ABSTRACT

BEACHLER, THERESA, MARIE. Metabolomic Profiling of Pregnant Mares with Experimental Ascending Placentitis. (Under the direction of Drs. Christopher Scott Bailey and Samuel Jones).

Equine ascending placentitis caused by migration of bacteria through the mare's reproductive tract remains a clinical challenge. Diagnosis is complicated by minimal to inconsistent clinical signs that are displayed in affected animals as well as the diagnostic modalities available for diagnosis early in the clinical course of disease. Currently, the diagnosis of ascending placentitis during pregnancy relies on a combination of clinical signs and transrectal ultrasonography to examine for thickening of the combined thickness of the uterus and placenta or the presence of placental separation near the level of the cervix. A host of other adjunctive diagnostic modalities such as hormonal profiling or the evaluation of markers of inflammation and infection such as serum amyloid A have been examined. Many of the changes noted, however, may also be detected in cases of systemic disease as well as conditions affecting other body systems or the fetoplacental unit in addition to ascending placentitis. Metabolomic profiling studies recently have been explored in both human medicine as well as veterinary medicine for the identification of biomarkers which may be used for diagnosing or identifying individuals at risk for developing a particular condition or disease. Therefore, the purpose of this research was to identify the metabolomic profile of biofluids from mares affected by experimental placentitis compared to that of healthy animals, with the goal of identifying individual metabolites or metabolite profiles that may be useful for identifying mares affected by the clinical disease.

In order to examine this, one-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectroscopic based metabolomics was performed on collected plasma and allantoic fluid to identify and quantify metabolites present. An experimental model of

Streptococcus equi subspecies *zooepidemicus* ascending placentitis was used to simulate disease in mares at 280 to 285 days of gestation. Plasma and allantoic fluid were serially collected from inoculated mares and healthy control animals at time points within the acute phase of infection until abortion, stillbirth, or foaling. Concurrently, whole blood lactate, serum amyloid A (SAA), and concentrations of progesterone, and estradiol-17β were collected along with the performance of transrectal and transabdominal ultrasonography at each time-point to compare the onset of metabolomic changes to those of commonly utilized diagnostic modalities.

Surprisingly, metabolomic alterations within the allantoic fluid were not identified between healthy and inoculated mares prior to or after the diagnosis of ascending placentitis. In contrast, in plasma two phases of metabolic changes were noted after experimental inoculation and infection. An immediate rise in the concentration of metabolites involved in energy, nitrogen, hydrogen, and oxygen metabolism were seen within four hours of inoculation and was followed by a decrease in metabolite concentrations involved in energy and nitrogen metabolism by day four coinciding with the ultrasonographic diagnosis of placentitis and prior to elevations in the systemic marker of inflammation, serum amyloid A. In summary, these studies serve to identify and expand the metabolomic profile of equine allantoic fluid and plasma respectively. Further research is needed to determine if these metabolic alterations could serve as a diagnostic target for the confirmation of disease in mares affected by experimental placentitis as well as in natural cases of infection.

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Metabolomic Profiling of Pregnant Mares with Experimental Ascending Placentitis

by
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DEDICATION

This thesis is dedicated to my husband Clifford for his unwavering support, patience, and love. Also, in honor of my late mother Audrey Gunter Beachler who taught that it takes courage and persistence to pursue your dreams.

BIOGRAPHY

Theresa Marie Beachler was born and raised in Sanford, North Carolina by her parents Robert and Audrey Beachler. She graduated from North Carolina State University with a Bachelor of Science in Animal Science in 2007 and a Doctorate of Veterinary Medicine in 2012. Following graduation of veterinary school, Theresa completed an internship and three-year residency at North Carolina State University College of Veterinary Medicine in comparative theriogenology and became board certified as a Diplomat of the American College of Theriogenologists in August of 2015. During completion of her residency, a dual master's program was transitioned to a Ph.D. in Comparative Biomedical Sciences under the guidance of Christopher Scott Bailey. The focus of her graduate research centered on the metabolomic investigation of equine biofluids in an experimental model of ascending placentitis utilizing nuclear magnetic resonance (NMR) spectroscopy, which is the work presented here.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Ascending Placentitis: Introduction and Review of the Current Literature

1.1 Overview of Placentitis

Placentitis is estimated to affect three to seven percent of all equine pregnancies and remains one of the most common causes of abortion, stillbirth, premature delivery, and neonatal mortality in the horse (Troedsson and Zent, 2004, Barr 2005, and Loef et al. 2010). In general, three types of placentitis have been described, including 1) ascending, 2) diffuse or hematogenous, and 3) focal or mucoid/Nocardioform placentitis (Williams et al. 2004 and Zent et al. 1999). Canisso and colleagues have more recently suggested the use of four categories based on the classification scheme of Williams et al. dividing the category focal into focal mucoid and multifocal as two separate entities (Canisso et al. 2015a and Williams et al. 2004). Our understanding of multifocal lesions and pathophysiology, however, remains scarce (Canisso et al. 2015a and Williams et al. 2004). Ascending placentitis is caused by transcervical bacterial migration through the caudal reproductive tract. As ascending placentitis is the most frequently diagnosed type of placentitis in mares in the United States and France, as well as the focus of this work, it will be described extensively below (Giles et al. 1993, Hong et al. 1993a, Hong et al. 1993b, and Laguire et al. 2011).

It has been estimated that ascending placentitis contributes to up to one third (9.8-33.5% depending location of investigation) of all late term abortions and causes of fetal loss in the United States (Giles et al. 1993, Hong et al. 1993a, Hong et al. 1993b, and Tengelsen et al. 1997). Most of these cases have been attributed to bacterial infections, accounting for up to 53% of submitted abortions, stillbirths, and neonatal losses (Giles et al. 1993). Commonly isolated bacteria include *Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus equisimilis*, *Staphylococcus* species, and *Enterobacter* species in ascending cases (Giles et al. 1993, Hong et al. 1993a, Hong et al. 1993b, and Tengelsen et al. 1997). *Streptococcus equi* subsp. *zooepidemicus* is the predominant bacterial pathogen identified regardless of the geographical location within the United States and in many locations worldwide, *Escherichia coli* is a close second (Platt 1975, Giles et al. 1993, Hong et al. 1993a, Hong et al. 1993b, Tengelsen et al. 1997, and Whitwell 1988).

While less understood than ascending placentitis, diffuse placentitis is believed to result from the hematogenous spread of microorganisms such as bacteria or fungi, secondary to sepsis from an underlying primary condition. The fundamental pathophysiology involved in this process of microorganism spread and dissemination to the placenta is not well understood. Although any bacteria are potentially capable of causing a diffuse placentitis following sepsis and hematogenous spread to the placenta, *Leptospira* species are a commonly identified regional etiology particularly within the central United States surrounding the state of Kentucky (Giles et al. 1993, Hong et al. 1993a, Hong et al. 1993b, and Donahue and Williams 2010). While there are over 250 known serovars of *Leptospira* bacteria, *Leptospira interrogans* serovar Pomona type kennewicki is diagnosed most frequently in North America, while L*eptospira interrogans* serovars Bratislava, Grippotyphosa, Copenhageni, Autumnalis, Hebdomadis, and Icterohaemorrhagiae are more common worldwide (Erol et al. 2015). Isolated cases of *Rhodococcus equi* and *Neorickettsia risticii* have also been reported (Coffman et al. 2008 and Patterson-Kane et al. 2002). Diffuse placentitis can also be attributed to fungal etiologies at an even lower incidence rate than bacterial cases (1.8 to 6.73%) with *Aspergillus* species as the predominant isolate (Hong et al. 1993a, Hong et al. 1993b, Giles et al. 1993, and Laguier et al. 2011).

Our understanding of focal mucoid placentitis - also referred to as nocardioform placentitis - has significantly improved over the last twenty years. An increasing number of retrospective studies and individual case reports have demonstrated its incidence as a cause of premature delivery and occasionally abortion across Europe, Australia, and New Zealand (Hong et al., 1993a, Hong et al., 1993b, Donahue and Williams 2010, Hanlon, McLachlan, and Gibson 2016, Erol et al. 2012, Christensen et al. 2006, and Cattoli et al. 2004). Some areas appear to have even higher incidence rates such as the state of Kentucky within the United States (Giles et al. 1993, Hong et al. 1993a, Hong et al. 1993b). Placental lesions located along the uterine body and base of the gravid uterine horn, consist of thickening with a thick tenacious exudate that often overlies white granular lesions on the chorioallantois (Canisso et al. 2015a). Cytology, histopathology, PCR, and culture has identified the gram positive, filamentous, branching bacilli *Crossiella equi* and *Amycolatopsis* species in addition to a few recent, but less frequent cases of *Streptomyces* species (Labeda et al. 2009 and Erol et al. 2012). Unlike ascending placentitis, fetal sepsis does not occur, and most foals are born with signs of prematurity alone. Attempts to investigate the pathophysiology behind the development of focal mucoid or nocardioform placentitis has thus far been unsuccessful when the organism has been delivered orally, intravenously, through pharyngeal administration, or within the uterus during the periovulatory period following breeding in inciting disease (Canisso et al. 2015b).

Finally, in many cases of abortion or stillbirth the cause remains unknown even after gross, histopathologic, and advanced molecular techniques are completed. Unknown or undiagnosed causes have ranged anywhere from 7.7-57.9% of all submissions depending the study (Murase et al. 2017, Giles et al. 1993, Hong et al. 1993a, Hong et al. 1993b, Tengelsen et al. 1997, and Smith et al. 2003).

Umbilical torsions are the most frequent etiology of abortion in the United Kingdom, accounting for up to 38.8% of documented cases in one study. This compares to an incidence rate of 4.5% within the United States (Smith et al. 2003 and Hong et al. 1993b). Umbilical cords near the upper end and beyond the upper end of the accepted normal length (36-83 cm) were the primary offenders whereas shorter umbilical cords were rarely associated with umbilical torsions (Smith et al. 2003 and Whitwell 1975). While both a genetic component (sire identity) as well as a uterine environmental component (mare age and parity) may contribute to umbilical cord length, it has been suggested that the discrepancy of incidence between the United Kingdom and the United States could possibly be attributed to the regional differences in the genetic selection for performance that may be concurrently selecting for longer umbilical cords in-utero. When increased cord length is combined with other risk factors such as an increased volume of fetal fluid or increased fetal movement, an increase in fetal rotation can occur resulting in an umbilical torsion (Lyle and Paccamonti 2010, Smith et al. 2003, Whitwell and Wood 1992, and Whitwell 1975). The exact mechanism or cause of umbilical torsions however has yet to be truly elucidated.

Abortion cases in France occur in similar frequency to those documented in the United States, with bacterial placentitis (*Streptococcus equi* subspecies *zooepidemicus* ascending cases) seen as the most common etiology. In France and the United States, viral causes were a less frequently diagnosed cause of abortion contributing to only 3 to 5.5% of all cases (Giles et al. 1993a and Laguier et al. 2011). This is drastically different than recent findings from other parts of Europe and Japan where of infectious cases of abortion, viruses were most frequently diagnosed at 21.3 and 10.1% respectively of all submissions (Marenzoni et al. 2012 and Murase et al. 2017).

1.2 Clinical Signs

For owners, cases of placentitis are frustrating emotionally as well as economically. Abortion or premature delivery of a compromised neonate without the presence of other premonitory signs of disease is a common occurrence (Macpherson and Bailey 2008). When present, precocious mammary gland development with or without the hastened production of mammary secretions is the most common clinical sign seen (Macpherson and Bailey 2008). Mammary development typically begins approximately one month (2 to 6 weeks) prior to foaling with substantial glandular development occurring over the final one to two weeks of gestation. Mammary development, however, is highly affected by parity and even potentially season, as many maiden mares do not develop appreciable glandular development until immediately prior to or even after parturition (Macpherson and Bailey 2008 and Canisso et al. 2015a). Therefore, when present precociously, the presence of mammary gland development frequently indicates an abnormality with the pregnancy, placenta, or fetus. This clinical sign is not specific to cases of ascending placentitis as any compromise to the fetoplacental unit may also produce premature mammary development - including the presence of twins, umbilical cord torsions, or any other cause of impending abortion and pregnancy loss. Vulvar discharge may also be seen but is more commonly noted in experimental models of placentitis compared to clinical cases. Discharge may also be quite difficult for owners to appreciate due to its potential scant nature, intermittent production, as well as the mare’s normal behaviors of urination, defecation, and movement of the tail over the vulva which can hide its appearance (**Figure 1.1**). In some experimental models, vulvar discharge has been noted as the first clinical sign consistent with placentitis and was seen in 100% (17/17) of mares (Bailey et al. 2010), while in others, it has been less consistently noted with 89% of mares diagnosed by day two post inoculation (Curcio et al. 2017), or only 69.2% of mares by the second to fifth day after inoculation (Morris et al. 2007). In each of these studies, $1x10^7$ colony forming units (CFU) of *Streptococcus equi* subspecies *zooepidemicus* bacteria were administered mid cervically, however many other groups utilize different inoculation dosages and methodologies which could also significantly impact the quality and occurrence of clinical signs seen. Other systemic signs are rarely seen with most mares appearing otherwise healthy.

As ascending placentitis is the most frequently documented category of placentitis in the United States as well as the focus of this work, the remainder of this review will focus on the pathophysiology, diagnostics, and treatment modalities available for this specific disease process.

1.3 Pathophysiology of Ascending Placentitis

Mares most commonly affected by ascending placentitis are pluriparous and middle-aged to geriatric, with poor vulvar confirmation that facilitates the development of a pneumovagina and potential bacterial migration (LeBlanc 2010, Whitwell 1988, and Macpherson and Bailey 2008). Other anatomical defects further predispose individual mares to developing placentitis including the presence of vestibulo-vaginal reflux, cervical tears, cervical adhesions, or cervical fibrosis that could lead to cervical incompetence during pregnancy. In many studies Thoroughbred mares are the most commonly diagnosed breed, however this breed is likely overrepresented in many of the aforementioned prevalence studies that have been performed due to the high number of Thoroughbred horses that are frequently located in localized geographical areas surrounding the Thoroughbred racing, breeding, and training industry, that allow for large populations to study as well as convenient sampling (Macpherson and Bailey 2008 and Murase et al. 2017).

Bacteria, which are often fecal, urinary, or environmental contaminants migrate through the mare's caudal reproductive tract and cervix and gain access to the chorioallantois. Platt noted that particular bacteria including *Streptococcus equi* subspecies *zooepidemicus*, caused a necrotizing inflammation of the chorioallantois with lesions that were most severe at the chorionic surface adjacent to the cervix and posterior uterine body (**Figure 1.2**) and first suggested that ascending infection migrated through the cervix and eventually to the fetus through colonization of the umbilical vein and/or allantoic or amniotic sacs (Platt 1975). Bacteria that have entered the amniotic fluid, likely through ascension of the umbilical cord, are then swallowed and/or aspirated into the fetal lung where the subsequent development of fetal sepsis may occur (Calderwood Mays et al. 2002). Direct ascension through the umbilicus itself may also occur.

Bacterial migration and subsequent inflammation lead to upregulation in pro-inflammatory pathways, alterations of the hormonal milieu that maintains pregnancy, and increased myometrial activity to eventually lead to abortion or premature delivery. Migration and colonization of bacteria induces a localized (rather than systemic) inflammatory response. The fetal membranes exhibit thickening and/or the separate from their attachment to the uterus (Platt 1975, LeBlanc et al. 2002, and Lyle et al. 2009). Experimental infection has specifically been shown to increase the expression of multiple proinflammatory cytokines including IL-6 and IL-8 within the fetal membranes at the location of the cervical star of the chorioallantois, IL-6, IL-18, and IL-1β in the chorioallantois at the placental body, and IL-1β and IL-8 in the maternal myometrium (LeBlanc et al. 2002, Lyle et al. 2009, LeBlanc et al. 2012, and El-Sheikh et al. 2018).

In the normal mare, the hormonal maintenance of pregnancy has been extensively studied by many groups and was found to differ substantially from that seen in other domestic animals such as ruminants or swine. Ovarian progesterone (P4) produced by the primary, secondary and accessory corporal lutea is the primary progestin responsible for uterine quiescence and maintenance of pregnancy during the first 150 days of equine pregnancy (Allen and Hadley 1947, Short 1959, van Niekerk et al. 1973, Squires, Wentworth, and Ginther 1974, and Holtan et al. 1974). After 150 days, progesterone becomes almost virtually undetectable and multiple progestagens are produced by the fetal placental unit in large quantities to take over the responsibility of pregnancy maintenance (Holtan et al. 1991, Chavette et al. 1997, and Ousey et al. 2003). A total of nine progestagens have been identified in the pregnant mare (**Table 1.1**). Fetal pregnenolone (P5) likely from the fetal adrenal gland, is first released into the umbilical artery and then converted into progesterone (P4) within the placenta and subsequently to 5α-pregnane,3,20-dione (5α-DHP) and 20α-hydroxy-5α-pregnan-3-one (20-α5P) by the maternal endometrium (Ousey 2004 and Reviewed Ousey 2006). Multiple other progestagens likewise are produced from these products within the uteroplacental tissues (**Figure 1.3**). While the exact biological activity of each progestagen remains unknown, 5α-DHP has been shown to bind more strongly than even P4 itself to the intrauterine P4 receptor *in vitro,* further supporting their role at maintaining uterine quiescence (Chavatte-Palmer et al. 2000). *In vitro* studies thus far however have been unable to confirm whether P4 or 5α-DHP were able to prevent oxytocin induced uterine contractions in strips of myometrium (Ousey et al. 2000). Over the last two to three weeks of pregnancy, the total systemic progestagen concentrations increase gradually and then precipitously decline within the hours to days prior to delivery in the normal mare (Hamon et al. 1991 and Rossdale et al. 1991). Concurrent with this rise in maternal progestagens, the onset of mammary development occurs with a classical change in mammary secretions consisting of an elevation of calcium greater than 40 mg/dL, an inversion in the electrolytes sodium (decrease) and potassium (increase), and the development of secretion acidity ($pH < 7$) indicating readiness for birth prior to parturition (Ousey, Dudan and Rossdale 1984, Rossdale et al. 1991, and Canisso et al. 2013).

In mares with fetoplacental compromise, three separate progestin profiles have been demonstrated, two of which may be seen in cases of placental disease including placentitis (Ousey et al. 2005). Ousey and colleagues proposed that in response to inflammation and placental pathology and/or fetal stress, an early rise in the progestin concentration may be seen prior to the normally expected rise at two to three weeks before parturition or a premature or rapid decline (Ousey et al. 2005). When present, the premature rise in progestin has been proposed to be associated with accelerated fetal adrenal gland maturation and activation of the hypothalamic-pituitary-adrenal axis, while a dramatic decline in progestin is seen in cases of imminent abortion (Lyle et al. 2009 and Ousey et al. 2005). Recent studies have also shown down-regulation in the mRNA expression encoding for the progesterone receptor concurrent with decreased progestin, within the uterine myometrium in mares euthanized following diagnosis with experimentally induced placentitis along with a significant reduction in the mRNA encoding for progestin converting enzymes (SRD5A1 and AKR1C23) and an upregulation in myometrial pro-inflammatory cytokines (El-Sheikh et al. 2018). These two profiles of placentitis contrast with the complete lack of a rise in the progestin concentration at parturition that is more commonly found in cases of fescue toxicity (Brendemuehl et al. 1990).

Large quantities of plasma estrogens are also found in maternal circulation after mid gestation due to hypertrophy of the fetal gonads. Dehydroepiandrosterone (DHEA) and 7-dehydroepiandrosterone (7-dehydro-DHEA) produced in large amounts in mid-pregnancy by the fetal gonads, serve as precursors for synthesis of the classic estrogens estrone, 17β-estradiol, 17α-estradiol and their sulfoconjugates as well as the unsaturated β-ring steroids equilin and equilenin and their derivatives 17α-dihyroequilin, 17α-dihyroequilenin, 17β-dihyroequilin and 17β-dihyroequilenin (Raeside, Liptrap, and Milne 1973, Pashen and Allen 1979, and reviewed by Canisso 2015a). Normally estrogens remain elevated for most of pregnancy, until they gradually decline over the final one to two months of gestation. In compromised pregnancies (not specific to placentitis) prior to day 280 (and the hormone’s natural decline in normal pregnancy), a reduction in plasma estrogens likely indicate severe fetal compromise and potential demise. Therefore, concentrations above 1000 ng/mL between days 150 and 280 are typically assumed to be normal while concentrations of less than 500 ng/mL are associated with severely compromised or deceased foals (LeBlanc 2010).

These alterations of the normal gestational hormonal profile, as well the concurrent pro-inflammatory environment may lead to a further upregulation in prostaglandin production, an alteration of the normal myometrial contractility, and activation of the hypothalamic-pituitary-adrenal (HPA) axis that precedes subsequent abortion or preterm delivery. LeBlanc and colleagues first proposed that the bacterial infection and upregulation of proinflammatory cytokines that was detected through upregulation in mRNA transcripts within the infected chorioallantois compared to healthy chorioallantois, leads to the release of prostaglandin E2

(PGE2) and prostaglandin F2alpha (PGF2α); increased concentrations of both prostaglandins were also detected by radioimmunoassay within the allantoic fluid of mares experimentally induced to develop *Streptococcal equi* subspecies *zooepidemicus* placentitis (LeBlanc et al. 2002, Lyle et al. 2009, and LeBlanc et al. 2012). Subsequent myometrial contractility studies in the same model of experimental placentitis illustrated an alteration in the myometrial contractility pattern from that of the late-term healthy periparturient mare (McGlothlin et al. 2004). This study was the first to demonstrate that uterine contractility in mares is similar to that seen in humans and primates. Pregnant mares experienced both **contractures**, or “epochs of myometrial activity” that are relatively long in duration with little to no generation of intrauterine pressure, as well as **contractions**, which are shorter in duration “epochs of myometrial activity”, and are associated with a large increase in intrauterine pressure. Healthy mares, primates, and humans progressively switched from a state of primary contractures (large spike bursts of myoelectrical activity) to increasing periods of contractions (short spike bursts of myoelectrical activity) as they exhibited progressively increasing numbers of contractions during the evening or night hours in the final days preceding parturition. Mares with experimentally induced placentitis showed an increase in both the duration and intensity of the large spike bursts of myometrial activity (contractures) within the last four days prior to parturition and/or abortion with a loss of the normal diurnal rhythm of contractile events seen in healthy pregnancies. Additionally, while the regulation of myometrial contractions in an equine infective model of preterm delivery is not completely understood, McGlothlin and colleagues proposed that the mechanism of premature delivery in mares occurs similar to that demonstrated in infective models of chorioamnionitis in the primate, following increased production of both prostaglandins and inflammatory cytokines (IL-6, IL-1, and TNF-α) (McGlothlin et al. 2004 and Gravett et al. 1994).

Furthermore, in response to infection, Lyle and colleagues showed an alteration in the HPA axis maturation in response to the proinflammatory milieu in mares prior to day 295 of gestation (Lyle et al. 2009). In the normal mare, fetal maturation occurs over the last five days of gestation with a rise in fetal cortisol detected during the last two days indicating maturation and activation of the HPA axis prior to normal parturition (Rossdale et al. 1973, Jeffcott and Rossdale 1977, Leadon et al. 1982, Rossdale and Silver 1982, Silver 1990, and Silver and Fowden 1994). In three of six mares inoculated to develop ascending placentitis, significant elevations were seen in intra-allantoic fetal cortisol concentrations in mares that had greater than a 25 fold increase in the expression of the proinflammatory cytokine IL-1β at the cervical star, suggesting that proinflammatory cytokines and the proinflammatory milieu may serve as the signal for premature HPA axis activation prior to 295 days of gestation (Lyle et al. 2009). Thus, the inflammation secondary to the bacterial colonization and migration appears to initiate the cascade in prostaglandin production and myometrial contractility responsible for preterm labor and/or abortion in cases of equine ascending placentitis.

Foals that are delivered alive from mares with ascending placentitis often require substantial medical intervention and expense ranging anywhere from $2,000 upwards to $10,000 or beyond due to prematurity, sepsis, or sequelae of both on the farm or in a hospitalized setting (Barr 2005 and Hughes et al. 2014). Despite substantial medical care, it is proposed by many that a large majority of foals affected by ascending placentitis will never be able to achieve productive performance careers or serve in their intended use as performance animals (LeBlanc 2010). While studies examining performance following placentitis are difficult and are only able to truly study the “survivors” rather than foals from all affected cases, other groups have examined foals affected by bacteremia, sepsis, or septic arthritis (Steel et al. 1999, Smith et al. 2004, Sanchez and Lester 2005, Sanchez 2008, Hemberg et al. 2010, and Corley and Corley 2012). When comparing studies of foals affected by bacteremia or septicemia, conflicting results were seen and discussed whether they may be attributed to genetic differences in individuals, the type of control population selected (full/half siblings vs. the general equine population), or the severity of clinical signs at presentation and treatment. In one study examining 393 Thoroughbred racing bred foals, a short-term survival of 57% was seen across cases, but within those survivors, the long-term outcome compared favorably with the foal’s maternal siblings regarding the percent of starters and winners seen (Sanchez and Lester 2005). In another retrospective study by the same group examining 434 Thoroughbred foals who presented for treatment of sepsis from 1982 to 2007, there was also no significant difference between the percent or number of starts, however, affected foals had a significantly lower number of wins and total earnings (Sanchez 2008). Axon and colleagues likewise saw a percentage of healthy control foals with larger total winnings compared to septic foals (Axon et al. 1999). When specifically examining septic arthritis, a sequela to sepsis and potential complication of ascending placentitis, 56% of affected Thoroughbred foals (35/62) survived long-term while only 29% (18/62) raced (Steel et al. 1999). In another study by Smith and colleagues, foals with septic arthritis admitted to a hospital were less likely to start in a race compared to sibling controls (OR 0.28; p=0.001 for all foals vs. OR 0.36 p=0.008 of the discharged survivors) (Smith et al. 2004). Further analysis revealed that foals discharged following treatment for septic arthritis had a significantly longer time period to start in their first race compared to sibling controls (1757 days vs. 1273 days) (Smith et al. 2004).

Surprisingly, a single retrospective study examining the long-term outcome of Thoroughbred foals produced from pregnancies affected by placentitis produced results contrary to what would have been expected based on many of the clinical impressions as well as reports published on sepsis and septic arthritis (Hughes et al. 2014). Retrospectively, cases of one hundred and ninety mares were examined who had suspected cases of clinical or subclinical placentitis based clinical signs of suspected placental failure including premature mammary development, loss of cervical plug, as well as the direct diagnosis of ultrasonographic thickening of the CTUP with or without placental separation. Unfortunately, gross placenta exams were performed only on those available, without the histopathological confirmation of placentitis. Likewise, mares treated with multimodal therapy were also included in the study without specific confirmation of placentitis on diagnostics or placental examination as well as mares who at parturition had premature separation of the chorioallantois (red bag) or meconium staining of foals. Of affected younger horses (2 years of age), there was no difference in the total number of starts, wins, or places compared to control foals. The authors suggest that this finding may be skewed however by one exceptional placentitis colt who far exceeded all the others, as well as many control foals, in earnings. Likewise, in examined older horses (greater than 3 years of age), no significant difference was seen in the number of starts, wins, or places, however, foals out of healthy control mares earned more total race winnings compared to those affected by gestational placentitis. What remains unknown is the number of histopathologically confirmed number of placentitis cases of the affected group. In regard to race findings, particularly to the potential lack of difference in the number of starts, wins etc., the authors also suggested that their findings could potentially have been affected by the degree of disease present in mares or offspring as many may not have been affected by severe cases of clinical placentitis or what they described as subclinical cases (Hughes et al. 2014).

1.4 Currently Available Diagnostics and Monitoring

1.4.1 Ultrasonography

Diagnosis of ascending placentitis is commonly based on a combination of clinical signs (precocial mammary development and/or vulvar discharge) and transrectal ultrasonography (Macpherson 2006, Macpherson and Bailey 2008, and LeBlanc 2010). Transrectal ultrasonography allows for assessment of the caudal reproductive tract specifically focusing on the area of chorioallantois surrounding the caudal pole or at the cervical star. Additionally, fetal activity and characterization of fetal fluids may also be performed. A 5 to 7.5-MHz linear ultrasound transducer is used to image the caudal reproductive tract following transrectal palpation and evacuation of feces. The combined thickness of the uterus and placenta (CTUP) is imaged by moving 2.5 to 5 cm cranial from the cervix and then laterally until the cranial branch of the vaginal artery (commonly referred to middle uterine artery) is visualized at the level of the uterine body (Renaudin et al. 1997 and Renaudin, Troedsson, and Gillis 1999). The area between the vessel and edge of the combined uterus and placenta is measured (**Figure 1.4**). Ideally, three measurements are taken, averaged, and compared to normal expected values (Macpherson 2006). In normal mares, the distinction of the uterus and placenta is frequently difficult as they are tightly apposed and indistinguishable from each other, however, this can depend on ultrasound and image quality. Normal values for CTUP has been described in light-breed, draft, and pony mares with relative minimal variance between breeds described (Renaudin et al. 1997, Kelleman et al. 2002, Bucca et al. 2005, Loef et al. 2010, Bailey et al. 2012, Loef et al. 2014, and Kimura et al. 2018). In light breed mares, the CTUP increases from 4 mm at four months of gestation to 1 to 1.2 cm at term (Renaudin et al. 1997) (**Table 1.2**). Mares with placentitis may display CTUP thickening as well as separation of the chorioallantois from the underlying uterine endometrium with or without hypo to hyperechoic exudate present between the two layers (**Figure 1.5**) (Renaudin et al. 1999, Kelleman et al. 2002, Morris et al. 2007, Loef et al. 2010, Bailey et al. 2012, Loef et al. 2014, and Kimura et al. 2018). As a caveat however, in some experimental models, abortion and clinical signs of placentitis such as vulvar discharge have been seen without, or prior to, thickening of the chorioallantois (Morris et al. 2007, Bailey et al. 2010, and Loef et al. 2014). When present, however, a clinical diagnosis of ascending placentitis is made and therapeutic intervention initiated as indicated. Transrectal ultrasonographic assessment of the CTUP finally is also a mainstay of monitoring in mares that are being treated for placentitis as the progression, stabilization, and/or resolution of elevated CTUP can be evaluated concurrently when assessing overall fetal well-being (Macpherson and Bailey 2008).

Assessment of fetal well-being and viability should also be performed during this examination. Fetal activity, as well as the character of both the allantoic and amniotic fluid, can be assessed during assessment of the CTUP at the cervical pole (Reef et al. 1995 and Bucca 2005). Increased echogenicity in either fluid compartment may indicate fetal compromise and/or placental pathology (LeBlanc 2010). Fetal fluids likewise can be evaluated on transabdominal ultrasonography during assessment fetal of heart rate assessment for a complete evaluation. Normal values for fetal heart rate have likewise been described as they change throughout gestation with episodes of tachycardia and bradycardia associated with fetal stress and compromise (**Table 1.3**) (Adams-Brendemuehl and Pipers 1987, Reef et al. 1995, Reef et al. 1996, and Bucca et al. 2005). Normal transabdominal parameters of the CTUP have also been published but are more helpful in diagnosing cases of mucoid (nocardioform) or potentially even diffuse placentitis (Bucca et al. 2005). Finally, transabdominal ultrasonography can be utilized to rule out the occurrence of twins by evaluating for the presence of two fetal thoraxes, which is another common differential diagnosis for clinical signs of precocial mammary development and/or lactation.

1.4.2 Hormonal Profiling

Endocrine profiling can also be performed. As mentioned above, profiling of progestin concentrations can be performed through gas or liquid chromatography-mass spectrometry or through direct progesterone analysis (ELISA or RIA) due to the cross-reactivity of the progestogens with the progesterone assay kit (Ousey et al. 2005, Morris et al. 2007, and Conley et al. 2016). However multiple studies mentioned above have found that these findings can be inconsistent and non-specific and identify any mare that is potentially experiencing placental compromise and are not necessarily only cases of ascending placentitis. Mares experiencing placentitis frequently exhibit a preterm rise in progestins or a precipitous drop (Ousey et al. 2005). Additionally, because of this, a single progesterone or progestin value may have little use and it has been recommended to analyze three or more samples of a minimum of at 48 to 72-hour intervals (Morris et al. 2007). Estrogens, specifically estradiol-17β recently has been suggested to serve as a diagnostic marker of ascending placentitis as estradiol-17β was significantly decreased in 17 untreated mares with experimental *Streptococcal equi* subspecies *zooepidemicus* placentitis compared to healthy control mares prior to delivery (n=17) (Canisso et al. 2017). Conflicting data on estrone sulfate have been produced with no difference detected in estrone sulfate concentrations between experimentally infected mares compared to normal controls in one model (Ball et al. 2013), whereas estrone sulfate was significantly reduced on the day prior to abortion only in another (Canisso et al. 2017). Earlier studies also questioned the use of estrogens as a reliable diagnostic marker due to their lack of specificity and inconsistent change in other experimental models (Santschi et al. 1991 and Stawicki et al. 2002). Finally, the hormone relaxin has also been investigated as a marker for placentitis and adjunct to the available diagnostic repertoire. Plasma relaxin produced from the equine placenta has been shown to decline in problematic pregnancies, but the large variability present between breeds as well as lack of a commercially available equine test and similar concerns with its specificity for only placentitis, limit its clinical applications (Stewart et al. 1982a, Stewart et al. 1982b, Ryan et al. 1999, and Ryan et al. 2009).

1.4.3 Vaginal Examination

Vaginal examination through digital palpation and/or vaginoscopy, in general, is not recommended as a screening tool for ascending placentitis and in fact, may be contraindicated. Its performance, however, may allow for culture and cytology of any cervical discharge to directly guide antibiotic therapy (LeBlanc 2010). Considerations against performing vaginal examination and procedures in the pregnant mare are due to the introduction or transfer of caudal microorganisms cranially to the cervix as well as avoiding manual and/or pneumonic vaginal stimulation and dilation of the cervix itself (Hinrichs et al. 1988, Handler et al. 2003). As the cervix and caudal reproductive tract can be visualized on transrectal ultrasonography and graded on transrectal palpation for tone and length, visual or digital examination is often not warranted except in the unusual mare (Bucca and Fogarty 2011).

1.4.4 Markers of Inflammation and Other Biomarkers

Recently, many groups have explored the use of various potential biomarkers of inflammation, however, they are also not specific for ascending placentitis and may be elevated in the face of any maternal systemic inflammatory process. The acute phase proteins haptoglobin and serum amyloid A have both been shown to be significantly increased in models of experimental ascending placentitis (Coutinho da Silva et al 2013 and Canisso et al. 2014). Serum amyloid A was additionally even increased in the fetal heart blood of aborted fetuses from naturally occurring cases of ascending placentitis, however, obtained values were significantly higher in acute compared to suggested chronic cases (Erol et al. 2016). Both proteins are considered moderate and major acute phase proteins respectively. Moderate acute phase proteins can always be found in the plasma in healthy patients but increase five to 10-fold in the face of inflammation. Major acute phase proteins, on the other hand, are virtually undetectable in healthy animals but can increase anywhere from 10 to even up to 1000 times that of baseline (Jacobsen and Andersen 2007 and Cray 2012). While serum amyloid A is not specific for placentitis or useful as a screening tool as a single modality, it has been shown to potentially aid in the monitoring and assessment to therapeutic intervention due to its short half-life and ability to decrease in parallel with resolution of disease (Jacobsen and Andersen 2007 and Coutinho da Silva et al. 2013). A single study to date has investigated the expression of alpha-fetoprotein in the fetal fluids of late gestational mares. In the same study, alpha-fetoprotein was elevated in the plasma of mares in an experimental model of ascending placentitis following inoculation with 5x10^6 CFU of *Streptococcus equi* subspecies *zooepidemicus* bacteria to induce a chronic rather than acute placentitis disease process (Canisso et al. 2015c). The significance of this protein, however, is unknown as it was found in both normal and abnormal mares and it is also unknown if alterations may be seen in clinical cases.

