2012

INGESTION OF ENDOPHYTE-INFECTED TALL FESCUE SEED INDUCES PERIPHERAL VASOCONSTRICTION BUT DOES NOT AFFECT CYCLICITY IN NON-PREGNANT MARES, AND A POPULATION OF BIOGENIC AMINE RECEPTORS RELATIVE TO VASOCONSTRICTION IS IDENTIFIED

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THESIS

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

By

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Lexington, KY

Director: Dr. Karen McDowell, Associate Professor of Veterinary Science

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2012

ABSTRACT OF THESIS

INGESTION OF ENDOPHYTE-INFECTED TALL FESCUE SEED INDUCES PERIPHERAL VASOCONSTRICTION BUT DOES NOT AFFECT CYCLICITY IN NON-PREGNANT MARES, AND A POPULATION OF BIOGENIC AMINE RECEPTORS RELATIVE TO VASOCONSTRICTION IS IDENTIFIED

Three experiments were conducted to explore the effect of fescue toxicosis on vasoconstriction and various parameters of the estrous cycle. In the experiment of Chapter 3, a 2x2 crossover experimental design with repeated measures was implemented to test whether the ingestion of endophyte-infected tall fescue seed would alter blood hormone concentrations of prolactin, progesterone, and estradiol, interovulatory intervals, and corpus luteum blood flow. Also, Doppler ultrasonography was used to assess whether palmar artery and palmar vein lumen diameter, area, circumference, and resistivity index could be altered by the ingestion of endophyte-infected tall fescue seed. Ingestion of endophyte-infected tall fescue seed significantly decreased palmar artery resistivity index, area, and circumference, and palmar artery and palmar vein lumen diameter, but did not alter interovulatory intervals, serum concentrations of prolactin, progesterone, estradiol, or corpus luteum blood flow. Experiments 1 and 2 from Chapter 4 profiled the dopaminergic, serotonergic, and α-adrenergic receptor types and subtypes, as expressed within the equine medial palmar artery, medial palmar vein, and uterine artery transcriptomes. Combined results from Chapters 3 & 4 imply that relative quantity of serotonergic receptor subtypes within a vessel may be related to with the severity of that vessel's vasoconstrictive response to endophyte-infected tall fescue.

KEYWORDS: Horse, Tall Fescue, Doppler Ultrasound, Vasoconstriction, Reproduction

Daniel Andrew Hestad

May 11, 2012

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To Nicole Brooke Stork-Hestad, my wife, and my best friend

ACKNOWLEDGEMENTS

In general, large and complex projects cannot be completed without help the help of others, and this one is no exception. Dr. Karen McDowell has been my most avid supporter, and I feel myself quite undeserving. Over the past two years, Dr. McDowell has always been there as both an academic and personal aid, and I feel that I have grown tremendously because of her guidance. In a similar manner, Dr. Jimmy Klotz has been a support for me as well. Experiment 2 was performed in Dr. Klotz's lab, where he decided to let in a student who had absolutely no prior experience with molecular biology. Because of that, I feel that I have a very well-rounded masters experience. Last in my thesis committee are Dr. Ball and Dr. Dwyer, who were patient with me in spite of my mistakes, and who were willing to put in the extra effort needed to pull this thesis together. In all, the guidance and support of these four people have helped to pull this project together.

In Experiment 1, our summer interns Elizabeth, Sarah, Emily, and Laura were a tremendous help. If they had not been there to help us, Dr. McDowell and I would probably still be at the farm. In Experiment 2, Adam Barnes was a lifesaver time and time again. It was his tireless dedication and his willingness to help correct my mistakes that helped us pull Experiment 2 together. The farm crew at Maine Chance Farm always deserves credit, as they are the individuals behind the scenes that take care of us and our research herd. The Reproduction Group at Gluck is a great resource, and Dr. Squires, Dr. Troedsson and all of my fellow students come together to create an academic atmosphere that is challenging and enriching. Dr. Neil Williams has been a great resource to me here in my last semester, and has graciously allowed me to take some of his valuable time. Of

course, this project could not have gotten underway without financial support from the USDA-ARS-FAPRU, the University of Kentucky College of Agriculture, and the Gluck Research Center.

