Giguere S, Sells SF & Donahue JM** 2006 Nocardioform placentitis with isolation of *Amycolatopsis* ssp in a Florida-bred mare. *J Am Vet Med Assoc* **228** 1234-1239.
- Christiansen DL, Moulton K, Hopper RM, Walters FK, Cooley AJ, LeBlanc MM & Ryan PL** 2010 Evidence-based medicine approach to develop efficacious therapies for late-gestation mares presenting with uterine infections using an experimentally induced placentitis model. *Animal Reprod Sci* **121** S345-346.
- Christiansen DL, Moulton K, Hopper RM, Walters FK, Cooley AJ, LeBlanc MM, & Ryan PL** 2010. Evidence-based medicine approach to develop efficacious therapies for late-gestation mares presenting with uterine infections using an experimentally induced placentitis model. *Animal Reprod Sci* **121** S345-346.
- Christoffersen M, Woodward E, Bojesen AM, Jacobsen S, Petersen MR, Troedsson MH & Lehn-Jensen H** 2012 Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis. *BMC Vet Res* **8** 41.
- Christoffersen M, Baagoe CD, Jacobsen S, Bojesen AM, Petersen MR & Lehn-Jensen H** 2010 Evaluation of the systemic acute phase response and endometrial gene expression of serum amyloid A and pro- and anti-inflammatory cytokines in mares with experimentally induced endometritis. *Vet Immunol Immunopathol* **138** 95-105.
- Coker MR, Rauw WM, Nieto NC, Thain D & Teglas MB** 2012 Hematologic and IgG responses of heifers experimentally infected with the agent of epizootic bovine abortion. *Vet Clin Pathol* **41** 344-52.
- Cox JE** 1975 Oestrone and equilin in the plasma of the pregnant mare. *J Reprod Fertil Suppl* **23** 463-8.
- Coutinho da Silva MA, Canisso IF, Macpherson ML, Johnson AE & Divers TL** 2013 Serum amyloid A concentration in healthy periparturient mares and mares with ascending placentitis. *Equine Vet J* **45** 619-624.
- Cray C, Belgrave RL** 2014 Haptoglobin quantitation in serum samples from clinically normal and clinically abnormal horses. *J Equine Vet Sci* **34** 337-340.

- Crisman MV, Scarratt WK & Zimmerman KL** 2008 Blood proteins and inflammation in the horse. *Vet Clin North Am Equine Pract* **24** 285-297.
- Dabrowski R, Wawron W, Kostro K** 2007 Changes in CRP, SAA, and haptoglobin produced in response to ovariohysterectomy in healthy bitches and those with pyometra. *Theriogenology* **67** 321-327.
- Daels PF, Chang GC, Hansen HO & Mohammed HO** 1996. Testosterone secretion during early pregnancy in mares. *Theriogenology* **45** 1211-1219.
- De Mees C, Laes JF, Bakker J, Smitz J, Hennuy B, Vooren PV, Gabant P, Szpirer J & Szpirer C** 2006. Alpha-fetoprotein controls female fertility and prenatal development of the gonadotropin-releasing hormone pathway through an antiestrogenic action. *Mol. Cell. Biol.* **26** 2012-2018.
- Deutsch HF** 1991. Chemistry and biology of alpha-fetoprotein. *Adv Cancer Res* **56**, 253-312.
- Denny HR** 1973 A review of brucellosis in the horse. *Equine Vet J* **5** 121-5.
- Donahue JM, Williams NM, Sells SF & Labeda DP** 2002 *Crossiella equi* sp nov., isolated from equine placentas. *Int J Syst Evol Microbiol* **52** 2169-2173.
- Donahue JM & Williams NM** 2000 Emergent causes of placentitis and abortion. *Vet Clin North Am Equine Pract* **16** 443-456.
- Douglas RH** 2004 Endocrine diagnostics in the broodmare: What you need to know about progestins and estrogens. In: *Proceedings of the Proceedings of the Society for Theriogenology/American College of Theriogenologists; Lexington, KY*. pp. 106-115.
- Ellis WA, O'Brien JJ, Neill SD & Bryson DG** 1986 Bovine leptospirosis: experimental serovar hardjo infection. *Vet Microbiol* **11** 293-299.
- El Idrissi AH, Benkirane A, el Maadoudi M, Bouskihane M, Berrada J & Zerouali A** 2001 Comparisons of the efficacy of *Brucella abortus* strain RB51 and *Brucella melitensis* Rev. 1 live vaccines against experimental infection with *Brucella melitensis* in pregnant ewes. *Rev Sci Tech* **3**, 741-7.
- Ellis WA** 1994 Leptospirosis as a cause of reproductive failure. *Vet Clin North Am Food Anim Pract* **10** 463-78.
- Erol E, Jackson CB, Steinman M, Meares K, Donahue J, Kelly N, Locke S, Smith JL & Carter CN** 2014. A diagnostic evaluation of real-time PCR, fluorescent antibody and microscopic agglutination tests in cases of equine leptospiral abortion. *Equine Vet J* in press doi:10.1111/evj.12281.
- Erol E, Sells SF, Williams NM, Kennedy L, Locke SJ, Labeda DP, Donahue JM & Carter CN** 2012 An investigation of a recent outbreak of nocardioform placentitis caused abortions in horses. *Vet. Microbiol.* **158** 425-430.
- Erol E, Williams NM, Sells SF, Kennedy L, Locke SJ, Donahue JM & Carter CN** 2012 Antibiotic susceptibility patterns of *Crossiella equi* and *Amycolatopsis* species causing nocardioform placentitis in horses. *J Vet Diagn Invest* **24**, 1158-1161.
- Esteller-Vico A, Troedsson MHT, Squires EL & Ball BA.** 2014 Inhibition of estrogen synthesis during the last trimester of gestation: changes in endocrine patterns, fetal growth and uterine artery hemodynamics in mares. Proceedings XI International Symposium on Equine Reproduction *J Equine Vet Sci* **34** 207 (abstract).
- Fahey JO** 2008 Clinical management of intra-amniotic infection and chorioamnionitis: a review of the literature. *J Midwifery Women's Health* **53** 227-35.