1.4.5 Histopathology and Gross Placental Examination

The true definitive diagnosis of ascending placentitis is performed through gross and histopathological assessment of the placenta examining for thickening at the area of the cervical star +/- exudate and signs of inflammation, necrosis, edema, and bacteria on histopathology that may also indicate acute versus chronic infection (Hong et al. 1993 and Williams et al. 2004). Lesions of acute infection are characterized by neutrophilic infiltration and/or chorionic villi necrosis whereas chronic cases of placentitis may exhibit necrosis of chorionic villi with the addition of mononuclear cells in addition to neutrophils within the intervillous areas (Hong et al. 1993 and Williams et al. 2004). Swabs of exudate, as well as samples of tissues themselves, can be submitted for microbiological culture (bacterial and/or fungal), and/or cytology or PCR testing for abortive agents (Reviewed Canisso et al. 2015). This, however, is of course following premature delivery and does not allow for medical management to attempt to prolong pregnancy and obtain a viable neonate. Therefore, transrectal ultrasonography, vigilant monitoring by owners for aberrant changes on physical exam, and/or frequent monitoring of high risk or previously diagnosed mares remain the commonly clinically utilized diagnostics to identify mares with ascending placentitis and highlights the need for additional methods of identification particularly early in the course of infection.

1.5 Treatment

Treatment of mares identified with ascending placentitis involves multimodal therapy consisting of combinations of broad-spectrum antimicrobial therapy to combat infection, anti-inflammatories to reduce and/or alleviate inflammation, and medications to promote uterine quiescence. Medications commonly administered as well as their dosages and routes of administration are presented in **Table 1.4**.

1.5.1 *Antimicrobials*

Broad spectrum antimicrobials are a mainstay of therapy in an attempt to reduce and eliminate any bacteria present and prevent further colonization and replication within the fetal membranes, fetal fluids, and fetus itself. Diaw and coworkers illustrated in 2010 that the uterine environment from naturally foaling mares was essentially sterile with no bacterial growth on uterine culture immediately post-delivery whereas mares affected by placentitis were diagnosed with the continued presence of intrauterine bacteria, often even despite aggressive antibiotic treatment (Diaw et al. 2010). Antimicrobial treatment approaches were initially largely anecdotal with common antibiotic therapy consisting of either trimethoprim sulfamethoxazole or a combination of penicillin and gentamicin (Macpherson and Bailey 2008). Early studies were performed to evaluate many of these commonly used antibiotics to penetrate the fetal membranes and fetal fluids (Sertich and Vaala 1992 and Santschi and Papich 2000). Sertich and Vaala administered daily potassium penicillin G (22,000 IU/kg IV q 6 hours) alone or in combination with gentamicin sulfate (2.2 mg/kg IV q 6 hours) or oral trimethoprim sulfadiazine (5 mg/kg po q 12 hours) to a total of 11 mares and measured the concentrations of each drug within the maternal serum and/or plasma and allantoic fluid on day 4 after daily administration, and amniotic fluid at the time of parturition (Sertich and Vaala 1992). While concentrations of all three drugs were detectable in expected concentrations in maternal serum or plasma, neither penicillin or gentamicin was detected within the allantoic or amniotic fluid of administered mares. Trimethoprim sulfadiazine, however, was detected within the allantoic fluid above the expected minimal inhibitory concentration (MIC) of *Streptococcus equi* subspecies *zooepidemicus*. These findings, particularly those regarding the allantoic and amniotic concentrations of penicillin and gentamicin may have been attributed to the assays used (bioassay measuring the inhibition of micrococcus luteus for penicillin and determination of gentamicin-glucose-60-phosphate dehydrogenase bound in a homogenous enzyme immunoassay for gentamicin sulfate) as well as potentially, the dosages utilized compared to those administered in later studies.

Santichi and Papich later examined the systemic pharmacokinetic profile of late-term pregnant mares following administration of gentamicin (6.6 mg/kg IV) and found that the plasma concentration versus time profile, distribution, and clearance was not different between pregnant and non-pregnant lactating mares (Santschi and Papich 2000). In three mares induced to foal within one hour of administration, gentamicin was not detectable within the serum of either of the three foals nor within amniotic fluid collected during birth from a single mare. Therefore, akin to Sertich and Vaala's conclusions in 1992, the authors proposed that gentamicin was unable to cross the fetal membranes and enter the fetal fluids, however with such a small sample size it is unknown if the administration and drug distribution following this single dose could have been detectable with a longer time to reach maximum distribution and allow for transfer with the analysis utilized.

Later work from the University of Florida utilized the technique of microdialysis to further determine if these antimicrobials were indeed able to cross the uterus and placenta and serve as an effective choice for treating cases of ascending placentitis (Murchie et al. 2006 and Rebello et al. 2006). *In vivo* microdialysis revealed that the concentrations of all three of the commonly utilized antibiotics were detectable within the allantoic fluid of pregnant mares indicating successful placental transfer and that both penicillin and trimethoprim sulfamethoxazole reached concentrations to be effective against *Streptococcus equi* subspecies *zooepidemicus* while gentamicin reached concentrations effective against *Klebsiella pneumoniae and Escherichia coli*. Additional successful transfer across the placenta of each of the antimicrobials was also seen in a small number of experimentally infected mares (Murchie et al. 2006 and Rebello et al. 2006). Furthermore, the use of trimethoprim sulfamethoxazole has further been supported as an effective antimicrobial choice against placentitis when used in combination with pentoxifylline and altrenogest (to be discussed below) as it resulted in 10 of 12 mares delivering live foals when initiated at 48 hours following experimental inoculation with 10^7 CFU of *Streptococcus equi* subspecies *zooepidemicus* bacteria in pony mares (Bailey et al. 2010). Therefore, trimethoprim sulfamethoxazole remains one of the most commonly utilized antimicrobials frequently at 30 mg/kg by mouth every 12 hours due to its ease of oral administration, cost effectiveness, reduced risk of injection reactions, and decreased risk for renal toxicity compared to combinations of gentamicin and penicillin (Van Duijkeren, Ensink, and Meijer 2002, Ensink, Bosch, and Van Duijkeren 2005, Macpherson and Bailey 2008, LeBlanc 2010, and Canisso et al. 2015a).

Recently, our lab has also been studying antibiotic efficacy in an *ex vivo* model. Aliquots of Muller Hinton broth, post-partum fluid, and purulent material were inoculated with either

Streptococcal equi subspecies *zooepidemicus* and *Escherichia coli* bacteria concurrent with either the antimicrobial penicillin, penicillin and gentamicin, or trimethoprim sulfamethoxazole (McKelvey et al. 2014). Surprisingly, no antibiotic was effective at reducing bacterial load in any fluid type against *Escherichia coli*. Against *Streptococcus equi* subspecies *zooepidemicus* however, bactericidal activity was seen within Muller Hinton broth and fetal fluids with trimethoprim sulfamethoxazole and the combination of penicillin and gentamicin whereas bactericidal activity in purulent fluid only occurred when a high dose of a combination of penicillin and gentamicin was added. These findings therefore, also question the use of trimethoprim sulfamethoxazole in the face of a highly cellular fluid environment within the study conditions, however clinically this antibiotic remains a mainstay of therapy despite its inability to potentially be able to completely clear all bacteria present due to its ease of use, safety for the dam and potential offspring, and continued perceived clinical efficacy (Bailey and Macpherson 2008, LeBlanc 2010, and Canisso et al. 2015a).

Ceftiofur sodium (Naxcel), a broad spectrum third-generation cephalosporin and β-lactam antibiotic was similarly evaluated by Macpherson and colleagues as it is an antimicrobial that is frequently utilized for a whole host of conditions and disease processes in equine medicine (Macpherson et al. 2017). Following administration of 2.2 mg/kg ceftiofur sodium q 24 hours in the muscle of six mares at and at 4.4 mg/kg q 24 hours in 5 mares, plasma was collected daily as well as allantoic fluid on day 4 of administration. There was no evidence that placental transfer occurred as the active metabolite desfuroylceftiofur acetamide was undetectable within allantoic fluid. Additionally, a subset of eight mares then received ceftiofur sodium at 4.4 mg/kg in the muscle q 24 hours until foaling. Similar to findings seen in early gestation, the ceftiofur sodium's active metabolite was again undetectable in the collected allantoic and amniotic fluid at the time of parturition, foal serum following delivery, as well as within the placental tissues themselves (Macpherson et al. 2017). Finally, the authors also investigated ceftiofur crystalline free acid (Exceed), a long-acting cephalosporin. Again, there was no evidence of transfer with the inability to detect the active metabolite desfuroylceftiofur acetamide within collected fetal fluids as well as within both fetal and placental tissues (Macpherson et al. 2012). Therefore, while treatment did not improve foal survival in either study, it was also illustrated that neither formulation of ceftiofur (ceftiofur sodium or ceftiofur crystalline free acid) were able to cross the fetal membranes and are likely ineffective in combatting infection in cases of ascending placentitis (Macpherson et al. 2012 and Macpherson et al. 2017). Furthermore, questioning ceftiofur's use as an effective treatment option, our lab has also applied the same *ex vivo* model as discussed above as to examine the antibiotic efficacy of ceftiofur compared to penicillin and gentamicin in Muller Hinton Broth and postpartum fluid (VonDollen et al. 2019). Bactericidal activity was seen with the combination of penicillin and gentamicin in both fluid types against both of the bacterial isolates. Ceftiofur was only bacteriostatic within postpartum fluid against *Streptococcus equi* subspecies *zooepidemicus* and was ineffective against *Escherichia coli* within fetal fluids (VonDollen et al. 2019).

Finally, enrofloxacin has also recently been investigated for its safety in pregnancy and as an antibiotic against placentitis. Enrofloxacin, a fluoroquinolone exhibits bactericidal activity against gram-negative and some gram-positive microorganisms through its inhibition of DNA gyrase enzymes (Giguere and Dowling 2013). Historically, its use in pregnant mares has been limited due to the potential for cartilage damage, arthropathies, and tendonitis in young, fast growing and developing horses (Vivrette et al. 2001). Ellerbrock and colleagues, however, have demonstrated that enrofloxacin when administered intravenously or orally in late-term gestational mares achieved concentrations above the minimal inhibitory concentration against many of the organisms within fetal fluids and fetal plasma (Ellerbrock et al. 2018a and Ellerbrock et al. 2018b). In this study, enrofloxacin was administered for a maximum of 11 days and mares were induced to deliver at 271 days of gestation. On examination of fetal cartilage from sacrificed foals, gross lesions were not seen, however, chondrocyte clustering was noted on histopathological examination of the articular cartilage. The significance of these lesions is unknown. Additionally, it is unknown how long term administration of enrofloxacin would impact the developing foal as well as what long term effects could be seen as the animals begin bearing weight following delivery. Further study is therefore required prior to this antibiotic becoming a mainstay of treatment in pregnant mares as well as cases of placentitis.

While the use of broad-spectrum antibiotics was shown to improve the clinical outcome as shown by Bailey et al., their use was unable to eliminate all bacterial organisms present following experimental infection and exhibited positive intrauterine cultures compared to a population of healthy foaling mares (Bailey et al. 2010 and Diaw et al. 2010). While a completely separate model of infection, Ensink et al. likewise demonstrated a trimethoprim sulfamethoxazole treatment failure in eliminating all present bacteria when it was used in a model of subcutaneous infection following inoculation *Streptococcus equi* subspecies *zooepidemicus* in a subcutaneous tissue chamber (Ensink, Bosch, and Van Duijerken 2005). Clinically the use of broad-spectrum antibiotics particularly trimethoprim sulfamethxazole remain as a key point in treatment. These studies, however, highlight the need for further antimicrobial options or routes of administration that may be more effective.

1.5.2 Anti-inflammatories

Anti-inflammatory therapy aims at reducing and halting the pro-inflammatory response and production of inflammatory cytokines and prostaglandins as each has been implicated in preterm labor in horses as well as a multitude of other species. Commonly selected non-steroidal anti-inflammatories that are utilized include flunixin meglumine, phenylbutazone, and recently firocoxib (**Table 1.4**). Prostaglandins are produced through the conversion of arachidonic acid by the cyclo-oxygenase enzymes (COX-1 and COX-2) (Reviewed Kynch 2017). Flunixin meglumine and phenylbutazone are both primarily COX-1 selective drugs and therefore have greater potential to cause deleterious effects on renal and gastrointestinal systems particularly when used long-term. In one study, Murchie et al. attempted to investigate if flunixin meglumine crossed the fetal membranes (Murchie et al. 2006). While flunixin meglumine was undetectable within the fetal fluid of pregnant mares, the authors hypothesized that this likely was due to their inability to detect the drug as flunixin tends to stay highly protein bound and if so, would be unable to flow into the microdialysis pores due to its size alone. While to this author's knowledge, other studies that specifically analyze for pregnancy outcome or the specific anti-inflammatory effects of flunixin meglumine in cases of either experimental or naturally occurring placentitis have not been performed, flunixin meglumine has been previously shown to mitigate the effects of endotoxin in mares in early gestation (Daels et al. 1991 and Daels et al. 1995). Specifically, flunixin meglumine was shown to alleviate prostaglandin production and promote pregnancy maintenance against cloprostenol (synthetic prostaglandin) induced abortion when administered between 90 and 150 days of gestation (Daels et al. 1991 and Daels et al. 1995). In an *ex vivo* model in our laboratory utilizing fetal membranes that were either harvested during early gestation at 34 days post ovulation or near term in late gestation, flunixin meglumine was also shown to significantly reduce prostaglandin production (PGF2α and PGE2) and the inflammatory response following treatment with *Escherichia coli* derived lipopolysaccharide, further supporting its use as an anti-inflammatory in response to potential placental infection (Bailey et al. 2013).

Recently the primarily COX-2 selective anti-inflammatory firocoxib has been investigated in the pregnant mare (Giguere et al. 2015 and Macpherson et al. 2018). Firocoxib ideally would have less deleterious effects on the gastrointestinal and renal systems of mares potentially making it an ideal anti-inflammatory in mares who require long term therapeutics to maintain a pregnancy to term. Initial work illustrated no adverse effects in mares treated with firocoxib at 0.1 mg/kg by mouth once daily, but the maximum concentration of the drug was significantly lower in pregnant mares compared to controls (Giguere et al. 2013). As firocoxib has a long half-life, the authors proposed that due to its accumulation, steady-state levels in pregnant mares could be reached by first utilizing a loading dose of 0.3 mg/kg PO akin to the practice performed in other musculoskeletal and gastrointestinal conditions in the horse (Giguere et al. 2013 and Kynch 2017). Macpherson and colleagues followed this work by utilizing the loading dose of 0.3 mg/kg PO q 24 hours for 3 days, followed by 0.1 mg/g PO q 24 hours in an experimental model of ascending placentitis (Macpherson et al. 2018). Mares who received firocoxib, displayed decreased concentrations of IL-1β, IL-10, and PGF2α within the allantoic fluid of treated infected mares compared to untreated infected animals, supporting the suppressive effect that firocoxib may have on both inflammatory cytokine and prostaglandin production within the fetal fluids as well as its use as a treatment modality in cases of placentitis.

Pentoxifylline is an immune modulator that is believed to have multifactorial actions including preventing the release of inflammatory cytokines, enhancement of uterine blood flow, and diminishing bacterial adhesion (Lauterbach and Zemballa 1996, Heller et al. 1999, Ousey et al. 2010, and Pozor et al. 2011). Rebello and colleagues illustrated that pentoxifylline was in fact able to cross the fetal membranes and was detected in high concentrations within the fetal fluids of both healthy and experimentally infected mares with no difference seen between groups (Rebello et al. 2006). A recent study by Bailey and colleagues was unable to detect an improvement in uterine arterial blood flow through doppler ultrasonography in pregnant pony mares when treated with pentoxifylline at 17 mg/kg PO q 24 hours daily between 18 and 190 days of gestation (Bailey et al. 2012 and Ousey et al. 2010). Regardless of the mechanism of action, Bailey and coworkers did, however, illustrate in a separate study, an improved pregnancy outcome when combination therapy of pentoxifylline was utilized in conjunction with trimethoprim sulfamethoxazole and altrenogest (Bailey et al. 2010).

Acetylsalicylic acid, more commonly known as aspirin in both human and veterinary medicine, is the final anti-inflammatory that has commonly been utilized in cases of placentitis for its anti-inflammatory properties as well as potential improvement of blood flow to the uterus in pregnant mares. In women, acetylsalicylic acid is known to inhibit platelet aggregation thereby increasing blood flow and is used as a preventative of pregnancy-induced hypertension, preeclampsia, and intrauterine growth restriction (Sibai et al. 1989, Vainio et al. 2002, and Meher et al. 2017). A recent 2018 study in horses investigated the use of acetylsalicylic acid on the uterine blood flow via Doppler ultrasonography and found that in late pregnancy the total arterial blood flow was significantly higher in mares receiving acetylsalicylic acid compared to controls receiving lactulose (Sielhorst et al. 2018). Its use has also been investigated in a single efficacy study, where Christiansen and coworkers administered acetylsalicylic acid, trimethoprim sulfamethoxazole, and altrenogest to 12 mares in an experimental model of ascending placentitis and produced 9 out of 12 viable foals in treated mares (Christiansen et al. 2010).

Clinically, flunixin meglumine remains the main anti-inflammatory in acute cases of placentitis while the selection of or transitioning mares to firocoxib in chronic cases of placentitis shows the most promise for both maintaining pregnancy and diminishing effects on the dam.

1.5.3 Tocolytics

Tocolytic therapy consisting of progestins and less commonly β-sympathomimetics in horses is the final aspect of multimodal therapy to prevent premature delivery and abortion by promoting uterine quiescence and preventing or halting uterine contractions. Progestins have been used to promote uterine quiescence in horses for the last thirty years, however, most of the evidence supporting their use preventing pregnancy loss was documented in the early stages of pregnancy. Altrenogest, a synthetic progestin was shown to prevent pregnancy loss in 100% of mares treated with the synthetic prostaglandin cloprostenol whereas mares treated with injectable progesterone following treatment with cloprostenol only maintained pregnancy in five out of eight mares (62.5%), while all cloprostenol only treated control mares aborted (Daels et al. 1996). Therefore, this study as well as the study by Bailey and coworkers demonstrating improved neonatal and pregnancy outcome when altrenogest was utilized as a parameter of multimodal therapy in an experimental placentitis have supported altrenogest use in cases of ascending placentitis as well as that of any mare at risk for abortion or experiencing systemic illness in late pregnancy (Bailey et al. 2010). Progestin use likewise has also been shown to promote pregnancy maintenance in a vast number of studies in the human literature (Meis et al. 2003, Hassan et al. 2011, and Romero et al. 2017).

Clenbuterol a β-sympathomimetic is another commonly utilized tocolytic in equine medicine. Card and Wood first illustrated in 1995 that 300 µg of clenbuterol resulted in transient elevations in fetal heart rate (10 to 69 beats per minute), maternal heart rate (4-50 beats per minute) as well as decreased uterine tone for up to 120 minutes following administration (Card and Wood 1995). However, when administered prior to foaling in another study, clenbuterol was unable to prolong natural parturition when administered at signs of fetal readiness through mammary electrolytes and potentially even caused mares foal earlier than those that did not receive clenbuterol (Palmer et al. 2002). This contrary effect was hypothesized by the authors to be due to cervical dilation following the relaxation of the cervical smooth musculature that resulted in a hastening of parturition (Palmer et al. 2002). Therefore, with this information, clenbuterol's use may be questionable at best in its benefit to maintaining equine pregnancy.

1.5.4 Adjunctive Therapy

Following the work by Canisso and colleagues that illustrated a reduction in estradiol-17β in an experimental model of ascending placentitis, the effectiveness of estradiol cypionate has been explored as an addition to the commonly utilized therapeutic regimens (Canisso et al. 2017). When estradiol cypionate was added to treatment regimen of trimethoprim sulfamethoxazole and flunixin meglumine, a decreased percentage of high-risk foals or those that required major assistance and showed signs of prematurity including silky hair, floppy ears, delay in sucking or standing, and an abnormal leukocyte: neutrophil ratio or evidence of sepsis was seen (Curcio et al. 2017). In another study, however, there was no difference seen in overall foal survival at parturition or at 60 days post-delivery regardless if they received a combination of trimethoprim sulfamethoxazole, flunixin meglumine, altrenogest, and estradiol cypionate, trimethoprim sulfamethoxazole, flunixin meglumine and estradiol cypionate, trimethoprim sulfamethoxazole, flunixin meglumine and altrenogest, or trimethoprim sulfamethoxazole and flunixin meglumine only (Muller et al. 2019). As cortisol was elevated in normal foaling mares and mares receiving therapy that consisted of a combination of estradiol cypionate, the authors proposed that potentially estradiol cypionate improved the endocrine response.

1.6 Summary

Ascending placentitis remains a common cause of pregnancy loss and neonatal morbidity and mortality in mares. Multiple diagnostic modalities are used to identify mares affected in addition to the presence of clinical signs, however, many mares abort prior to identification while others are identified late in the course of disease and upregulation of the inflammatory cascade. Likewise, multiple treatment options are available with most consisting of multimodal therapeutic combinations of antimicrobials, anti-inflammatories, and tocolytics.

Table 1.1 Abbreviations, systematic name and common names of progestogens in the pregnant mare.

Systematic Name	**Common Name**	**Abbreviation**
4-Pregnene-3,20-dione	Progesterone	P_4
3β-Hydroxy-5-pregnene-20-one	Pregnenolone	P_5
5-Pregnene-3β,20β-dione	_____	P5-ββ
5α-Pregnane-3,20-dione	_____	5α-DHP
3β-Hydroxy-5α-pregnan-20-one	_____	3β-5P
20α-Hydroxy-5α-pregnan-3-one	_____	20α-5P
5α-Pregnane-3β,20β-diol	_____	ββ-diol
5α-Pregnane-3β,20α-diol	_____	βα-diol
5α-Pregnane-3α,20α-diol	_____	αα-diol

Table 1.2 Normal combined thickness of the uterus and placenta (CTUP) values for light-bred mares commonly used to evaluate mares for ascending placentitis. *Adapted from Renaudin et al 1997*.

Gestational Age	**CTUP**
Days 271-300	< 7 mm
Days 301-330	< 10 mm
Greater than Day 330	> 12 mm

Table 1.3 Normal values for fetal heart rate analyzed on transabdominal ultrasound examinations. *Adapted from Bucca et al. 2005*.

Gestational Age	**Fetal Heart Rate at Rest (beats per minute)**	**Fetal Heart Rate at Activity (beats per minute)**
Days 241-270	91 ± 6.6	106.6 ± 9.6
Days 271-300	86.6 ± 7.9	110 ± 14.9
Days 301-330	72.5 ± 8	90.7 ± 16
Days 331-360	66.4 ± 6.5	86 ± 10.8

Table 1.4 Commonly utilized therapeutics for treatment of mares with ascending placentitis. Abbreviations: milligram (mg), kilogram (kg), international unit (IU), per os (PO), intravenous (IV). *Adapted from LeBlanc 2012 and Canisso et al. 2015a.*

Therapeutic Agent	**Suggested Dose**	**Mechanism of Action**
Trimethoprim Sulfamethoxazole	15-30 mg/kg, PO, q 12 hours	Antimicrobial
Potassium penicillin G	22,000 IU/kg, IV, q 6 hours	Antimicrobial
Gentamicin	6.6 mg/kg, IV, q 24 hours	Antimicrobial
Flunixin meglumine	1.1 mg/kg, IV or PO, q 12 hours	Anti-inflammatory
Phenylbutazone	2.2 mg/kg, PO, q 12 hours	Anti-inflammatory
Pentoxifylline	8.5 mg/kg, PO, q 12 hours	Anti-inflammatory
Acetylsalicylic acid	50 mg/kg, PO, q 12 hours	Anti-inflammatory
Altrenogest	0.088 mg/kg, PO, q 24 hours	Tocolytic/Progestin
Clenbuterol	0.8 µg/kg, PO/IV, q 12 hours	Tocolytic/β-sympathomimetic

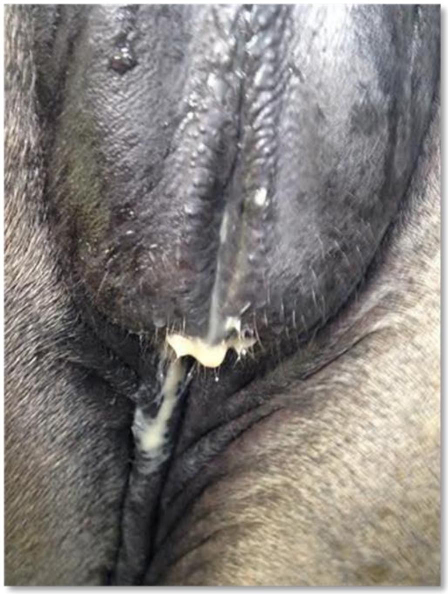

Figure 1.1 Representative photograph of purulent vulvar discharge that may be seen in cases of ascending placentitis from mare at 287 days of gestation following experimental inoculation with *Streptococcus equi* subspecies *zooepidemicus* bacteria.

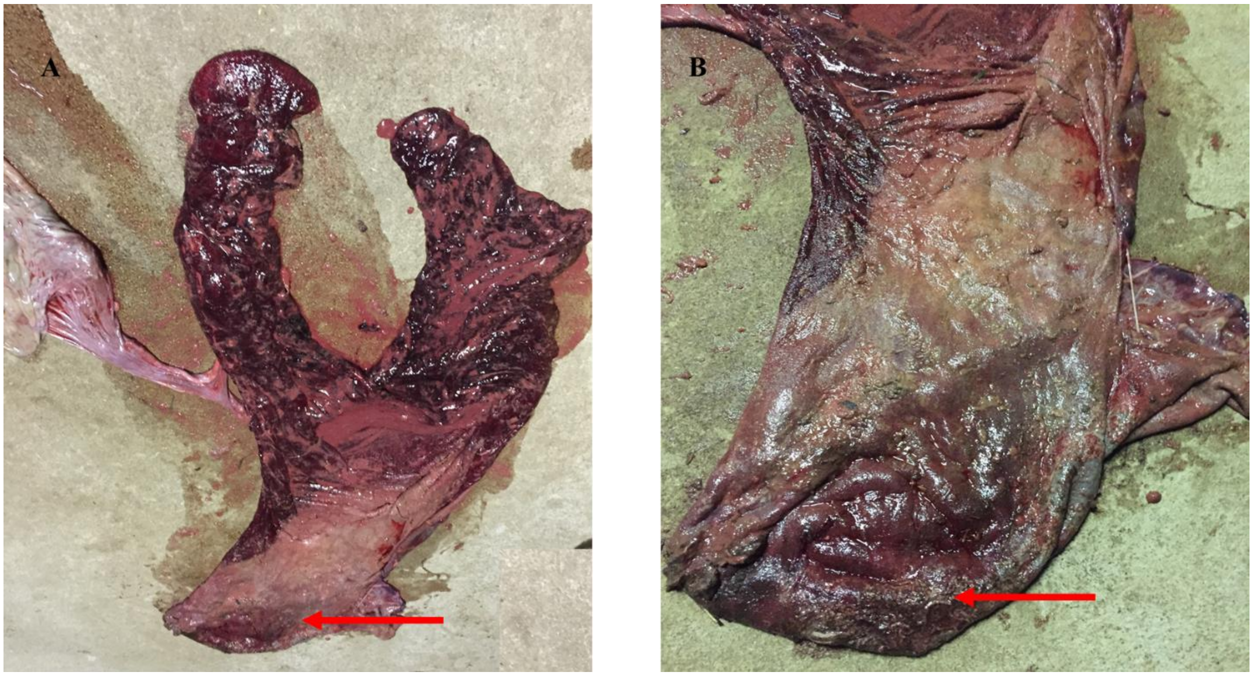

Figure 1.2 Representative photograph of placenta affected by ascending placentitis, depicting focally extensive thickening of the chorioallantois at the area of the cervical star (**red arrows**) extending to the caudal uterine body with purulent exudate and necrosis present. Depicted placenta is from a mare who experienced preterm delivery at 301 days of gestation following experimental inoculation with *Streptococcus equi* subspecies *zooepidemicus* bacteria. Entire placenta is depicted in panel **A**, with magnified image of cervical star and area of the uterine body in panel **B**.

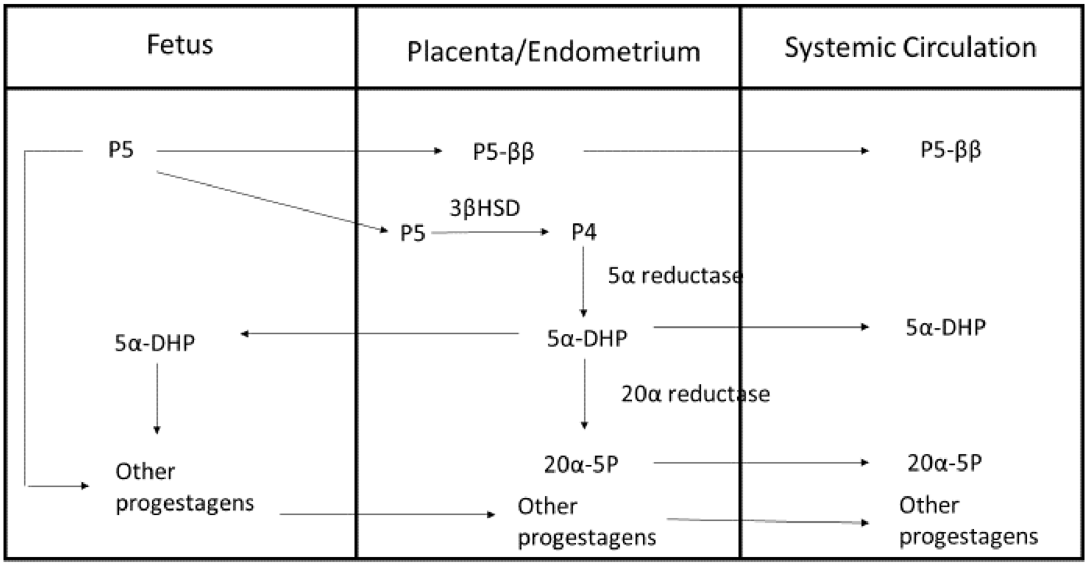

Figure 1.3 Diagram depicting progestogen synthesis pathway in the pregnant mare within the fetoplacental unit and maternal endometrium that occurs after the second half of gestation. *Adapted from Ousey et al. 2006.*

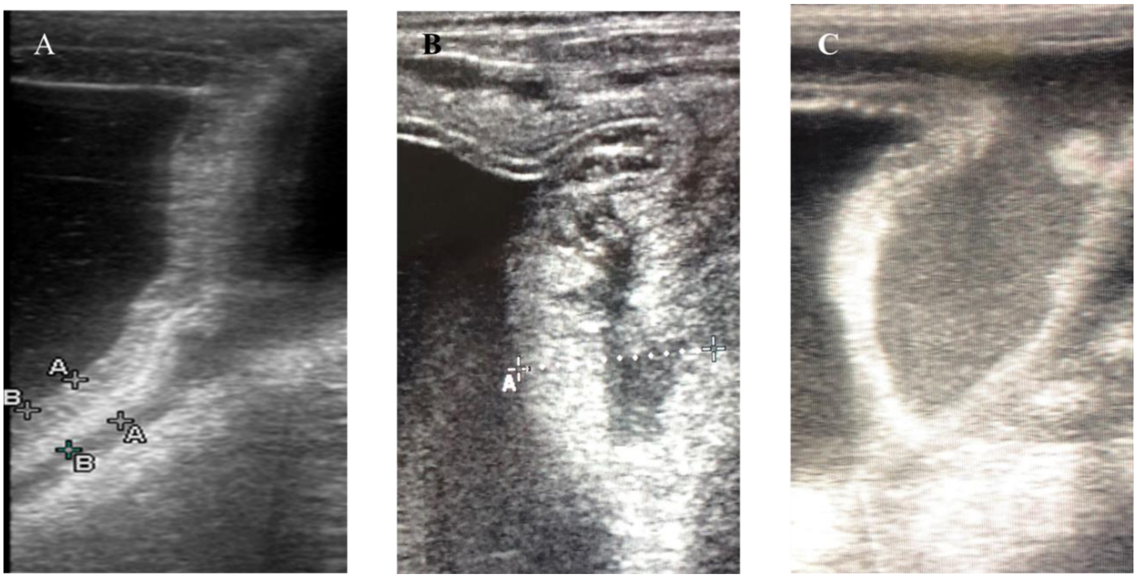

Figure 1.4 Transrectal ultrasonography of the combined thickness of the uterus and placenta (CTUP) at the caudal pole near the area of the cervix. Panel **A** depicts a normal CTUP from a mare at 305 days of gestation (< 1 cm) with no evidence of thickening or separation. Panels **B** and **C** depict CTUP images consistent with ascending placentitis following experimental inoculation with *Streptococcus equi* subspecies *zooepidemicus* bacteria with evidence of separation and a small amount of hypoechoic to mixed echogenic exudate between the chorioallantois and uterine endometrium in panel **B**, two days following inoculation, and severe separation with mixed echogenic exudate extending to the dorsally between the chorioallantois and uterine endometrium in panel **C** by six days following inoculation.

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Chapter 2

Introduction to Metabolomics and its Application to Veterinary Medicine

2.1 Metabolomics

Metabolomics is one of the exponentially expanding areas within the field of “Omics” which also includes genomics, transcriptomics, and proteomics. Metabolomics specifically involves the study of all endogenous and exogenous low molecular weight (< 1500 Daltons) compounds or metabolites in a system. Collectively, these metabolites are known as the metabolome (Dunn and Ellis 2005 and Villas-Boas et al. 2007). Each metabolite functions as either intermediates or as end products in the various metabolic processes and reactions that occur within a biofluid, cell, tissue, or even an entire organism (Dunn and Ellis 2005 and Mamas et al. 2011). The metabolome is therefore composed of a wide variety of compounds ranging from amino acids, organic acids, carbohydrates, ketones, lipids, fatty acids, nucleotides, and small proteins. As the metabolome is the final product of transcription, translation, and post-translational processing, it is the closest to the phenotype of an individual or system and responds the quickest to any present stressors such as disease, nutritional change, toxins, or alterations in the environment compared to the genome, proteome, or transcriptome (Viant 2007, Lewis, Asnani, and Gerszten 2008, and Kirwan 2013). Additionally, while thousands to hundreds of thousands of metabolites exist, their numbers are inherently fewer compared to a system’s genes, transcripts, or proteins and may allow for the identification of meaningful statistically significant patterns within a biological system (Dona et al. 2016).