Finally, I am greatly indebted to the personal support that I received from my friends and family. From farm manager to master's student to veterinary student, my friends and family have always been there to support me, let me bounce ideas off of them, and encourage me to step outside of my comfort zone. Dr. Zent and Mrs. June, in particular, have been an amazing driving force in my recent past, and truly believed in my capabilities. Most deserving of special mention is my wife, Nicole, who is singularly responsible for making me a better person. Without her delicate sensibilities and will of iron constantly by my side, I would most certainly be lost. Thank you.

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CHAPTER ONE: Introduction

"Fescue toxicosis" is the singular term describing a multitude of adverse physiological conditions afflicting several animal species which ingest endophyte (*Neotyphodium coenophialum*; Glenn et al., 1996) -infected (E+) tall fescue (*Lolium arundinaceum*). Tall fescue is a commonly utilized pasture forage in the Southeastern United States, with an estimated 700,000 horses maintained on tall fescue pasture (Ball et al., 2002). With a desirable advantage in its capability for hearty growth in adverse conditions, such as drought, high and low temperatures, ill-suited soil, and heavy grazing (Ball et al., 1987; Siegel et al., 1987; Bacon, 1995), tall fescue is still heavily propagated today. Nevertheless, livestock ingesting E+ tall fescue can exhibit signs of an ergotism-like toxicity, signs not seen when livestock ingest endophyte-free (E-) tall fescue. The endocrine action of E+ tall fescue causes late pregnant mares to exhibit well established signs, such as agalactia, decreased blood hormone concentrations of prolactin (PRL) and progesterone (P4), increased blood hormone concentrations of estradiol (E2), increased gestational intervals, birth of dysmature foals, and increased foal and placental weights (Loch et al., 1987; Monroe et al., 1988; Putnam et al., 1991; McCann et al., 1992).

Producers often fail to recognize problems with fescue toxicosis, however, as many of the signs are not overtly severe and have the potential to go undetected. This is especially true for horses, as many fescue toxicosis signs such as reduced heat tolerance, decreased growth rates, and decreased fertility are not consistently reported (Putnam et al., 1991; Aiken et al., 1993; Brendemuehl et al., 1994; Arns et al., 1997). In addition, inconsistent observations plague studies concerning even well known signs in horses, such as those observed in pregnant mares grazing E+ tall fescue, as the severity of signs

from will vary study to study (Loch et al., 1987; Monroe et al., 1988; Ireland et al., 1991; Putnam et al., 1991). Thus, a high rate of between-study variation coupled with the many signs of fescue toxicosis that are not overtly severe could result in a producer or veterinarian often misdiagnosing a fescue toxicosis related problem.

Investigations into early reproductive efficiency in horses is one such area fraught with between-study variation. In 1988, Monroe et al. observed that when mares were rebred after experiencing gestational failure due to fescue toxicosis, if kept on E+ tall pasture, they were likely to exhibit decreased fertility when compared to their counterparts grazing E- tall fescue. However, Brendemuehl et al. (1994) found that while mares would exhibit increased interovulatory intervals, increased P4 concentrations, and a tendency for increased early embryonic death while ingesting E+ tall fescue, 60 day pregnancy rates did not differ between mares ingesting E+ and E- tall fescue. This variation is likely due to differences in animals, differences in time of year of the studies, and differences in ergot alkaloid concentrations between the studies. Although the former two causes of variation may depend on differences in experimental design, the latter variation source is an area which should be controlled in future studies in order to provide producers and veterinarians a sound basis for the recognition of potential problems due to fescue toxicosis.

Between-study variability in ergot alkaloid concentration in treatment diets is a problem which has the potential to be remedied in present day studies. Ergot alkaloid measurement methods used within studies have increased in precision from quantitation of percent endophyte infection in E+ tall fescue via tandem mass spectrometry (Yates et al., 1985) to methods which identify specific ergot alkaloids – and the concentration of

those alkaloids – within the plant via high-performance liquid chromatography (Rottinghaus et al., 1991). An increase in the sensitivity of ergot alkaloid identification at the chemical level would concomitantly suggest a need for the determination of both minimal and maximal levels of ergot alkaloid in the diet required to elicit signs of fescue toxicosis within the horse. As of yet, attempts at the establishment of at least baseline levels have not been successful (Loch et al., 1987; Arns et al., 1997; Smith et al., 2009), leaving investigators with no reliable means of controlling between-study variability in ergot alkaloid concentration. Efforts to improve repeatability and remove variation in fescue toxicosis research must therefore include a method of assessing baseline toxicity levels in animals ingesting E+ tall fescue.