- Ferrazzi E, Muggiasca ML, Fabbri E, Fontana P, Castoldi F, Lista G, Primerano L, Livio S & Di Francesco S** 2012. Assessment of fetal inflammatory syndrome by “classical” markers in the management of preterm labor: a possible lesion from metabolomics and system biology. *J Matern Fetal Neonatal Med* **S5** 54-61
- Finger IS, Dos Santos RS, Pazinato FM, De Vita B, Prester NC, Nogueira CEW & Curcio BR** 2014. Evaluation of biochemical composition on amniotic fluid in mares with placentitis. In: Proceedings XI International Symposium on Equine Reproduction. *J Equine Vet Sci* **34** 240 (abstract).
- Gabant P, Forrester L, Nichols J, Van Reeth T, De Mess C, Pajack B, Watt A, Smitz J, Alexandre H, Szpirer C & Szpirer J** 2002. Alpha-fetoprotein, the major fetal serum protein, is not essential for embryonic development but is required for female fertility. *PNAS* **99** 12865-12870.
- Giles RC, Donahue JM, Hong CB, Tuttle PA, Petrites-Murphy MB, Poonacha KB, Roberts AW, Tramontin RR, Smith B & Swerczek TW** 1993. Causes of abortion, stillbirth, and perinatal death in horses: 3,527 cases (1986-1991). *J Am Vet Med Assoc* **203** 1170-1175.
- Giles RC, Hong CB, Donahue JM, Swerczek TW, Poonacha KB, Tuttle PA & Tramontin, RR** 1988 Equine abortion caused by a gram-positive filamentous bacterium. In *Proceedings 37th Annual meeting, American College of Veterinary Pathologists* Monterey CA p65 (abstract).
- Ginther OJ** 1992 Reproductive biology of the mare. *Equiservices, Crossplans*; 625.
- Grogono-Thomas R & Woodland RM** 1996 An experimental model of *Campylobacter fetus fetus* induced abortion in sheep. Proceedings 8th International Workshop Campylobacters, Helicobacters, and Related Organisms, Newell DG, Ketley JM, Feldman RA pp 351-354.
- Han X, Rossdale PD, Ousey J, Holdstock N, Allen WR, Silver M, Fowden AL, McGladdery AJ, Labrie F & Belanger A** 1995 Localization of 15-hydroxy prostaglandin dehydrogenase (PGDH) and steroidogenic enzymes in the equine placenta. *Equine Vet J* **27** 334-9.
- Holdstock NB, McGladdery AJ, Ousey J & Rossdale PD** 1995. Assessing methods of collection and changes of selected biochemical constituents in amniotic and allantoic fluid throughout equine pregnancy. *Biol. Reprod. Monograph* **1**, 21-38.
- Hong CB, Donahue JM, Giles RC, Petrites-Murphy MB, Poonachar KB, Roberts AW, Smith BJ, Tramontin RR, Tuttle PA & Swerczek TW** 1993. Etiology and pathology of equine placentitis. *J Vet Diagn Invest* **5** 56-63.
- Henderson LL, Warren JC** 1984 Purification and characterization of epimeric estradiol dehydrogenases (17 alpha and 17 beta) from equine placenta. *Biochemistry* **23** 486-91.
- Hall MR, Hanks D, Kvasnicka W, Bosomworth A, Smith H, Stott JL, Blanchard MT, Anderson ML** 2002. Diagnosis of epizootic bovine abortion in Nevada and identification of the vector. *J Vet Diagn Invest* **14** 205-210.
- Hart KA, Barton MH** 2012 Adrenocortical insufficiency in horses and foals. *Vet Clin North Am Equine Pract* **27** 19-34.
- Hong CB, Donahue JM, Giles RC, Petrites-Murphy MB, Poonachar KB, Roberts AW, Smith BJ, Tramontin RR, Tuttle PA & Swerczek TW** 1993 Etiology and pathology of equine placentitis. *J Vet Diagn Invest* **5** 56-63.

- Hong CB, Donahye JM, Giles RC Jr, Petrites-Murphy MB, Poonacha KB, Roberts AW, Smith BJ, Tramontin RR, Tuttle PA & Swerczek TW** 1993. Equine abortion and stillbirth in central Kentucky during 1988 and 1989 foaling seasons. *J Vet Diagn Invest* **5** 560-6.
- Hendricks WK, Colenbrander B, van der Weijdgen GC & Stout TAE** 2009 Maternal age and parity influence of fetal growth in Dutch Warmblood mares. *Anim Reprod Sci* **115** 110-123.
- Hulten C, Gronlund J, Hirvonen J, Tulamo RM, Suominen MM, Marhaug G, Forsberg M** 2002. Dynamics in serum of the inflammatory markers serum amyloid A (SAA), haptoglobin, fibrinogen and α 2-globulins during induced noninfectious arthritis in the horse. *Equine vet J* **34** 699-704.
- Hulten C, Johansson E, Fossum C & Wallgren P** 2003. Interleukin 6, serum amyloid A and haptoglobin as markers of treatment efficacy in pigs experimentally infected with *Actinobacillus pleuropneumoniae*. *Vet Microbiol* **95** 75-89.
- Hillier SL, Krohn MA, Kiviat NB, Watts DH & Eschenbach DA** 1991 Microbiologic causes and neonatal outcomes associated with chorioamnion infection. *Am J Obstet Gynecol* **165** 955-61.
- Hinrichs K, Sertich PL, Palmer E, Kenney RM** 1987. Establishment and maintenance of pregnancy after embryo transfer in ovariectomized treated with progesterone. *J Reprod Fert* **80** 395-401.
- Henderson JT, Whitlock EP, O'Conner E, Senger CA, Thompson JH & Rowland MG** 2013. Low-dose aspirin for the prevention of morbidity and mortality from preeclampsia: a systematic evidence review for the U.S. preventive services task force. U.S. Preventive Services Task Force Evidence Synthesis Report No: 14-05207-EF-1.
- Jacobsen S, Kjelgaard-Hansen M, Petersen HH & Jensen AL** 2006 Evaluation of a commercially available human-serum amyloid A (SAA) turbidometric immunoassay for determination of equine SAA- concentrations. *Vet. J.* **172**, 315-319.
- Jungersen G, Sorensen V, Giese SB, Stack JA & Riber U** 2006 Differentiation between serological responses to *Brucella suis* and *Yersinia enterocolitica* serotype O:9 after natural or experimental infection in pigs. *Epidemiol Infect* **134** 347-357.
- Jacobsen S & Andersen PH** 2007. The acute phase protein serum amyloid A (SAA) as a marker of inflammation in the horse. *Equine vet Educ* **19** 38-46.
- Jeffcott LB, Hyland JH, MacLean AA, Dyke T & Roberstson-Smith G.** 1987 Changes in maternal hormone concentrations association with induction of fetal death a day 45 of gestation in mares. *J Reprod Fert Suppl* **35** 461-7.
- Kashman LH, Hughes JP, Stabenfeldt GH, Starr MD & Lasley BL** 1988 Estrone sulfate concentrations as an indicator of fetal demise in horses. *Am J Vet Res* **49** 184-187.
- King DP, Chen CI, Blanchard MT, Aldridge BM, Anderson M, Walker R, Mass J, Hanks D, Hall M & Stott JF** 2005 Molecular identification of a novel *Deltraproteobacterium* as the etiologic agent of bovine abortion (Foothill abortion). *J Clin Microbiol* **43** 604-609.
- Klein C & Troedsson MH** 2013 Macrophage migration inhibitory factor is expressed by equine conceptuses and endometrium. *Reprod Domest Anim* **48** 297-304.
- Klein J, Buffin-Meyer B, Mullen W, Carty DM, Delles C, Vlahou A, Mischack H, Decramer S, Bascands JL & Schanstra JP** 2014. Clinical proteomics in obstetrics and neonatology. *Expert Rev Proteomics* **11** 75-89.