Samples that can be analyzed in metabolomics vary greatly. Biofluids are often easily collected and can range from plasma, urine, saliva, bile, or cerebrospinal fluid, amongst many others. Entire cells such as bacteria, yeast, or even cell lines (epidermal cells, endometrial cells, hepatic cells, etc.) can also be analyzed to determine the intracellular and/or extracellular or excreted metabolites present. Cells do however require additional processing prior to analysis. Tissue samples and even *in vivo* organisms likewise can be analyzed but may require even more substantial processing or even special machinery for analysis (Jones and Cheung 2007 and Villas-Boas et al. 2007). Sample preparation protocols utilized, therefore, vary substantially based on the specific composition as well as the identity of the organism the sample is obtained from (Villas-Boas 2007).

Regardless of sample type, quenching must be performed during or immediately after sample harvesting to halt all continued biochemical processes. Quenching allows for the analysis of the *in vivo* metabolism and not the metabolism that may continue to occur subsequent to sample acquisition. In general, quenching rapidly inactivates metabolism through either a rapid change in temperature (< -40 °C or > 80 °C) or pH (pH < 2.0 or pH > 10) and can be performed by multiple methodologies (Villas-Boas et al. 2005 and Villas-Boas et al. 2007). The most commonly utilized modalities include rapid freezing or the addition of perchloric acid or cold methanol (Villas-Boas et al. 2005). Rapid freezing at -80 °C on dry ice or at -196 °C in liquid nitrogen are commonly performed for many biofluids, as the samples can then be stored until analysis at these temperatures in a -80 °C freezer or liquid nitrogen tank respectively. Rapid freezing preserves sample integrity with minimal alteration of the present *in vivo* metabolites. Acids such as perchloric acid are also frequently utilized, particularly to quench the metabolism of microbial cells. These acids may, however, allow for the loss of intracellular metabolites due to the cell membrane damage that can occur in response to the acidic pH (Villas-Boas et al. 2007). Cold methanol likewise is frequently utilized as it additionally separates the intracellular and extracellular metabolites present in addition to quenching the sample, but akin to perchloric acid after separation, loss of metabolites can also occur due to continued leakage from membrane damage (Villas-Boas et al. 2007).

2.2 Targeted and Untargeted Approaches to Metabolomic Analysis

In metabolomics two approaches for analysis are typically pursued. Some research may be hypothesis-driven where alterations in specific individual metabolites or known metabolic pathways by a specific disease or physiologic processes are proposed. In those cases, *metabolite targeted analysis* or *metabolomic fingerprinting* may be performed examining for differences within a small number or even a single metabolite of interest (Fiehn 2001). When available, targeted and labeled internal standards can then be used to further allow for clear identification, quantification, and comparison between groups of interest. Conversely, due to the nature of metabolomics, experiments likewise can be performed without the specific understanding of all of the potential alterations that could occur within the system of interest. A *global metabolomic profiling* approach is then used to first identify all possible present metabolites (Fiehn 2001). Investigators can then compare the pattern or fingerprint of metabolites that may occur in response to a specific environmental change, toxin, insult, or disease process (Fiehn 2001). Metabolomic profiling is therefore associated with biomarker discovery, development of potential diagnostics, and generation of hypotheses, into the effects a characteristic or stressor can affect a system (Bjerrum 2015). These two approaches are not necessarily mutually exclusive of each other particularly when utilizing the platform of nuclear magnetic resonance spectroscopy, as information concerning the entire metabolome is also obtained while examining for the specific or individual metabolites of interest.

2.3 Analytical Techniques

The two analytical platforms utilized in metabolomics include mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Both platforms can be performed with targeted and untargeted techniques, however, each has its own inherent advantages and disadvantages for these purposes. Both mass spectrometry and NMR spectroscopy are considered high-throughput and can, therefore, be used to analyze large numbers of samples at a given time. NMR spectroscopy is non-destructive allowing for individual samples to be analyzed in a variety of NMR experiments or saved for future analysis by other platforms (Emwas 2015). Mass spectrometry is inherently more sensitive with the ability to detect up to picomolar concentrations of metabolites compared to micromolar concentrations in NMR spectroscopy (Emwas 2015 and Gromski et al. 2015). It can also be utilized to look for compounds that do not contain hydrogens or carbons, which is typically a requirement for the most common NMR spectroscopic experiments. However, due to the need for separation techniques prior to analysis as discussed below, mass spectrometry also requires substantially more sample preparation whereas samples intended for NMR spectroscopy frequently require none or only minimal initial preparation.

A brief introduction to mass spectrometry will be described below with a more detailed description of NMR spectroscopy as it is the platform utilized in this work. Mass spectrometry specifically measures the mass-to-charge ratio (m/z) of ionized particles within a vacuum under a strong magnetic field (Villas-Boas et al. 2005 and Bjerrum 2015). Mass spectrometry is therefore first paired with a separation platform such as gas chromatography (GC-MS) or liquid chromatography (LC-MS) to isolate aspects of the sample that investigators are most interested in and reduce unnecessary overlap of results. Gas chromatography is typically chosen for samples that are volatile or who are easily rendered volatile while liquid chromatography is reserved for those which are mobile in liquid states (Jones and Cheung 2007, Villas-Boas et al. 2005, and Villas-Boas et al. 2007). Samples are then ionized within the mass spectrometer and subsequently separated by their mass-to-charge ratio within the magnetic field as heavier ions are not deflected as far as lighter ions, to produce a mass spectrum that is characteristic of both molecular weight and structure (Villas-Boas et al. 2007 and Bjerrum 2015). Mass spectrometry spectra are therefore plots of relative ion abundance on the y-axis and the m/z ratio on the x-axis (Wang and Griffiths 2008).

Nuclear magnetic resonance (NMR) spectroscopy works by applying strong magnetic and radio-frequency pulses to atoms with an odd atomic mass or odd atomic number who have a spin quantum number equal to ½ and thus exist in either the spin states of - 1/2 or + 1/2 (Purcell, Torrey, and Pound 1946, Bloach, Hansen, and Packard 1946, Villas-Boas et al. 2007 and Jones and Cheung 2007). The most commonly utilized nuclei include ^{1}H, ^{13}C, ^{31}P, ^{15}N, and ^{19}F due to their natural abundance and the atomic properties described above. Under the application of short pulses of high-energy radio frequencies, these nuclei will absorb energy and transition from a low energy state into a high energy state. As each nucleus then relaxes and returns to its previous lower energy state, radio frequency radiation is emitted and detected as peaks producing an NMR spectrum. The amount of energy absorbed as well as the radiofrequency emitted is related to the number of nuclei present as well as the local environment surrounding the nucleus within a specific molecule (Dunn and Ellis 2005). Each chemically distinct nucleus exhibits an NMR signal at a characteristic resonance frequency that is measured in terms of chemical shift (parts per million (ppm) (Dona et al. 2016). The location of individual peaks or resonance frequency is reflective of their specific chemical moieties as well as the moiety of any surrounding neighbors

(**Figure 2.1**). NMR spectra are therefore plots of the relative intensity (or abundance) of a particular nucleus on the y-axis and chemical shift (ppm) on the x-axis. Composite spectra for a biofluid, cell, tissue, or organism are consequently comprised of the superimposition of the spectra of all individual metabolites within a sample (Nicholson and Lindon 2008).

In addition to the property of chemical moiety, the location of each resonance can be affected by external factors such as pH, particularly when dealing with biofluids (Dona et al. 2016). Therefore, the addition of buffers to samples such as urine which may have variable pH values helps control for these shifts prior to analysis (Jones and Cheung 2007). Additionally, each resonance frequency is typically referenced to a known standard compound that is added during processing. These references are frequently exogenous compounds that are not naturally present within the system undergoing analysis (Dona et al. 2016). For example, for samples analyzed in water or a combination of deuterated water (D20) and water, the standard trimethylsilyl propionate (TSP) is added while tetramethylsilane (TMS) is added to the unknown samples processed in chloroform. Each of these standards displays single peaks (singlets) located at 0 ppm.

One dimensional (1-D) hydrogen or proton-NMR spectroscopy is the most commonly utilized technique or experiment within NMR spectroscopic investigations as most metabolites contain hydrogen atoms in their chemical structure (Villas-Boas et al. 2005). The multiplicity or pattern of peaks observed for each individual hydrogen signal exhibit an n+1 splitting rule where n is equal to the number of the atoms immediately surrounding the hydrogen of interest. Ratios of the signals present follow the pattern of Pasqual’s triangle, where a doublet (hydrogen next to a single neighbor) has a 1:1 splitting pattern, while a triplet (hydrogen surrounded by two total neighbors) displays a 1:2:1 ratio. For example, for the molecule lactate which is a metabolite commonly utilized as a biomarker of gastrointestinal inflammation in the horse and was examined as a marker of inflammation in experimental ascending placentitis in this work (Chapter 4), two distinct groups of hydrogens are present (**Figure 2.2**) (Reviewed by Li, Gu, and Zhou 2015, Johnston, Holcombe, and Hauptman 2007, and Petersen et al. 2016).

The typical chemical shift for proton NMR spectroscopy ranges from 0 to 10 ppm. Metabolites with relatively high intensity that are located in uncrowded areas within a spectrum may be identified with one-dimensional techniques alone by comparing each resonance frequency to frequencies within a known database (Dona et al. 2016). Direct comparisons can be made through online databases such as the Human Metabolome Database (HMDB), BioMagResBank (BMRB), Birmingham Metabolomic Library Nuclear Magnetic Resonance database (BML-NMR), or Madison Metabolomics Consortium Database (MMCD). Databases are also available which are specific to individual microorganisms such as the *Escherichia coli* Metabolome Database (ECMDB) and the Yeast Metabolome Database (YMDB). Spectra can also be imported into computer programs such as Chenomx NMR Suite (Chenomyx, Edmonton, Alberta, Canada) in which peaks can be identified utilizing the program’s internal database of standard metabolites (**Figure 2.3**) (Dona et al. 2016). Many of these programs are able to account for potential differences in pH when the pH of the sample is known and specified. While many metabolites are frequently conserved between different species and sample types, resonance frequencies or peak locations have not been described in every mammalian or microbial system. Small variations may, therefore, be present.

Occasionally due to the nature of metabolites having similar structures or composition such as the presence of methyl or methylene groups, peaks can be located in areas that may be extremely crowded or even overlap hindering their ability to be identified on 1-D H-NMR spectroscopy with a high degree of confidence (Dona et al. 2016, Villas-Boas et al. 2005, and Villas-Boas et al. 2007). Two-dimensional (2-D) experiments can then be performed to not only confirm identifications deduced on standard 1-D H-NMR spectroscopy but also to determine the identity of unknown peaks within these areas as well. Homonuclear experiments can be performed correlating the protons or hydrogens within individual metabolites to elucidate their structure. Homonuclear experiments include correlated spectroscopy (COSY) that examines hydrogens within two to three bonds of each other and total correlation spectroscopy (TOCSY) that is able to identify a larger interconnected group of spin coupled hydrogens located within a coupled chain. Heteronuclear experiments can provide further information on metabolite identity by comparing and correlating the relationship of protons to the carbon backbone of each molecule. Heteronuclear experiments include heteronuclear single quantum correlation spectroscopy (HSQC) that looks at the direct correlation of each hydrogen to its attached carbon and heteronuclear multiple bond correlation spectroscopy (HMBC) which provides correlations between carbons and protons that are separated by two to four bonds. Both TOCSY and HMBC provide information regarding connectivity within each molecule or metabolite. Examples of bonds identified by each of the above experiments are depicted in **Figure 2.4** with a representative HSQC spectrum presented in **Figure 2.5**. Finally, if desired, experiments utilizing other nuclei (carbon (^{13}C), nitrogen (^{15}N), fluorine (^{19}F), and phosphorous (^{31}P)) can also be performed.

The area under each peak is again representative of the abundance or intensity of that chemical group within a compound. Therefore, the peak height or area under the curve (AUC) of each peak, is directly proportional to the number of nuclei present (Jungnickel and Forbes 1963). In the field of metabolomics, this value is referred to as an integral and allows for comparisons between samples, individuals, and groups. Additionally, as discussed above, exogenous compounds are frequently added as reference compounds including TSP or TMS. When these compounds are added in known amounts and related to the peak height present, concentrations of other compounds can be inferred. Therefore, NMR spectroscopy can produce results that are truly quantitative, compared with semi-quantitative comparisons between samples, individuals, or phenotypes.

2.4 Methods for Statistical Analysis

Most of the NMR spectroscopy studies that are focused on metabolomic profiling generate extremely large data sets. Therefore, multivariate techniques are most frequently used to reduce the complexity of the data as well as to examine for the presence of multiple existing collinearities which could be overlooked when utilizing univariate techniques (Jones and Cheung 2007). Multivariate techniques may be applied to identified and integrated individual metabolites or to the spectrum as a whole. When analyzing the spectrum as a whole, it is typically divided and split into integral regions which are called buckets or bins and range between 0.01 to 0.04 ppm in width (Halouska and Powers 2006 and Jones and Cheung 2007). All the signals within each bucket are integrated as described above, summed, and analyzed following data normalization. Following analysis, metabolites of interest can be identified from the bins contributing to any interesting variance within the dataset.

Multivariate analysis consists of both unsupervised and supervised techniques. The primary utilized method is the unsupervised technique of principal component analysis (PCA) that examines for the presence of intrinsic clustering within the data set according to its intrinsic properties with no consideration of class or groups (for example healthy versus diseased) (Dona et al. 2016). Principal component analysis specifically reduces the data in an unbiased manner and expresses the variance as a small number of factors or the data's principal components (**Figure 2.6**). The output returned from statistical analysis programs displays the data as score and loading plots. Score plots display the coordinates of the main variance of all the samples within the analyzed system. Score plots, therefore, display an overview of all samples and enable for visualization of groupings, trends, and for the presence of any outliers (Dona et al. 2016). Loading plots, on the other hand, define the magnitude and manner of correlation in which the principal components and variance contribute to the difference in the score (Lindon, Holmes, and Nicholson 2003).

Supervised multivariate techniques are also commonly utilized and consist of either partial least squares discriminate analysis (PLS-DA) or random forest analysis. PLS-DA takes into account class membership (i.e. groups; specific treatments or control group versus disease) by identifying variables within a linear subspace that are associated with and maximize the separation between classes (Jones and Cheung 2007 and Gromski et al. 2015). Random forest analysis estimates which variables are important to distinguish groups similar to PLS-DA analysis but does so by utilizing a bootstrap sampling technique where the data is randomly chosen, and decision trees are created based on the data's random features for both classification and to estimate a particular variable's (metabolite or bins) importance (Liland 2011 and Gromski et al. 2015).

2.5 Application of Metabolomics to Veterinary Medicine

While veterinary metabolomic studies continue to lag behind the modalities of more widespread use in human medicine and research, animal-related metabolomic investigations and publications have been increasing steadily. As the metabolome is the final response to both genetic modifications and the combination of environmental and internal factors, metabolomics is a field well suited for the study of disease, the effects of toxins or nutritional insults, identification of biomarkers or drug targets, or to improve our understanding of basic physiology within a variety of species and organ systems. Biofluids such as plasma, serum, whole blood, saliva, urine, or feces are easily obtainable from most veterinary patients with others such as cerebrospinal or synovial fluid being slightly more invasive. Disease processes ranging from cancer, heart disease, and inborn errors of metabolism have been examined for evidence of metabolomic change in human medicine (Arn 2007, Kind et al. 2007, Lewis, Asnani, and Gerszten 2008, and Leichtle et al. 2013). Biomarkers identified from similar studies have been proposed to serve as indicators of disease or even as risk factors for developing a particular condition such as prostatic adenocarcinoma in men and may be able to predict aggressiveness as well as the potential for recurrence in some cases (Kelly et al. 2016, Liang et al. 2017, and Perez-Rambla et al. 2017). Similar work has been performed in the horse in a model of gastrointestinal sepsis where significant elevations in the metabolite citrulline were found to be a poor prognostic indicator and identified horses at an increased risk of developing laminitis (Steelman et al. 2014). Targets that may be elevated or decreased depending on the pathophysiological process involved, may then be able to serve as an avenue for pharmaceutical interventions (Robertson and Reily 2012). Additionally, the use of metabolomics in drug development can allow for the study of pharmacokinetics, toxicity, and interactions with the use of fewer research subjects (Jones and Cheung 2007 and Robertson and Reily 2012). Finally, with a finite view of the ongoing metabolism within a system, changes can even be proposed to improve the management or welfare of an animal. For example, in a recent study by Jang et al., specific metabolic patterns were seen in the muscle, plasma, and urine of racehorses before and after exercise. The authors proposed that understanding of, and potentially being able to balance or counter, these metabolic changes, could allow for the optimization of their racing performance through changes to their diet and overall management (Jang et al. 2017).

2.6 Summary

In summary, metabolomics involves the study of all metabolites involved in metabolism within a cell, tissue, organism, or biofluid. Metabolomic studies have the potential to impact a wide range of fields including veterinary medicine and show significant potential particularly in the areas of biomarker and diagnostic investigation, toxicology, as well as in the understanding of the basic metabolic processes that may be unique to individual animal species.

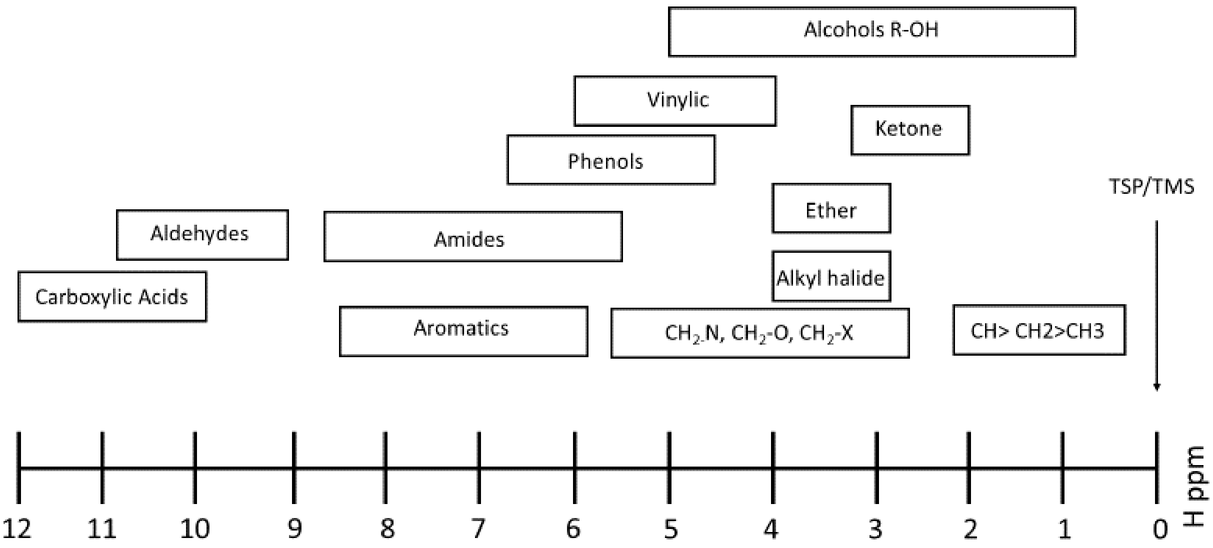


Figure 2.1 Visual representation of associated proton chemical shifts detected on 1-D proton nuclear magnetic resonance (NMR) spectroscopy of various metabolites in relation to the reference standards TSP or TMS at 0 ppm. The x-axis represents chemical shift in parts per million (ppm).

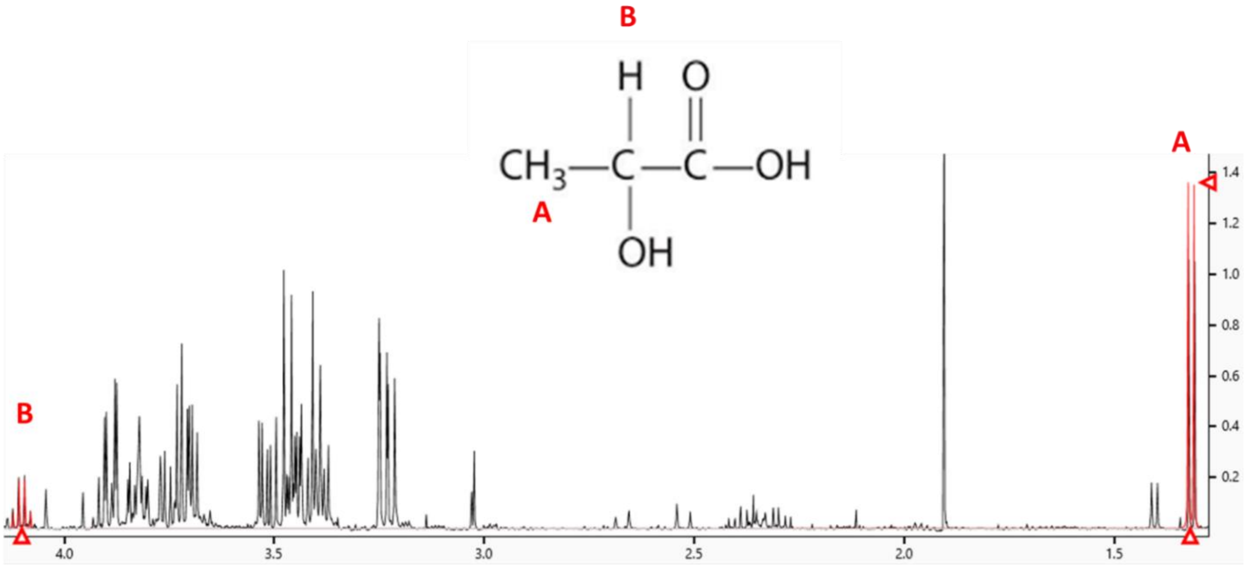
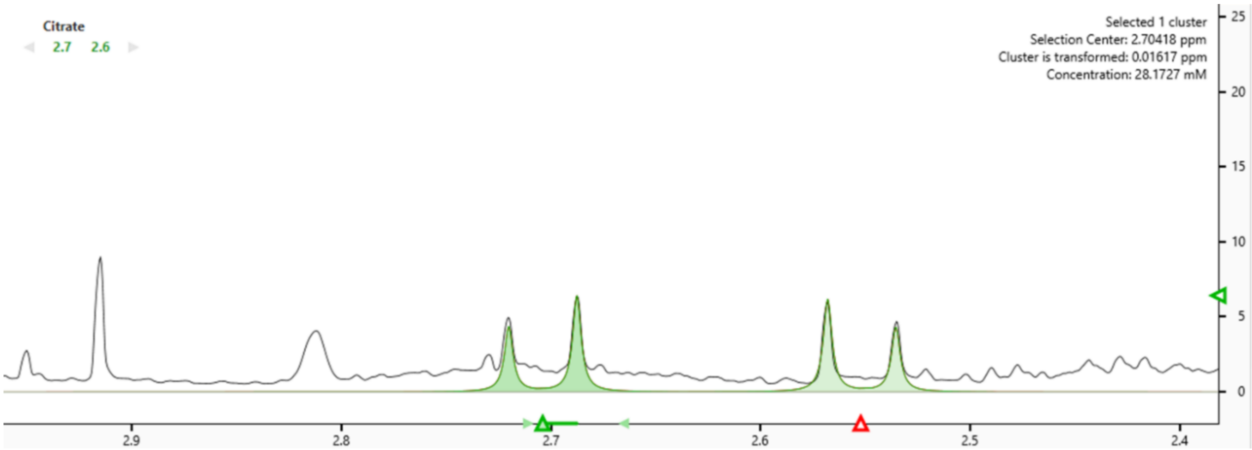


Figure 2.2 Chemical structure and one dimensional H-NMR spectrum of lactate (red) depicting the multiplicity pattern due to splitting. Representative spectrum from the polar fraction of equine plasma (Chapter 5) from a healthy mare at 285 days of gestation. Methyl group (A) is represented as a doublet due to two surrounding neighbors while the methine group (B) is represented as a quartet due to its immediate three surrounding neighbors.



Selected 1 cluster
Selection Center: 2.70418 ppm
Cluster is transformed: 0.01617 ppm
Concentration: 28.1727 mM

Figure 2.3 Representative spectrum from equine allantoic fluid of mare at 280 days of gestation with the compound citrate matched from the internal database in the program Chenomyx NMR Suite (Chenomyx Inc, Edmonton, Alberta, Canada). The y-axis depicts relative intensity and the x-axis chemical shift in parts per million (ppm).

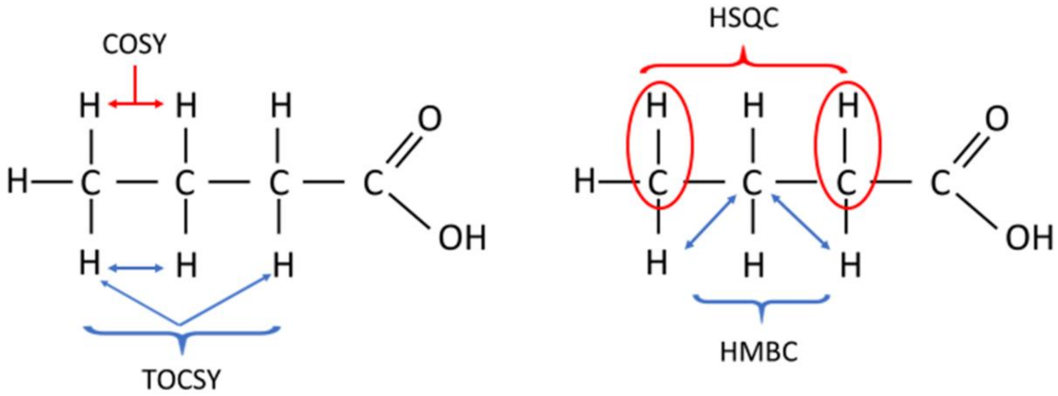


Figure 2.4. Diagram depicting the bonds identified in the commonly performed 2-D nuclear magnetic resonance (NMR) spectroscopy experiments. Correlated spectroscopy (COSY) identifies direct correlation between neighboring hydrogen groups or atoms while total correlation spectroscopy (TOCSY) identifies the correlation of hydrogens present with the entire spin system of a molecule. Heteronuclear experiments identify correlations between hydrogens and carbon molecules within a molecule. Heteronuclear single quantum correlation (HSQC) spectroscopy experiments identifies the correlation of each hydrogen and its directly attached carbon on the carbon backbone while heteronuclear multiple bond correlation spectroscopy experiments identifies the correlations of all the hydrogens and carbons within two to four bonds distance within a molecule.

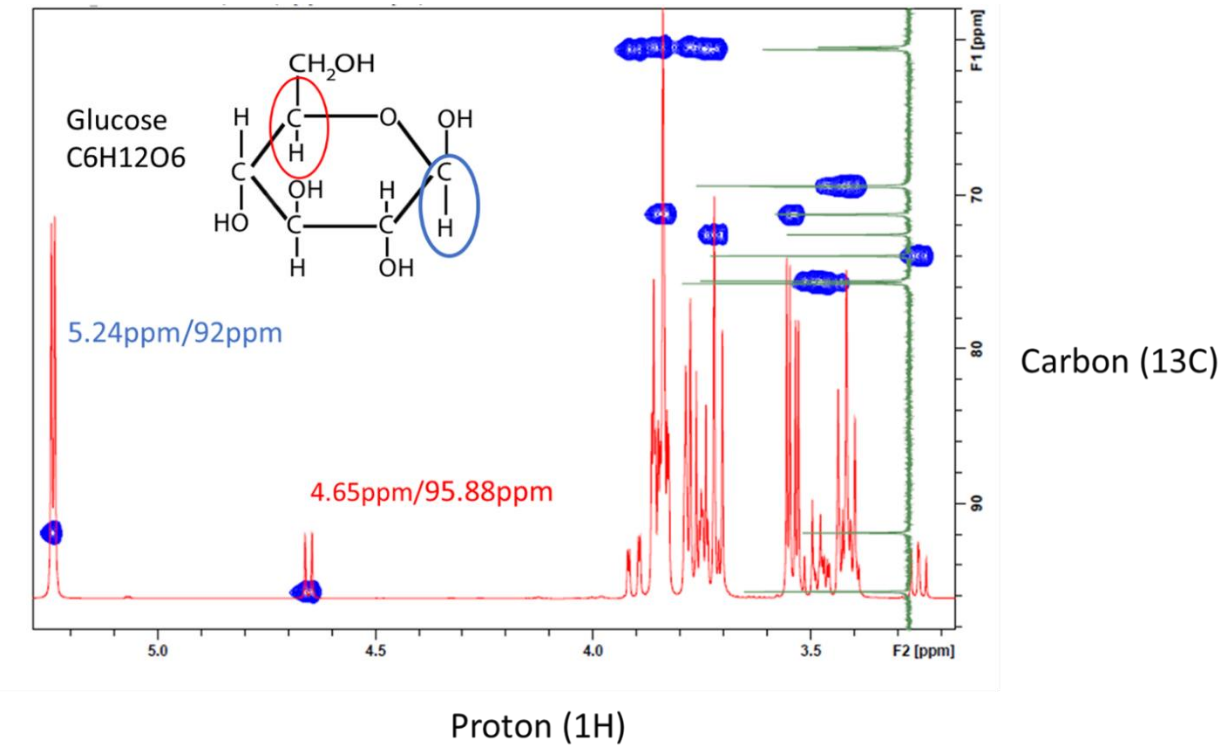


Figure 2.5 Representative 2-D heteronuclear single quantum correlation (HSQC) spectroscopy (Blue) overlaid with 1-D proton spectrum demonstrating the proton to carbon coupling on carbon #1 (Blue) and carbon #5 (Red). Each respective carbon and hydrogen are circled in their respective color and identified with their respective proton and carbon chemical shifts respectively. The y-axis represents carbon chemical shift in parts per million (ppm) and the x-axis proton chemical shift in parts per million (ppm). Spectrum depicted is from a representative allantoic fluid sample from a healthy control mare at 285 days of gestation (Chapters 3 and 4).

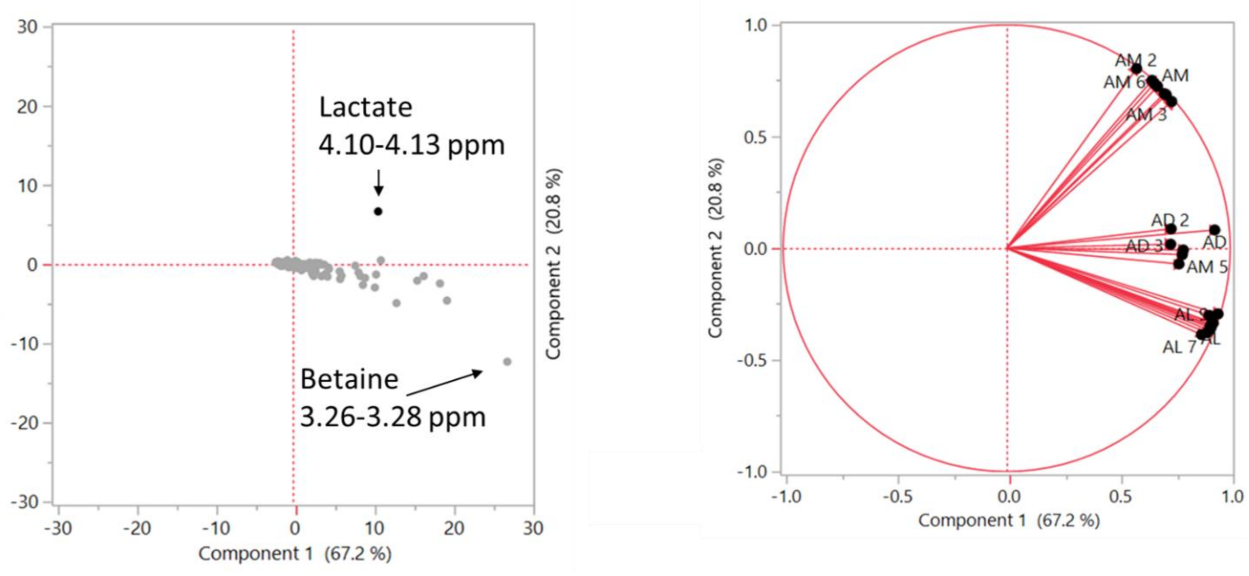


Figure 2.6 Representative Principal Component Analysis depicting separation between allantoic fluid (AL samples for mares 1-7), amniotic fluid (AM samples for mares 1-6), and admixed fluid (AD mares 1-3) as described in Chapter 3. Variance identified distinguishing amniotic fluid is attributed to differences in the metabolite lactate while the variance distinguishing allantoic fluid is attributed to the metabolite betaine.

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Chapter 3

Metabolomic Profile of Allantoic and Amniotic Fluid in Late Term Gestational Mares Characterized by ^{1}H-Nuclear Magnetic Resonance Spectroscopy

3.1 Introduction

Over the last century, the intricacies of equine placentation including the anatomy, histologic properties, and function of the fetal membranes as well as their associated fetal fluids have been studied by many to expand our overall understanding of pregnancy in the horse as well as to provide potential avenues for the assessment of gestational well-being. The equine embryo first enters the uterus on days five and a half to six post ovulation to begin its migratory phase throughout the luminal uterine surface (Ginther 1983 and Battut et al. 1997). By day six and a half, glycoproteins produced by the trophectoderm surround the spherical embryo to form the embryonic capsule and remain until beyond fixation to approximately day 21 of gestation (van Niekerk 1965, Denker, Betteridge, and Sirois 1987, Betteridge 1989, and Oriol, Sharom, and Betteridge 1993). As the capsule then begins to break down, the fetal membrane of the allantois is first distinguishable as an outgrowth of the fetal hindgut (van Niekerk 1965). During this early period of embryonic development, the amniotic fetal membrane has also become well established and discernable, forming from folds of ectoderm that envelop and surround the developing fetus (Ewart 1897, van Niekerk 1965, and Samuel, Allen and Steven 1976). Allantoic fluid and amniotic fluid are contained within each of these compartments respectively and increase in volume as gestation progresses. As the allantois continues to grow and expand, replacing the early choriovitelline placenta and regressing yolk sac, it will begin to oppose and fuse with the chorionic membrane to form the chorioallantois or what is considered the true equine placenta for the remainder of gestation (Ewart 1897, Samuel, Allen and Steven 1976, and Favaron et al. 2015). Following the unique endometrial cup reaction of the horse, the expanding chorioallantois and associated chorioallantoic villi oppose and begin to interdigitate with the endometrial villi. The villi continue to elongate and develop until day 120 of gestation when the functional microcotyledon is formed (Samuel, Allen, and Steven 1974, Samuel, Allen, and Steven 1976, and Samuel, Allen, and Steven 1977).

The biochemical composition of the allantoic and amniotic fluid has been described in early, mid, and late-term gestational mares, as well as at parturition in both normal foaling mares as well as those experiencing dystocia (Schott 1988, Williams et al. 1992, Williams et al. 1993, Paccamonti et al. 1995, Holdstock et al. 1995, Kochar et al. 1997, and Zanella et al. 2014). Allantoic and amniotic fluid in late gestation expressed inherent differences in many commonly examined electrolytes including sodium, chloride, calcium, and phosphorous on biochemistry panels, indicators of kidney function such as creatinine, and in enzymes utilized to assess liver function such as alkaline phosphatase and γ-glutamyltransferase (Williams et al. 1993 and Zanella et al. 2014). In human obstetrics and fetal medicine, metabolomics has further been utilized through both mass spectrometric and nuclear magnetic resonance (NMR) spectroscopic approaches to identify metabolites present within amniotic fluid, cervical secretions, and plasma that may be predictive of individuals at risk for the development of spontaneous preterm birth syndrome or preeclampsia (Menon et al. 2004, Romero et al. 2010, and Auray-Blais et al. 2011) as well as to explore the normal metabolome associated with pregnancy (Orczyk-Pawilowicz et al. 2016). While the lipidomic profile of equine amniotic fluid has recently been described by a mass spectrometric approach, a detailed description of the metabolomic profile of allantoic and amniotic fluid in the horse has not yet been performed (Wood et al. 2018). Therefore, the objective of this study was to characterize the metabolomic profile through non-targeted NMR spectroscopy of equine allantoic and amniotic fluid in late-term pregnant mares.