In short, future studies in fescue toxicosis require investigators to have a complete and well rounded knowledge of the history of fescue toxicosis, a thorough knowledge of ergot alkaloid identification methods, the endocrine mode of action of fescue toxicosis, and a definitive method of minimizing variation within studies. To date, review of the literature reveals inconsistencies in the reporting of the effects of E+ tall fescue on early reproductive efficiency and fertility in the horse (Monroe et al., 1988; Brendemuehl et al., 1994; Arns et al., 1997). One source of variation between these studies was the concentration of ergot alkaloid within the diets of treated animals, and the establishment of toxicity through some baseline measurement. The objective of this thesis is to explore the effect of fescue toxicosis on certain parameters of the estrous cycle, including differences in interovulatory interval, serum concentrations of PRL, P4, and E2, and blood flow to the corpus luteum (CL), while simultaneously attempting to establish a model for assessing baseline levels of toxicity in treatment mares.

CHAPTER TWO: Literature Review

The History of Ergotism and Its Relation to Fescue Toxicosis

An ergot is a type of fungus which produces mycotoxins called alkaloids (Peraica et al., 1999). Ergotism, therefore, is the poisoning of an individual by an ergot, usually by the means of an ergot-infected plant. As far back as 1100 BC, authenticated reports indicate that ergot refined from rye (*Scale cereale* L.) was used in obstetrics as a method of controlling blood loss (Schiff, 2006). Effects of ergotism in humans are traceable throughout history (van Dongen and de Groot, 1995; Schiff, 2006), but it was not until the middle ages that ergotism would reach epidemic proportions. Ergotism in these epidemics struck in two different forms throughout the middle ages: convulsive and the gangrenous forms (Schiff, 2006). In cases where resolution would not occur, death would result, and ergot-related deaths across history range in the tens of thousands (van Dongen and de Groot, 1995; Schiff, 2006). Soon, however, it became known that a trek to St. Anthony's parish could prevent death and alleviate the sufferer's symptoms, and the disease soon became known as St. Anthony's Fire (van Dongen and de Groot, 1995; Schiff, 2006; Strickland et al., 2011). Modern knowledge has revealed, however, that the changing geological locations (*i.e.* trekking to St. Anthony's parish for care) resulted in a removal of the causative agent of the affliction, which was mainly bread made from ergot-infected rye (Schiff, 2006).

In 1597 A.D. the causative agent of these ergotism epidemics was identified by scientists at Marburg University (Marburg, Germany), who discovered that ergotism

would appear soon after ergot (*Claviceps purpurea*) infected rye was eaten (Schiff, 2006; Strickland et al., 2011). Despite a scientific understanding of ergotism, individuals suffering from ergotism may have thought themselves to be victims of witchcraft, and the Salem Witch Trials are cited as an instance where ergotism would neatly explain the signs of the supposed victims (Caporael, 1976). A lack of foodstuff regulations allowed ergotism epidemics to persist (Strickland et al., 2011), as epidemics of the convulsive form of ergotism were documented even as late as 1928 (Schiff, 2006). Today, ergotism outbreaks are more likely to develop on an individual basis, due to the overdose of an ergot based drug such as ergotamine (Strickland et al., 2011), or perhaps in developing countries due to individual farming practices and slack grain cleaning protocols (Peraica et al., 1999).

St. Anthony's Fire is now a malady of the past, even though the possibility of such outbreaks among the human population still exists (Peraica et al., 1999). However, its containment to history has been accomplished mainly through stricter regulations placed on grains used for human food (Peraica et al., 1999; Strickland et al., 2011). This still leaves livestock managers and forage producers at a loss, as scientists have learned to refine ergot alkaloids and use them for medicinal purposes (Peraica et al., 1999; Schiff, 2006; Strickland et al., 2011), but are unable to negate their effects while the ergot alkaloids remain in the forage. Attempts to remove the negative economic impact of fescue toxicosis while still producing a resilient forage therefore requires a thorough understanding of the alkaloids produced by the endophytic fungus *Neotyphodium coenophialum*, such as the natural sources, chemistry, analysis, absorption, metabolism, clearance, toxicodynamics, and physiological signs associated with fescue toxicosis.