- Kolarich DD, Weber A, Turecek PL, Schwarz HP & Altmann F** 2006 Comprehensive glycol-proteomic analysis of human alpha-antitrypsin and its charge isoforms. *Proteomics* **6** 3369-3380.
- Kolm G, Klein D, Knapp E, Watanabe K & Walker I.** 2006 Lactoferrin expression in the horse endometrium: relevance in persistent mating-induced endometritis. *Vet Immunol Immunopathol* **114** 159-167.
- Kotlabova K, Doucha J & Hromadnikova I.** 2011 Placental-specific microrna in maternal circulation - identification of appropriate pregnancy-associated micrnas with diagnostic potential. *J Reprod Immunol* **89** 185-191.
- Lavoie-Lamoureux A, Leclere M, Lemos K, Wagner B & Lavoie JP** 2012 Markers of systemic inflammation in horses with heaves. *J Vet Intern Med* **26** 1419-26.
- Labeda DP, Donahue JM, Williams NM, Sells SF & Henton MM** 2003 Amycolatopsis kentuckyensis sp. nov., Amycolatopsis lexingtonensis sp. nov. and Amycolatopsis pretoriensis sp. nov., isolated from equine placentas. *Int. J Syst Evol Microbiol* **53**, 1601-5.
- Labeda DP, Price NP, Kroppenstedt RM, Donahue JM, Williams NM & Sells SF** 2009 Streptomyces atriruber sp. nov. and Streptomyces silaceus sp. nov., two novel species of equine origin. *Int. J. Syst. Evol. Microbiol.* **59**, 2899-903.
- Labeda DP, Donahue JM, Williams NM, Sells SF & Henton MM** 2003 Amycolatopsis kentuckyensis sp. nov., Amycolatopsis lexingtonensis sp. nov. and Amycolatopsis pretoriensis sp. nov., isolated from equine placentas. *Int J Syst Evol Microbiol* **53**, 1601-5.
- Labeda DP, Price NP, Kroppenstedt RM, Donahue JM, Williams NM & Sells SF** 2009 Streptomyces atriruber sp. nov. and Streptomyces silaceus sp. nov., two novel species of equine origin. *Int J Syst Evol Microbiol* **59** 2899-903.
- Lichenstein HS, Lyons DE, Wurfel MM, Johnson DA, McGinley MD, Leidli JC, Trollinger DB** 1994. Afamin is a new member of the albumin, alpha-fetoprotein, and vitamin D-binding protein gene family. *J Biol Chem* **269**, 18149-54.
- Lyle SK, Paccamonti DL, Huberta JD, Schlafer DH, Causey RC, Eilts B & Johnson JR,** 2006. Laparoscopic placement of an indwelling allantoic catheter in the mare: biochemical, cytologic, histologic, and microbiologic findings. *Anim Reprod Sci* **94** 428-431.
- Luft AJ, Lai PC, Roberston HA, Saunders NR & Lorscheider FL** 1984. Distribution of alpha-fetoprotein in fetal plasma and in amniotic and allantoic fluids of the pig. *J. Reprod. Fert.* **70**,605-7.
- LeBlanc MM, Giguere S, Brauer K, Paccamonti DL, Horohov DW, Lester GD, O'Donnell, LJ, Sheerin BR, Pablo L, Rodgerson DH & Evans MJ** 2002. Premature delivery in ascending placentitis is associated with increased expression of placental cytokines and allantoic fluid prostaglandins E2 and F2alpha. *Theriogenology* **58** 841-844.
- LeBlanc MM, Giguere S, Brauer K, Paccamonti DL, Horohov DW, Lester GD, O'Donnell LJ, Sheerin BR, Pablo L, Rodgerson DH & Evans MJ** 2002 Premature delivery in ascending placentitis is associated with increased expression of placental cytokines and allantoic fluid prostaglandins E2 and F2alpha. *Theriogenology* **58** 841-844.
- LeBlanc MM** 2010 Ascending placentitis in the mare: an update. *Reprod Domest Anim* **45** 228-34.

- Ladomery MR, Maddockws DG & Wilson ID** 2011. Micrnas: Their discovery, biogenesis, function and potential use as biomarkers in non-invasive prenatal diagnostics. *Int J Mol Epidemiol Genet* **3** 253-260.
- Laugier C, Foucher N, Sevin C, Leon A, Tappet J:** A 24-year retrospective study of equine abortion in Normandy (France). *J Equine Vet Sci* 2011; 31:116-123.
- LeBlanc MM, Giguere S, Lester GD, Brauer K & Paccamonti DL** 2012 Relationship between infection, inflammation and premature parturition in mares with experimentally induced placentitis. *Equine Vet J* **S41** 8-14.
- Ladomery MR, Maddockws DG & Wilson ID** 2011. Micrnas: Their discovery, biogenesis, function and potential use as biomarkers in non-invasive prenatal diagnostics. *Internat J Mol Epidemiol Genet* **2(3):253-260.**
- Lee SY, Park KH, Jeong EH, Oh KJ, Ryu A & Park KU** 2012. Relationship between maternal serum C-reactive protein, funisitis and early-onset neonatal sepsis. *J Korean Med Sci* **27** 674-80.
- Mays MB, LeBlanc MM & Paccamonti D** 2002. Route of fetal infection in a model of ascending placentitis Route of fetal infection in a model of ascending placentitis. *Theriogenology* **58** 791-792.
- Mizejewski GJ** 1995. Alpha-fetoprotein signal sequences: a proposed mechanism for subcellular localization and organelle targeting. *J Theor Biol* **176** 103-113.
- Morris S, Kelleman AA, Stawicki RJ, Hansen PJ, Sheerin PC, Sheerin BR, Paccamonti DL & LeBlanc MM** 2007. Transrectal ultrasonography and plasma progesterin profiles identifies fetoplacental compromise in mares with experimentally induced placentitis. *Theriogenology* **67** 681-691.
- McDowell KJ, Webb, B.A., Williams, N.M., Donahue, J.M., Newman, K.E., Lindemann, MD & Horohov DW** 2010 The role of caterpillars in mare reproductive loss syndrome: a model for environment causes of abortion. *J Anim. Sci.* **88** 1379-87.
- Mittelbrunn M, Gutierrez-Vasquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzales MA, Bernad A & Sanchez-Madrid F** 2011 Unidirectional transfer of microRNA loaded exosomes from T cells to antigen-presenting cells. *Nat Commun* **2:282**.doi:10.1038/ncomms1285.
- Montenegro D, Romero R, Pineles BL, Tarca AL, Kim YM, Draghici S, Kusanovic JP, Kim JS, Erez O, Mazaki-Tovi S, Hassan S, Espinoza J & Kim CJ.** 2007 Differential expression of microRNAs with progression of gestation and inflammation in the human chorioamniotic membranes. *Am J Obstet Gynecol* **197** 289.e1-6.
- Mayor-Lynn K, Toloubeydokhti T, Cruz AC & Chegini N.** 2011 Expression profile of micrnas and mrnas in human placentas from pregnancies complicated by preeclampsia and preterm labor. *Reprod Sci* **18** 46-56.
- Morris S, Kelleman AA, Stawicki RJ, Hansen PJ, Sheerin PC, Sheerin BR, Paccamonti DL & LeBlanc MM** 2007 Transrectal ultrasonography and plasma progesterin profiles identifies fetoplacental compromise in mares with experimentally induced placentitis. *Theriogenology* **67** 681-691.
- Meador VP & Deyoe BL** 1986 Experimentally induce *Brucella abortus* infection in pregnant goats. *Am J Vet Res* **47** 2337-42.
- Meinecke B & Gips H** 1990. The endocrine status of clinically conspicuous mares during the peripartum period. *Tierarztl Prax* **5** 513-23.