3.2 Materials and Methods

Fetal fluid was collected from a total of 12 pregnancies in seven mares between 270 and 295 days of gestation over two breeding seasons. Mares were five to fourteen years of age and were maintained on pasture at a university-owned farm in central North Carolina. All procedures were carried out in accordance with North Carolina State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals (IACUC # 16-209). Prior to sample collection, all mares were examined to ensure a healthy pregnancy through a routine physical examination and through transrectal ultrasonographic measurement of the combined thickness of the uterus and placenta (CTUP) and transabdominal assessment of echogenicity of fetal fluids, fetal activity, and fetal heart rate. Parameters were determined to be within normal limits for all mares.

3.2.1 Fetal Fluid Collection

Fetal fluids were collected from each mare through ultrasound-guided transabdominal puncture, utilizing a modified technique developed by Canisso and co-workers as described below (Canisso, et al. 2014). The ventral abdomen was clipped on both sides from the xiphoid process of the sternum to the mammary gland and laterally to the level of the stifle. The abdomen was then imaged using a 2.5-MHz ultrasound transducer (Micromaxx® Ultrasound System; 2.5-MHz curvilinear sector probe; Sono-Site, Inc. Bothell, WA), to locate an area of fetal fluid that was free of the fetus and subsequently aseptically prepared with chlorhexidine scrub and alcohol. Prior to allantocentesis, each mare was sedated utilizing detomidine (0.2 mg/kg, IV, Zoetis, Pasippany, New Jersey). An 18-gauge, 20 cm echogenic tip needle (Chiba Needle G17897, Cook Medical LLC, Bloomington, IN) was then passed under ultrasound guidance into the fetal fluid pocket. Two to six mLs of fluid were obtained via gravity flow into sterile cryovials. Fluid was immediately quenched and flash-frozen in liquid nitrogen in 2 mL aliquots and stored at -196 ^{o}C until analysis.

At the time of sample collection, allantoic fluid was first differentiated from amniotic fluid based on its characteristic translucent, golden amber color compared to the clear to opaque white of amniotic samples. Samples noted to be light yellow were denoted as potentially admixed combinations of allantoic and amniotic fluid. Fluid type was subsequently analyzed for creatinine concentration utilizing methods routinely used for biofluid biochemistry at North Carolina State University for confirmation of type in addition to gross color characteristics. Briefly, 150 µL were thawed and analyzed by the Jaffé calorimetric method to determine creatinine concentration (Cobas c501, Roche Diagnostics USA, Indianapolis, IN).

3.2.2 Metabolomic Processing

Fetal fluid samples were first thawed at room temperature and 1 mL aliquots lyophilized to remove all water (Labconco FreeZone 2.5-L, Labconco, Kansas City, MO). Seven hundred µL of 0.1-mM deuterated trimethylsilyl propionate (TSP) (Trimethylsilyl Propionate, Sigma Aldrich, St. Louis, MO) in 10% deuterium oxide (D20) and 90% water (D2O, Sigma Aldrich, St. Louis, MO) was then added to the dried samples, and centrifuged at 3500 x g for 15 minutes to remove any remaining particulate matter. The supernatant was removed and pipetted into 5-mm borosilicate NMR tubes (Wilmad Labglass, Fisher Scientific, Waltham, MA) for NMR spectroscopic analysis.

3.2.3 ^{1}H-NMR and 2D NMR spectroscopy and spectral pre-processing

One-dimensional (1D) proton (^{1}H) NMR spectroscopy was performed using a 500-MHz spectrometer (Bruker AVANCE, Billerica, MA) with Oxford Narrow Bore Magnet, HP XW 4200 Host Workstation, and integrated software (Topspin 3.2 Software) at the BioNMR Facility (North Carolina State University, Raleigh, NC). A 5-mm proton broadband inverse probe with Z gradients was used for all 1D ^{1}H presaturation experiments and 2D ^{1}H-^{13}C experiments and was tuned to the ^{1}H frequency of 500.193 MHz and ^{13}C frequency of 125.77 at the 500-MHz spectrometer with all spectra obtained at 294K with a 2.04 total second acquisition time and 128 number of transients.

3.2.4 Spectral processing, identification, and quantification

All 1D- and 2D-NMR spectra were processed using an NMR processor (ACD/Labs 12.0, Advanced Chemistry Department, Inc., Toronto, Ontario, Canada). The spectra were zero-filled to 16,384 points and Fourier transformed. Baseline and phasing correction was performed automatically, and each was referenced to the internal standard TSP peak at 0 ppm chemical shift. Peak identification was performed using Chenomyx (Chenomx NMR Suite 8.1, Chenomx, Edmonton, Alberta, Canada) and databases of known compounds (Human Metabolome Database (HMDB)). To help confirm and validate peak identity, two-dimensional (2D) proton experiments (homonuclear correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY)), proton-carbon experiments (heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond experiments (HMBC) were performed on representative allantoic and amniotic samples (**Figure 3.1**). Spectra were integrated and the common integrals of peak height obtained were weighted to the TSP integral, which was added at a known concentration to perform quantitative comparisons between fluid type.

3.2.5 Statistical Analysis of NMR Data

Partial least squares discriminant analysis (PLS-DA) was performed on the integrals obtained from intelligent bucketing to identify metabolites that contributed to variation between groups at each time-point (MetaboAnalyst 4.0[k]) (Xia et al. 2015). Integrals of identified metabolites were compared between fluid types using a Kruskal-Wallis test followed with a Dunn's multiple comparison post-hoc test, with a significance level set at 0.05 (STATISTIX 8.1, Statistix, Inc., Tallahassee, FL). Data are presented as ratios of endogenous peak area to the peak area of TSP, as an appropriate internal standard, per 1 mL of amniotic, allantoic, or admixed fluid (mean ± standard error of the mean).

3.3 Results

A total of 24 samples were collected from fetal fluid puncture. While a mild variation in color was seen between mares, allantoic fluid was characteristically amber to golden brown in nature (n=10) while amniotic fluid was clear to opaque white (n=9). Admixed samples collected were light yellow in color (n=5). Biochemical analysis revealed statistically significant elevations in the concentration of creatinine within allantoic fluid compared to amniotic samples (**Table 3.1**; p=0.003). All samples produced spectra upon NMR spectroscopy with identifiable peaks and were used for quantification. Characteristic spectra were seen for allantoic and amniotic samples with admixed samples appearing as combinations of each (**Figure 3.2**). A total of 28 metabolites were identified within allantoic and admixed fluid samples (**Figure 3.3**) whereas 23 metabolites were identified within amniotic samples (**Figure 3.4**). Metabolite assignment for both fluid types is illustrated in **Table 3.2**.

PLS-DA analysis showed a clear separation between allantoic and amniotic samples with admixed samples overlapping with characteristics of each (**Figure 3.5**). Upon metabolite integration of individually identifiable peaks within each spectrum, allantoic fluid contained significant elevations in the metabolites betaine, creatine, creatinine, citrate, histidine, myo-inositol, nitrophenol, and unknown #1 (**Table 3.3**). Lactate was significantly elevated within amniotic fluid compared to allantoic and admixed samples (p=0.003). No difference was seen in the amino acid tryptophan between fluid types.

3.4 Discussion

This study is the first to describe the non-targeted metabolomic profile of both allantoic and amniotic fluid in the normal pregnant mare utilizing ^{1}H-NMR spectroscopy. Amniotic fluid originates from the combined secretions of the respiratory, urogenital, and gastrointestinal tracts, amniotic epithelium, as well as fetal skin prior to its keratinization and is thought to provide both a source of mechanical support or cushion to the developing fetus, an avenue for the development of coordinated movement and neurologic function, and a minor source of nutrients (Bonnet 1889, Assheton 1906, Zietzschmann 1924, Brden, Evans, and Binns 1972, Baetz et al. 1976, and Nutrition Reviews 1976). Allantoic fluid in late stages of pregnancy is primarily composed of secretions from the mesonephros, metanephros, and fetal kidneys through the anatomic structure of the urachus (Assheton 1906, Zietzschmann 1924, Brden, 1972). Fetal urine as maturation and patency of the urethra is completed, will additionally enter the amniotic fluid cavity as well after mid-gestation in the equine pregnancy (Mellor and Slater 1971, and McCance 1972 , Holdstock et al. 1995). These factors in addition to individual differences in the fetus and dam, likely contribute to the difference in metabolic composition seen between the two fluid compartments.

While highlighting a few of the metabolites identified within allantoic and amniotic fluid in this study, the metabolite creatinine has previously been detected within the fetal fluids of many mammalian species including those of the horse (Mellor 1971, Schott, 1988, Williams et al. 1992, Williams et al. 1993, Paccamonti et al. 1995, Holdstock et al. 1995, Kochar et al. 1997, and Zanella et al. 2014). In women, an increasing concentration of creatinine has been demonstrated within amniotic fluid throughout gestation due to increasing degrees of fetal kidney maturity, as well as progressive muscle development, and has been used commonly to assess prenatal kidney functionality (Jones 1971 and Oliveria, Barros, and Magalhaes 2002). Similar to human pregnancies, creatinine has also been shown to increase throughout gestation within both amniotic fluid and allantoic fluid of the pregnant mare when analyzed from 37 days of gestation to term upon biochemical analysis (Holdstock et al. 1995). Holdstock and colleagues showed similar findings to those of this study as creatinine was found to be significantly elevated in allantoic fluid compared to amniotic fluid throughout the entire period of gestation in thoroughbred mares. While increasing levels were also found likewise throughout gestation in pony mares, elevated levels within allantoic fluid became statistically significant compared to amniotic fluid after day 200 of gestation until term (Holdstock et al. 1995). Pony mares utilized in this study similarly were analyzed in late gestation between 270 and 295 days.

Both the metabolites creatine and its degradation product creatinine have also previously been identified in both the urine and plasma of adult thoroughbred horses through NMR spectroscopy (Escalona et al. 2015). Creatine is known to be found in high concentrations in skeletal and heart muscle of adults and fetuses and is involved in the production of ATP by the enzyme creatine kinase. Creatinine is commonly considered as a waste product created from the non-enzymatic degradation of creatine and is subsequently excreted most commonly through the urine (Wyss 2000). Therefore, it is unsurprising that in this study we likewise found that both creatinine and creatine were present in allantoic and amniotic fluid and with similar correlations in each fluid compartment, as both compartments have a contribution of fetal urine.

Myo-inositol is a critical component of many of the phospholipids found within cells and serves as a precursor for phosphoinositide signaling (Hayashi et al. 1974 and Harris et al. 2011). L-Myo-inositol-1-Phosphate synthase, the enzyme responsible for producing myo-inositol has been recently detected within the fetal liver and its main metabolite myo-inositol is believed to help prevent irregularities in liver development and function during human embryonic development as well as serve as a precursor for myelin within the developing nervous system (Hayashi et al. 1974, Harris et al. 2011, and Chhetri et al. 2012). Hormonally, myo-inositol in humans has also been shown to be an insulin sensitizer across multiple systems and is hypothesized to function in this fashion during gestation as well (Pkhaldze, Brbakadze, and Kyashilaya 2016 and Fruzzetti et al. 2017).

Betaine a metabolite that was shown to be found in higher concentrations of allantoic fluid than the amniotic fluid in this study has also been identified in the allantoic fluid of the chicken embryo (Feng et al. 2007). Betaine is produced from the oxidation of choline and can also be obtained directly from the diet (Feng et al. 2007 and Kirsch et al. 2010). Early in pregnancy, betaine has been shown to actively accumulate within early murine embryos from the dam via the SIT1 transporter as well as to protect early embryos against increased osmolarity in vitro (Biggers, Lawitts, and Lechene 1993, Anas et al. 2008, and Imbard et al. 2013). Additionally, betaine also serves as a methyl donor for many of the necessary methylation reactions that along with choline may assist in the promotion of normal development and protect against the occurrence of developmental anomalies (Karunamuni et al. 2017). Supplemental betaine has been shown to alleviate cardiac developmental anomalies in an ethanol-induced model mimicking prenatal alcohol syndrome in quail embryos (Karunamuni et al. 2017). While the exact role of this metabolite is unknown in equine development, it is plausible that it potentially could display a similar role to that in other species as well as humans.

3.5 Conclusions

In summary, by comparing the metabolomic profile of amniotic fluid and allantoic fluid we observed that the concentration of multiple metabolites including betaine, creatine, and creatinine, citrate, histidine, myo-inositol, nitrophenol, and unknown #1 were increased in allantoic fluid in late gestation while lactate comparatively was elevated within the amniotic fluid. Therefore, these results begin to establish a database of metabolites of the fetal fluids within the normal equine pregnancy.

Table 3.1 Mean creatinine concentration determined by biochemical analysis in allantoic, amniotic, and admixed fluids presented as mean ± standard error of the mean in mg/dL. *Significant at $p < 0.005$.

	Allantoic Fluid	Amniotic Fluid	Admixed Fluid
Creatinine (mg/dL)	50.91 ± 4.95	12.49 ± 4.75*	34.65 ± 11.36

Table 3.2 List of metabolites identified in confirmed allantoic (ALF) and amniotic fluid (AMF) of pregnant mares using 1D and 2D NMR spectroscopy experiments with their respective proton chemical shifts and multiplicity (bs-broad singlet, d-doublet, dd-doublet of doublets, dt-doublet of triplets, m-multiplet, s-singlet, t-triplet, q-quartet).

Compound	Chemical Formula	Chemical Shifts (ppm)	Fluid Type
Acetate	C2H4O2	1.92 (s)	ALF and AMF
Allantoin	C4H6N4O3	5.39 (s), 6.0 (s), 6.8(s), 7.34 (d), 10.6 (s)	ALF and AMF
Betaine	C5H11NO2	3.26 (s), 3.89 (s)	ALF and AMF
Carnosine	C9H14N4O3	2.68 (dt), 3.03 (dd), 3.19 (dd), 3.24 (dt), 4.48 (m), 7.26 (s), 8.52 (s)	ALF and AMF
Citrate	C6H8O7	2.53 (d), 2.65 (d)	ALF and AMF
Creatine	C4H9N3O2	3.04 (s), 3.93 (s)	ALF and AMF
Creatinine	C4H7N3O	3.05 (s), 4.07 (s)	ALF and AMF
Creatine phosphate	C4H10N3O5P	3.04 (s), 3.93 (s)	ALF and AMF
Formate	CH2O2	8.43 (s)	ALF and AMF
Galactose	C6H12O6	3.48 (dd), 3.6 (dd), 3.72 (d), 3.82 (d), 3.92 (d), 3.99 (d), 4.07 (t), 4.60 (d), 5.23 (d)	ALF
Glucose	C6H12O6	3.2 (dd), 3.36 (m), 3.46 (m), 3.63 (dd), 3.72 (m), 3.82 (m), 3.88 (dd), 5.25 (d), 5.36 (d)	ALF and AMF
Glycolate	C2H4O3	3.93 (s)	ALF and AMF
Histidine	C6H9N3O2	3.15 (dd), 3.32 (dd), 3.79 (d), 7.26 (s), 8.52 (s)	ALF and AMF
Lactate	C3H6O3	1.33 (d), 4.11 (q)	ALF and AMF
Maltose	C12H22O11	3.27 (dd), 3.40 (t), 3.57 (m), 3.62 (m), 3.65 (m), 3.70 (m), 3.75 (m), 3.85 (m), 3.90 (dd), 3.92 (d), 3.95 (m), 5.34 (d), 5.40 (d)	ALF
Mannose	C6H12N2O2	3.40 (dd), 3.56 (t), 3.64 (m), 3.72 (m), 3.79 (m), 3.87 (dd), 3.90 (m), 5.15 (d)	ALF
Methanol	CH4O	3.38 (s)	ALF and AMF
π-methyl histidine	C7H11N3O2	3.07 (dd), 3.15 (dd), 3.68 (s), 3.95 (dd), 7.00 (s), 7.67 (s)	ALF and AMF
Methylmalonate	C4H6O4	1.22 (d), 3.16 (q)	ALF

Table 3.2 (continued).

Myo-inositol	C6H12O6	3.20 (t), 3.47 (dd), 3.70 (t), 4.06 (t)	ALF and AMF
N-methylhydantoin	C4H6N2O2	2.92 (s), 4.08 (s)	ALF and AMF
Nitrophenol	C6H5NO3	6.80 (m), 8.10 (m)	ALF and AMF
Trimethylsilyl propionate	C6H1402Si	0.00 (s)	ALF and AMF
Tryptophan	C11H12N202	3.29 (dd), 3.47 (dd), 4.04 (dd), 7.19 (m), 7.274 (m), 7.36 (s), 7.53 (d), 7.73 (d)	ALF and AMF
Unknown #1	Unknown	0.52 (s), 2.29 (s), 3.6 (s), 3.83 (s)	ALF and AMF
Unknown #2	Unknown	1.2 (s), 1.8 (s), 4.46 (s), 5.45 (s)	ALF
Urea	CH4N2O	6.0 (s)	ALF and AMF
Water	H20	4.77 (bs)	ALF and AMF

Table 3.3 Mean metabolite integral value in allantoic, amniotic, and admixed fluids presented as mean ± standard error of the mean. * Significant at $p < 0.05$; ** significant at $p < 0.005$.

Metabolite	Allantoic Fluid	Amniotic Fluid	Admixed Fluid
Betaine	95.81 ± 18.18**	8.60 ± 3.27	27.78 ± 14.27
Creatine	14.31 ± 2.14**	2.29 ± 0.81	5.13 ± 2.22
Creatinine	27.03 ± 4.22*	4.64 ± 1.10	9.50 ± 5.04
Citrate	5.97 ± 0.90**	0.96 ± 0.27	2.27 ± 0.87
Histidine	8.94 ± 1.56**	1.23 ± 0.75	3.62 ± 1.50
Lactate	11.59 ± 3.37	31.19 ± 5.14**	7.75 ± 0.83
Myo-Inositol	18.05 ± 3.67	3.00 ± 1.07	8.53 ± 4.69
Nitrophenol	0.78 ± 0.13*	0.22 ± 0.10	0.56 ± 0.46
Tryptophan	0.47 ± 0.07*	0.26 ± 0.10	0.48 ± 0.19
Unknown #1	1.91 ± 0.30**	0.36 ± 0.13	0.85 ± 0.27
π-methyhistidine	0.70 ± 0.13**	0.19 ± 0.10	0.46 ± 0.17

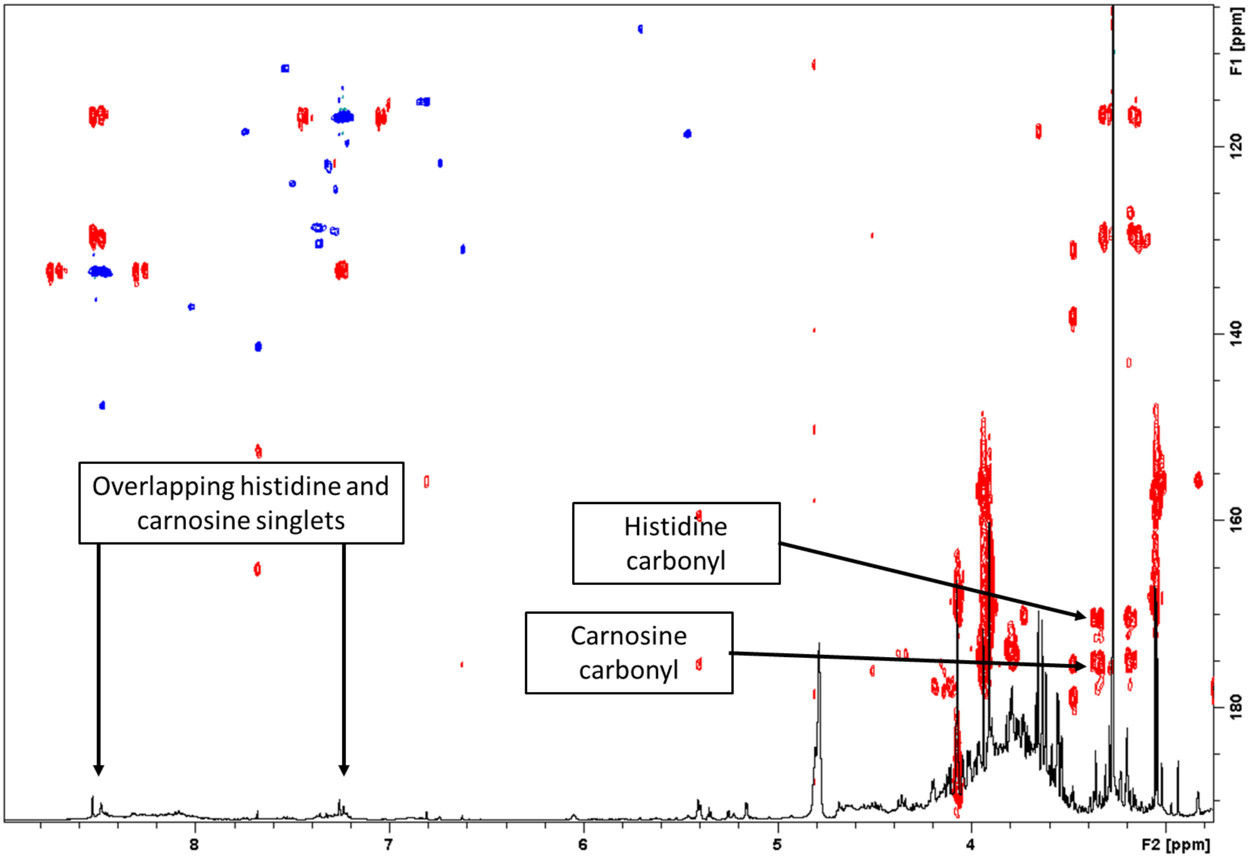


Figure 3.1 Two-dimensional spectral analysis of allantoic fluid. Representative 2-D overlay of HSQC (blue) and HMBC (red) experiments depicting one bond and long-range correlation utilized to confirm the identification of individual metabolites identified on 1-D analysis. Identification of the overlapping metabolites histidine and carnosine are depicted. The presence of two carbonyls illustrated the presence of more than one chemical component at the hydrogen resonance. X-axis depicts proton chemical shift in parts per million (ppm); Y-axis depicts carbon chemical shift in parts per million (ppm).

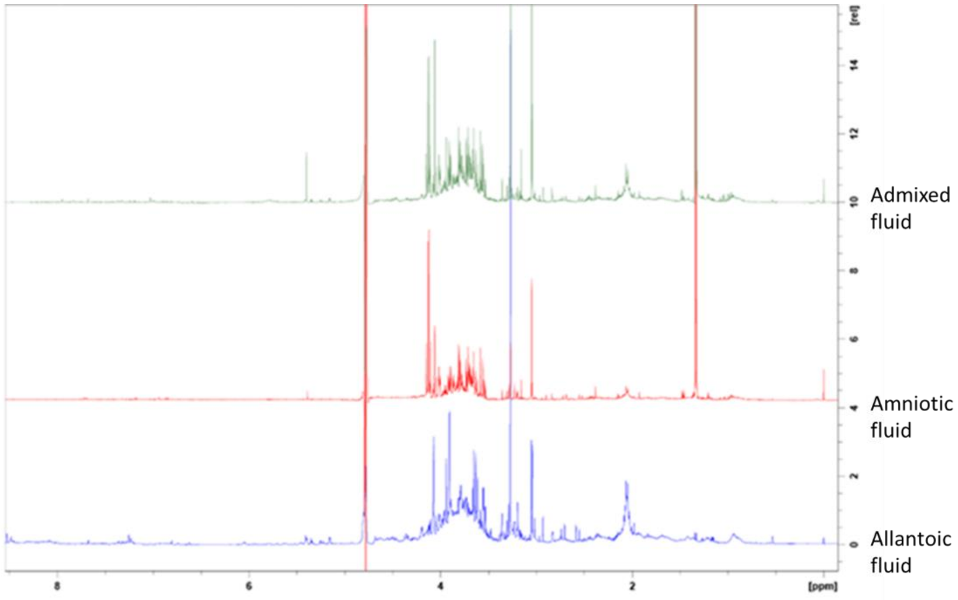
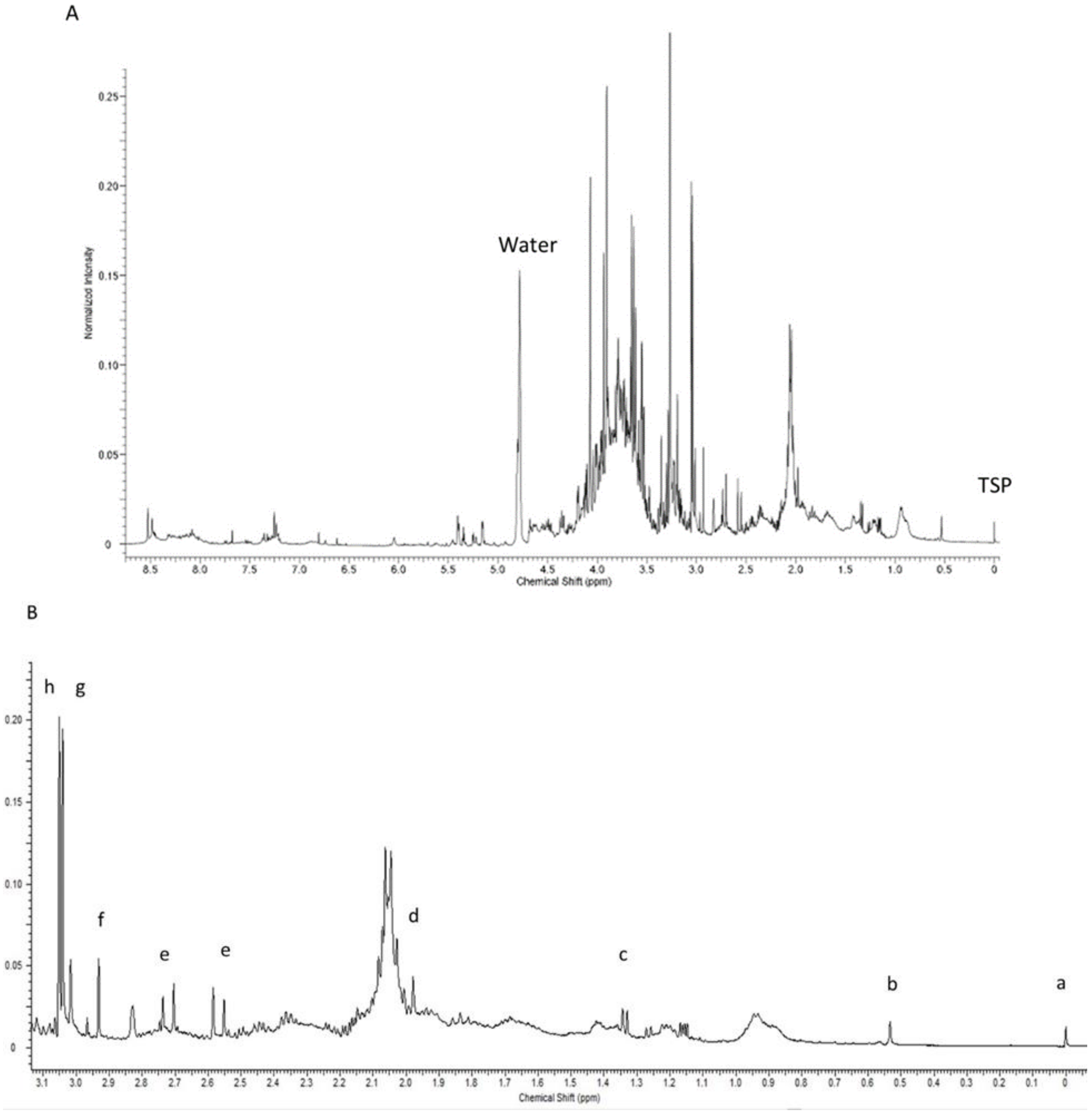


Figure 3.2 Representative ^{1}H-NMR spectra from allantoic, amniotic, and admixed fluid samples. The y-axis is indicative of peak intensity and x-axis of chemical shift in parts per million (ppm).

Figure 3.3 Representative ^{1}H-NMR spectrum of allantoic fluid. Adapted from Beachler et al. 2018 (Chapter 4). The x-axis depicts chemical shift in parts per million (ppm); y-axis depicts peak intensity (a) trimethylsilyl propionate, (b) unknown 1, (c) lactate, (d) acetate, (e) citrate, (f) N-methylhydrantoin, (g) creatine, (h) creatinine, (i) betaine, (j) methanol, (k) myo-inositol, (l) glucose, (m) glucosamine, (n) mannose, (o) galactose, (p) allantoin, (q) tryptophan, (r) histidine and carnosine, (s) formate, (t) π-methylhistidine.


A
Water
TSP
Normalized Intensity
0.25
0.20
0.15
0.10
0.05
0
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0
Chemical Shift (ppm)
B
h
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e
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0.20
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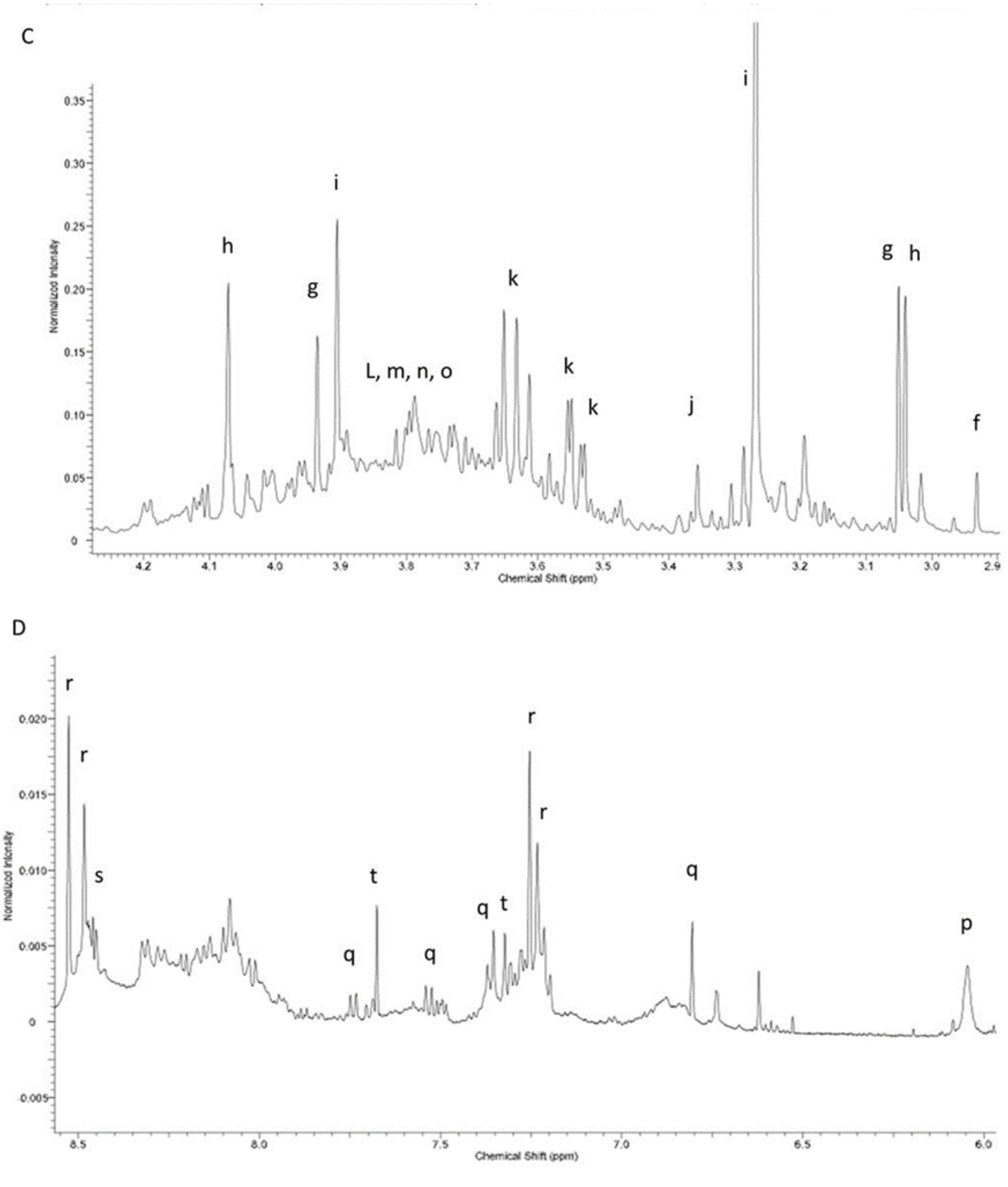
0.05
0
3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0
Chemical Shift (ppm)


C
Normalized Intensity
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
h
g
i
L, m, n, o
k
k
k
j
i
g
h
f
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
Chemical Shift (ppm)
D
Normalized Intensity
0.020
0.015
0.010
0.005
0
-0.005
r
r
s
q
t
q
q
t
r
r
q
p
8.5
8.0
7.5
7.0
6.5
6.0
Chemical Shift (ppm)