The History of Tall Fescue and Fescue Toxicosis

Originally a European grass introduced to the United states in the 1800's (Buckner et al., 1979), tall fescue (*Lolium arundinaceum*) was re-discovered on a Kentucky farm in Menifee County in 1931 by Dr. E.N. Fergus (Ball et al., 1987; Bacon, 1995). After much testing at the University of Kentucky, the grass was released in 1943 with distinguishing characteristics that made the cultivar particularly hardy (Buckner et al., 1979). During the 1940's and 1950's, the grass was promoted vigorously to a southeastern public in need of a grass hardy enough to withstand its harsh summers. Its reception, and subsequent widespread popularity, resulted in the official recognition of the Kentucky 31 (KY31) cultivar in 1972 (Fergus and Buckner, 1972). Presently, KY31 has been adapted for use in most of the southern United States, with current estimates suggesting that between 14 and 19 million hectares are occupied by tall fescue (Bacon, 1995; Bouton, 2007; Hannaway et al., 2009).

The characteristics which fueled its promotion have allowed the endophyte-infected (E+) tall fescue cultivar to out compete endophyte-free (E-) cultivars of grass. Not long after the initial release of the grass, reports of a disease in cattle resembling ergotism began to appear (Cunningham, 1948; Ball et al., 1987; Strickland et al., 2011). As time went on, livestock grazing tall fescue pastures demonstrated adverse physiological signs (Strickland et al., 2011), yet no cause was identified, despite rigorous study (Bacon, 1995). Terms such as "summer slump," "summer syndrome," "fescue toxicity," and "fescue toxicosis," began to describe the signs observed by livestock producers. The first concise listing of physiological signs affecting cattle was in 1963 (Jacobson et al., 1963), and although scientists were rapidly pursuing an ergot-related

component within tall fescue, the physiological agent(s) responsible for the signs had not yet been identified (Bacon, 1995).

Not until 1973 did a breakthrough occur in fescue toxicosis research, when in northern Georgia scientists began isolating a number of species of fungi (Bacon, 1995). Studies on the grass revealed a fungus that was endophytic, *Claviceps* - related, and capable of producing several toxic ergot alkaloids (Bacon et al., 1975; Porter et al., 1979). Cattle toxicity data was reported in 1980 (Hoveland et al., 1980), when cattle grazing E+ and E- pastures were found to have different physiological signs. The association between endophyte and fescue toxicity has been confirmed numerous times (Schmidt et al., 1982; Monroe et al., 1988; Gadberry et al., 2003), firmly establishing that E+ tall fescue is toxic to livestock. Later research garnered a name for the fungus, *Acremonium coenophialum* (Morgan-Jones and Gams, 1982) (now *Neotyphodium coenophialum*; Glenn et al., 1996).

Neotyphodium coenophialum and Alkaloid Production

Produced mainly from the *Neotypodium* or *Claviceps* genera of fungi, ergot alkaloids are a naturally occurring, diverse group of compounds (Evans, 2011). These compounds therefore generally fall into three groups, or classes of alkaloid: ergopeptines, lysergic acid derivatives, and clavines (Komarova and Tolkachev, 2001; Schardl et al., 2006). Of these compounds, it seems that ergovaline – of the ergopeptine class – is the most physiologically active alkaloid (Evans, 2011; Strickland et al., 2011). However, it should be noted that the scientific community has not ascribed total responsibility for fescue toxicosis to ergovaline (Hill et al., 2001; Gadberry et al., 2003), as it is still not

clear whether or not it is one alkaloid, several alkaloids acting in harmony, or a specific combination of alkaloids that cause fescue toxicosis (Strickland et al., 2009). Furthermore, several theories such as additive effects, bioaccumulation, resistivity, or increased sensitivity, have yet to be explored in detail (Klotz et al., 2008, 2009). Nevertheless, when compared to alkaloids such as lysergic acid, ergovaline was not outperformed in producing an adverse physiological response (Klotz et al., 2007; Strickland et al., 2009).