- McCullum M, Rhyan J, Coburn S, Ewalt D, Lahr C, Nol P, Keefe T, Kimberling C, & Salman M** 2013 Clinical, culture, serology, and histopathology outcomes of the bighorn sheep experimentally infected with *Brucella ovis* *J Wild Dis* **49** 900-10.
- Marques PX, O'Donovan J, Williams EJ, Souda P, Gutierrez J, Worrall S, McElroy M, Proctor A, Brady C, Sammin D, Basset H, Whitelegge JP, Markey BK, Nally JE.** 2010 Identification of immunologically relevant proteins of *Chlamydia abortus* using sera from experimentally infected pregnant ewes. *Clin Vaccine Immunol* **17** 1274-1281.
- McGlothlin JA, Lester GD, Hansen PJ, Thomas M, Pablo L, Hawkins DL & LeBlanc MM** 2004. Alteration in uterine contractility in mares with experimentally induced placentitis. *Reproduction* **127** 57-66.
- Moreira N, Kruger ER, Warth JFG, Biesdorf SM, Goularte MMM & Weiss RR** 1998. Aspectos etiologicos e epidemiologicos do aborto equine. *Arch Vet Scienc* **3** 25-30.
- Marcolongo-Pereira, C, Adrien ML, Ladeira SRL, Soares MP, Assis-Brasil ND & Schild AL.** Abortos em equinos na regio sul do Rio Grande do Sul: estudo de 72 casos. *Pesq Vet Bras* **32** 22-26.
- Munro C & Stabenfeldt G** 1984. Development of a microlite plate enzyme immunoassay for the determination of progesterone. *J Endocrinol* **101**, 41-9.
- Okano A, Shioya Y, Obata T & Fukuhara R** 1978. Specific protein in bovine allantoic fluid and amniotic fluid. *Jap J Zootech Sci* **49** 276-282.
- Ousey JC, Houghton E, Grainger L, Rossdale PD & Fowden AL** 2005. Progesterone profiles during the last trimester of gestation in Thoroughbred mares with normal and compromised pregnancies. *Theriogenology* **64** 1844-1856.
- Olsen SC & Johnson C** 2011 Comparison of abortion and infection after experimental challenge of pregnant Bison and Cattle with *Brucella abortus* strain 2308. *Clin Vaccine Immunol* **18** 2075-2078.
- Ousey JC, Houghton E, Grainger L, Rossdale PD & Fowden AL** 2005. Progesterone profiles during the last trimester of gestation in Thoroughbred mares with normal or compromised pregnancies. *Theriogenology* **63**, 1844-56.
- Ousey JC, Rossdale PD, Cash RS & Worthy K** 1987 Plasma concentrations of progesterone, oestrone sulphate and prolactin in pregnant mares subjected to natural challenge with equid herpesvirus-1. *J Reprod Fert (Suppl.)* **35**, 19-28.
- Ousey JC** 2011 Endocrinology of pregnancy. In: *Equine Reproduction*. Ed: A.O. McKinnon, E.L. Squires, W. Vaala, and D. D. Varner. Wiley-Blackwell Philadelphia. pp. 2222-2231.
- Ousey JC, Forhead AJ, Rossdale PD, Grainger L, Houghton E & Fowden AL** 2003 Ontogeny of uteroplacental progesterone production in pregnant mares during the second half of gestation. *Biol Reprod* **69**, 540-548.
- Ousey JC, Houghton E, Grainger L, Rossdale PD & Fowden AL** 2005 Progesterone profiles during the last trimester of gestation in Thoroughbred mares with normal or compromised pregnancies. *Theriogenology* **63** 1844-56.
- Ousey JC, Rossdale PD, Cash RS & Worthy K** 1987 Plasma concentrations of progesterone, oestrone sulphate and prolactin in pregnant mares subjected to natural challenge with equid herpesvirus-1. *J Reprod Fert (Suppl.)* **35**, 19-28.
- Pashen RL & Allen WR** 1979 The role of the fetal gonads and placenta in steroid production, maintenance of pregnancy and parturition in the mare. *J Reprod Fert Suppl* **27**, 499-509.

- Paccamonti DL, Swiderski CE, Marx B, Gaunt S & Blouin D** 1995. Electrolytes and biochemical enzymes in amniotic and allantoic fluid of the equine fetus during late gestation. *Biol Reprod Monograph Series* **1** 39-48.
- Peck AB, Murgita RA & Wigzell H** 1978. Cellular and genetic restrictions in the immunoregulatory activity of alpha-fetoprotein. *J Exp Med* **147** 667-683.
- Palmer MV, Cheville NF & Jensen AE** 1996. Experimental infection of pregnant cattle with the vaccine candidate *Brucella abortus* strain RB51: pathologic, bacteriologic, and serologic findings. *Vet Path* **33** 682-691.
- Poester FP, Samartino LE, Santos RL** 2013. Pathogenesis and pathobiology of brucellosis in livestock. *Rev Sci Tech Off Int Epiz* **32** 105-115.
- Popowski T, Goffinet F, Maillard F, Schmitz T, Leroy S & Kayem G** 2011 Maternal markers for detecting early-onset neonatal infection and chorioamnionitis in cases of premature rupture of membranes at or after 34 weeks of gestation: a two-center prospective study. *BMC Preg Childbirth* **26** 11-26.
- Raeside JI, Liptrap RM, McDonell WN & Milne FJ** 1979 A precursor role for DHA in a feto-placental unit for oestrogen formation in the mare. *J Reprod Fertil Suppl.* **27**,493-7.
- Raeside JI & Rosskopf EM** 1980. Simulation of pregnancy levels of plasma oestrogen sulphate by infusion in the non-pregnant mare: a preliminary study. *Anim Reprod Sci* 1980; **3**, 101-106.
- Raeside JI, Liptrap RM, McDonell WN & Milne FJ** 1979 A precursor role for DHA in a feto-placental unit for oestrogen formation in the mare. *J Reprod Fertil Suppl* **27**,493-7.
- Raeside JI** 2004 Estrogens in the pregnant mare: a review Equine placental pathology: Kentucky perspective. In: *Proc 1st workshop on the equine placenta, University of Kentucky*, Lexington, KY, pp.42-48.
- Raeside JI, Renaud RL & Christie HL** 1997 Postnatal decline in gonadal secretion of dehydroepiandrosterone and 3 beta-hydroxyandrost-5,7-dien-17-one in the newborn foal. *J. Endocrinol.* **155**, 277-82.
- Romero R, Espinoza J, Rogers WT, Moser A, Nien JK, Kusanovic JP, Gotsch F, Erez O, Gomez R & Edwin S** 2008. Proteomic analysis of amniotic fluid to identify women with preterm labor and intra-amniotic inflammation/infection: The use of a novel computational method to analyze mass spectrometric profiling. *J Matern Fetal Neonatal Med* **21** 367-388.
- Ruetschi U, Rosen A, Karlsson G, Zetterberg H, Rymo L, Hagberg H & Jacobsson B** 2005. Proteomic analysis using protein chips to detect biomarkers in cervical and amniotic fluid in women with intra-amniotic inflammation. *J Proteome Res* **4** 2236-2242.
- Ryan PL, Christiansen DL, Hopper RM, Bagnell CA, Vaala WE & LeBlanc MM** 2009. Evaluation of systemic relaxin blood profiles in horses as a means of assessing placental function in high-risk pregnancies and responsiveness to therapeutic strategies. *Ann NY Acad Sci* **1160**, 169-78.
- Rosdale PD, Ousey JC, Cottrill CM, Chavatte P, Allen WR & McGladdery AJ** 1991. Effects of placental pathology on maternal plasma progesterone and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. *J. Reprod. Fert. Suppl.* **44** 579-590.
- Rance TA & Park BK** 1978. Measurement of estrone, equilin and dehydroepiandrosterone in peripheral plasma of pregnant pony mares by radioimmunoassay *J Steroid Biochem* **10** 65-9.