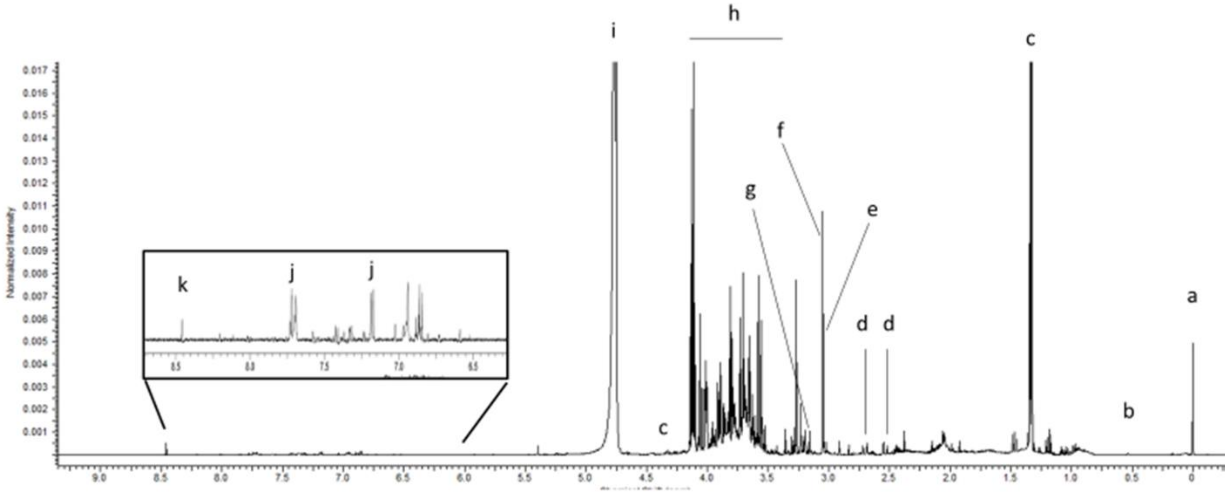
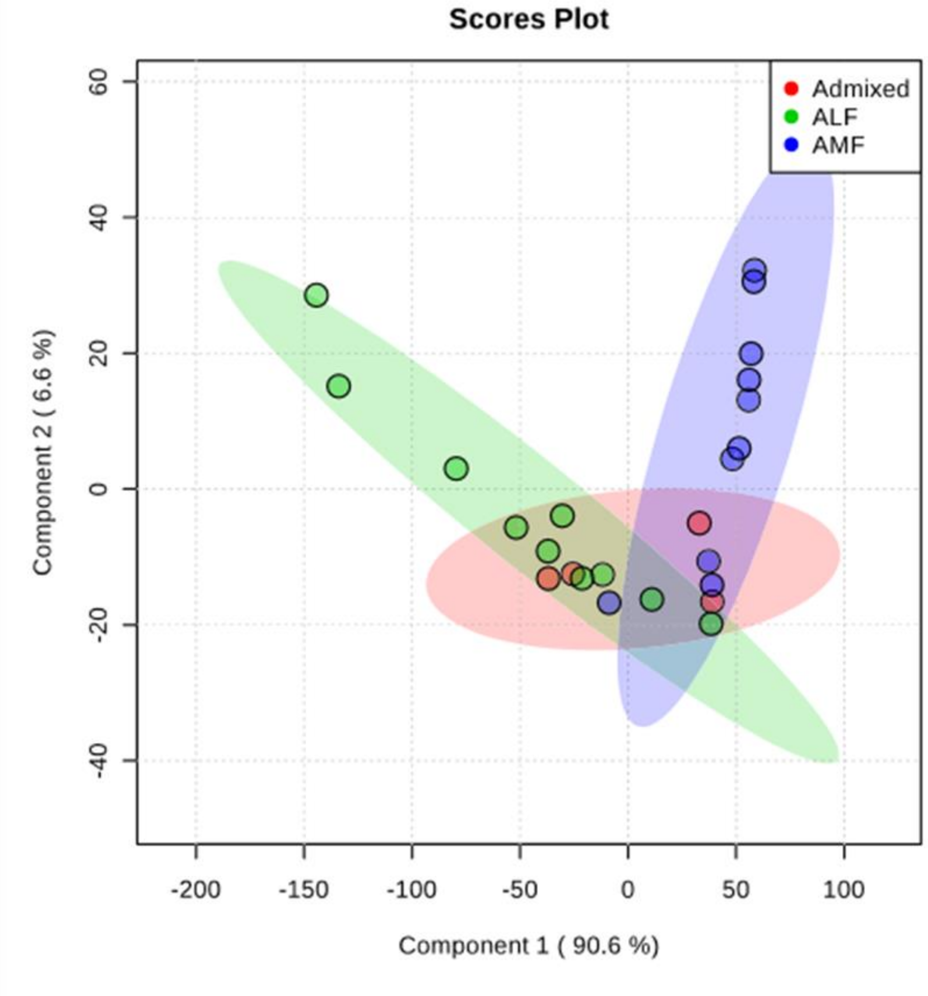


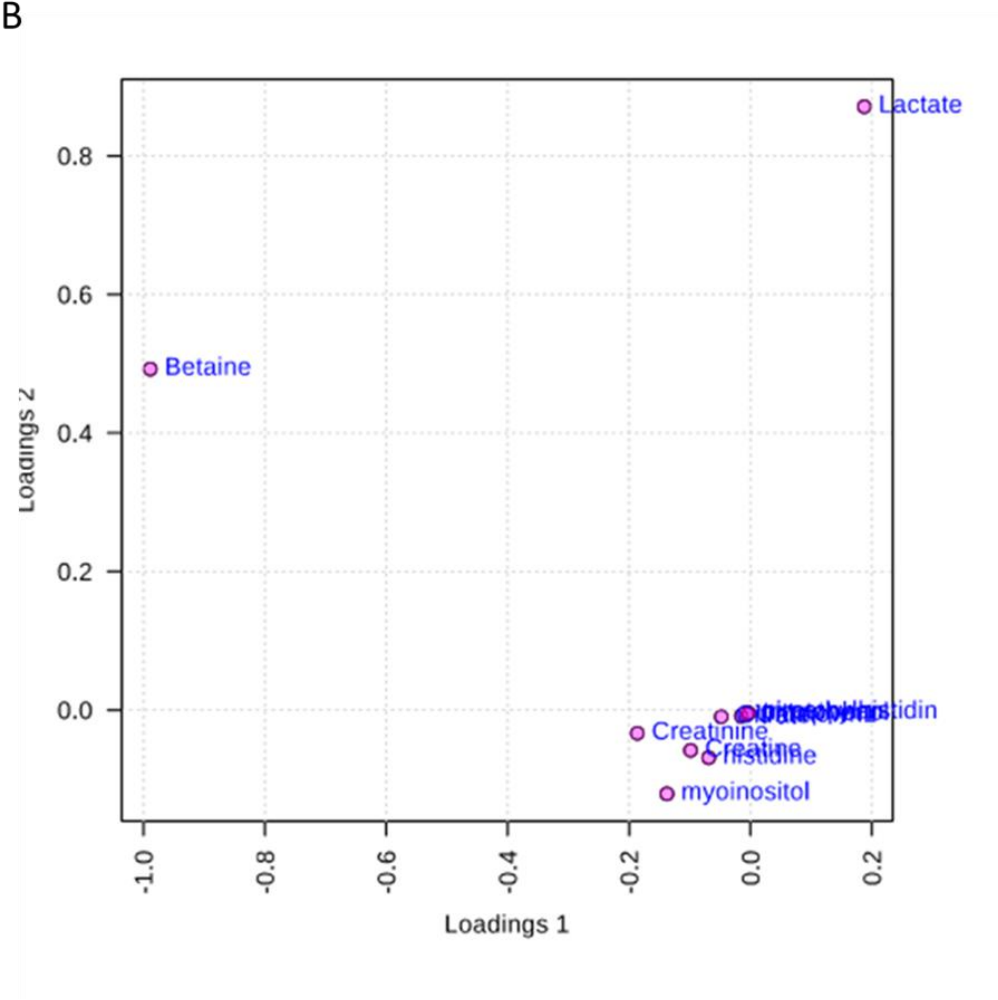
Figure 3.4 Representative ^{1}H-NMR spectrum of amniotic fluid. The x-axis depicts chemical shift in parts per million (ppm); y-axis depicts peak intensity. Selected identified peaks are as follows (a) trimethylsilyl propionate, (b) unknown 1, (c) lactate, (d) citrate, (e) creatine (f) creatinine, (g) myo-inositol, (h) glucose, (i) water, (j) histidine and carnosine, (k) formate.

Figure 3.5 Scores plot (A) and loading plot (B) from partial least squares determinate analysis depicting summary of the components contributing to variance seen between fluid types (MetaboAnalyst 4.0). The metabolite betaine contributes to the majority of the variance in allantoic fluid while lactate contributes to the variance seen in amniotic fluid separating each of these fluid types into two distinct classes. Admixed fluid (red) exhibits characteristics that are similar between both allantoic and amniotic fluid.

A


Scores Plot
Admixed
ALF
AMF
Component 2 (6.6 %)
Component 1 (90.6 %)
60
40
20
0
-20
-40
-200
-150
-100
-50
0
50
100

B


Lactate
Betaine
Creatinine
Creatine
Histidine
myoinositol
0.8
0.6
0.4
0.2
0.0
Loadings 2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
Loadings 1

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Chapter 4

Allantoic Metabolites, Progesterone, and Estradiol-17β Remain Unchanged After Infection in an Experimental Model of Equine Ascending Placentitis.

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4.1 Abstract

The objective of this study was to characterize the metabolomic profile of equine allantoic fluid in the pregnant mare with and without experimentally induced ascending placentitis, with the goal of identifying biomarkers of this disease. We compared the onset of metabolomic changes to common modalities for diagnosis of ascending placentitis, including measurement of the combined thickness of the uterus and placenta (CTUP), hormonal profiling, and measurement of serum acute phase proteins. Ten pregnant pony mares were randomly divided into two groups: five healthy control mares (CONT), and five mares induced to develop ascending placentitis (PLAC) via inoculation with *Streptococcus equi* subsp. *zooepidemicus* bacteria at days 280 to 285 of gestation. Allantoic fluid, whole blood, and serum were collected from both groups at 270 to 275 days of gestation, and at the following time points post-inoculation: 4 hours, days 2, 4, 6, and 10. Differences between groups in identified metabolites, progesterone, estradiol-17β, lactate, and serum amyloid A (SAA) were assessed using an ANOVA with repeated measures. A total of 27 metabolites were identified in allantoic fluid. No differences were detected between groups at any time point ($p>0.05$) for any identified metabolite, progesterone, estradiol-17β, or lactate concentrations. Significant elevations in CTUP ($p=0.003$) and SAA ($p=0.0001$) were detected by days four and six post inoculation, respectively. The results of this study established a database of equine allantoic fluid metabolites and confirmed the utility of uteroplacental ultrasound for detection of placentitis prior to the onset of hematologic changes.

Key Words: Equine; ascending placentitis; metabolomics; allantoic fluid

4.2 Introduction

Ascending placentitis is one of the most common causes of late term abortions in horses, contributing to approximately one third of all late term losses (Giles et al. 1993, LeBlanc et al. 2002, Macpherson 2006, Cummins et al. 2008, and Laugier et al. 2011). The disease is commonly caused by migration of single isolates or mixed populations of bacteria, such as *Streptococcus equi* subsp. *zooepidemicus*, *Escherichia coli*, *Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* through the cervix. This leads to necrotizing inflammation of the chorioallantois, colonization of the allantoic and/or amniotic membranes, fetal sepsis, and abortion (LeBlanc et al. 2002 and Cummins et al. 2008). Unfortunately, abortion or preterm delivery can frequently be seen without the presence of premonitory signs. When present, clinical signs noted include precocious mammary development, lactation, and occasionally vaginal discharge (LeBlanc et al. 2002, Cummins et al. 2008, and Bailey et al. 2010). Accepted, and commonly available screening tools for pregnant mares are limited to serial ultrasonographic measurement of the uteroplacental thickness (CTUP), and serial monitoring for alterations in progestagen or estrogen production. Normal parameters of CTUP have been described for light horse, warmblood, draft, and pony mares, with minimal variance between breeds (Renaudin et al. 1997 and Bucca 2006). As the hormones progesterone and estrogen are produced by the fetoplacental unit and released into the maternal circulation, hormonal alterations may occur in response to fetal and/or placental compromise (Macpherson 2006, Stawicki et al. 2002, Macpherson and Bailey 2008, Canisso et al. 2017, and Shikichi et al. 2017). Additionally, the acute phase protein serum amyloid A (SAA) has recently been shown to increase in an experimental model of placentitis (Coutinho da Silva et al. 2013, Canisso et al. 2014a, and Canisso et al. 2015). These diagnostics however are relatively insensitive to early disease both clinically and experimentally, and frequently go undetected prior to fetal loss (Bucca 2006, Macpherson 2006, and Macpherson and Bailey 2008).

In the present study, we sought to compare the onset of changes in CTUP, hormones, SAA, and serum lactate with the onset of changes to the metabolome of allantoic fluid in a case-controlled experimental model of disease. Metabolomics has been used to gain new insight into the metabolic changes associated with a multitude of normal and abnormal physiologic states including exercise, altered nutritional states or hormonal profiles, or specific diseases (Oliver et al. 1998). Mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy represent different modalities for performing global or targeted metabolomic profiling (Oliver et al. 1998). In human medicine, the field of metabolomics is becoming more widely used in both research and clinical settings. It has also been useful in identifying biomarkers of disease to indicate treatment efficacy, and as a prognostic indicator for many conditions ranging from congenital errors of metabolism, cardiovascular disease, and numerous cancers (Arn 2007, Kind et al. 2007, Lewis et al. 2008, Mamas and Dunn 2011, and Leichtle et al. 2013). In the field of obstetrics, metabolomics has specifically been used to identify risk factors for preterm labor and pre-eclampsia (Romero et al. 2010, Auray-Blais et al. 2011, and Menon et al. 2014). In veterinary medicine, metabolomic technology is still relatively novel and not widely available, however several studies have documented its usefulness in both research and clinical situations (Steelman et al. 2014, Li et al. 2015, and Luck et al. 2015). Although metabolomics has not been used in the field of equine reproduction to date, this technology could have potential applications in the diagnosis of infertility and other reproductive pathologies, including the diagnosis of equine placentitis.

As the allantoic space is acutely involved in the pathophysiology of ascending bacterial placentitis, metabolic changes within this compartment may occur earlier than the metabolic profile of other biofluids such as serum, urine, or saliva. Therefore, the objective of this study was to characterize the metabolomic profile of equine allantoic fluid in the pregnant mare with and without experimentally induced ascending placentitis, with the goal of identifying biomarkers of disease that are produced prior to ultrasonographic alterations or hematologic and hormonal alterations in response to infection.

4.3 Material and Methods

4.3.1 Animals

Ten pregnant pony mares were enrolled in this study at 270 to 275 days of gestation between the months of February to July. Mares were five to thirteen years of age and were maintained on pasture at a university owned farm in central North Carolina. All procedures were carried out in accordance with North Carolina State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals. All mares were examined to ensure a healthy pregnancy through a routine physical exam and by measurement of CTUP, echogenicity of fetal fluids, fetal activity, and fetal heart rate. All parameters were within normal limits for gestational age prior to onset of the trial.

At the time of enrollment, mares were randomly divided into two separate groups. Five mares were experimentally induced to develop ascending placentitis by intracervical inoculation of *Streptococcus equi* subsp. *zooepidemicus* bacteria (Group=PLAC) while five mares served as normal healthy controls (Group=CONT).

4.3.2 Bacterial Inoculation

Between days 280 to 285 of gestation, mares in the PLAC group were inoculated mid-cervically with *Streptococcus equi* subsp. *zooepidemicus* bacteria originally obtained from a clinical isolate submitted to the microbiology laboratory at the University of Florida, College of Veterinary Medicine in 1999 (Murchie et al. 2006). These bacteria were minimally passaged and remained frozen at -80 °C. Two days prior to inoculation, an aliquot of banked *Streptococcus equi* subsp. *zooepidemicus* was thawed and re-plated on Columbia agar with 5% sheep blood at 37 °C and 5% carbon dioxide to confirm pure culture. On the day of inoculation, a 1 mL inoculate was prepared containing 10^7 colony forming units (CFU) using the McFarland standards for microbiology dilutions (McFarland Standard 0.5, Hardy Diagnostics, Santa Maria, CA). At the time of inoculation, mares were placed in stocks with tails tied laterally and their perineum was washed thoroughly with water and an iodine-based soap and dried. The inoculum was drawn up into an insemination pipette, alternating with air and two 0.5 mL 0.9% saline cushions to prevent bacterial loss during deposition. Inoculation was then performed in accordance with standard techniques described by MacPherson and co-workers (Stawicki et al. 2002, Bailey et al. 2010, and Bailey et al. 2012). Briefly, using a sterile gloved arm and clean technique, an AI pipette was manually guided through the vagina and passed into the cervix using digital guidance. The inoculum was then placed 2.5 cm beyond the external cervical os. Confirmation of placement was obtained through the presence of cervical plug on the operators' fingers and subsequent transrectal ultrasonographic assessment of a fluid line in the cervix, combined with an absence of fluid between the endometrium and fetal membranes. Control mares received no cervical procedures at this time. Standard bacteriologic quantification of each inoculate was performed by plating serial dilutions of the bacterial suspension on Columbia blood agar with 5% sheep blood at 37 °C and 5% carbon dioxide and determining the number of colony forming units in each mL of fluid (Clinical and Laboratory Standards Institute 1999).

4.3.3 Allantocentesis

Fetal fluid was collected from both groups at 270 to 275 days of gestation (baseline) and at the following time points following inoculation (group PLAC): 4 hours, day 2, 4, 6, and 10, and then weekly until foaling. Collections were performed in control mares at the same intervals, starting between day 280 and 285. Fetal fluids were collected from each mare through ultrasound-guided transabdominal puncture, utilizing a modified technique developed by Canisso and co-workers as described below (Canisso et al. 2014b). The ventral abdomen was clipped from the xyphoid process of the sternum to the mammary gland and laterally to the level of the stifle on both sides of the abdomen. The abdomen was then imaged using a 2.5-MHz ultrasound transducer (Micromaxx® Ultrasound System; 2.5-MHz curvilinear sector probe; Sono-Site, Inc. Bothell, WA), to locate a fluid pocket that was free of the fetus and amniotic membrane and then aseptically prepared with chlorhexidine scrub and alcohol. Prior to allantocentesis, each mare was sedated utilizing detomidine (0.2 mg/kg, IV, Zoetis, Pasippany, New Jersey). An 18-gauge, 20 cm echogenic tip needle (Chiba Needle G17897, Cook Medical LLC, Bloomington, IN) was then passed into fetal fluid under ultrasound guidance. Two to five mL of fluid were obtained via gravity flow into sterile cryovials. Fluid was immediately flash-frozen in liquid nitrogen in 2 mL aliquots and stored at -196 ºC until analysis. Allantoic fluid was differentiated from amniotic fluid and peritoneal fluid based on characteristic amber color and creatinine concentration (Cobas c501, Roche Diagnostics USA, Indianapolis, IN) (Williams et al. 1993 and Zanella et al. 2014).

Only samples consistent with pure allantoic fluid were utilized for subsequent principal component and multivariate analysis.

4.3.4 Mare Monitoring and Treatment

At the time of inoculation, the time of each sample collection, and 12 and 24 hours after inoculation, a complete physical examination and jugular venipuncture (5 mL sample) were performed. Feto-placental health was assessed by transrectal and transabdominal ultrasonographic measurement of the CTUP and fetal heart rate. Detection of CTUP greater than 10 mm was considered consistent with placentitis between 270 to 300 days of gestation, while a CTUP greater than 12 mm was considered diagnostic for placentitis between 300 and 330 days of gestation (Renaudin et al. 1997 and Bucca 2006). At the time of diagnosis, standard multimodal therapy of trimethoprim sulfamethoxazole (30 mg/kg, q12 h, PO, Aurobindo Pharma Inc., Dayton NJ), flunixin meglumine (1.1 mg/kg q12 h, PO, Banamine, Merck Animal Health, Elkhorn, NE), and altrenogest (0.088 mg/kg, q24 h, PO, Regumate, Merck Animal Health, Summit, NJ) as previously described was initiated (Macpherson 2006, Macpherson and Bailey 2008, and Bailey et al. 2010). All treatments were continued until foaling.

Sampled blood was used for lactate and SAA measurements and hormonal (progesterone and estradiol-17β) analyses. A calibrated hand-held electrochemical lactate oxidase biosensor (Lactate Plus, Nova Biomedical, Waltham MA) was used to immediately analyze the concentration of extracellular lactate in a 0.7 µL sample of whole blood. The analyzer has a measuring range of 0.3-25 mmol/L in human whole blood. Serum amyloid A was analyzed through an immunoturbidity assay in 5 µL of serum (StableLab Eq Serum Amyloid A EQ-1 reader, Stablelab, Sligo, Ireland) on flash-frozen serum. The hand-held test has been validated by the manufacture with a reported mean precision and mean accuracy of 98.6 and 95.6% respectively, and a detection range of 0-3000 µg/mL. Progesterone and estradiol-17β analyses were performed through radioimmunoassay (MP Biologicals, Santa Anna, CA). Progesterone analysis was performed in a single assay with an intra-assay CV of 7.8%. Estrodiol-17β was analyzed in three assays with an inter-assay CV of 9.6% and intra-assay CV of 8.6%.

Mares in both groups were assessed regularly for signs of impending foaling, including mammary development, lengthening vulva, and softening of the tail head and gluteal musculature. The milk electrolyte calcium was assessed daily (Predict-A-Foal, Animal Health Care Products, Williston, VT) after initial detection of cloudy mammary fluid, and all mares were monitored for foaling using an electronic foaling sensor (Foalert, Foalert Inc., Acworth, GA). At the time of foaling, mares were allowed to foal normally unless assistance was deemed clinically necessary due to presence of dystocia or premature separation of the fetal membranes.

4.3.5 Assessment of Mares and Foals

Full physical examination was performed on all live foals at the time of parturition. Foals that were unable to breathe without assistance, unresponsive to stimuli, or were unable to obtain sternal recumbency, or displayed evidence of prematurity or dysmaturity on physical examination were deemed non-viable. Non-viable foals were euthanized using an overdose of a barbiturate (1 mL/5kg, once, IV; pentobarbital sodium and phenytoin in combination-Beuthanasia®, Schering-Plough Animal Health, Kenilworth, NJ, USA). Foals that were able to right themselves, breathe without mechanical assistance, and respond to stimuli were deemed viable at birth. Viable foals were monitored closely for normal expected behavior following delivery, including a normal time to stand and nurse, as well as the ability to nurse effectively. A complete blood count (CBC) (Vetscan HM5, Abaxis, Union City, CA, USA) and assessment of immunoglobulins (IgG) (Snap®Foal IgG test, Idexx, Westbrook, ME, USA) was performed on all viable foals at 12-24 hours of age to examine for evidence of adequate passive immunoglobulin transfer. Foals showing signs of systemic compromise and/or sepsis were administered the systemic antimicrobials ceftiofur sodium (5 mg/kg, q12 h, IM; Naxcel, Zoetis, Parsippany, New Jersey) and/or amikacin (25 mg/kg, q24 h, IV; Amikacin Sulfate Injection, USP, Heritage Pharmaceuticals Inc., Eatontown, NJ) as indicated. All viable foals were monitored regularly and received full physical examinations for the first 5 to 7 days of life post-foaling.

Each mare was assessed for passage of the fetal membranes in its entirety, and a uterine culture was performed within six hours post-partum. Samples were collected through sharp dissection from the fetal membranes of all mares for histopathology and placed into 10% neutral buffered formalin until analysis. A complete gross necropsy and culture of fetal stomach contents were performed on deceased foals.

4.3.6 Metabolomic Sample Preparation

A 1 mL allantoic fluid aliquot from each mare at each time-point was thawed at room temperature and lyophilized to remove all water (Labconco FreeZone 2.5-L, Labconco, Kansas City, MO). Lyophilized samples were then dissolved in 700 µL of 10% deuterium oxide (D20) and 90% water (D2O, Sigma Aldrich, St. Louis, MO) with 0.1-mM trimethylsilyl propionate (TSP) (Trimethylsilyl Propionate, Sigma Aldrich, St. Louis, MO). Samples were centrifuged at 3500 x g for 15 minutes to remove any remaining particulate matter. The supernatant (699 µL) was removed and pipetted into 5-mm borosilicate NMR tubes (Wilmad Labglass, Fisher Scientific, Waltham, MA) for NMR spectroscopic analysis.

4.3.7 ^{1}H-NMR and 2D NMR spectroscopy and spectral pre-processing

One-dimensional (1D) proton (^{1}H) NMR spectroscopy was performed using a 500-MHz spectrometer (Bruker AVANCE, Billerica, MA) with Oxford Narrow Bore Magnet, HP XW 4200 Host Workstation, and integrated software (Topspin 3.2 Software) at the BioNMR Facility (North Carolina State University, Raleigh, NC). To help identify and confirm peaks, two-dimensional (2D) proton experiments (homonuclear correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY)), proton-carbon experiments (heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond experiments (HMBC)) were performed on representative allantoic samples. A 5-mm proton broadband inverse probe with Z gradients was used for all 1D ^{1}H presaturation experiments and 2D ^{1}H-^{13}C experiments and was tuned to the ^{1}H frequency of 500.193 MHz and ^{13}C frequency of 125.77 at the 500-MHz spectrometer with all spectra obtained at 294K with a 2.04 total second acquisition time and 128 number of transients.

4.3.8 Spectral processing, identification, and quantification

All 1D- and 2D-NMR spectra were processed using an NMR processor (ACD/Labs 12.0, Advanced Chemistry Department, Inc., Toronto, Ontario, Canada). The spectra were zero-filled to 16,384 points and Fourier transformed. Baseline and phasing correction were performed automatically. The spectra were then referenced to the internal standard TSP peak at 0 ppm chemical shift. Peak identification was performed using database of known compounds

(Chenomx NMR Suite 8.1, Chenomx, Edmonton, Alberta, Canada and the Human Metabolome Database (HMDB)).

4.3.9 Histopathology

At the time of foaling and/or abortion, samples of amnion, umbilical cord, and the chorioallantoic membrane at the areas of the gravid horn, non-gravid horn, uterine body, and cervical star were collected into 10% neutral buffered formalin for histologic analysis. Tissue samples were processed paraffin-embedded tissues were sectioned (5 μm) and stained with hematoxylin and eosin for histologic analysis. Histologic analysis was performed by a veterinary pathologist, who was blinded to experimental groups. At each site, a subjective score of 0-3 was applied for edema, necrosis, inflammation, and bacteria as follows: Edema: 0=normal, 1=minimal separation of chorioallantoic membrane by clear spaces, 2=moderate separation of tissue by clear spaces that thickens the chorioallantoic membrane less than twice normal, 3= marked separation of tissue by clear spaces that thickens the chorioallantoic membrane greater than twice normal; Necrosis: 0=normal, 1=mild multifocal areas of necrosis that affects chorioallantoic villi only, 2=moderate necrosis which results in blunting and loss of villi, 3=severe necrosis with villous loss and erosion; Inflammation: 0=normal, 1=mild multifocal infiltration of by non-degenerative neutrophils and macrophages, 2=moderate multifocal infiltration by degenerative and non-degenerative neutrophils and macrophage, 3=marked to severe infiltration by mostly degenerative neutrophils and macrophages; Bacteria: 0=no bacteria visualized, 1=rare scattered cocci observed, 2=multifocal colonies of cocci, and 3=large confluent mats of cocci. A placentitis score was calculated by tabulating the cumulative score in each of the respective categories at the area of the cervical star and the ventral uterine body.

4.3.10 Statistical Analysis

Gestational age was compared between groups using one-way ANOVA while differences in CTUP, fetal heart rate, lactate, SAA, progesterone, and estradiol-17β were assessed between groups and over time using a repeated measures analysis with treatment, day, and their interaction in the model statement (STATISTIX 8.1, Statistix, Inc., Tallahassee, FL). The scores obtained from histopathology of the placenta were compared between groups at both the cervical star area and the uterine body using a One-way ANOVA. Chi Square analysis was performed to evaluate the likelihood of obtaining a viable foal. For all tests, significance was set at 0.05.

Two methods of analysis were used for metabolomic data. Common integrals from intelligent bucketing were normalized by dividing each bin by the sum to make the integrals for each mare sum to one. Principal component analysis (PCA) was then used to compare groups and identify metabolomics integrals that contributed to variance between groups and assist identification of pertinent metabolites (JMP PRO12 (SAS Institute, Inc., Cary, NC, USA). For PCA analysis, intelligent bucket integration was utilized with selected dark regions ranging from −3.19 to 0.4 ppm, 4.77 to 5.08 ppm, and 9.0 to 12.82 ppm. These regions eliminated areas that did not contain metabolites and contained the NMR standard TSP or water from analysis.

During the second method of analysis, the common integrals of identified peaks were weighted to the TSP integral, which was added at a known concentration to perform quantitative comparisons between mares, individual time points, and groups. Obtained integrals of identified metabolites were then compared between groups and over time using a repeated measures analysis with a significance level set at 0.05.

4.4 Results

4.4.1 Clinical Outcome

Mean gestational age at foaling was significantly decreased in infected mares compared to healthy controls (303.8 ± 12.87 days PLAC vs. 328.6 ± 10.14 days CONT; p=0.01). Three of five mares induced to develop ascending placentitis (group PLAC) developed ultrasonographic signs of placentitis within four days of inoculation (CTUP was greater than 10 mm on day two in two mares and on day four in one mare), while one mare aborted on day two prior to an elevation in CTUP, and one mare failed to develop an elevated CTUP greater than 10 mm, but delivered a dead fetus at 11 days post inoculation (Stawicki et al. 2002). By day four, the mean CTUP of infected mares was significantly greater than control mares (14.7 ± 6.9 mm PLAC vs. 6.4 ± 0.4 mm CONT; p=0.003) (**Figure 4.1A**). Mean CTUP in control mares remained stable during the period of all assessments. Fetal heart rates did not differ between groups at any time point (**Figure 4.1B**).

Live foals were produced from two of five infected mares, and four of five control mares (40% PLAC vs. 80% CONT; p= 0.057). Of the deceased foals, all foals within group PLAC had pure growth of *Streptococcus equi* subsp. *zooepidemicus* in fetal stomach contents. Fetal stomach content culture from the single deceased filly from group CONT showed evidence of *Aeromonas caviae* in the enriching growth media thioglycolate only. In addition to this, uterine cultures were obtained from mares within six hours after foaling. Mares in group PLAC were more likely to have *Streptococcus equi* subsp. *zooepidemicus* on uterine culture than control mares (5/5 PLAC; 3/5 were pure cultures vs. 2/5 CONT; p= 0.016).

4.4.2 Hematologic and Hormonal Profiling

No significant differences were detected at any time-point for lactate, progesterone, or estradiol-17β concentrations (**Figures 4.1C, 4.1E, and 4.1F**). Serum amyloid A concentrations (**Figure 4.1D**) were significantly elevated in infected mares beginning on day six (670 ± 490 µg/mL PLAC vs. 0 ± 0 µg/mL CONT; p=0.0001).

4.4.3 Histopathology

A statistically significant increase in the mean histopathological placentitis score was detected in the chorioallantoic membrane at the level of the cervical star in infected mares compared to healthy control mares (8.20 ± 2.77 PLAC vs. 2.40 ± 1.67 CONT; p=0.0039). All PLAC mares had histologic evidence of placentitis, including the mare that aborted prior to day two and the mare that did not have a rise in CTUP above published normal measurements. No histologic differences were seen grossly between groups in the uterine body, where repeat allantocentesis had been performed (5.60 ± 3.847 PLAC vs. 2.80 ± 2.683 CONT; p=0.2187).

4.4.4 Allantoic Fluid Sampling

A total of 55 samples were collected from 10 mares at six time-points. Sixty-seven percent of samples were golden brown to amber colored and consistent with allantoic fluid (n=37) while 18% were clear, consistent with amniotic fluid (n=10). Seven samples (12%) were a light-yellow color, most consistent with admixing of the two fluid-types. Creatinine concentration results were significantly different between colors (**Table 4.1;** p-value=0.0074), confirming identification of fluid type by color. Samples not consistent with allantoic fluid (n=17) were eliminated from metabolomic processing. All allantoic samples produced spectra upon ^{1}H-NMR spectroscopy with identifiable peaks used for subsequent multivariate analysis.

4.4.5 Metabolomic Profiling

Twenty-seven unique metabolites, in addition to the NMR standard TSP, were identified from distinct peaks in the spectra from allantoic fluid in pregnant mares using 1D and 2D methods (**Table 4.2, Figure 4.2**). Two-dimensional analysis confirmed identification of all metabolites identified in one-dimensional experiments (**Figure 4.3**).

4.4.6 Metabolomic Analysis

When principal component analysis (PCA) was applied to the spectral peaks without identification of specific metabolites, there was no consistent separation detected between groups at any time point (**Figure 4.4**). Also, no difference was detected between groups at any time point ($p > 0.05$) for any individually identified metabolite (**Figure 4.5**). Across groups, a sequential decrease was seen over time in the metabolite betaine ($p=0.0002$). An initial decrease across groups was also seen in the “unknown #1” metabolite from the time points baseline to day six, that reversed and began to increase by day 10 ($p=0.0007$).

4.5 Discussion

As has been previously described by this and other investigational groups, the experimental model of infection produced a reliable and reproducible model of equine ascending placentitis, with 80% of mares either aborting or developing characteristic ultrasonographic evidence of placentitis within four days of inoculation, and all infected mares developing histologic evidence of placentitis.

In this study, changes were noted first using transrectal ultrasonographic measurement of the CTUP, prior to alterations in SAA and in the absence of alterations in estradiol-17β, progesterone, lactate, or allantoic metabolites. Alterations in CTUP have been well documented in both experimentally induced models of disease and in natural infection but remain controversial due to the high degree of subjectivity (Renaudin et al. 1997, Bucca 2006, and Macpherson and Bailey 2008). Likewise, elevations in SAA have previously been documented in mares with experimentally induced and naturally occurring placentitis (Coutinho da Saliva et al. 2013, Canisso et al. 2014a, and Canisso et al. 2015). In our model of infection, the uteroplacental thickness increased by an average of 0.97 mm (range 0.3 to 1.49 mm), and clinical diagnosis could be achieved using CTUP prior to any change in SAA. Lactate, serum progesterone, and serum estrogen concentrations have previously been proposed as diagnostic tools for placentitis. Several retrospective studies of treatment outcomes in mares suspected to have naturally occurring placentitis have suggested that progesterone and estrogen concentrations – either singly or in combination – could be used to predict disease. However, few retrospective studies were able to obtain confirmatory diagnostics at foaling or abortion, and it is not known whether natural variation in hormone levels could have played a role in noted changes or whether conditions other than placentitis could be confounding factors. Additionally, one recent study reported a reduction in the hormone estradiol-17β prior to abortion in untreated mares that were experimentally induced to develop placentitis. The authors suggested estradiol-17β may serve a potential biomarker of ascending placentitis, but data were not correlated to other diagnostic markers of placentitis, such as CTUP (Canisso et al. 2017). The diagnostic potential of estrogens may also be supported by findings of Shikichi and coworkers, who reported lower estradiol-17β concentrations in mares with suspected compromised pregnancy (Shikichi et al. 2017). However, in that study, histologic confirmation of placentitis was not performed at foaling. Decreased serum concentrations of estradiol-17β were not found by Macpherson and coworkers or in our laboratory after experimental infection, even when feto-placental compromise was demonstrated by changes in fetal fluid echogenicity or immediately prior to fetal death (Bailey 2009). In the absence of a reliable, inducible change in an experimental model of disease, or repeatable changes paired with histologic diagnosis of disease in clinical cases, the validity of progesterone and estradiol assay for diagnosis of ascending placentitis remain controversial.

While pure allantoic samples were obtained from allantocentesis in many cases, some samples appeared consistent with amniotic fluid or admixed fluid visually, as well as based on creatinine content and spectral profile. The difficulty of obtaining pure allantoic fluid may be attributed to positioning of the foal following sedation, continued fetal movement during the procedure, and the close proximity of the two fluid compartments themselves (Schott and Mansman 1988, Williams et al. 1993, Allen and Wilsher 2009 and Zanella et al. 2014).