Levels of ergot alkaloid – particularly ergovaline – in treatment diets appear to offer more sources of scientific variation when assessing toxic potential. Concentrations of ergovaline are indeed maximal in the seed head of the plant (Smith et al., 2009), yet the sheath, leaf, and stem of the plant still contain ergovaline and other alkaloids (Fayrer-Hosken et al., 2008). Similarly, while a pasture might be determined E+ or E-, concentrations of ergovaline within the total available forage can vary from as little as 100 ppb to as much as 1171 ppb (Oliver et al., 1993; Breuhaus, 2003; Matthews et al., 2005). Additionally, the concentration of alkaloid within an infected plant can vary based on conditions such as drought, fertilization rate, and growth stage (Belesky and Hill, 1997; Fayrer-Hosken et al., 2008). Ultimately, for the equine farm manager, total ergot alkaloid ingested by the animal becomes important in assessing a management strategy, as strategies such as replanting, reseeding, herbicide treatments, or domperidone treatment, can dramatically increase the cost of management as herd size and farm size increase. However, a safe concentration of ergot alkaloid in tall fescue has not been established for either non-pregnant, pregnant, or growing horses, as a threshold

concentration of alkaloid needed to produce fescue toxicosis signs has not been established (Smith et al., 2009).

Ergot Alkaloid Disruption of Hormonal Homeostasis

Ergot alkaloids have an ergoline ring structure (Komarova and Tolkachev, 2001) with a molecular architecture similar to various biogenic amines, including dopamine, serotonin, norepinephrine, and epinephrine (Berde, 1980; Weber, 1980; Komarova and Tolkachev, 2001). This structural similarity allows them to bind to the biogenic amine (dopaminergic, serotonergic, and α-adrenergic) receptors (Porter and Thompson, 1992; Abney et al., 1993; Dyer, 1993; Oliver et al., 1993). As a result, endocrine responses, such as decreased circulating blood concentrations of prolactin and vasoconstriction are observed. The agonistic interaction of ergot alkaloids on dopaminergic, serotonergic, and α-adrenergic receptors has an endocrine impact which is observed across a wide range of biological systems and species (Strickland et al., 1992; Abney et al., 1993; Schmidt and Osborn, 1993; Cross, 2011), and warrants review.

In cattle, the interaction of the ergot alkaloids of E+ tall fescue with α-adrenergic and serotinergic receptors causes a peripheral vasoconstriction. Several *in vitro* studies have confirmed that ergot alkaloids will induce a significant contractile response in bovine vasculature when compared to the contractile ability of epinephrine (Klotz et al., 2007; Klotz et al., 2010) and serotonin (Dyer, 1993; Oliver et al., 1993). In addition, *in vivo* experiments have shown cattle to have a significant peripheral vasoconstriction when ingesting E+ tall fescue (Rhodes et al., 1991; Aiken et al., 2007; Aiken et al., 2009). *In vitro* contractility assays using equine vasculature showed a significant ability

of ergot alkaloids to induce a contractile response when compared to various biogenic amine compounds (Abney et al., 1993; Klotz and McDowell, 2010). This result has been confirmed *in vivo* using Doppler ultrasound (Moore et al., 2008; McDowell et al., 2009).

Of utmost importance for understanding the fescue toxicosis syndrome is the endocrine control of prolactin. Detailed reviews of prolactin control are available (Ben-Jonathan et al., 1985; Benker et al., 1990), but a brief review will be given here. Prolactin is secreted from the lactotrophs of the anterior pituitary, where tonic inhibition by dopamine (released from the hypothalamus) regulates its release (Ben-Jonathan and Hnasko, 2001). Certain compounds – such as E2, cholecystokinin, and serotonin – have been shown to stimulate PRL, but this mode of action is mainly through an inhibition of dopamine secretion (Ben-Jonathan and Hnasko, 2001). Dopamine arrives at the anterior pituitary via the hypothalamic-hypophysial portal system, where it exerts inhibition of prolactin by interacting with the dopamine type-2 (D2) receptor of the lactotroph (Strickland et al., 1992). It is through interaction with this receptor that ergopeptines mediate inhibition of prolactin release.