- Riddler AL, Smith SL & West DM** 2014. Seroconversion and semen shedding in rams experimentally infected with *Brucella ovis*. *N Z Vet J* **62** 47-50.
- Ruetschi U, Rosen A, Karlsson G, Zetterberg H, Rymo L, Hagberg H & Jacobsson B** 2005. Proteomic analysis using protein chips to detect biomarkers in cervical and amniotic fluid in women with intra-amniotic inflammation. *J Proteome Res* **6** 2236-2242.
- Renaudin CD, Troedsson MHT, Gills VL, King A & Bodena A.** 1997 Ultrasonographic evaluation of the equine placental by transrectal and transabdominal approach in the normal pregnant mare. *Theriogenology* **47** 559-573.
- Ryu A, Park KH, Oh KJ, Lee SY, Jeong EH, Park JW** 2013 Predictive value of combined cervicovaginal cytokines and gestational age at sampling for intra-amniotic infection in preterm premature rupture of membranes. *Acta Obstet Gynecol Scand* **92** 517-24.
- Rosdale PD, Ousey JC, Cottrill CM, Chavatte P, Allen WR & McGladdery AJ** 1991 Effects of placental pathology on maternal plasma progestagen and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. *J Reprod Fert Suppl* **44**, 579-90.
- Santschi EM, LeBlanc MM & Weston PG** 1991 Progestagen, oestrone sulphate, and cortisol concentrations in pregnant mares during medical and surgical disease. *J Reprod Fert (Suppl)* **44** 627-634.
- Scott F, Peters H, Boogert T, Robertson R, Anderson J, McLennan A, Kesby G & Edelman D** 2002. The loss rates for invasive prenatal testing in a specialized obstetric ultrasound practice. *Aust N Z J Obst Gynaecol* **42** 55-58.
- Stabenfeldt GH, Daels PF, Munro CJ, Kindahal H, Hughes JP & Lasley B** 1991 An oestrogen conjugate enzyme immunoassay for monitoring pregnancy in the mare: limitations of the assay between days 40 and 70 of gestation. *J Reprod Fert Suppl* **44**, 37-44.
- Stott JL, Blanchard MT, Anderson M, Mass J, Walker RL, Kennedy PC, Norman BB, BonDurant RH, Oliver MN, Hanks D & Hall MR** 2002. Experimental transmission of epizootic bovine abortion (foothill abortion). *Vet Microbiol* **88** 161-173.
- Schmidt AR, Williams MA, Carleton CL, Darien BJ & Derken FJ** 1991. Evaluation of transabdominal ultrasound-guided amniocentesis in the late gestation. *Equine Vet J* **23** 261-265.
- Simpson KS, Adams MH, Behrendt-Adam CY, Baker CB, McDowell KJ** 2000. Differential gene expression in day 12 and day 15 equine conceptuses. *J Reprod Fert Suppl* **56**, 539-47.
- Smith KM, Lai PCW, Roberston HA, Church RB & Lorscheider FL** 1979. Distribution of alpha-fetoprotein in fetal plasma, allantoic fluid, amniotic fluid and maternal plasma of cows. *J Reprod Fert* **57**, 235-238.
- Sorensen K, Neely DP, Read W & Grappel PM** 1990. Measurement and clinical significance of equine fetal protein in pregnant mares serum. *J Equine Vet Sci* **10** 417-421.
- Sells S.** Molecular diagnostics of medically important bacterial infections of the equine placenta caused by members of the order actinomycetales. PhD dissertation. University of Madras, Chennai, India.
- Sheoran AS, Nally JE, Donahue JM, Smith BJ & Timoney JF** 2000 Antibody isotypes in sera of equine fetuses aborted due to *Leptospira interrogans* serovar Pomona-type kennewicki infection. *Vet Immunol Immunopathol* **77** 301-9.

- Silberzahn P, Zwain I & Martin B** 1984 Concentration increase of unbound testosterone in plasma of the mare throughout pregnancy *Endocrinol* **115** 416-9.
- Smith CR, Ketterer PJ, McGowan MR & Corney BG** 1994. A review of laboratory techniques and their use in the diagnosis of *Leptospira interrogans* serovar hardjo infection in cattle. *Aust Vet J* **9** 290-4.
- Shivaprasad HL, Sundberg JP, McEntee K, Gordon L, Johnstone AC, Lombardo de Barros CS & Hoffman RL** 1994 Cystic adenomatous hyperplasia of the equine allantois: a report of eight cases *J Vet Diagn Invest* **6** 107-10.
- Sereepapong W, Limpongsanurak S, Triratanachat S, Wannakrairot P, Charuruks N & Krailadsiri P** 2001 The role of maternal serum C-reactive protein and white blood cell count in the prediction of chorioamnionitis in women with premature rupture of membranes. *J Med Assoc Thai* **84** 360-6.
- Sherman DJ, Tovbin J, Lazarovich T, Avrech O, Reif R, Hoffmann S, Caspi E & Boldur I** 1997 Chorioamnionitis caused by gram-negative bacteria as an etiologic factor in preterm birth. *Eur J Clin Microbiol Infect Dis* **6** 417-23.
- Smith EJ, Muller CL, Sartorius JA, White DR & Maslow AS** 2012 C-reactive protein as a predictor of chorioamnionitis. *J Am Osteopath Assoc* **112** 660-4.
- Taylor BD, Holzman CB, Fichorova RN, Tian Y, Jones NM, Fu W & Senagora PK:** 2013 Inflammation biomarkers in vaginal fluid and preterm delivery *Hum Reprod* **28** 942-952.
- Sorensen K, Neely DP, Read W & Grappel PM** 1990. Measurement and clinical significance of equine fetal protein in pregnant mares serum. *J Equine Vet Sci* **10** 417-421.
- Smith KC, Blunden AS, Whitwell KE, Dunn KA & Wales AD** 2003 A survey of equine abortion, stillbirth and neonatal death in the UK from 1988 to 1997. *Equine Vet J* **35**, 496-501.
- Tambor V, Kacerovsky M, Lenco J, Bhat G & Menon R** 2013 Proteomics and bioinformatics analysis reveal underlying pathways of infection associated histologic chorioamnionitis in pPROM *Placenta* **34** 155-61.
- Tambor V, Kacerovsky M, Andrys C, Musilova I, Hornychova H, Pliskova L, Link M, Stulik J & Lenco J** 2012. *PLoS One* **7** e41164.
- Tait AD, Hodge LC & Allen WR** 1985. The biosynthesis of 3 beta-hydroxy-5,7-androstadien-17-one by the horse fetal gonad. *FEBS Lett* **182** 107-110.
- Tan AC, Ward M Goodfellow.** (2006) Exploration of Amycolatopsis diversity in soil using genus-specific primers and novel selective media. *Syst Appl Microbiol* **29** 557-569.
- Thompson ME, Zhou A & Knott CD** 2012 Low testosterone correlates with delayed development in male Orangutans. *PLOS One* **7** e47282.
- Timoney JF, Kalimuthusamy N, Velineni S, Donahue JM, Artiushin SC & Fettinger M** 2011 A unique genotype of *Leptospira interrogans* serovar Pomona type kennewicki is associated with equine abortion. *Vet Microbiol* **150** 349-53.
- Tita ATN & Andrews WW** 2009 Diagnosis and management of clinical chorioamnionitis. *Clin Perinatol* 2010; **37**:339-35
- Troedsson MH & Macpherson ML** 2011. Placentitis. In AO McKinnon, EL Squires, WE Vaala, DD Varner (Eds.). Equine Reproduction. 2011, Wiley-Blackwell, Hoboken NJ, pp. 2359-67.
- Troedsson MH & Zent WW** 2004. Clinical ultrasonographic evaluation of the equine placenta as a method to successfully identify and treat mares with placentitis. In K