All mares that were diagnosed with placentitis based on increased CTUP in this study were also treated using a standard multimodal treatment approach, including systemic antimicrobials, anti-inflammatories, and a synthetic progestin from the onset of disease until parturition. A single PLAC mare with elevated CTUP and SAA by days three and six respectively experienced remission of clinical signs, reduction in the CTUP and normalization of SAA six days after onset of treatment. This mare delivered a viable foal 40 days after the onset of treatment, at 325 days of gestation. A second PLAC mare maintained pregnancy in the presence of elevations in CTUP and placental separation and delivered a compromised foal with numerous health issues, including neonatal sepsis, failure of passive transfer and angular limb deformities at 300 days of gestation. This foal was treated in the field with systemic antimicrobials including ceftiofur sodium and amikacin and survived. In both cases, placentitis was subsequently confirmed histologically, while uterine culture revealed heavy, pure growth of *Streptococcus equi* subsp. *zooepidemicus*. Clinical resolution of disease and positive neonatal outcome in the absence of bacterial clearance from the uterus or histologic resolution suggests a protective effect of ongoing therapy, which may act to contain bacterial migration to the fetus or fetal fluids. However, similar to outcomes of other work from our laboratory, as well as clinical experience of the authors, treatment of mares after elevations in CTUP have been associated with unsatisfactory outcomes, with three of five infected mares producing dead or nonviable foals and one mare producing a severely compromised dysmature foal with numerous health issues. These findings contrast with a recent clinical study by Shikichi et al. where 459 mares were monitored and treatment with a similar regimen of progestins (altrenogest or medroxyprogeterone) and the tocolytic agent ritodrine was initiated either on detection of clinical signs consistent with impending abortion, such as precocious mammary development, galactorrhea, and/or vulvar discharge, and/or the presence of elevated progestins during screening analysis (n=89) (Shikichi et al. 2017). Mares subsequently diagnosed with placentitis received antimicrobial trimethoprim-sulfoxazole. In that study, nine mares that aborted had histologic evidence of placentitis, despite treatment, however, it is unknown what percentage of all treated mares had this condition. In that study, confirmatory diagnostics for ascending placentitis, such as histologic examination of the placenta or obtaining culture samples from the uterus or neonatal blood, were not reported on all enrolled mares. Thus, it is not known what percentage of animals that were treated in fact had ascending placentitis. This conundrum further serves to emphasize the need for a sensitive and specific test that could rapidly diagnose placentitis.

While this study did not identify specific alterations in allantoic metabolites, which could serve as biomarkers of equine ascending placentitis, it is the first metabolomic investigation in the field of equine reproduction and establishes feasibility and methodology for analysis of equine fetal fluid. Furthermore, the results of this study are the first to establish a database of equine allantoic fluid metabolites that could serve as a foundation for future metabolomic research in horses. Other work has illustrated that ^{1}H-NMR spectroscopy can identify, quantify, and characterize products of cellular metabolism that are less than 1800 Daltons in size within cells, tissues, or biofluids (Jones and Cheung 2007 and Fiehn 2002). ^{1}H-NMR spectroscopy therefore may serve as a comprehensive means of analyzing the health status of an individual through unbiased quantification of metabolites in low micro-molar concentrations. In our experiment we conclusively identified 27 metabolites and spectral and statistical analysis did not reveal any differences between groups. Our inability to detect significant metabolomic differences between CONT and PLAC groups may result from one or more characteristics in our model. Repeated allantoic sampling was performed in both CONT and PLAC groups in an effort to identify early changes in metabolites. This alone may have obscured alterations by creating an inflammatory environment via the sampling regimen. In support of this, two metabolites (betaine and unknown #1) were seen to change over time across both groups and it is unknown whether they may be related to sampling procedures, or to the progress of gestation alone. Betaine continued to decrease with increasing gestational age, while unknown metabolite #1 increased and then returned to previous levels by day 10, when sampling frequency was low. However, as all mares underwent the same procedures with equal frequency at equivalent gestational age, it would have been expected that bacterial infection of the allantoic fluid and/or fetus would result in unique spectral differences in the PLAC vs. CONT group.

Other factors that may have adversely affected our ability to identify changes are the large volume of allantoic fluid produced in equine pregnancy and variations in the location of sampling. Dilution of bacterial and inflammatory metabolites in allantoic fluid and variations in sampling location (i.e. via transabdominal puncture through the ventral uterus close to the uterine bifurcation), may have obscured changes that would have been apparent from cervical or vaginal sampling. Further analysis of metabolomic spectra of fluid samples, vaginal swabs, and of bacterial samples themselves may identify a sampling site which is both more predictive of disease and safer than allantocentesis. In line with previous publications, one uninfected control mare in our study delivered a stillborn, premature fetus at 311 days of gestation. This may have resulted from inflammation or iatrogenic infection related to the repeated allantocentesis. The number of allantocenteses performed in this study ranged from two to eleven, with a median of eight procedures performed per mare. The control mare that experienced a stillbirth had an elevated histological score in the region of the placenta that could have been affected by the allantocenteses, but a normal histologic score at the level of the cervical star. Uterine culture results returned a mixed population light growth of coagulase negative *Staphylococcus spp.* and *Streptococcus* equi subsp. *zooepidemicus.* Fetal stomach culture results returned with the contaminant *Aeromonas caviae* in the enriching growth media thioglycolate. Therefore, while an overt cause of pregnancy loss and fetal loss was not detected on gross fetal necropsy and/or diagnostic testing, the allantocentesis attempts themselves cannot be ruled out as the cause.

4.6 Conclusions

In conclusion, this is the first study to evaluate the use of ^{1}H-NMR spectroscopy to characterize the metabolome of an equine biofluid other than whole blood or plasma, urine or feces, and is also the first study to investigate potential alterations of the equine metabolome in response to infection (Keller et al. 2011, Escalona et al. 2015, Jang et al. 2017, and Niemuth et al. 2017). It establishes methodology and a database of known metabolites for future work. Further work characterizing the metabolome of allantoic fluid in healthy mares at broader stages of gestation, as well as naturally occurring cases of placentitis would further expand this database of allantoic metabolites in the healthy mare as well as those affected by ascending placentitis. In this study, an elevation in the transrectal CTUP was the first detectable change in mares with ascending placentitis preceding an elevation in the acute phase protein SAA, while other proposed hematologic indicators of placentitis, including lactate, progesterone, and estradiol-17β were not diagnostic for disease in our model.

4.7 Funding: This work was supported by the Grayson-Jockey Club Research Foundation.

Table 4.1 Creatinine content of fetal fluids obtained during transabdominal fetal fluid puncture in 10 mares (n=5 PLAC, n=5 CONT) on biochemical analysis. Data expressed as mean ± standard deviation.

Fluid Type	**Color**	**Creatinine (mg/dL)**
Allantoic	Brown	80.2 +/- 24.5
Amniotic	Clear	16.0 +/- 5.7
Admixed	Yellow	28 +/- 13.951

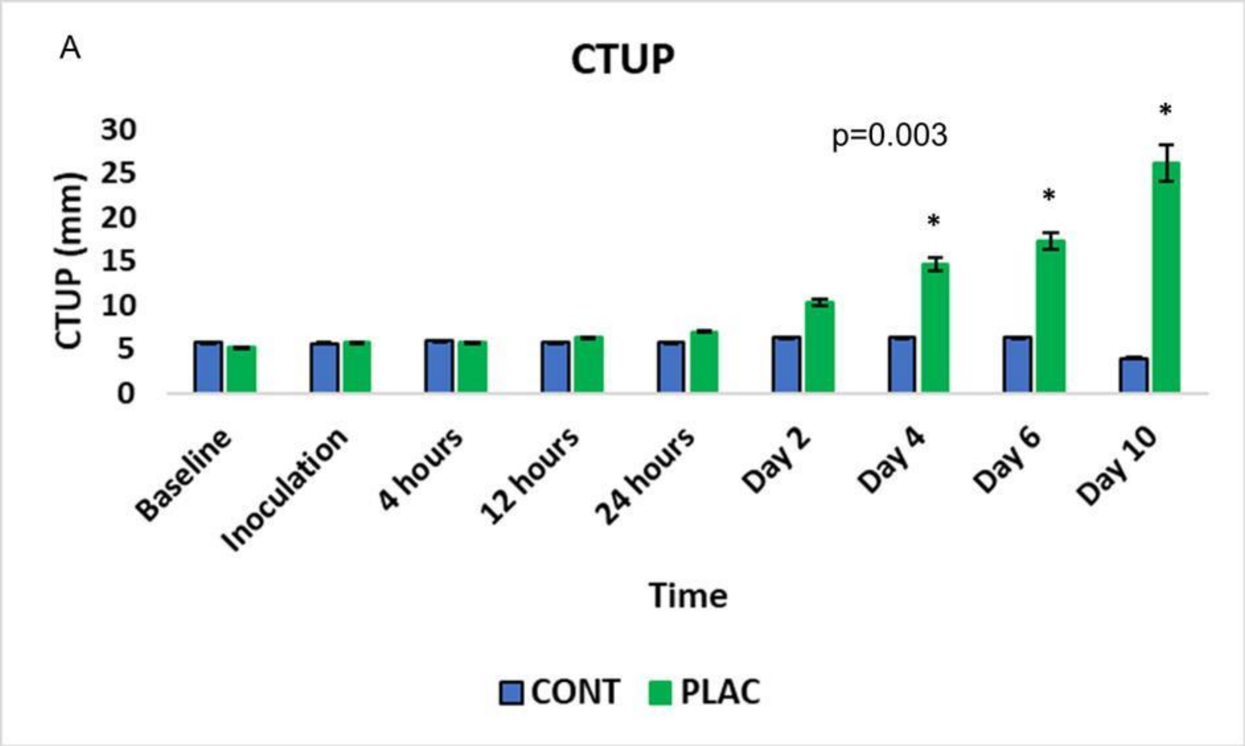
Table 4.2 List of metabolites identified in confirmed allantoic fluid of pregnant mares with and without ascending placentitis (groups PLAC and CONT) using 1D and 2D NMR spectroscopy experiments with their respective proton chemical shifts and multiplicity (bs-broad singlet, d-doublet, dd-doublet of doublets, m-multiplet, s-singlet, t-triplet, q-quartet).

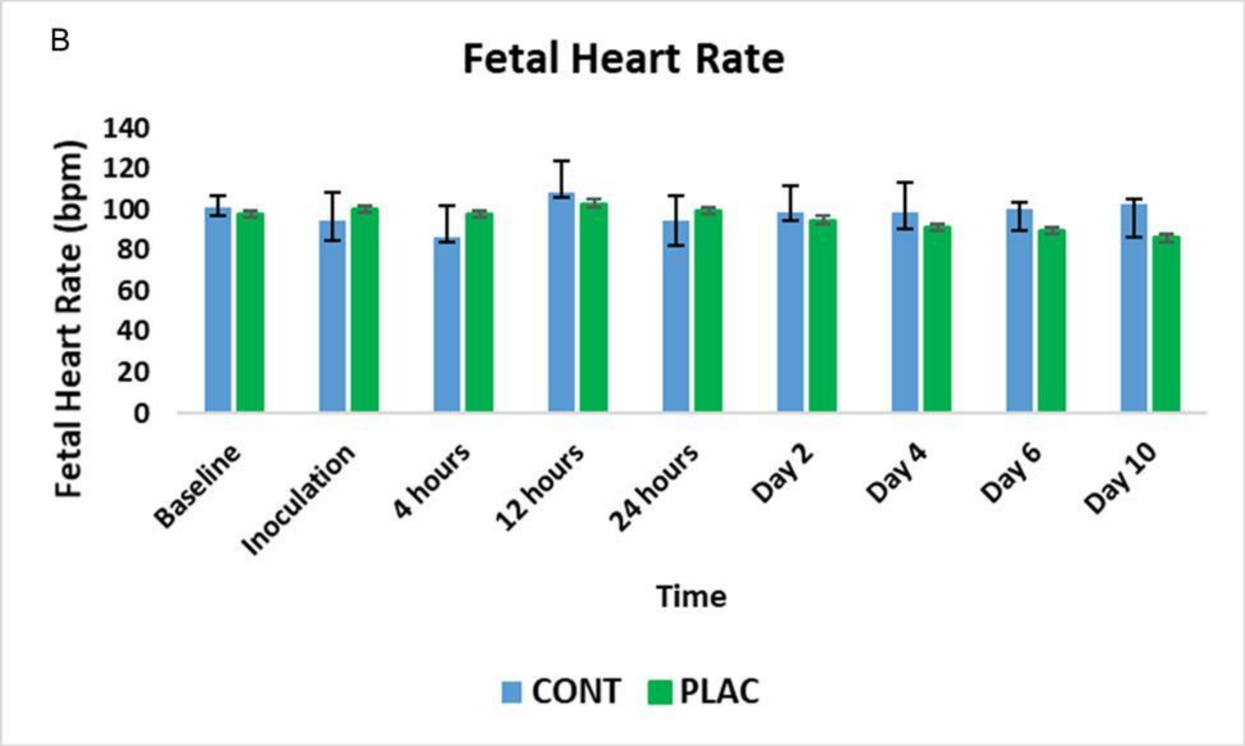
Compound	Chemical Formula	Chemical Shifts (ppm)
Acetate	C2H4O2	1.92 (s)
Allantoin	C4H6N4O3	5.39 (s), 6.0 (s), 6.8(s), 7.34 (d), 10.6 (s)
Betaine	C5H11NO2	3.26 (s), 3.89 (s)
Citrate	C6H8O7	2.53 (d), 2.65 (d)
Creatine	C4H9N3O2	3.04 (s), 3.93 (s)
Creatinine	C4H7N3O	3.05 (s), 4.07 (s)
Creatine phosphate	C4H10N3O5P	3.04 (s), 3.93 (s)
Formate	CH2O2	8.43 (s)
Galactose	C6H12O6	3.48 (dd), 3.6 (dd), 3.72 (d), 3.82 (d), 3.92 (d), 3.99 (d), 4.07 (t), 4.60 (d), 5.23 (d)
Glucose	C6H12O6	3.2 (dd), 3.36 (m), 3.46 (m), 3.63 (dd), 3.72 (m), 3.82 (m), 3.88 (dd), 5.25 (d), 5.36 (d)
Glycolate	C2H4O3	3.93 (s)
Histidine	C6H9N3O2	3.15 (dd), 3.32 (dd), 3.79 (d), 7.26 (s), 8.52 (s)
Lactate	C3H6O3	1.33 (d), 4.11 (q)
Maltose	C12H22O11	3.27 (dd), 3.40 (t), 3.57 (m), 3.62 (m), 3.65 (m), 3.70 (m), 3.75 (m), 3.85 (m), 3.90 (dd), 3.92 (d), 3.95 (m), 5.34 (d), 5.40 (d)
Mannose	C6H12N2O2	3.40 (dd), 3.56 (t), 3.64 (m), 3.72 (m), 3.79 (m), 3.87 (dd), 3.90 (m), 5.15 (d)
Methanol	CH4O	3.38 (s)
π-methylhistidine	C7H11N3O2	3.07 (dd), 3.15 (dd), 3.68 (s), 3.95 (dd), 7.00 (s), 7.67 (s)
Methylmalonate	C4H6O4	1.22 (d), 3.16 (q)
Myoinositol	C6H12O6	3.20 (t), 3.47 (dd), 3.70 (t), 4.06 (t)
N-methylhydantoin	C4H6N2O2	2.92 (s), 4.08 (s)
Nitrophenol	C6H5NO3	6.80 (m), 8.10 (m)

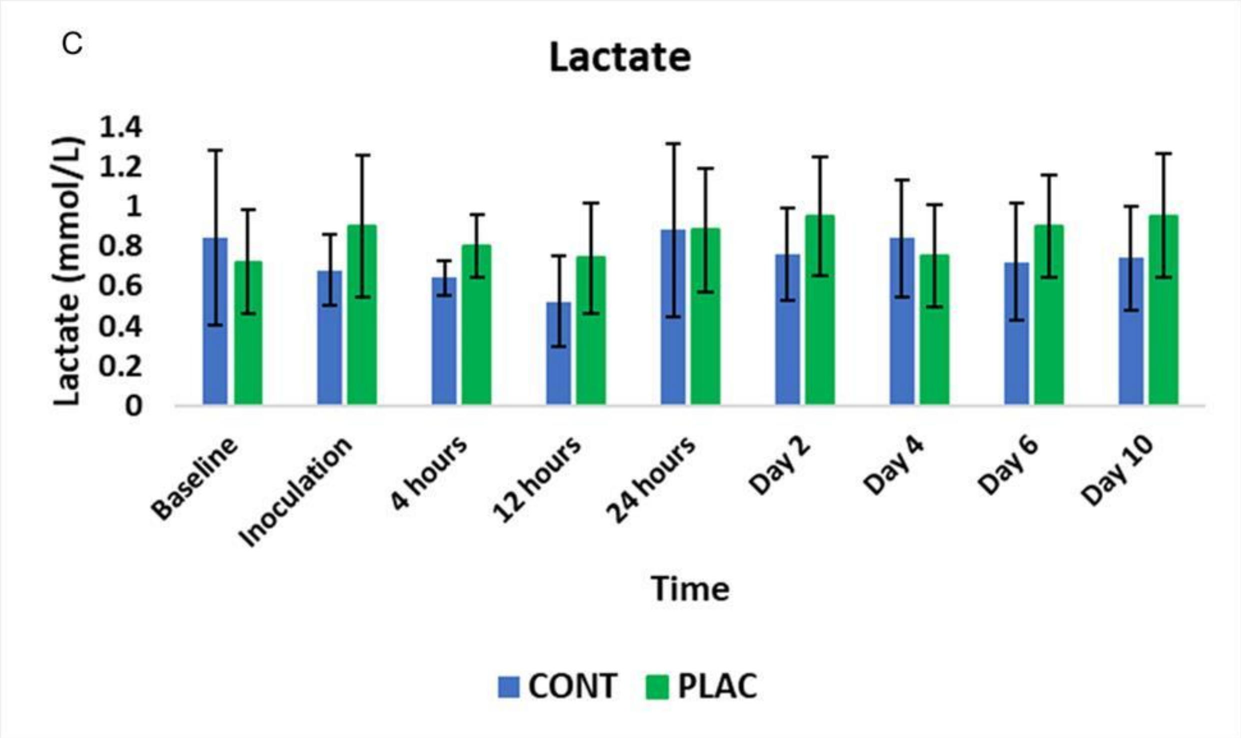
Table 4.2 (continued).

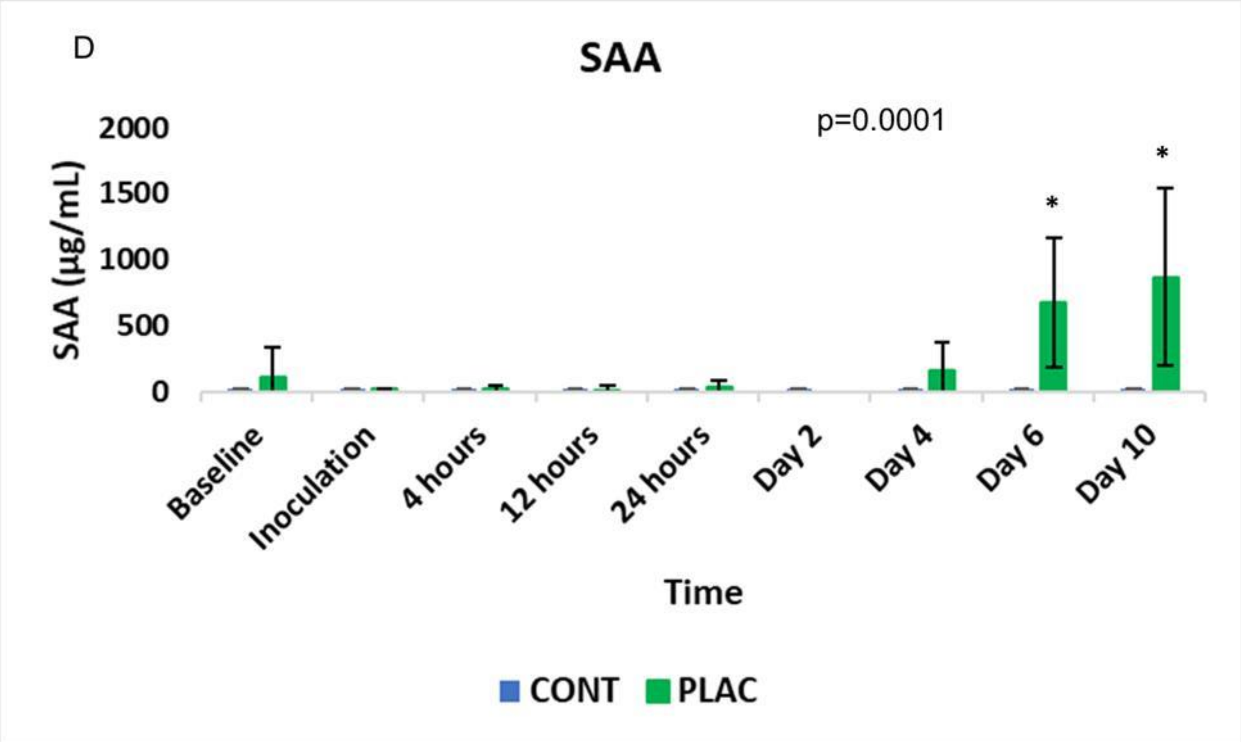
Trimethylsilyl propionate	C6H1402Si	0.00 (s)
Tryptophan	C11H12N202	3.29 (dd), 3.47 (dd), 4.04 (dd), 7.19 (m), 7.274 (m), 7.36 (s), 7.53 (d), 7.73 (d)
Unknown #1	Unknown	0.52 (s), 2.29 (s), 3.6 (s), 3.83 (s)
Unknown #2	Unknown	1.2 (s), 1.8 (s), 4.46 (s), 5.45 (s)
Urea	CH4N2O	6.0 (s)
Water	H20	4.77 (bs)

Figure 4.1 Combined thickness of the uterus and placenta (CTUP in mm; panel A), fetal heart rate (beats per minute (bpm); panel B), whole blood lactate (mmol/L; panel C), serum SAA (µg/mL; panel D), serum progesterone (ng/mL; panel E) and serum estradiol-17β concentrations (pg/mL; panel F) obtained at time points baseline, inoculation, 4, 12, and 24 hours, and on days 2, 4, 6, 10 from 10 pregnant mares (n=5 group CONT; n=5 group PLAC). Data reported as mean ± standard deviation; * denotes significant at $p<0.05$.


A
CTUP
p=0.003
*
*
*
30
25
20
15
10
5
0
CTUP (mm)
Baseline
Inoculation
4 hours
12 hours
24 hours
Day 2
Day 4
Day 6
Day 10
Time
CONT
PLAC


B
Fetal Heart Rate
140
120
100
80
60
40
20
0
Fetal Heart Rate (bpm)
Baseline
Inoculation
4 hours
12 hours
24 hours
Day 2
Day 4
Day 6
Day 10
Time
CONT
PLAC


C
Lactate
Lactate (mmol/L)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Baseline
Inoculation
4 hours
12 hours
24 hours
Day 2
Day 4
Day 6
Day 10
Time
CONT
PLAC


D
SAA
p=0.0001
*
*
SAA (µg/mL)
2000
1500
1000
500
0
Baseline
Inoculation
4 hours
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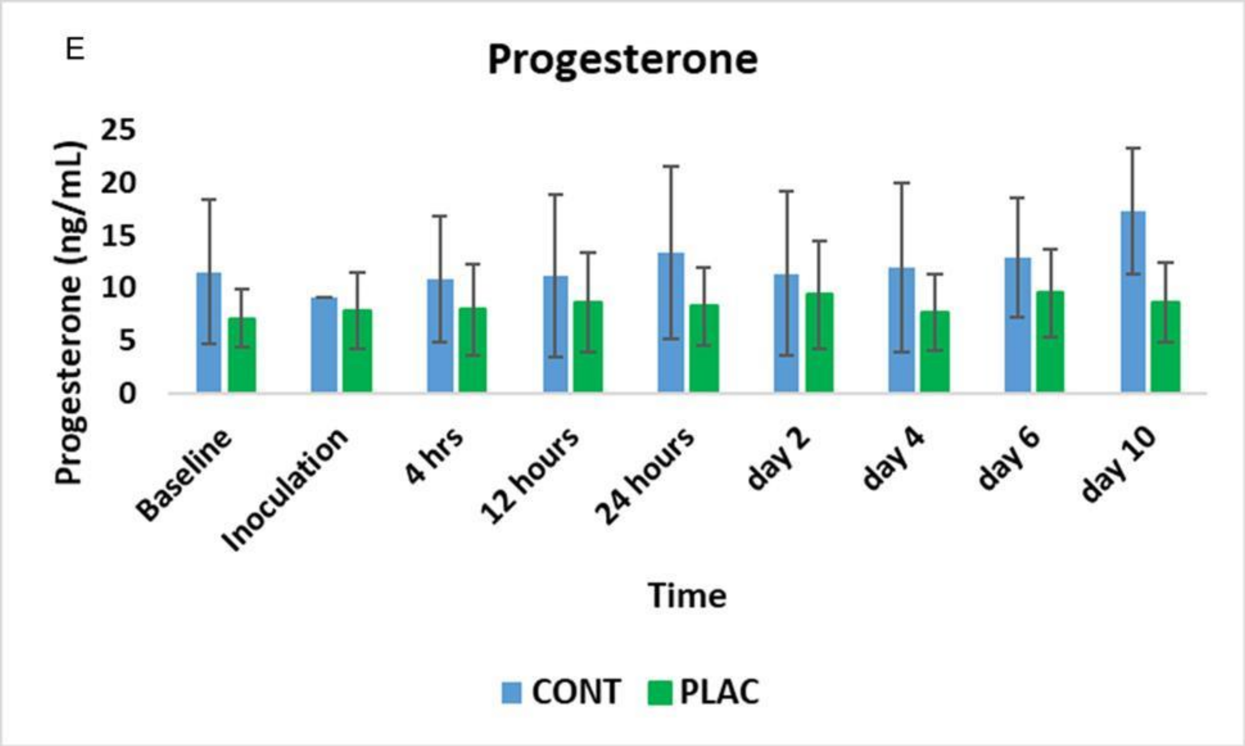
Day 2
Day 4
Day 6
Day 10
Time
CONT
PLAC


E
Progesterone
Progesterone (ng/mL)
25
20
15
10
5
0
Baseline
Inoculation
4 hrs
12 hours
24 hours
day 2
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day 6
day 10
Time
CONT
PLAC

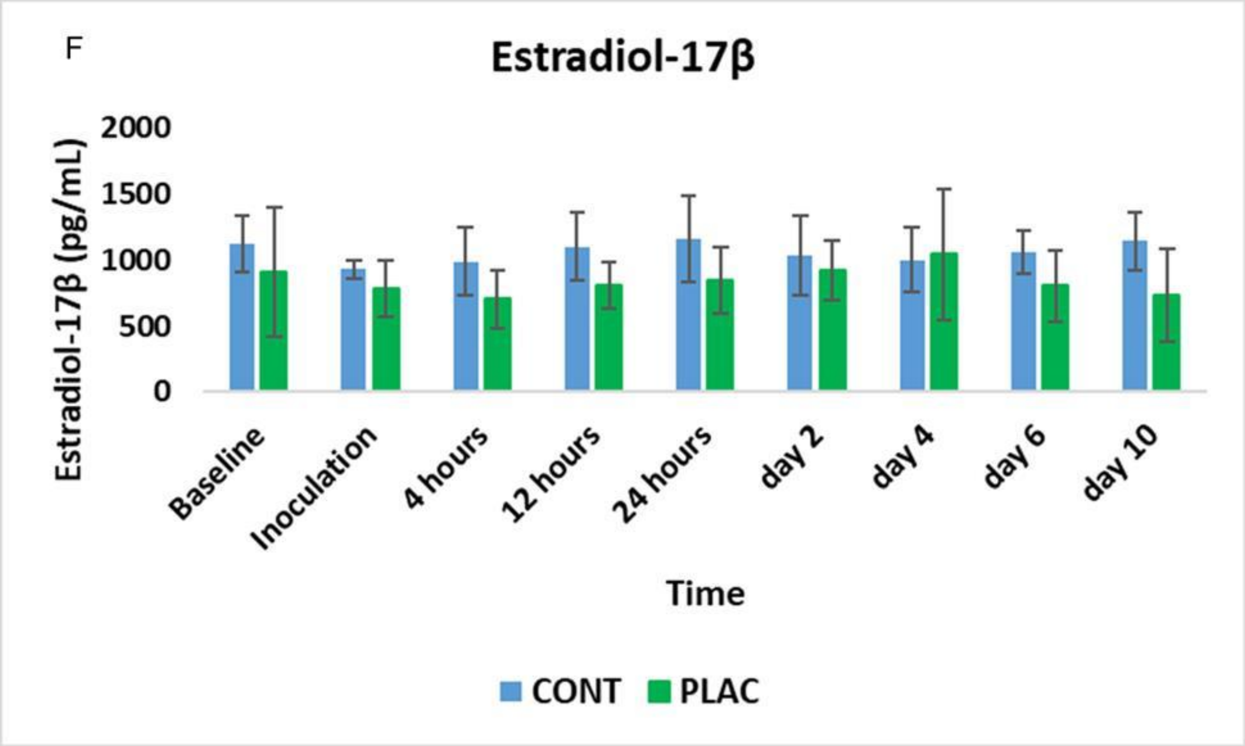
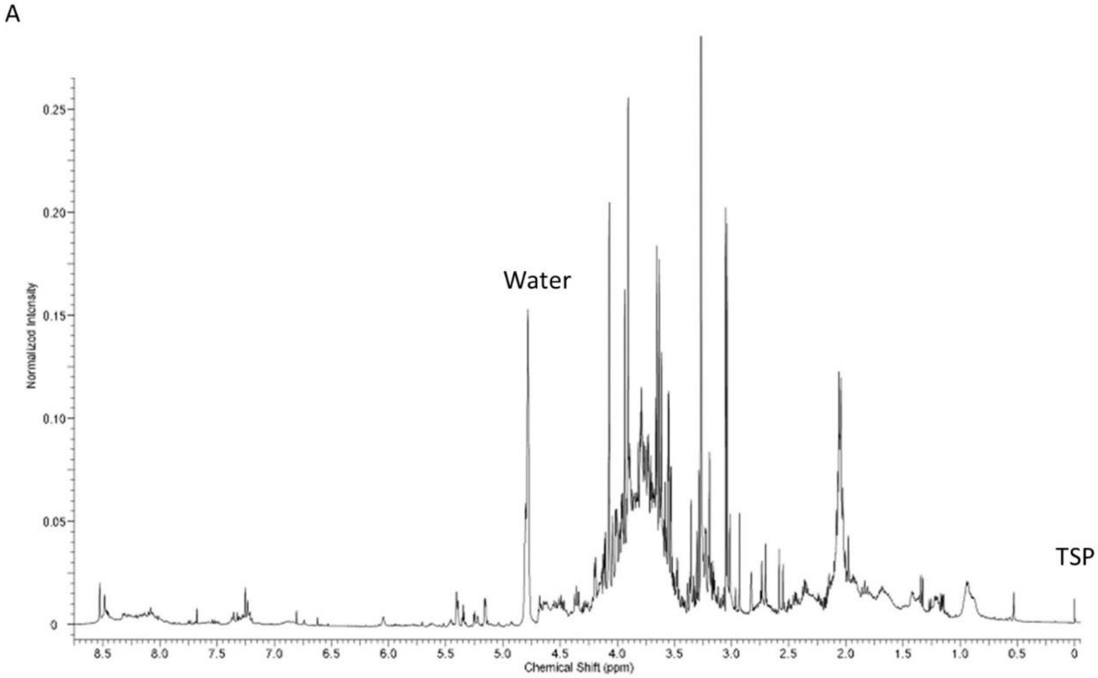

F
Estradiol-17β
Estradiol-17β (pg/mL)
2000
1500
1000
500
0
Baseline
Inoculation
4 hours
12 hours
24 hours
day 2
day 4
day 6
day 10
Time
CONT
PLAC

Figure 4.2 Representative ^{1}H-NMR spectrum of allantoic fluid. The x-axis depicts chemical shift in parts per million (ppm); y-axis depicts peak intensity. Panel A displays full 1H-NMR spectrum; 0 to 3 ppm chemical shift magnified in panel B, 3 to 4 ppm in panel C, and 6 to 8.5 ppm in panel D. Identified peaks are as follows: (a) trimethylsilyl propionate, (b) unknown 1, (c) lactate, (d) acetate, (e) citrate, (f) N-methylhydrantoin, (g) creatine, (h) creatinine, (i) betaine, (j) methanol, (k) myo-inositol, (l) glucose, (m) glucosamine, (n) mannose, (o) galactose, (p) allantoin, (q) tryptophan, (r) histidine, (s) formate, (t) π-methylhistidine.