Historically, the endocrine hallmark of fescue toxicosis in several species has been the decrease in circulating blood concentrations of prolactin (Neal and Schmidt, 1985; Bond and Bolt, 1986; Monroe et al., 1988; Gadberry et al., 2003). In late pregnant mares, the decrease in prolactin concentrations can be observed in as little as three days (McCann et al., 1992), while concentrations of prolactin in cattle will decline to near zero within assay detection limits (Parish et al., 2003b). In cattle and sheep, this decrease in prolactin causes a decrease in milk production (Burke et al., 2010), while horses and rabbits may exhibit a complete agalactia (Stidham et al., 1982; Monroe et al., 1988; Cross

et al., 1995). Administration of dopamine D2 receptor (DRD2) antagonists, such as domperidone, will alleviate agalactia in foaling mares (Redmond et al., 1994; Jones et al., 2003), providing evidence that decreased milk production is caused by the agonistic action of ergot alkaloids on the D2 receptors of the lactotrophs of the anterior pituitary gland.

Other endocrine effects are evident, especially in the mare, where the impact of E+ tall fescue has the most deleterious effect during late pregnancy. In a healthy mare not grazing tall fescue, concentrations of estrogens decrease steadily in the final four months of gestation leading up to parturition (Palme et al., 2001), and relaxin increases steadily from day 240 to foaling (Stewart and Stabenfeldt, 1981). Progestagens (primarily 5α - pregnanes) increase in the final four to six weeks of gestation before decreasing precipitously in the final days – or even hours – before parturition (Ousey, 2004). However, in mares grazing E+ tall fescue, progestagens and relaxin are significantly decreased, and estradiol is increased (Monroe et al., 1988; Ryan et al., 2001). These alterations could potentiate the prolonged gestation seen in horses, as the initiation of parturition is dependent upon the functional maturation of the fetal organs (Ousey et al., 2000). Of particular concern are the organs of the hypothalamic – pituitary - adrenal axis. The progestagens which increase in the final four to six weeks of gestation are derived from pregnenolone, which is produced in high quantities by the fetal adrenal gland at this time (Ginther, 1992; Ousey et al., 2000; Ousey, 2004). Thus, the decreased progestagen concentrations observed in mares ingesting E+ tall fescue may indicate that the function of the fetal hypothalamic -pituitary-adrenal axis is impaired, and this impaired function may be the cause of the prolonged gestations observed in mares grazing E+ tall fescue.

Endocrine function may also be altered in non-pregnant mares and cows ingesting E+ tall fescue, but reports in the literature are inconsistent. Varying rates of pregnancy reduction have been reported in cows consuming E+ tall fescue (7.6%-39.2%) (Gay et al., 1988; Brown et al., 1992). Others, however, have failed to reproduce this effect in both cows (Burke et al., 2001a) and heifers (Mahmood et al., 1994). Similarly, studies involving non-pregnant mares ingesting E+ tall fescue have been unclear concerning endocrine function alterations. One study found that while mares were ingesting E+ tall fescue, luteal phases were prolonged, while embryonic losses during the first 14-21 days of gestation were not different from mares grazing E- tall fescue (Brendemuehl et al., 1994). However, another study found that mares grazing E+ tall fescue had progesterone concentrations during the luteal phase which were similar to mares receiving E- diets (Arns et al., 1997). Ultimately, the effect of ergot alkaloids on the endocrine function of non-pregnant mares and cows should be investigated further in order to elucidate the mechanism for these inconsistencies.

The Physiological Signs of Fescue Toxicosis

Signs of fescue toxicosis manifest themselves across several different species (Hoveland et al., 1980; Monroe et al., 1988; Rhodes et al., 1991; Settivari et al., 2009), both sexes (Monroe et al., 1988; Fayrer-Hosken et al., 2008), and in varying degrees depending on the season (Burke et al., 2001b). In addition, signs seem to segregate themselves in accordance with the endocrine alterations imposed upon the animal, as ergot alkaloids have been shown to act agonistically on dopaminergic, serotonergic, and α-adrenergic receptors (Strickland et al., 1992; Dyer, 1993). A thorough understanding of

the manifestations observed when animals ingest E+ tall fescue will help understand the biology of this syndrome.