- Betteridge (Ed.) Workshop on the Equine Placenta, University of Kentucky Agricultural Station, Lexington, KY 2004, pp.66-67.
- Todhunter KH, Muscatello G, Blishen AJ, Chicken C, Perkins NR, Gilkerson JR, & Begg AP** 2013 Bacteria isolated from field cases of equine amnionitis and fetal loss. *Aust Vet J* **91**, 138-142.
- Urieli-Shoval S, Cohen P, Eisenberg S & Matzner Y** 1998 Widespread expression of serum amyloid A in histologically normal human tissues. Predominant localization to the epithelium. *J. Histochem Cytochem* **46**1377-84.
- Volkman DH, Williams JH, Henton JH, Donahue JM & Williams NM** 2001. The first reported case of equine nocardioform placentitis in South Africa. *J S Afr Vet Assoc* **72**, 235-8.
- Verma A, Stevenson B & Adler B** 2013. Leptospirosis in horses. *Vet Microbiol* **167** 61-66.
- Weng, Q., Tanaka, Y., Taniyama, H., Tsunoda, N., Nambo, Y., Watanabe, G, and Taya, K.** (2007) Immunolocalization of the steroidogenic enzymes in equine fetal adrenal glands during mid-late gestation. *J Reprod Dev* **53**, 1093-8.
- Williams NM, Donahue JM, Bolin DC, Giles RC, Harrison LR, Hong CB, Jackson CB, Poonacha KB & Vickers ML** 2004. Equine placental pathology: Kentucky perspective. In: K Betteridge (Ed.) Workshop on the Equine Placenta, University of Kentucky Agricultural Station, Lexington, KY 2004, pp.88-92.
- Williams MA, Wallace SS, Tyler JW, McCall CA, Gutierrez A & Spano JS** 1993. Biochemical characteristics of amniotic and allantoic fluid in late gestational mares. *Theriogenology* **40**, 1251-1257.
- Wolfsdorf KE, Williams NM & Donahue M** 2000 Bacterial placentitis attributable to a gram-positive filamentous branching bacillus organism. *J Am Vet Med Assoc* **216**, 1915-1916.
- Wu HC, Shen CM, Wu YY, Yuh YS & Kua KE** 2009 Subclinical histological chorioamnionitis and related clinical and laboratory parameters in preterm deliveries. *Pediatr Neonatol* **50** 217-21.
- Yamada H, Kato EH, Morikawa M, Shimada S, Saito H, Watari M, Minakami H & Nishihira J** 2003 Decreased serum levels of macrophage migration inhibition factor in miscarriages with normal chromosome karyotype *Hum Reprod* **18** 616-20.
- Yoon BH, Jun JK, Park KH, Syn HC, Gomez R, Romero R** 1996. Serum C-reactive protein, white blood cell count, and amniotic fluid white blood cell count in women with preterm premature rupture of membranes. *Obstet Gynecol* **88**1034-40.
- Zanella, L.F., Takahira, R.K., Melo-Ona, C.M., Ona-Magalhaes, L.C., Prestes, N.C.,** 2014. Biochemical profile of amniotic and allantoic fluid during different gestational phases in mares. *J. Equine Vet. Sci.* **34**, 403-406.
- Zanella LF** 2008 Biochemical analyses of the amniotic and allantoic fluids throughout gestation in *Equus caballus*. Dissertation thesis, São Paulo State University. Botucatu, São Paulo, Brazil 2008.
- Zavy, M.T., Vernon, M.W., Sharp, D.C. III and Bazer, F.W.** (1984) Endocrine aspects of early pregnancy in pony mares: a comparison of uterine luminal and peripheral plasma levels of steroids during estrous cycle and early pregnancy. *Endocrinology* **115**, 214-219.

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PROFESSIONAL ADDRESS & POSITION

Assistant Professor of Theriogenology, Section of Equine Medicine & Surgery, Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois.

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EDUCATION

PhD candidate in Veterinary Science, Reproduction Laboratory, The Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington KY, USA. Thesis: Studies on Equine. Advisor: Dr. Barry A. Ball (2011-2014).

Residency in Theriogenology, Cornell University College of Veterinary Medicine, Ithaca NY, USA. Two-year program with multi-species approach for clinical training, with particular emphasis in equine theriogenology (July-2009-July 2011).

Visiting Scholar, Aberystwyth University, Institute of Rural Sciences, Aberystwyth, Wales, United Kingdom. Field: Equine Reproduction & Stud Farm Management, Advisor: Dr. Mina Davies-Morel. (June 2008 - January 2009).

Master of Science, Federal University of Viçosa, Department of Animal Sciences, Viçosa, MG, Brazil. Field: Equine Reproductive Physiology & Biotechnology. (2006-2008).

DVM, Federal University of Paraná College of Veterinary Medicine, Parana, Brazil. (1999-2003).

BOARD CERTIFICATIONS

- I** Diplomate, American College of Theriogenologists (Comparative Animal Reproduction), Milwaukee, Wisconsin USA, 2011.
- II** Diplomate, European College of Animal Reproduction (Equine Reproduction), 2012, Budapest, Hungary European Union, 2012.

EMPLOYMENTS

- 2014-till University of Illinois Urbana-Champaign, College of Veterinary Medicine, Department of Veterinary Medicine Clinical Sciences, Urbana, IL.
- 2011-14 Graduate Research Assistant University of Kentucky, Maxwell H. Gluck Equine Research Center, Lexington, KY.
- 2011-13 Staff Veterinarian (research herd 450-600 horses and 80 goats), University of Kentucky, Maxwell H. Gluck Equine Research Center, Lexington KY.
- 2009-11 Theriogenology Resident, Cornell University Hospital for Animals, Department of Clinical Sciences, Ithaca NY.
- 2006-08 Graduate Research Assistant Federal University of Viçosa and Stud & Mule Farm Veterinarian (Taruma Stud Farm), Viçosa/Ponte Nova, MG, Brazil.
- 2007 Lecturer, Animal Reproduction, University of Viçosa College of Veterinary Medicine, Viçosa MG, Brazil.
- 2004 Lecturer, Veterinary Obstetrics, Federal University of Mato Grosso Department of Veterinary Clinical Sciences, Cuiabá MT, Brazil
- 2003-08 Self-employed large animal practitioner (Equine, Bovine and Ovine), Brazil.