A
Water
TSP
Normalized Intensity
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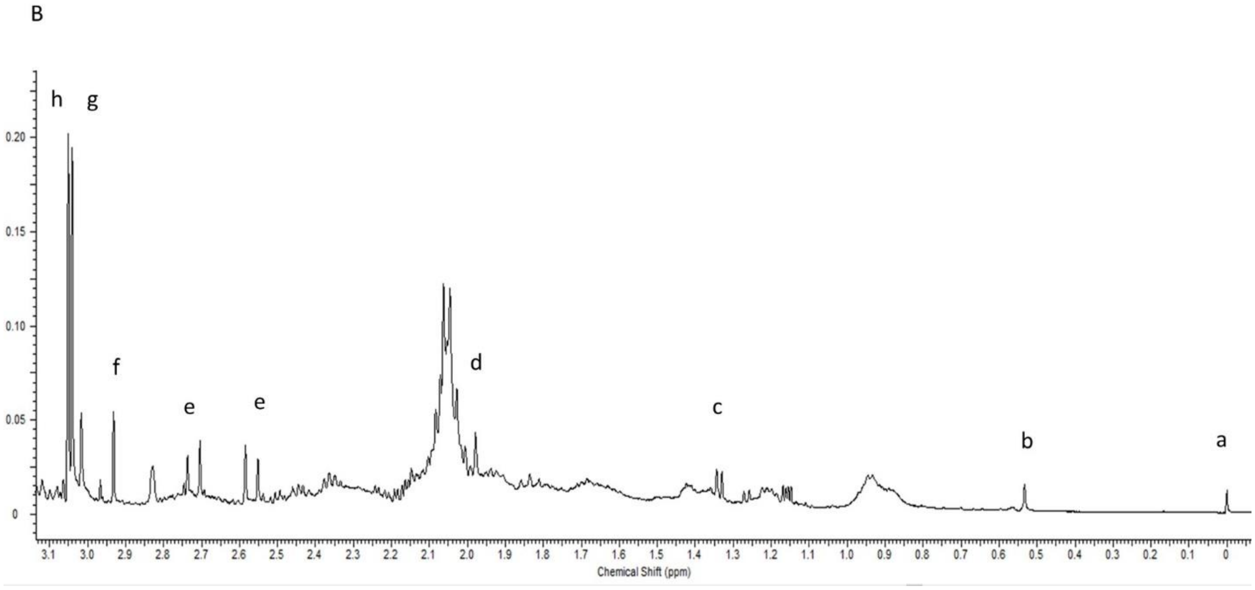
0.20
0.15
0.10
0.05
0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Chemical Shift (ppm)


B
h
g
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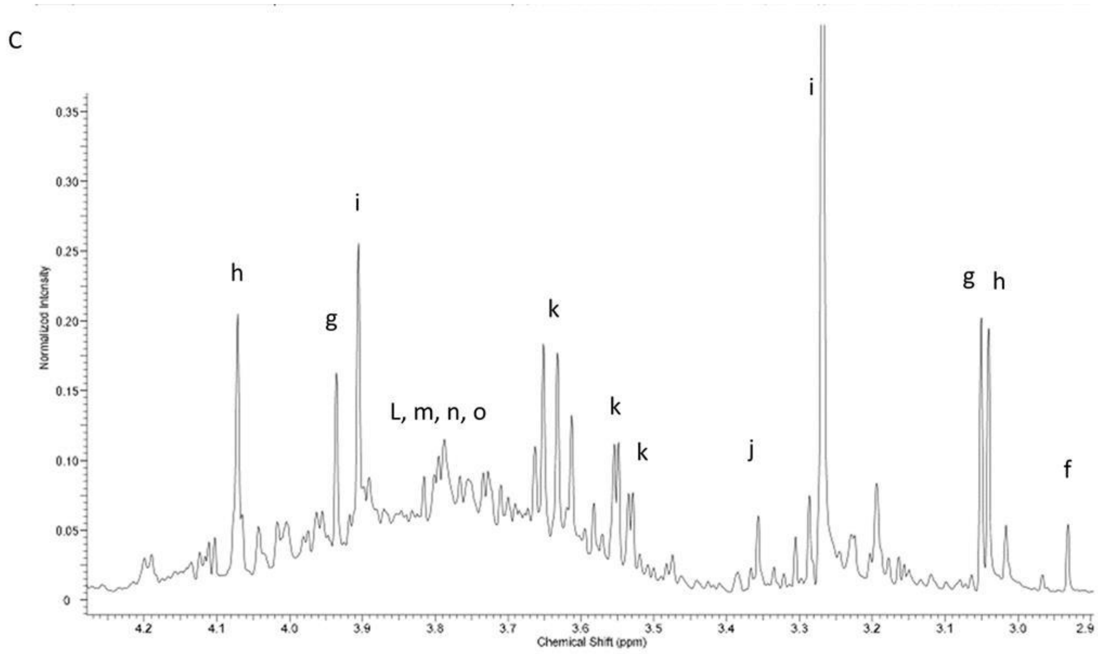
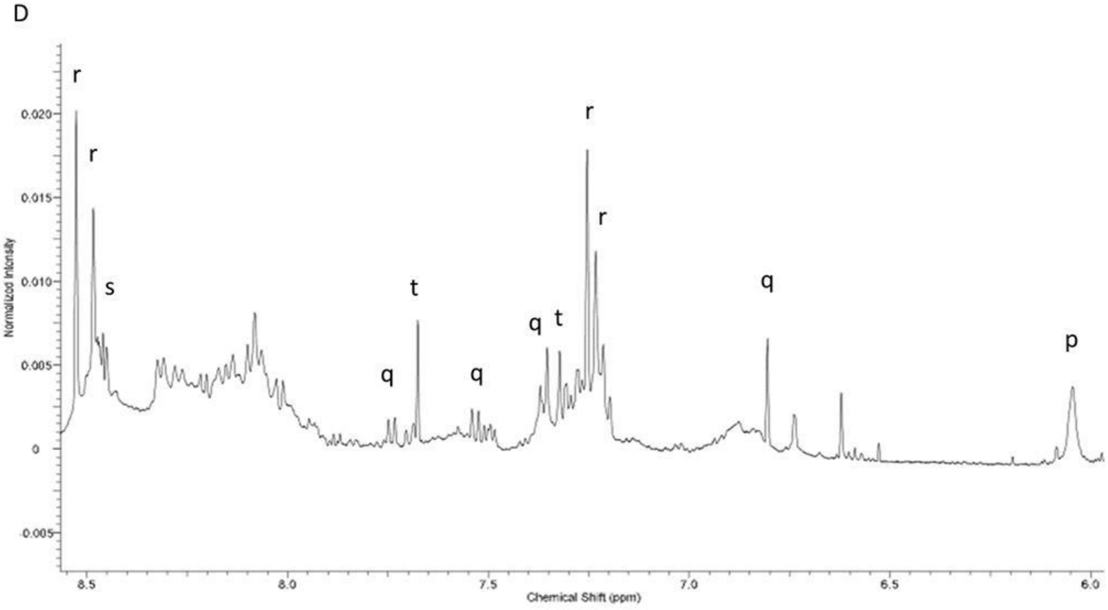
e
d
c
b
a
0.20
0.15
0.10
0.05
0
3.1
3.0
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Chemical Shift (ppm)


C
i
i
h
g
k
L, m, n, o
k
k
j
g h
f
Normalized Intensity
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
Chemical Shift (ppm)


D
r
r
s
r
r
t
q
t
q
q
q
p
Normalized Intensity
0.020
0.015
0.010
0.005
0
-0.005
8.5
8.0
7.5
7.0
6.5
6.0
Chemical Shift (ppm)

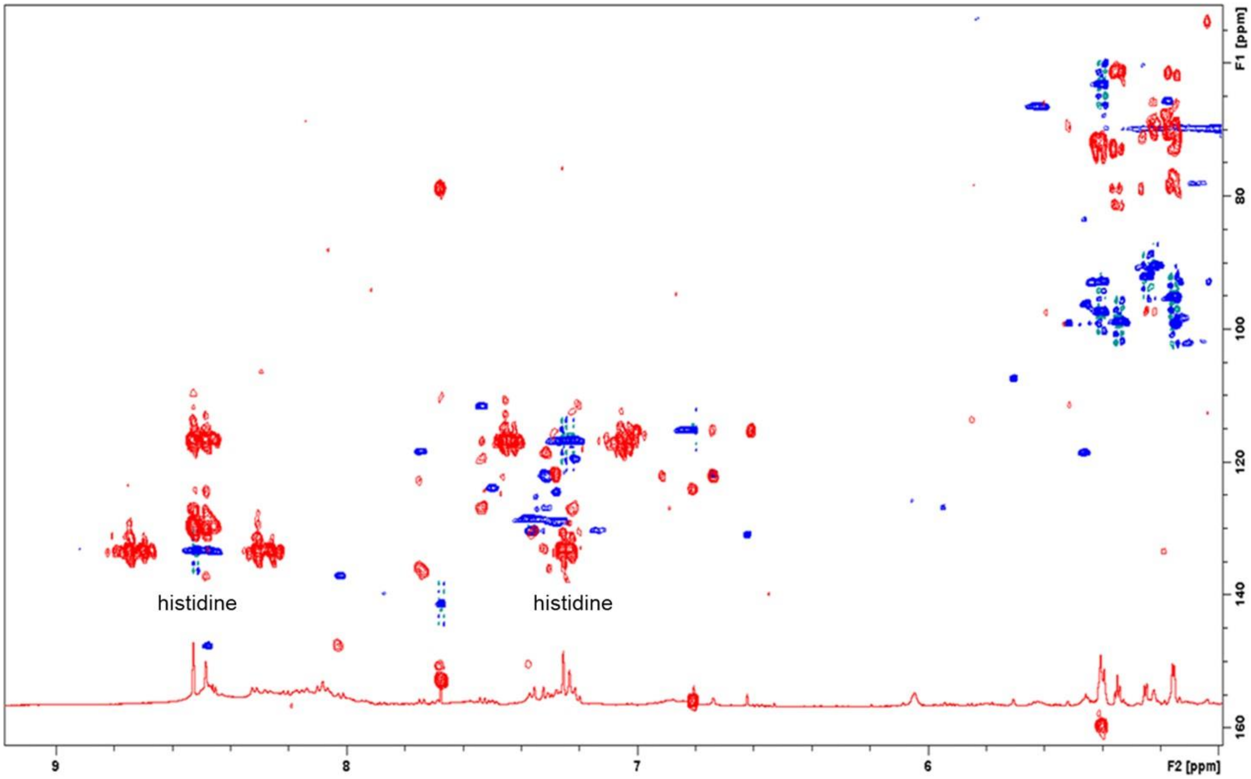


Figure 4.3 Two-dimensional spectral analysis of allantoic fluid. Representative 2-D overlay of HSQC (blue) and HMBC (red) experiments depicting one bond and long-range correlation utilized to confirm the identification of individual metabolites identified on 1-D analysis. Identification of the amino acid histidine is depicted. X-axis depicts proton chemical shift in parts per million (ppm); Y-axis depicts carbon chemical shift in parts per million (ppm).

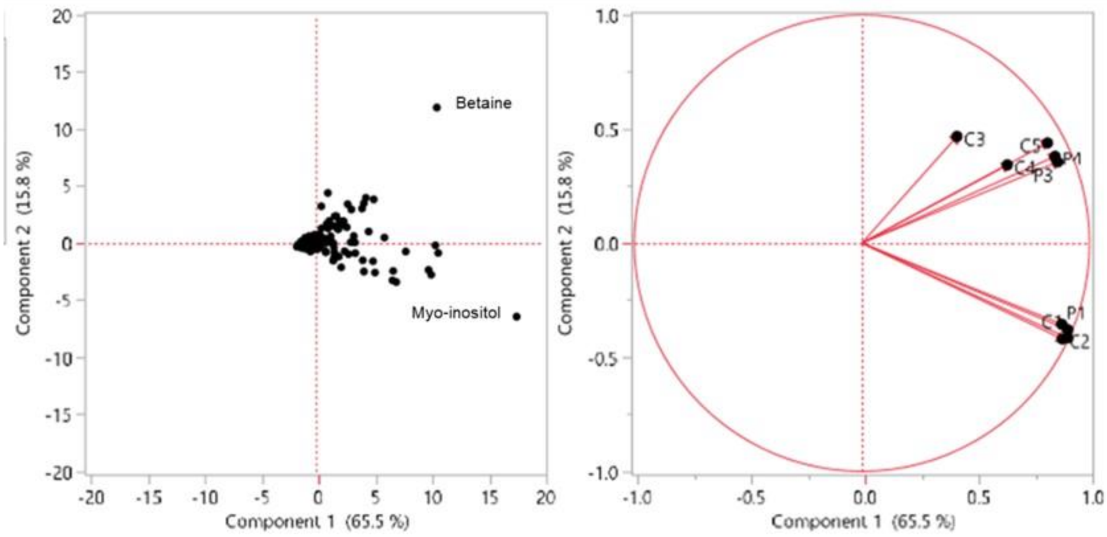
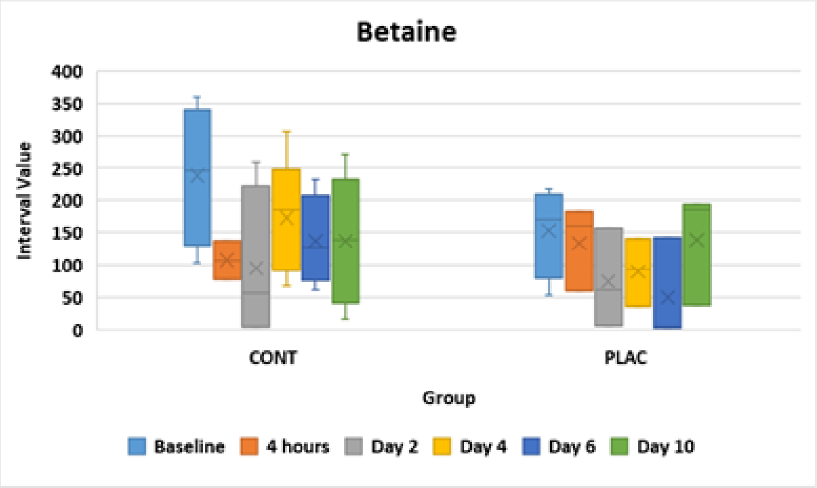
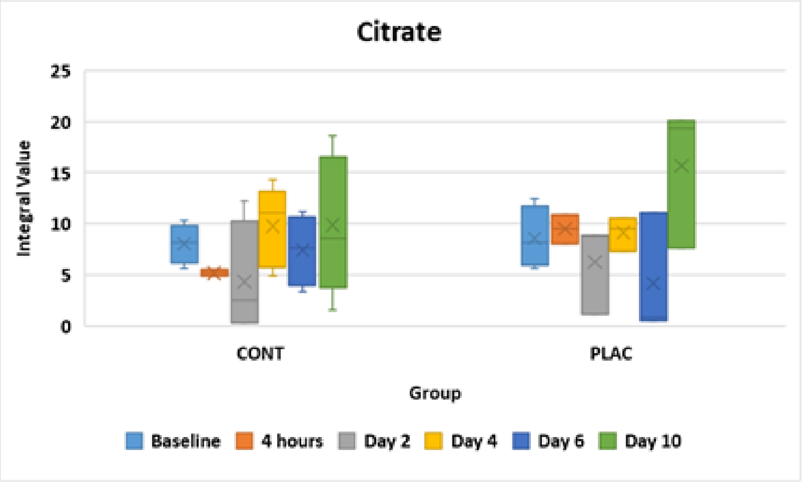
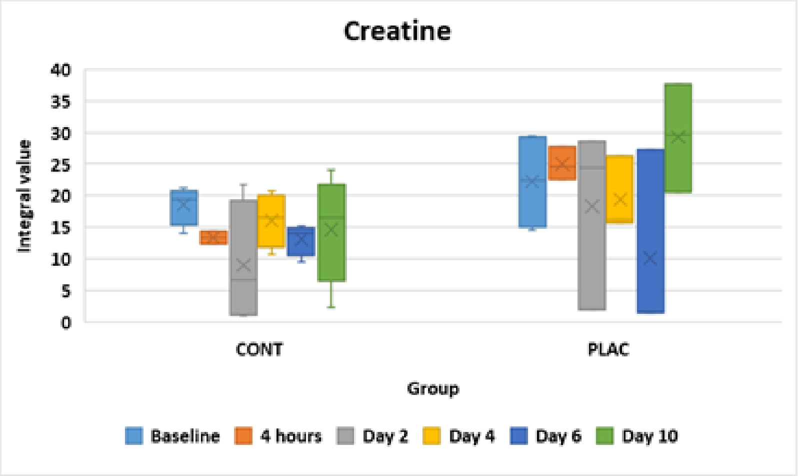


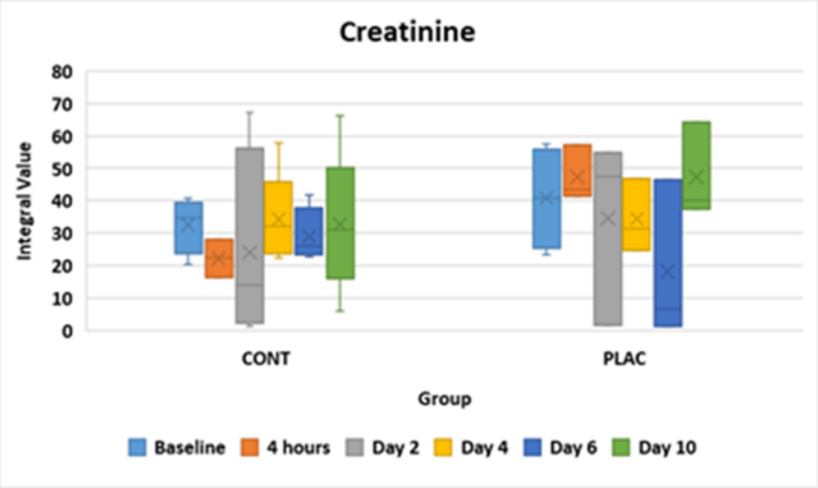
Figure 4.4 Representative Principal Component Analysis (PCA) summary plot (left) and loading plot (right) of spectral segments (bins - 0.03 ppm) obtained from proton NMR analysis of allantoic fluid. The summary plot identifies bins contributing to variance across all mares. The loading plot identifies individual animals affected by sources of variance. P= individual PLAC mares (n=5). C= individual CONT mares (n=5). No evidence of separation detected between groups for any bin analyzed.

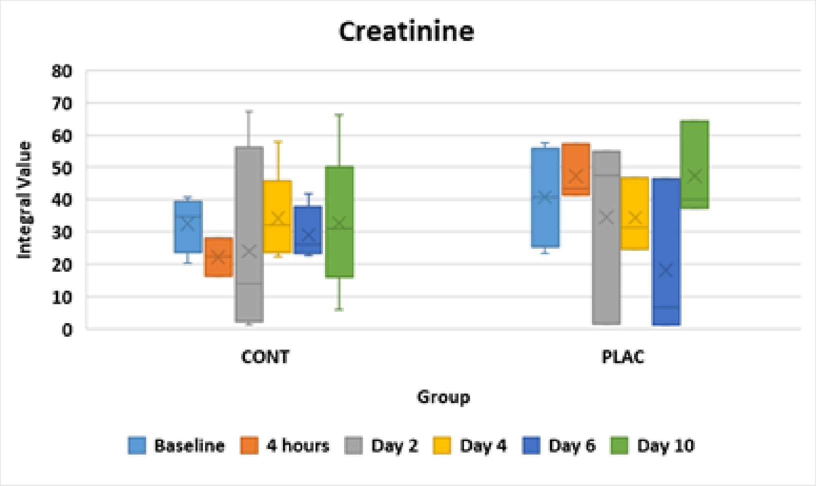
Figure 4.5 Box and whisker plot of peak intensity of identified metabolites in allantoic fluid by group and timepoint (n=5 group CONT; n=5 group PLAC) at the time points baseline, 4 hours, and on days 2, 4, 6, and 10.

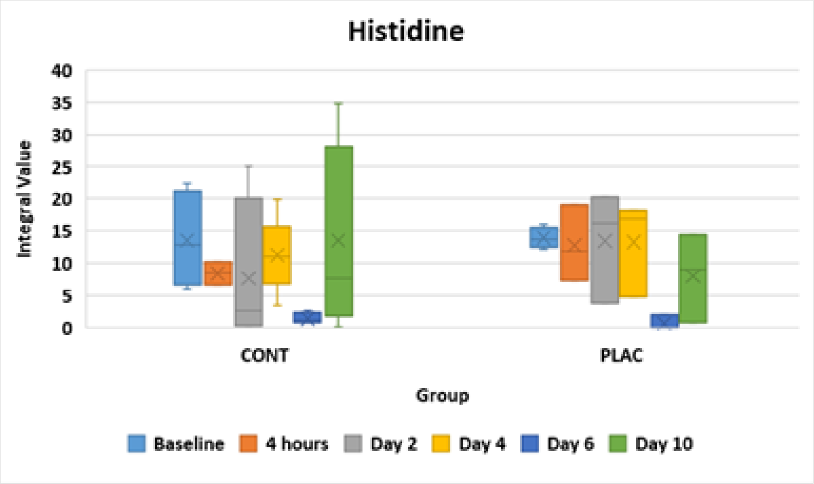

Betaine
Interval Value
400
350
300
250
200
150
100
50
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10

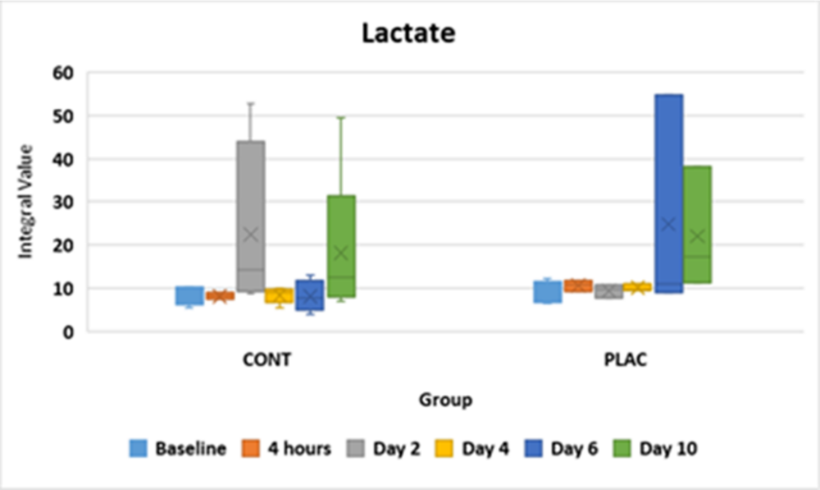

Citrate
Integral Value
25
20
15
10
5
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10

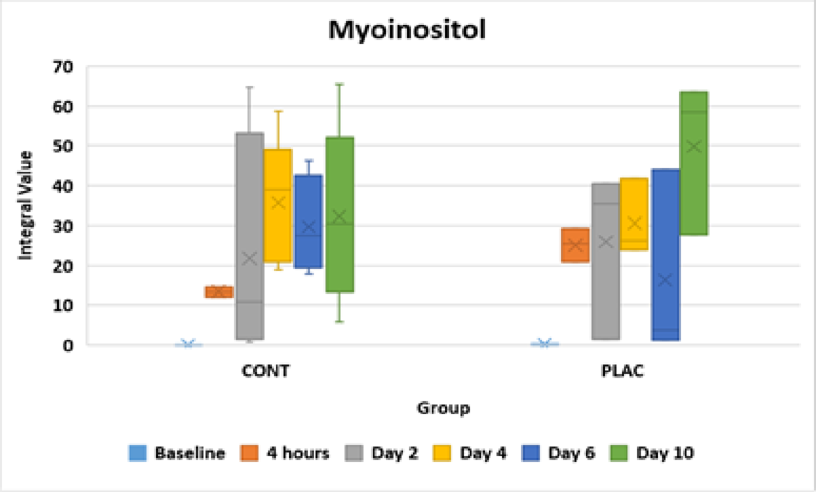

Creatine
Integral value
40
35
30
25
20
15
10
5
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10

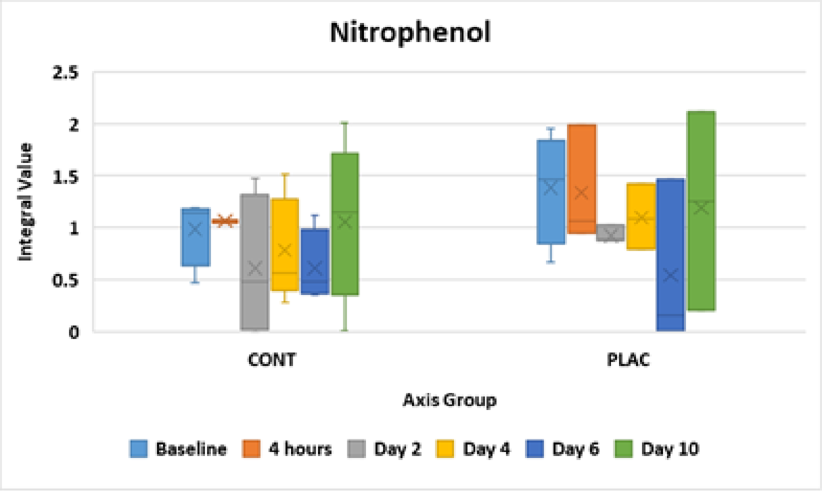

Creatinine
Integral Value
80
70
60
50
40
30
20
10
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10

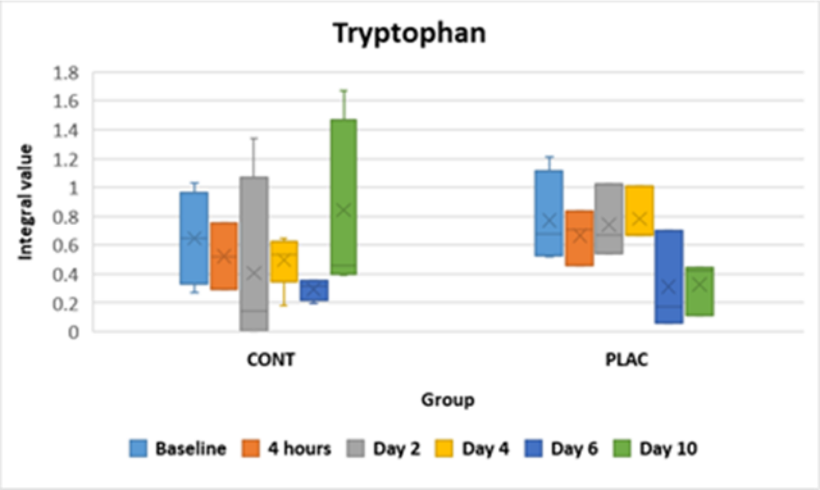

Creatinine
Integral Value
80
70
60
50
40
30
20
10
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10

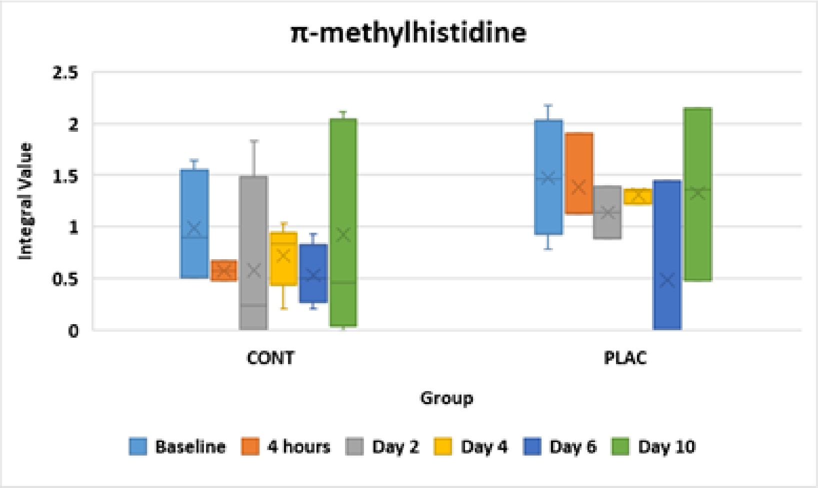

Histidine
Integral Value
40
35
30
25
20
15
10
5
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10

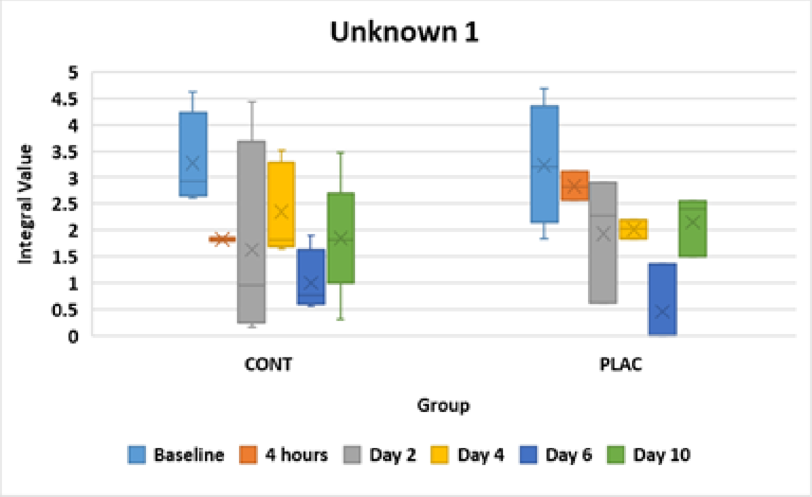

Lactate
Integral Value
60
50
40
30
20
10
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10


Myoinositol
Integral Value
70
60
50
40
30
20
10
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10


Nitrophenol
Integral Value
2.5
2
1.5
1
0.5
0
CONT
PLAC
Axis Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10


Tryptophan
Integral value
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10


π-methylhistidine
Integral Value
2.5
2
1.5
1
0.5
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10


Unknown 1
Integral Value
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CONT
PLAC
Group
Baseline
4 hours
Day 2
Day 4
Day 6
Day 10

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Chapter 5

Plasma metabolomic profiling in an experimental model of ascending placentitis

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5.1 Summary

Background: Metabolomics may represent an avenue for early diagnosis of equine ascending placentitis.

Objectives: The objective of the study was to characterize the metabolomic profile of plasma in healthy pregnant mares and mares with ascending placentitis.

Study Design: Ten late-term pregnant pony mares were used in a controlled experiment.

Methods: Placentitis was induced via intracervical inoculation in five mares between days 280 and 285 of gestation, while five mares served as healthy controls. Repeated ultrasound exams and jugular venipuncture were performed to obtain combined thickness of the uterus and placenta (CTUP), fetal heart rate, and plasma for NMR spectroscopy. Mares with an increased CTUP were diagnosed with placentitis and treated in accordance with published therapeutic recommendations. NMR metabolomic analysis was performed to identify plasma metabolites and metabolite concentrations at each time-point. Concentrations were compared using ANOVA with repeated measures and PLS-DA analysis.

Results: Four hours post-inoculation, a significant increase was detected in the metabolites alanine, phenylalanine, histidine, pyruvate, citrate, glucose, creatine, glycolate, lactate, and 3-hydroxyisobutyrate that returned to baseline by 12 hours. On day 4, a significant reduction in the metabolites alanine, histidine, phenylalanine, tyrosine, pyruvate, citrate, glycolate, lactate, and dimethylsulfone was seen in infected mares compared to controls.

Main Limitations: This study serves as a foundation for the investigation of systemic metabolite changes in response to intrauterine infection in the mare. Findings from this experimental model should be confirmed in naturally infected mares prior to any potential application as a diagnostic tool.

Conclusions: Two phases of metabolite changes were noted after experimental infection: An immediate rise in metabolite concentration involved in energy, nitrogen, hydrogen, and oxygen metabolism within 4 hours after inoculation that was followed by a decrease in metabolite concentrations involved in energy and nitrogen metabolism at 4 days, coinciding with ultrasonographic diagnosis of placentitis.

5.2 Introduction

Ascending placentitis is among the most important infectious cause of equine pregnancy loss worldwide, contributing to approximately one-third of all late-term abortions, stillbirths, and neonatal loss in the horse (Giles et al. 1993, Laguier et al. 2011). Placental infection is most frequently attributed to ascending migration of bacteria through the cranial reproductive tract to incite a necrotizing inflammation of the chorioallantois at the level of the cervical star (Platt 1975). Common bacteria include β-hemolytic *Streptococcus* species, of which *Streptococcus equi* subspecies *zooepidemicus* is the most commonly diagnosed isolate, as well as *Escherichia coli, Klebsiella pneumoniae*, or *Pseudomonas aeruginosa* (Giles et al. 1993, Laguier et al. 2011, and Platt 1975). Diagnosis of ascending placentitis is made based on clinical signs (premature mammary development and/or vulvar discharge) and evidence of thickening of the combined thickness of the uterus and placenta (CTUP) as detected on transrectal ultrasonography (Renaudin et al. 1997 Renaudin et al. 1999). Other diagnostics, including hormonal profiling and measurement of serum amyloid A, may also be performed. However, changes may not be seen early in the disease course and may also be seen with a variety of different fetoplacental pathologies (Ousey et al. 2005, Canisso et al. 2017, Santschi et al. 1991, Stawicki et al. 2002, and Canisso et al. 2014).

In the present study, nuclear magnetic resonance (NMR) metabolomic analysis was used to assess the metabolic profiles of plasma from pony mares with normal pregnancies and after experimental induction of ascending placentitis with *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*). A growing number of studies have applied this technology to quantify alterations in low molecular weight metabolites of blood, tissues and body fluids in response to a variety of conditions such as exercise, genetic abnormalities, or disease (Dunn and Ellis 2005, Arn 2007, and Jang et al. 2017). In the healthy horse common metabolites of urine, feces, and plasma have been described (Jang et al. 2017 and Escalona et al. 2015). Plasma biomarkers have also been identified that are sufficiently sensitive to identify horses at risk for laminitis following gastrointestinal sepsis or to rapidly diagnose acute laminitis (Stellman et al. 2014 and Keller et al. 2011). To date, only one equine reproductive metabolomic study has been performed; however, potential plasma metabolic alterations occurring in response to equine ascending placentitis have not been described (Beachler et al. 2019). Therefore, the objective of this study was to characterize the metabolomic profile of plasma in mares using NMR spectroscopy with and without experimentally induced ascending placentitis, with the goal of identifying potential biomarkers of disease that occur in response to placental infection.

5.3 Materials and Methods

5.3.1 Experimental model

Ten systemically and reproductively healthy pony mares between 270 and 275 days of gestation were enrolled in this experimental study. Mares were 5 to 13 years of age, weighed between 181.8 and 386.4 kgs, and were maintained on pasture with supplemental hay and pelleted ration at a university-owned farm in central North Carolina. All procedures were carried out in accordance with North Carolina State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals. Mares were divided randomly into two separate groups: five mares were experimentally inoculated with *S. zooepidemicus* bacteria to induce ascending placentitis (PLAC), while five mares served as uninfected control animals (CONT).

Mares in group PLAC were inoculated as previously described (Beachler et al. 2019, Bailey et al. 2010, Bailey et al. 2012, and Murchie et al. 2006). Briefly, at days 280 to 285 days of gestation, mares were inoculated mid-cervically with 1 x 10^7 CFU (mean 9.4 x 10^6) of a clinical isolate of *S. zooepidemicus*, which has been widely used for the study of placentitis across institutions (Beachler et al. 2019, Bailey et al. 2010, Bailey et al. 2012, Murchie et al. 2006, Coutinho da Silva et al. 2013, and Lyle et al. 2009). The inoculum was drawn into an artificial insemination pipette and deposited 2.5 cm beyond the external cervical os under digital guidance. Confirmation of placement was obtained through the presence of the cervical plug on the operator's fingers, as well as the presence of a fluid line within the cervix on subsequent transrectal ultrasound assessment. No cervical procedures were performed in control mares. All mares were subjected to the same diagnostic regimen, which included a complete physical exam, transrectal and transabdominal ultrasound examination, and allantocentesis one to two days prior to inoculation (baseline), and at the following time-points after inoculation 4 hours, days 2, 4, 6, and 10, and then weekly until delivery of a foal. Mares were diagnosed with placentitis based on detected changes in the CTUP: greater than 8 mm between 270 to 300 days of gestation, greater than 10 mm between 300 and 330 days, or greater than 12 mm after 330 days of gestation (Renaudin et al. 1997 and Renaudin et al. 1999). Upon diagnosis, standard multimodal therapeutics of trimethoprim sulfamethoxazole[b] (30 mg/kg bwt, per os q. 12 h), altrenogest[c]

(0.088mg/kg bwt, per os q. 24 h), and flunixin meglumine[c] (1.1 mg/kg bwt, per os q. 12 h) were administered until abortion or delivery of a live foal. All mares were regularly assessed for signs of foaling including mammary development, lengthening of the vulva, and softening of the tail head and gluteal musculature. The milk electrolyte calcium was assessed daily after detection of cloudy mammary secretions (Predict-A-Foal[d]), and all mares were monitored for foaling using an electronic foaling sensor (Foalert[e]). Following delivery, tissue samples of the fetal membranes were immediately collected from the chorioallantois (cervical star, uterine body, gravid horn, and nongravid horn), amnion, and mid-umbilicus as well as any other grossly abnormal areas and placed into formalin. All samples were processed by the clinical laboratory at North Carolina State University and evaluated by a pathologist who was blinded to the status of the animals for the presence of bacteria, inflammation, edema, and necrosis. Allantocentesis results compared to clinical and other hematologic data have previously been described (Beachler et al. 2019).

5.3.2 Blood collection and processing

Jugular venipuncture was performed at 270 to 275 days of gestation (baseline) and at the following time-points following inoculation (PLAC): 4, 12, and 24 hours, day 2, 4, 6, 10, and then weekly until foaling. Collections were performed at the same intervals in control mares. Five mLs of whole blood were collected into lithium heparin tubes, centrifuged for 10 minutes at 3500 x g and 4 °C to obtain the plasma fraction, and then flash-frozen in 2-mL aliquots at -196 °C in liquid nitrogen. Samples were subsequently stored at -80 °C until analysis.