"Summer Slump" is Heat Stress and Vasoconstriction

During summer months, many observed problems caused by the ingestion of E+ tall fescue are termed "summer slump" (Schmidt and Osborn, 1993). Summer slump is associated with increased rectal temperatures, increased respiratory and heart rates, excessive salivation, and decreased average daily gain (ADG) in cattle (Jacobson et al., 1963; Rhodes et al., 1991), while lambs displayed varying rates of increased rectal temperatures (Aldrich et al., 1993; Gadberry et al., 2003) depending on the study. Increased rectal temperatures in affected animals are coupled with observed behaviors such as seeking shade and watering holes to cool themselves (Hemken et al., 1979; Bond et al., 1984; Rhodes et al., 1991). Recently, vasoconstriction has been posited as a causal link to "summer slump" due to the action of ergot alkaloids on α-adrenergic and serotonergic receptors (Dyer, 1993; Strickland et al., 2009; Foote et al., 2011; Strickland et al., 2011). A decrease in the ability of the individual to dissipate heat away from the body (due to vasoconstriction) would exacerbate the heat stress experienced during the warm months of the year (Burke et al., 2001b). Vasoconstriction therefore results in an inability of that individual to adequately thermoregulate, resulting in the signs of summer slump.

Decreased ADG is another aspect of summer slump that may be exacerbated by heat stress due to excessive vasoconstriction during summer months. Cattle may exhibit decreased feed intake when consuming E+ tall fescue, but only when ambient

temperatures are in excess of 32 °C (Burke et al., 2001b), an effect which is similar in mice (Larson et al., 1994). Lambs may exhibit up to 66% lower than normal weight gain when fed E+ tall fescue (Parish et al., 2003a), and feed intake is lower under heat-stress conditions (Rhodes et al., 1991). In livestock, the inability of an individual to cool itself predisposes it to decreased grazing during the day due to excessive shade seeking, and therefore decreased ADG (Aldrich et al., 1993; Thompson and Stuedemann, 1993). Still, other studies have not noted a temperature-dependent decrease in ADG (Boling et al., 1984), allowing for other speculation as to a causal link of the decrease in ADG (Thompson and Stuedemann, 1993).

Horses do not display the signs that are attributed to summer slump (Cross et al., 1995). Yearling geldings had decreased weight gains when fed E+ tall fescue (Aiken et al., 1993), but weight change was not seen in mature geldings (Redmond et al., 1991b) or mares (Monroe et al., 1988; Arns et al., 1997). Increased ease in sweating – and therefore cooling – is one of the proposed reasons why this sign is not observed in the horse (Putnam et al., 1991; Cross et al., 1995). Indeed, horses injected with intravenous ergot alkaloid exhibited increased sweating (Bony et al., 2001). As sweating is the primary means of heat dissipation in the horse (Scott et al., 2001), increased sweating while consuming E+ tall fescue may mitigate observations of summer slump in horses. Nevertheless, an alternative explanation for the mitigation of summer slump in horses may exist, as other studies have not observed increased sweating in horses ingesting E+ tall fescue (Webb et al., 2008; Parks, 2009).

"Fescue Foot" is Cold Stress and Vasoconstriction

A more striking clinical sign observed in livestock grazing E+ tall fescue is "fescue foot" (Garner and Cornell, 1978). This is commonly observed in cattle and sheep (Tor-Agbidye et al., 2001), and is associated with a dull hair coat, loss of tail switch, lameness, gangrenous tissue, and sloughing of the hoof and tips of ears, with extreme cases resulting in loss of the whole limb (Garner and Cornell, 1978; Schmidt and Osborn, 1993; Tor-Agbidye et al., 2001). Given the proper conditions – such as high alkaloid content of the pasture – livestock can become lame very quickly in cold weather. Cattle can become lame in as little as 18 days (Jensen et al., 1956), and sheep in 21 days (Tor-Agbidye et al., 2001). Sloughing of the hoof is commonly preceded by the formation of lesions above the coronary band. During the late fall/early winter season, the ingestion of E+ tall fescue hay can elicit lesions in as little as six weeks, with the tissue below the lesions eventually becoming gangrenous and sloughing of tissue occurs (Jensen et al., 1956).