1. PUBLICATIONS

2. Selected peer review
3. Silva ES, Scoggin KE, **Canisso IF**, Troedsson MH, Squires EL, Ball BA. Expression of receptors for ovarian steroids and prostaglandin E2 in the endometrium and myometrium of mares during estrus, diestrus and early pregnancy. *Animal Reproduction Science* 2014; 151:169-181.
4. **Canisso IF**, Ball BA, Cray C, Williams NM, Scoggin KE, Davolli GM, Squires EL, Troedsson MH. Serum amyloid A and haptoglobin concentrations are increased in plasma of mares with ascending placentitis in the absence of changes in peripheral leukocyte counts or fibrinogen concentration. *American Journal of Reproductive Immunology* 2014, 72:376-85.
5. **Canisso IF**, Ball BA, Squires EL, Troedsson MH. How to perform transabdominal ultrasound guided fetal fluid sampling. *Journal of Equine Veterinary Science* 2014; 34: 1143-1147.
6. **Canisso IF**, Ball BA, Cray C, Squires EL, Troedsson MH. Use of a qualitative horse-side test to measure serum amyloid A in mares with experimentally induced ascending placentitis. *Journal of Equine Veterinary Science* 2014- in press.
7. **Canisso, IF**, Coffee LC, Ortved K, Fubini S, Schlafer DH, Gilbert, R.O. Bilateral Sertoli and Interstitial cell tumors in abdominal testes of a goat with Polled Intersex Syndrome (PIS). *Reproduction in Domestic Animals* 2014; 49:64-9.
8. **Canisso IF**, Ball BA, Erol E, Claes A, Scoggin KE, McDowell KJ, Williams NM, Dorton AR, Wolfsdorf KE, Squires EL, Troedsson MH. Attempts to induce nocardioform placentitis (*Crossiella equi*) experimentally in mares. *Equine Veterinary Journal*, 2014 doi: 10.1111/evj.12249.

9. **Canisso IF**, Ball BA, Troedsson MH, Silva ES, Davolli GM. Decreasing pH of mammary gland secretions is associated with parturition and is correlated with electrolyte concentrations in prefoaling mares. *Veterinary Record* 2013. doi:10.1136/vr.101658.
10. **Canisso IF**, Gallacher K, Gilbert M-A, Korn AE, Bedford-Guaus SJ, Schweizer CM, Gilbert, RO. Preovulatory progestagen treatment in mares fails to delay ovulation. *The Veterinary Journal* 2013, 197: 324-328.
11. Coutinho da Silva MA, **Canisso IF**, Macpherson M, Johnson AEM. Serum amyloid A concentration in healthy periparturient mares and mares with experimentally ascending placentitis. *Equine Veterinary Journal* 2013; 45:619-624.
12. **Canisso IF**, Pinn T, Gerdin JA, Olivett TL, Buckles EL, Schweizer CM, Ainsworth DM. B-cell multicentric lymphoma as a probable cause of abortion in a Quarter Horse broodmare. *Canadian Veterinary Journal* 2013; 61: 288-291.
13. **Canisso IF**, Rodriguez JA, Sanz M, Coutinho da Silva MA. A clinical approach to the diagnosis and treatment of retained fetal membranes with an emphasis placed on the critically ill mare. *Journal of Equine Veterinary Science* 2013; 33:570-579.
14. **Canisso IF**, Beltaire K, Bedford-Guaus SJ. Premature luteal regression in a pregnant mare and subsequent pregnancy maintenance with the use of oral altrenogest. *Equine Veterinary Journal* 2013; 45: 97-100.
15. **Canisso IF**, Carvalho GR, Davies-Morel MCG, Ker PG, Rodrigues AL, Silva EC, Coutinho da Silva. Seminal parameters and field fertility of cryopreserved donkey jacks semen after insemination of horse mares. *Equine Veterinary Journal* 2011; 43: 179-83.
16. **Canisso IF**, Carvalho GR, Davies-Morel MCG, Guimarães JD, McDonnell SM. Sexual behavior and ejaculate characteristics in Pêga donkeys (*Equus asinus*) mounting estrous horse mares (*Equus caballus*). *Theriogenology* 2010; 73: 56-63.
17. **Canisso IF**, Coutinho da Silva MA, Davies-Morel MCG, McDonnell SM. How to manage jacks to breed mares. Proceedings Annual Convention AAEP 2009; 55, 342-348.
18. **Canisso IF**, Davies-Morel MCG, McDonnell SM. Strategies for the management of donkey jacks in intensive breeding systems. *Equine Veterinary Education* 2009; 12: 652-59.
19. **Canisso IF**, Carvalho GR, Silva EC, Rodrigues AL, Ker PG, Guimarães JD. Some biometric aspects of the external genital tract from donkeys Pêga breed semen donors. *Ciência Rural* 2009; 39:2556-2562. (In Portuguese).
20. **Canisso IF**, Souza FA. Entrapment of the small intestine in the epiploic foramen in a stallion. *Ciência Veterinária Trópicos*. 2009; 12:60-64. (In Portuguese).
21. **Canisso IF**, Souza FA, Silva EC, Rodrigues AL. Vaginal prolapse, dystocia, necrotic vaginitis, induced by a foreign body in a primiparous cow. *Brazilian Ruminants Journal*, 2008; 3,7-14. (In Portuguese).
22. **Canisso IF**, Souza FA, Palhares MS. Equine neonatal isoerythrolysis. *Brazilian Journal of Equine Medicine*, 2008; 18: 30-36. (In Portuguese).
23. **Canisso IF**, Souza FA, Carvalho GR, Silva EC, Guimarães JD, Lima AL. (2008) Artificial Insemination in horses: raw, extended cooled & shipped semen. *Academic Journal Agricultural Sciences* 2008; 8: 30-47. (In Portuguese).

24. **Canisso IF**, Souza FA, Escobar JMO, Carvalho GR, Davies Morel MC, Da Silva EC, Guimarães JD, Lima AL. Freezing of donkey semen (*Equus asinus*). *Revista Investigaciones Veterinarias del Peru*, 2008; 19:113-125. (In Spanish).
25. **Canisso IF**, Souza FA, Carvalho GR, Guimarães JD, Silva EC, Lima AL. Breeding soundness examination of donkey jacks (*Equus asinus*). *Revista Brasileira de Reprodução Animal* 2008; 32:233-239. (In Portuguese).
26. Souza FA, **Canisso IF**. Biological aspects of aging and diminution of reproductive efficiency in mares. *Ciência Veterinária Trópicos* 2008; 11:9-15. (In Portuguese).
27. Souza FA, **Canisso IF**, Borges AM, Vale Filho VR, Lima AL, Silva EC. Uterine environment roles in embryonic losses in mares. *Revista Conselho Federal Medicina Veterinaria*. 2008; 22, 25-35. (In Portuguese).