5.3.3 *Sample Preparation and NMR Spectroscopy*

Immediately prior to analysis, plasma samples were thawed at room temperature. Two hundred µL was dissolved in 400 µL of 10% deuterium oxide[f] (D_2O) and 90% water containing 0.1-mM trimethylsilyl propionate[f] (TSP), centrifuged at 3500 x g for 10 minutes and the supernatant transferred into 5-mm borosilicate NMR tubes[g]. Standard one-dimensional (1D) proton (^1H) NMR spectroscopy was performed using a 500-MHz spectrometer[h] with Oxford Narrow Bore Magnet, and ZGPR pulse sequence. All samples were also analyzed with a Carr-Purcell-Meiboom-Gill (CPMG) experiment. To confirm peak identity, two-dimensional (2D) proton experiments (homonuclear correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY)) and proton-carbon experiments (heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond (HMBC)) experiments were performed on representative samples. To assist the elucidation of non-polar compounds, a methanol-chloroform-water extraction was performed on representative samples for protein precipitation and separation of the hydrophilic and lipophilic fractions (Stringer et al. 2011). Briefly, 0.5 mL of plasma was mixed with 1 mL of chloroform-methanol[f] (1:1 ratio) and centrifuged at 1300 x g for 15 min. The supernatant was removed, pellet resuspended with 0.5 mL of chloroform-methanol, and the pellet re-centrifuged. The supernatants were then combined, 0.5 mL of ice-cold water was added, and samples were allowed to sit for 15 minutes at -20 °C. After 15 minutes, the lower lipophilic and upper aqueous fractions were removed, vacuum evaporated, and stored overnight at -80 °C. The dried water-soluble extracts were dissolved in 600 µL of 10% D_2O/90% water with 0.1-mM TSP while the dried lipid-rich extract was dissolved in 600 µL of deuterated chloroform with 0.3% tetramethylsilane[f] (TMS) prior to 1D ^1H-NMR spectroscopic analysis. A 5-mm proton broadband inverse probe with Z gradients was used for all 1D presaturation experiments and 2D ^{1}H-^{13}C experiments and was tuned to the ^{1}H frequency of 500.193 MHz and ^{13}C frequency of 125.77 at the 500-MHz spectrometer. All spectra were obtained at 294 K with a 2.04 total second acquisition time and 128 number of transients.

5.3.4 Spectral processing, identification, and quantification

Spectra were processed using ACD/Labs 12.0[i]. Baseline and phasing correction were performed manually. Spectra were referenced to the internal standards TSP (aqueous samples/fractions) or TMS (lipophilic fractions) at 0 ppm chemical shift. Each 1D ^{1}H-NMR spectrum from samples processed in 10% D20/90%H20 was divided into 0.03 ppm chemical shift bins through intelligent bucketing and was integrated to compensate for small shifts in the signals and reduce the total number of variables. Dark regions ranged from -0.1 to 0.1 ppm, 4.6 to 5.1, and 9.0 to 12.9 ppm to eliminate regions from analysis that did not contain peaks of interest or contained TSP or water. The total spectral area was calculated and bins subsequently normalized by dividing each integral by this total area. Peak identification was performed with Chenomx NMR Suite 8.1[j], databases of known compounds (Human Metabolome Database (HMDB)), and the literature (Jang et al. 2017, Fathi et al. 2017, Nicholson and Foxall 1995, and Oostendorp et al. 2006). Preliminary identification based on those sources was then confirmed with 2D spectral analysis. To perform quantitative comparisons between mares, individual time-points, and groups, a second method of integration was performed in which metabolite specific peaks were manually integrated and weighted to the TSP integral.

5.3.5 Data analysis

Partial least squares discriminant analysis was performed on the integrals obtained from intelligent bucketing to identify metabolites that contributed to variation between groups at each time-point (MetaboAnalyst 4.0[k]) (Xia et al. 2015). Metabolites with integrals that could be specifically identified without overlap were compared between groups and over time using a repeated measures analysis. Mean gestational age was assessed by a one-way ANOVA, while the histologic presence of ascending placentitis was assessed with a Fisher's exact test. Significance for integrals, mean gestational age, and presence of histological placentitis was set at 0.05 (Statistix 10.0[l]). All data are presented as mean ± s.d.

5.4. Results

5.4.1 Clinical and pregnancy outcome

Three of five inoculated mares (Group PLAC) were diagnosed with ascending placentitis based on changes in ultrasonographic measurements (CTUP) within four days of inoculation, while one mare aborted on day 2, prior to an elevation in CTUP. One additional mare delivered a stillborn fetus at 11 days post-inoculation but failed to develop an elevated CTUP greater than 10 mm during the study. The CTUP of control mares remained stable throughout examination and fetal heart rates of both groups did not differ at any time-point. Two of five inoculated mares produced live foals while four of five control mares delivered live foals. Inoculated mares foaled significantly earlier than control mares (mean gestational age 303.8 ± 12.9 days PLAC vs. 328.6 ± 10.1 days CONT; p=0.01). Histological lesions consistent with ascending placentitis at the area of the cervical star occurred only in inoculated mares compared to controls (5/5 PLAC vs. 0/5

CONT; p=0.008. Histologic evaluation of the placenta from the control mare that aborted revealed evidence of inflammation limited to the body in the region of allantocentesis site.

5.4.2 Metabolite profiles

All samples produced spectra with identifiable peaks. Thirty-five metabolites were assigned for samples processed in 10% D20/90% H20 (Fig 1, Supplementary Item 1), whereas 19 metabolites were assigned in the lipophilic fraction in deuterated chloroform (Fig 2). Metabolite identity was confirmed with 2D spectral analysis (Supplementary Items 2 and 3). Partial least squares discriminate analysis showed a clear separation between treatment groups at 4 hours and 4 days post-inoculation (Fig 3). No clear separation was seen at any other time-point analyzed.

A significant increase was seen in 10 individual metabolite concentrations (alanine, phenylalanine, histidine, pyruvate, citrate, glucose, creatine, glycolate, lactate, and 3-hydroxyisobutyrate at 4 hours in infected mares (Table 1). Four days after inoculation, however, a significant decrease was seen in nine individual metabolite concentrations in infected mares compared to healthy controls (alanine, histidine, phenylalanine, tyrosine, pyruvate, citrate, glycolate, lactate, and dimethylsulfone) (Table 1). These metabolites were also suppressed in a mare that aborted on day 11 without an elevation in CTUP compared to healthy controls. No significant differences were seen at other time-points (p>0.05).

5.5. Discussion

This study is the first to describe the plasma metabolome of pregnant mares. It further demonstrates intriguing differences between mares with experimental ascending placentitis and healthy control mares. In this work, a distinct peripheral response to inoculation was seen at four hours following inoculation, with significantly higher concentrations of plasma metabolites involved in energy metabolism (pyruvate, glucose, lactate, and acetate), as well as those involved with nitrogen metabolism (histidine, tyrosine, and phenylalanine); however, plasma metabolites returned to baseline by 12 hours post-inoculation in the absence of other therapeutic interventions. On day 4, when four of the five inoculated mares were diagnosed with ascending placentitis based on an elevated CTUP or the occurrence of abortion (4/5), metabolites involved in energy metabolism (pyruvate, glycolate, lactate, formate, and citrate) and nitrogen metabolism (histidine, tyrosine and phenylalanine) were significantly *decreased* in infected mares. Metabolite concentrations returned to baseline values by day 6, with no significant difference in any of the analyzed metabolites seen between mares regardless of pregnancy outcome at other time-points. The abatement in metabolic changes by day 6 coincided with the initiation of multimodal therapy including systemic antimicrobial, anti-inflammatory, and progestin therapy in infected mares, whereas control mares received no systemic medications.

The reduction in many of the metabolite concentrations seen in this study has been described in other experimental models of infection, including dimethylsulfone, amino acids involved in nitrogen metabolism, and metabolites involved in energy. Dimethylsulfone, a metabolite that was significantly reduced on day 4 post infection, is produced from the endogenous transamination of the amino acid methionine and has also been identified in the plasma, serum, and cerebrospinal fluid of humans and in a variety of domestic animals (Basoglu et al. 2016 and Engelke et al. 2005). It may be obtained from the diet from a variety of plants or through intestinal bacterial production of the normal gastrointestinal microbiome (Basoglu et al. 2016, Engelke et al. 2005, and He and Slupsky 2014). In naturally occurring cases of respiratory infection in calves, Basglou et al. found a decreased concentration in serum dimethylsulfone and suggested that the reduction was due to the presence of pathogenic organisms associated with bronchopneumonia within the respiratory tract (Basoglu et al. 2016). Reduced concentrations of amino acids, including histidine, have been found in the amniotic fluid and serum of women at risk for preterm labor, a condition analogous to equine ascending placentitis, and both in plasma and urine of people affected by bacterial pneumonia (Virgiliou et al. 2017 and Laikis et al. 2010). Similarly, decreased concentrations of pyruvate have also been described in the amniotic fluid of women at risk for preterm delivery, as well within the serum of sheep experimentally infected with *Trypanosoma congolense* compared to healthy controls (Virgiliou et al. 2017 and Neils et al. 2007).

The presence of bacteria and subsequent inflammation within the placenta, fetal fluids, and the fetus itself likely contribute to the metabolomic profile demonstrated in this study. Bacterial colonization and replication may alter both local and systemic pyruvate and amino acids in mares with experimental *S. zooepidemicus* infection due to their increased utilization as an energy source as well as due to increased demands by the fetus in response to infection (Virgiliou et al. 2017, Laiakis et al. 2010, and Neils et al. 2007). Bacterial colonization and replication in experimental cases of ascending placentitis are known to cause upregulation in both proinflammatory cytokines and prostaglandins in the fetal fluids and placenta. The inflammatory response affects the maternal hormonal profile and uterine contractility and causes activation of the hypothalamic-pituitary-adrenal axis, resulting in abortion or premature delivery of affected foals (Lyle et al. 2009, Stawiki et al. 2002, Canisso et al. 2017 and Ousey et al. 2005). Bacterial metabolism, inflammation, tissue damage, and increased metabolic utilization all likely contribute to the altered metabolic profile of serum from mares with experimentally induced ascending placentitis. It was interesting to note that these changes were also observed in one mare which did not have ultrasonographically detectable disease.

In this study, all of the infected mares were treated with standard multimodal therapy after placentitis was diagnosed on day 4 and had similar metabolic profiles to control mares after treatment was initiated for the remainder of the study. Other models of infection have suggested that abatement of metabolic changes may occur in response to the initiation of therapeutics. Niels and coworkers demonstrated that serum pyruvate concentrations in sheep decreased after experimental infection *Trypanosoma congolense,* but returned to baseline in a subset of sheep treated with diminazene aceturate (Neils et al. 2007). Therefore, while there were no infected, untreated mares in this study to allow for a similar comparison, the metabolic return to baseline seen after day 6 may also be a treatment-related response.

In summary, this study described alterations of the plasma metabolome in mares with experimental ascending streptococcal placentitis. Additionally, it is the first report to expand the known metabolomic profile of equine plasma through a methanol-chloroform-water extraction protocol. Surprisingly, the peracute infection was associated with a marked increase in the concentration of plasma metabolites associated with energy metabolism, while a significant reduction in plasma concentrations of amino acids and metabolites involved in energy metabolism was observed in mares with active established infections. These changes occurred concurrently with the clinical diagnosis of placentitis based on transrectal ultrasonography on day 4 post-inoculation and may represent a diagnostic target for the confirmation of infectious disease in high-risk mares. Following the initiation of standard multimodal therapeutics, these alterations returned to baseline with no differences displayed between infected and healthy mares.

5.6. Manufacturer Details

[a]Hardy Diagnostics, Santa Maria, CA, USA.

[b]Aurobindo Pharma Inc., Dayton NJ, USA.

[c]Merck Animal Health, Summit, NJ, USA.

[d]Animal Health Care Products, Williston, VT, USA.

[e]Foalert Inc., Acworth, GA, USA.

[f]Sigma Aldrich, St. Louis, MO, USA.

[g]Wilmad Labglass, Fisher Scientific, Waltham, MA, USA.

[h]Brucker Avance, Billerica, MA, USA.

[i]Advanced Chemistry Department, Inc., Toronto, Ontario, Canada.

[j]Chenomx, Edmonton, Alberta, Canada.

[k]Xia Lab, McGill University, Montreal, Quebec, Canada.

[l]Analytical Software Inc., Tallahassee, FL, USA.

Table 5.1: Mean integrals of individually identified metabolites in mares with and without ascending placentitis.

Data for plasma integrals reported as mean ± s.d. for the time-points four hours and day 4 post-inoculation in control mares (CONT) and mares experimentally inoculated to develop *Streptococcus equi* subspecies *zooepidemicus* ascending placentitis (PLAC). Data were analyzed for normality with a Shapiro-Wilk test and data with a non-normal distribution were rank transformed. Differences between groups over time were analyzed using ANOVA with repeated measures. Superscripts indicate significant group*time interactions of * $p < 0.05$; ** $p < 0.005$.

	Days Post-inoculation			
	4 hours		Day 4	
	CONT	PLAC	CONT	PLAC
Acetate	3.63 ± 0.57	5.18 ± 0.57	3.98 ± 0.61	3.48 ± 0.19
Acetone	1.32 ± 0.19	2.36 ± 1.18	1.55 ± 0.24	1.35 ± 0.30
Alanine	4.22 ± 0.51	5.98 ± 0.58*	4.56 ± 0.41	3.82 ± 0.32*
Citrate	2.00 ± 0.37	2.83 ± 0.24*	2.24 ± 0.18	1.71 ± 0.19*
Creatine	0.77 ± 0.12	1.02 ± 0.04*	1.03 ± 0.06	0.86 ± 0.10
Creatinine	0.85 ± 0.12	1.22 ± 0.06	0.74 ± 0.06	0.64 ± 0.08
Dimethylsulfone	1.08 ± 0.31	1.31 ± 0.17	1.12 ± 0.15	0.85 ± 0.17**
Formate	0.28 ± 0.05	0.45 ± 0.13	0.35 ± 0.04	0.28 ± 0.03
Glucose	3.30 ± 0.45	4.57 ± 0.31*	3.49 ± 0.28	3.18 ± 0.47
Glycolate	0.85 ± 0.12	1.19 ± 0.05*	0.97 ± 0.07	0.82 ± 0.10*
Histidine	0.97 ± 0.15	1.37 ± 0.16*	1.15 ± 0.11	0.92 ± 0.04*
3-Hydroxyisobutyrate	1.85 ± 0.24	3.28 ± 1.53*	1.45 ± 0.21	1.23 ± 0.24
Lactate	1.74 ± 0.26	2.54 ± 0.34*	1.89 ± 0.16	1.61 ± 0.17*
Phenylalanine	1.22 ± 0.21	1.70 ± 0.24*	1.47 ± 0.17	1.14 ± 0.07*
Pyruvate	0.87 ± 0.13	1.28 ± 0.05**	1.02 ± 0.09	0.82 ± 0.07**
Tyrosine	5.25 ± 0.87	7.56 ± 0.91	6.33 ± 0.63	5.17 ± 0.28*
Valine	3.03 ± 0.30	4.10 ± 0.41	2.99 ± 0.26	2.64 ± 0.23

Table 5.2: Metabolites identified using ^{1}H-nuclear magnetic resonance spectroscopy of equine plasma processed in 10% D20/90% H20.

Acetate	Terminal -CH3 lipids
Acetone	-CH2 lipids
Alanine	-CH2CH2CO lipids
Betaine	-CH=CH-CH2 lipids
Choline and choline metabolites	=CH-CH2CH= lipids
Citrate	-CO-CH2 lipids
Creatine	-CH=CH- lipids
Creatinine	
Dimethylsulfone	
Formate	
Glucose	
Glutamine	
Histidine	
Isoleucine	
Lactate	
Leucine	
N-acetyl glycoproteins	
Phenylalanine	
Pyruvate	
Threonine	
Trimethylsilyl propionate (TSP)	
Tyrosine	
Urea	
Valine	
Water	
2-hydroxyisobutyrate	
3-hydroxyisobutyrate	
3-hydroxyvalerate	

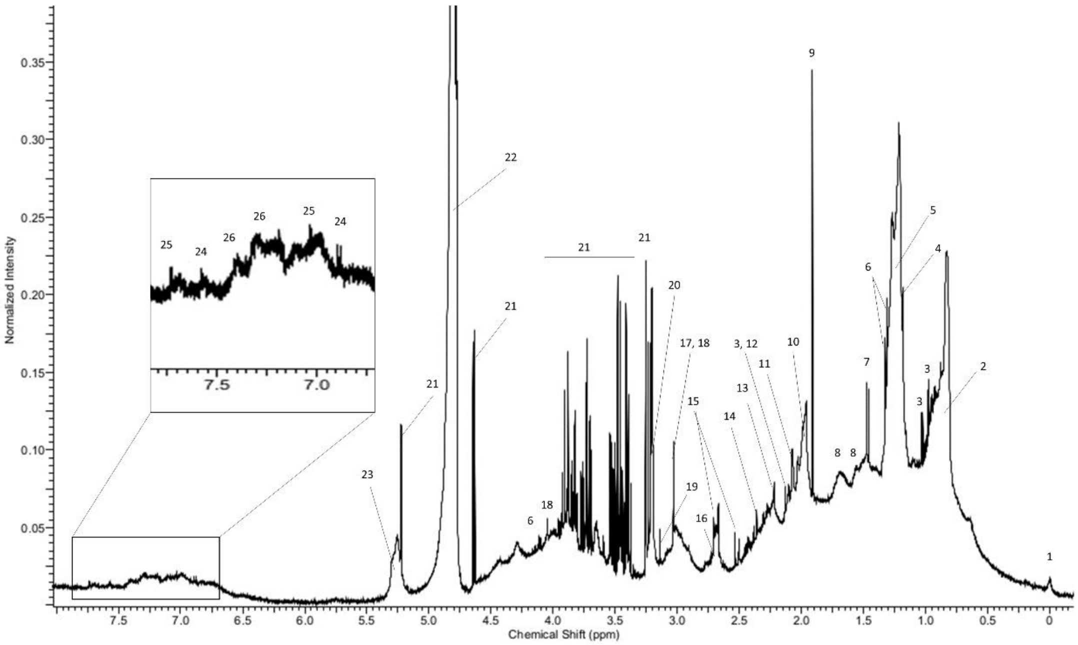


Figure 5.1: Representative ^{1}H-nuclear magnetic resonance spectrum of equine plasma processed in 10% D20/90% H20. The x-axis depicts chemical shift in parts per million (ppm); y-axis depicts peak intensity. Assigned peaks are as follows: 1: trimethylsilyl propionate (TSP), 2: terminal $-CH_3$ lipids, 3: valine, 4: 3-hydroxyisobutyrate, 5: $-CH_2$ lipids, 6: lactate, 7: alanine, 8: $-CH_2CH_2CO$ lipids, 9: acetate, 10: N-acetyl glycoproteins, 11: $-CH=CH-CH_2$ lipids, 12: glutamine, 13: acetone, 14: pyruvate, 15: citrate, 16: $-CO-CH_2$ lipids, 17: creatine, 18: creatinine, 19: dimethylsulfone, 20; choline and choline metabolites, 21: glucose, 22: water, 23: -CH=CH- lipids, 24: phenylalanine, 25: histidine, and 26: tyrosine.

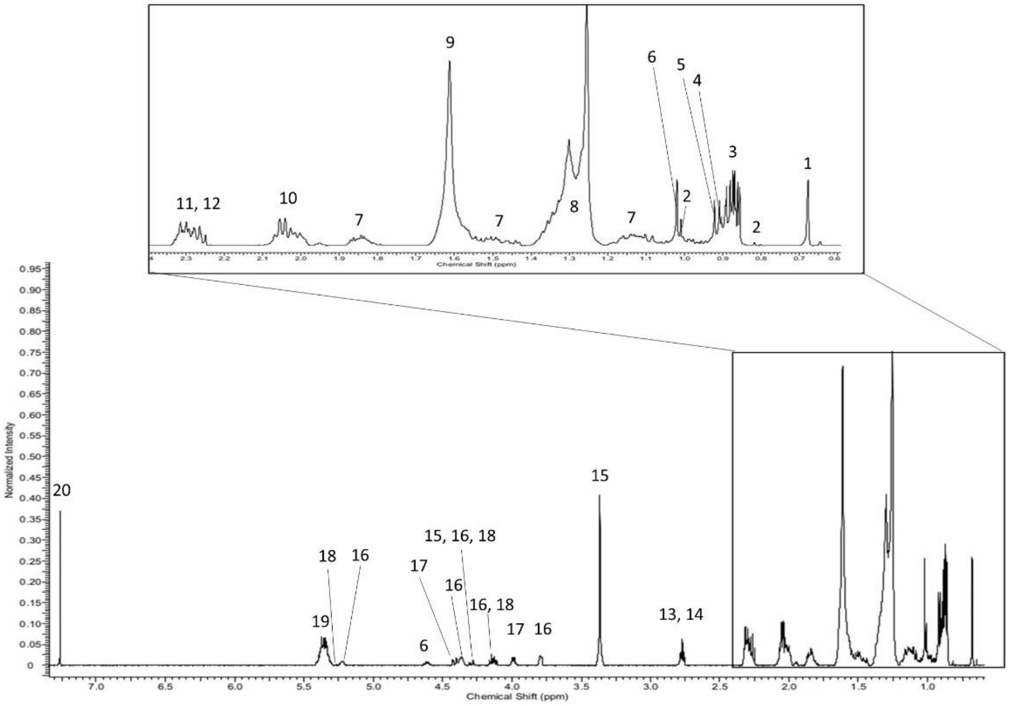
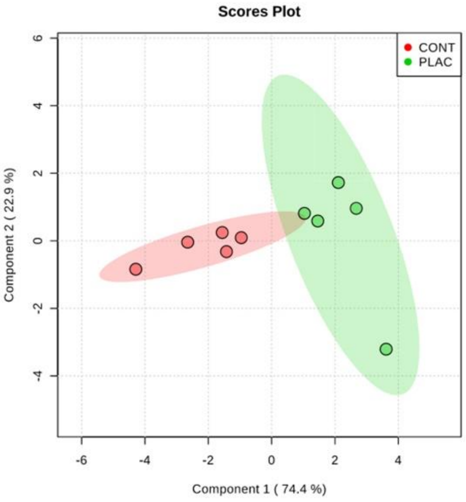


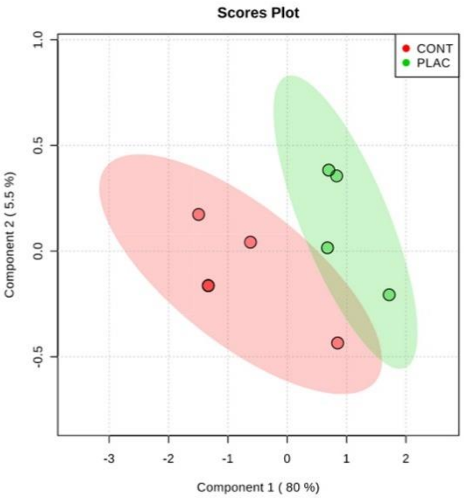
Figure 5.2: Representative ^{1}H-NMR spectrum of the non-polar extract of equine plasma in deuterated chloroform (CDCL3). The x-axis depicts chemical shift in parts per million (ppm); y-axis depicts peak intensity. Identified peaks are as follows: 1: total cholesterol C18, 2: free cholesterol, 3: total cholesterol C26/C27, 4: CH_3 fatty acyl chains, 5: Linoleic CH_3 acyl chains, 6: esterified cholesterol, 7: multiple cholesterol protons, 8: $(CH_2)_n$ fatty acyl chains, 9: CH_2CH_2CO fatty acyl chains, 10: $CH_2CH=$ fatty acyl chains, 11: CH_2CO fatty acyl chains, 12: linoleic acid CH_2CO fatty acyl chains, 13: $=CHCH_2CH=$ fatty acyl chains, 14: linoleic $=CHCH_2CH=$ fatty acyl chain, 15: choline, 16: diacylglycerol (DAG) glycerol backbone, 17: phosphatidylcholine, 18: triacylglycerol (TAG) glycerol backbone, 19: -CH=CH fatty acyl chains, and 20: chloroform.

Figure 5.3: PLS-DA scores scatter plot for ^{1}H-NMR plasma spectra of inoculated (PLAC) and control mares (CONT) at 4 hours (Fig 3a) and day 4 (Fig 3b) post-inoculation.

A


Scores Plot
CONT
PLAC
Component 2 (22.9 %)
6
4
2
0
-2
-4
-6
-4
-2
0
2
4
Component 1 (74.4 %)

B


Scores Plot
CONT
PLAC
Component 2 (5.5 %)
1.0
0.5
0.0
-0.5
-3
-2
-1
0
1
2
Component 1 (80 %)

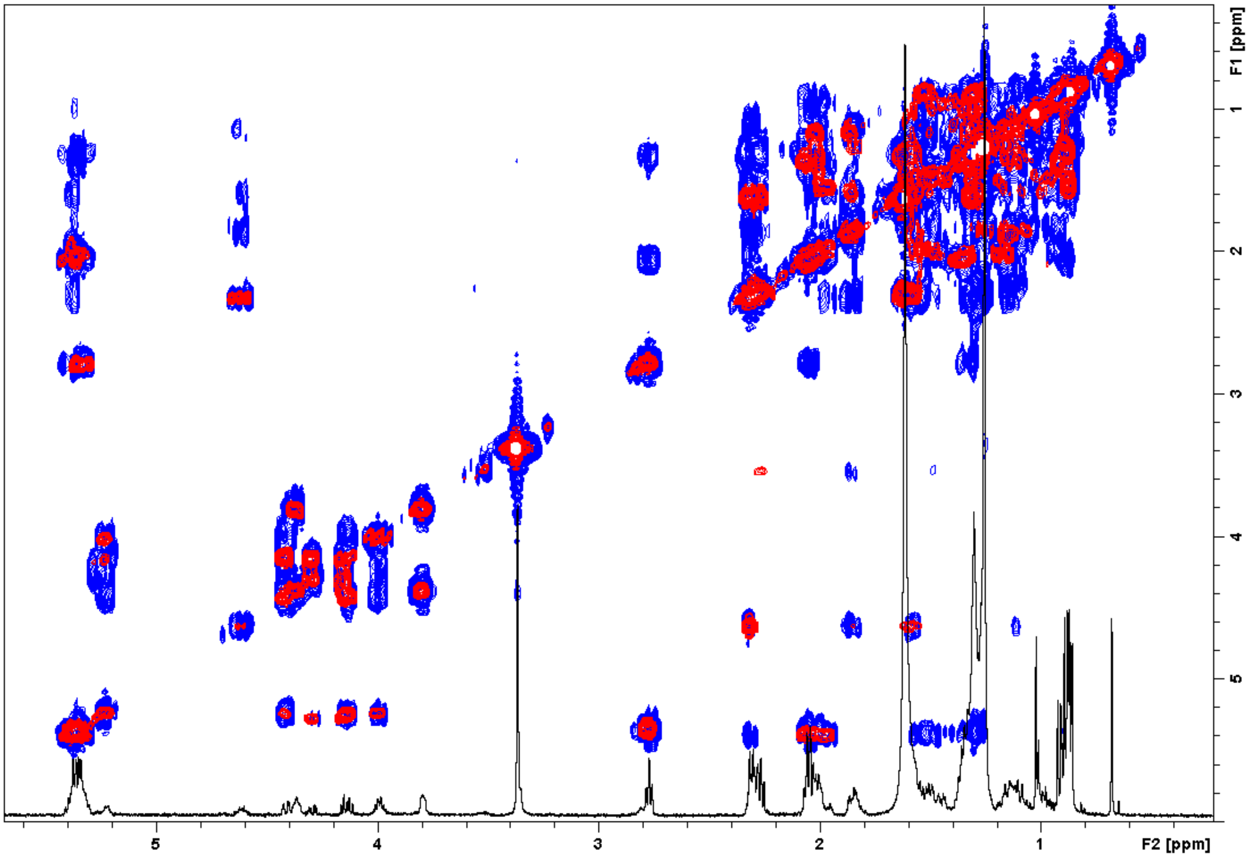


Figure 5.4: Combined (^{1}H-^{1}H)-NMR COSY (red) and (^{1}H-^{1}H)-NMR TOCSY (blue) spectra of the non-polar extract of equine plasma in deuterated chloroform (CDCL3). The proton (^{1}H) chemical shift in parts per million (ppm) are on the x and y-axes. The 1-D ^{1}H-NMR spectrum (black) of this extract is on the x-axis.

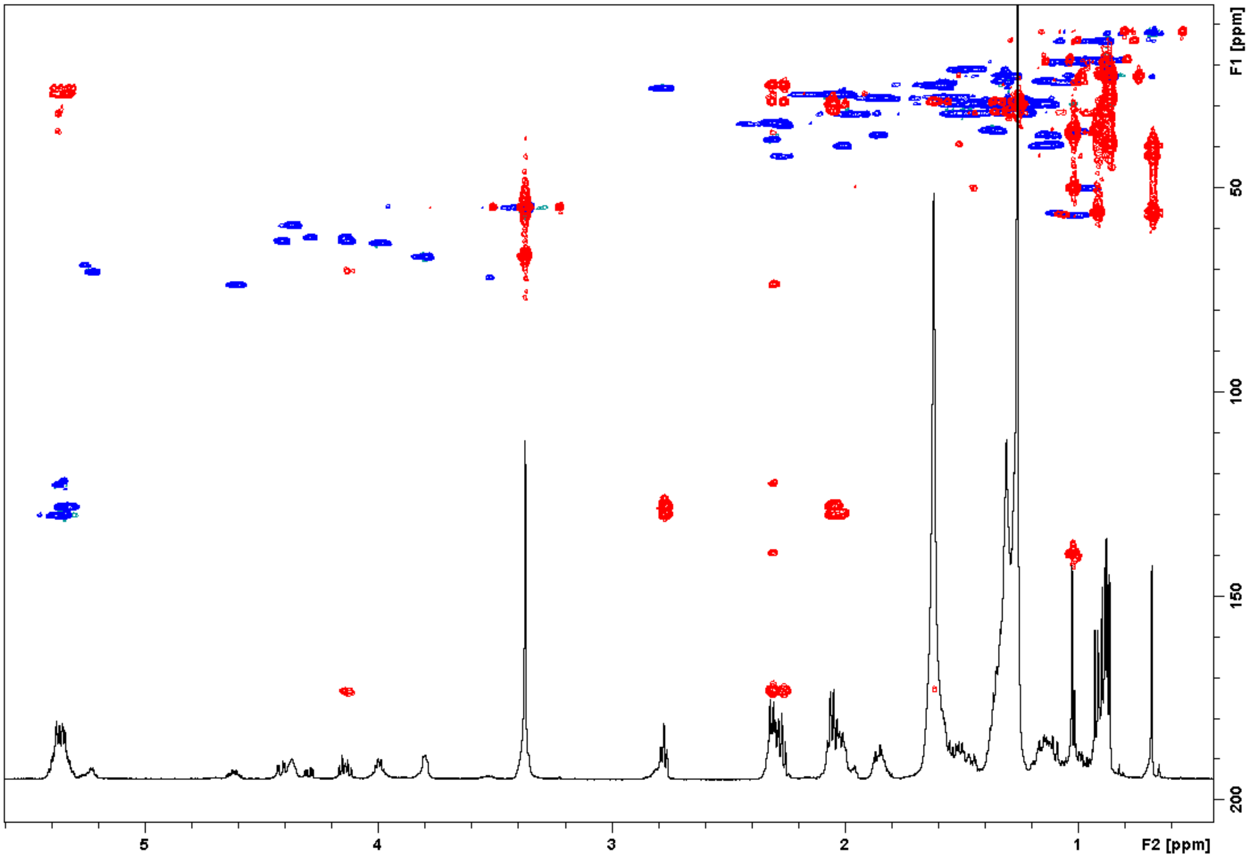


Figure 5.5: Combined (^{1}H-^{13}C)-NMR HSQC (red) and (^{1}H-^{13}C)-NMR HMBC (blue) spectra of the non-polar extract of equine plasma in deuterated chloroform (CDCL3). The x-axis depicts the proton (^{1}H) chemical shift while the y-axis depicts the carbon (^{13}C) chemical shift in parts per million (ppm). The 1-D ^{1}H-NMR spectrum (black) of this extract is over laid on the x-axis.

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Chapter 6

Conclusions and Future Directions

This work expands the known database and metabolomic profile of plasma in the horse with the performance of a methanol-chloroform-water extraction to identify the polar and nonpolar components of plasma including lipids. It is the first to identify the NMR-based metabolomic profile of amniotic fluid and allantoic fluid and to specifically compare their differences. Finally, this work is the first to attempt to demonstrate the metabolomic profile of mares with experimental ascending placentitis compared to healthy cohorts. While the allantoic fluid compartment is inherently involved in the pathophysiology of bacterial migration and colonization, as well as the subsequent inflammatory cascade, we were surprised to not be able to identify metabolomic differences consistent with either inflammation or infection in this study. Potential reasons for the lack of metabolic alterations first may be due to the dilution of any present inflammatory or bacterial metabolites within the large fluid volume that is present in late gestation. The sampling location itself may also contribute to these findings as metabolic alterations may more likely be seen within cranial vaginal or cervical secretions during the initial stage of an experimental infection compared to those within the allantoic fluid. Additionally, while serial sampling was performed in both groups obtaining data over time in order to be able to compare to the onset of clinical disease and findings of other diagnostic modalities, the potential for the creation of an inflammatory environment in both groups is possible that could have obscured any innate metabolite alterations that could be related to infection alone. Finally, it is possible that similar to the lack of systemic changes on a complete blood count (CBC) and biochemistry panel, these data on the allantoic fluids may represent a true lack of metabolomic difference within this compartment.

Plasma metabolic changes were detected in two phases following inoculation. Initially, an increased concentration in metabolites involved in energy, nitrogen, oxygen, and hydrogen metabolism was seen in inoculated mares at four hours that returned to baseline by the 12-hour time-point. All parameters then remained unchanged between groups until day four post inoculation where multiple metabolites were significantly decreased in inoculated mares compared to healthy animals involved in energy metabolism (pyruvate, glycolate, lactate, formate, and citrate) and nitrogen metabolism (histidine, tyrosine, and phenylalanine). Interestingly, this coincided with the ultrasonographic diagnosis of disease and the initiation of multimodal systemic therapy in infected mares. Further research would be needed to confirm these findings in a larger number of mares due to the small numbers used in this study. Additionally, as we hypothesized that the lack of continued metabolic alterations between groups seen on days six and 10 is due to the administration of the multimodal therapeutics trimethoprim sulfamethoxazole, altrenogest, and flunixin meglumine, these findings would also need to be confirmed in an untreated cohort to examine if the reduction in metabolites involved in energy and nitrogen metabolism remain for prolonged periods of gestation or until foaling and/or abortion. Similarly, before plasma metabolomics could be considered useful as a diagnostic screening tool in addition to transrectal ultrasonography, its findings would likewise need to be confirmed in cases of naturally occurring infection. Consistent metabolic signatures could then be used to create a multivariable statistical model for the prediction of disease or identification of mares who should undergo transrectal ultrasonography for the screening of fetoplacental heath. Statistical modeling options to be evaluated include regression analysis utilizing linear, logistic, or COX models. For example, backward linear regression modeling was applied to the experimental plasma metabolites that were noted to be statistically different between groups on day 4 following inoculation in this work (Statstix 10.0) and each model was analyzed for fit. The model with the lowest Akaike information criterion (AIC) value was selected to produce the following best fit model for the plasma metabolomic prediction of placentitis: y (presence of disease) = -0.570 + 0.368 * (acetate) – 1.15 * (citrate) – 0.748 * (dimethylsulfone) + 0.581 * (glucose) + 2.143 * (formate). Once validated, plasma metabolite alterations in the specifically identified metabolites for a mare in question could again be utilized to screen for mares who may be at high risk for, are asymptomatic, or are in an early stage of developing ascending placentitis.