Abstracts

1. **Canisso IF** How to treat retained placenta in mares. Annual Meeting European Society for Domestic Animals Reproduction, Helsinki Finland. Reproduction in Domestic Animals 2014.
2. **Canisso IF**, Ball BA, Esteller-Vico A, Squires EL, Troedsson MH. **Dehydroepiandrosterone sulphate (DHEA-S) concentrations in mares with experimentally induced ascending placentitis**. Annual Meeting European Society for Domestic Animals Reproduction, Helsinki Finland. Reproduction in Domestic Animals 2014.
3. **Canisso IF**, Ball BA, Esteller-Vico A, Squires EL, Troedsson MH. Dehydroepiandrosterone sulfate and testosterone concentrations in mares carrying normal pregnancies. Society for Theriogenology 2014.
4. **Canisso IF**, Ball BA, Scoggin KE, Troedsson MHT. Alpha-fetoprotein is highly expressed and appears to be increased in the foetal fluids of mares with placentitis. Proceedings European Society for Domestic Animals Reproduction, Bologna, Italy. Reproduction Domestic Animals 2013.
5. **Canisso IF**, Ball BA, Troedsson MHT, Cray C, Davolli GM, Squires EL, Williams NM. Acute phase proteins and leukocyte counts in mares with experimentally induced ascending placentitis. Proceedings XI International Symposium on Equine Reproduction New Zealand, January (2014 accepted).
6. **Canisso IF**, Ball BA, Troedsson MHT, Silva EM, Davolli GM. Decreasing pH of mammary gland secretions predict foaling and are correlated with electrolyte concentrations in prefoaling mares. Proceedings Annual Convention American Association of Equine Practitioners 2013 (December 2013-accepted)..
7. Ball BA **Canisso IF**, Vico AE, Troedsson MHT, Stanley SD. Estrogens as potential diagnostic markers in mares with experimentally induced ascending placentitis. Proceedings Annual Convention American Association of Equine Practitioners 2013 (December 2013-accepted).
8. **Canisso IF**, Ball BA, Troedsson MHT, Claes A, Scoggin KE, Erol E, Williams, NM, Squires EL. Experimental induction of nocardioform placentitis in mares. In Proceedings Annual Meeting Society for Theriogenology/American College Theriogenologists August 2013.
9. **Canisso IF**, Ball BA, Troedsson MHT, Cray C. Serum Amyloid A and Haptoglobin concentrations in pregnant mares with experimentally induced ascending

- placentitis. Proceedings Annual Convention American Association Equine Practitioners 2012 58; 524-525.
10. **Canisso IF**, Gallacher K, Gilbert M-A, Korn AE, Bedford-Guaus SJ, Schweizer CM, Gilbert, RO.et al. Progestin treatment of preovulatory mares fails to delay ovulation and may impair fertility. Proceedings Annual Meeting Society for Theriogenology/American College Theriogenologists *Clinical Theriogenology Journal* 2012.
 11. Souza IF, Canisso IF et al. Association of IGF-1 concentrations in seminal plasma and puberty in Gir Zebu bulls. Proceedings Annual Meeting Society for Theriogenology/American College Theriogenologists. *Clinical Theriogenology Journal* 2012.
 12. **Canisso IF**, Ball BA, Troedsson MHT, Klein C, Stanley SD. Diagnostic markers for experimentally induced ascending placentitis in mares. Proceedings XI International Symposium on Equine Infectious Diseases. *Journal Equine Veterinary Science* 2012; 32:S73.
 13. Coutinho da Silva MA, **Canisso IF**, Pinto CRF, Macpherson ML, Forshey BS, Howell DG. Serum amyloid A concentration in mares with experimentally induced ascending placentitis. Proceedings 8th International Symposium on Equine Embryo Transfer. *Journal Equine Veterinary Science* 32; 407, 2012.
 14. Coutinho da Silva MA, **Canisso IF**, Johnson AE, Divers T. Parturition increases serum amyloid A concentration in healthy pregnant mares. Proceedings Society for Theriogenology/American College of Theriogenologists. *Clinical Theriogenology* 3, 355, 2011.
 15. **Canisso IF**, Carvalho GR, Coutinho MA. Donkey jack semen cryopreservation and seminal parameters. Proceedings Society for Theriogenology/American College of Theriogenologists 2009- 2nd place Abstract Competition *Clinical Theriogenology* 3, 2009.
 16. **Canisso IF**, Coutinho da Silva, Davies-Morel MG, McDonnell SM. How to manage jacks to breed mares. Proceedings Annual Convention American Association of Equine Practitioners 2009; 55:342-348.
 17. **Canisso IF**, Carvalho GR, Torres CAA, Guimarães JD, Souza FA, Silva EC, Martins LF. Sexual behavior of jacks when an estrous mare is used in semen collection. Proceedings 5th International Symposium on Stallion Reproduction. *Animal Reproduction Science* 2008; 107:314.

BOOK CHAPTERS CO-AUTHORED

1. **Canisso IF**, Coutinho da Silva MA. Bacterial Endometritis. Current Therapy in Equine Medicine Ed. E Robinson, K. Sprayberry. Elsevier Saint Louis, MO 2013; 7th edition, Chapter 164 – in press.
2. **Canisso IF**, Coutinho da Silva MA. Ovarian Enlargement, Physiology. In The Horse Clinical Veterinary Advisor Ed. Wilson DA, Section editor Juan Samper. Elsevier Saint Louis, MO 2011; p421-23.
3. **Canisso IF**, Coutinho da Silva MA. Infertility, chromosomal or genetic. In The Horse Clinical Veterinary Advisor. Ed. Wilson DA, Section editor Juan Samper. Elsevier Saint Louis, MO 2011; p 295-296.

4. **Canisso IF**, McDonnell SM Donkey breeding behavior with an emphasis on the Pêga breed. In: Veterinary Care of Donkeys. Eds. Matthews & Taylor. 2010
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GUEST LECTURERS

2013 The road trip to the development of a diagnostic panel for equine placentitis. Veterinary Seminar Series, Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY.

2012 What theriogenologists should know about donkey reproduction. Annual Meeting, Society for Theriogenology/American College of Theriogenologists Baltimore, MA.

2011 Donkey Reproductive Behavior, Section of Reproduction and Behavior, New Bolton Center, University of Pennsylvania, PA.

2009 How to manage jacks to breed mares. Annual Covention, American Association of Equine Practitioners Las Vegas NV (“How to lecturer”).

2007 Stallion semen cryopreservation. Academic Veterinary Week University of Viçosa, Minas Gerais, Brazil.

2007 Beef cattle breeding management and reproductive efficiency, College of Veterinary Medicine, University of Viçosa-UNIVIÇOSA, Viçosa, Minas Gerais, Brazil.

2006 Breeding and production system of the Pantaneiro Horse, Equine Students’ Group, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil.

2007 Reproductive Endocrinology, Federal University of Mato Grosso College of Veterinary Medicine, Federal University Mato Grosso, Cuiaba, Mato Grosso, Brazil. Two lecturers of 3-h.