Fescue foot was once simply hypothesized to have been caused by a peripheral vasoconstriction (Jensen et al., 1956; Yates et al., 1979). It was known that livestock ingesting E+ tall fescue and suffering from fescue foot exhibit blood vessels of the hoof which were congested, affected with thrombosis, and thickened cell walls and constricted lumens (Jensen et al., 1956), however no direct cause could be confirmed. Recent studies, however, have demonstrated a unique vasoconstrictive potential of the alkaloids produced by *Neotyphodium coenophialum* (Klotz et al., 2007; Strickland et al., 2009; Foote et al., 2011; Klotz et al., 2010; Klotz et al., 2011). Thus the vasoconstriction which occurs during cold weather in order to conserve body heat (Curtis, 1983), combined with

the vasoconstriction caused by the ergot alkaloids, can produce an excessive peripheral vasoconstriction resulting in fescue foot.

Horses, unlike sheep and cattle, do not exhibit the sloughing of any peripheral tissue, and fescue foot is not seen in horses grazing E+ tall fescue (Cross et al., 1995). It has been hypothesized that the incidence of laminitis will be increased in areas with the confirmed presence of E+ tall fescue (Rohrbach et al., 1995), however, studies elucidating other effects to E+ tall fescue on the horse have noted that laminitis was not an issue in treatment horses (Monroe et al., 1988; Putnam et al., 1991; Brendemuehl et al., 1994). Nevertheless, equine peripheral vasculature has been shown *in vitro* to be vasoconstrictive when stimulated with ergot alkaloids (Abney et al., 1993; Klotz and McDowell, 2010), and studies with Doppler ultrasound have confirmed that E+ tall fescue will indeed elicit a vasoconstrictive response *in vivo* (Moore et al., 2008; McDowell et al., 2009). Still, no direct evidence links laminitis and ingestion of E+ tall fescue. Additionally, carbohydrate overload during spring and summer exists as an alternative explanation for when there is a correlation between laminitis and E+ tall fescue consumption (Rohrbach et al., 1995; Johnson et al., 2010). Taken together, these data would indicate that while sound in theory, there is no causal link between laminitis and the ingestion of E+ tall fescue in the horse.

Reproduction in the Late Pregnant Mare

Reproductive effects incurred by late pregnant mares grazing E+ tall fescue are perhaps the best documented of all fescue toxicosis signs (Ball et al., 1987; Monroe et al., 1988; Putnam et al., 1991), as they are quite severe and not exhibited by other livestock

species. Prolonged gestation is well documented, with mares consuming E+ tall fescue prolonging gestation by 20-27 days (Monroe et al., 1988; Putnam et al., 1991; Redmond et al., 1994). This is likely the result of ergot alkaloid interference with a cascade of hormones, rather than with any one hormone alone. Mares suffering with prolonged gestation due to ingestion of E+ tall fescue often give birth to foals who are referred to as dysmature (Porter and Thompson, 1992), as they are carried past full term, abnormally large framed, and yet exhibit signs of being premature. Additionally, dysmature foals typically have poor muscling, long, fine coats, overgrown hooves, and irregular incisors. These large framed foals may be rotated 90 to 180 degrees from the normal dorsal-sacral presentation at parturition (Monroe et al., 1988; Porter and Thompson, 1992). Dysmature foals are thus captured in a parturition event for which neither they nor the maternal uterine environment is prepared. As such, up to 91% of mares grazing E+ tall fescue pastures may experience a dystocia, with only one out of 11 foals surviving past birth (Putnam et al., 1991).

Dystocia – or difficult births – can produce a range of ill consequences, ranging from cervical tears to death. Mares grazing E+ tall fescue during gestation often have placentas which are thickened, red in appearance, and retained longer compared to mares grazing E- tall fescue (Monroe et al., 1988). This form of dystocia is colloquially referred to as a "red bag" delivery due to the reddish hue observed of the placenta when it is expelled (Cross et al., 1995). In a normal parturition, the placenta (chorioallantois) is breached at the cervical star, then expelled inside-out after the fetus has been delivered (Ginther, 1992), revealing a shiny interior. However, in cases of fescue toxicosis in pregnant mares, the microcotyledon/microcaruncle unit of the chorioallantois

prematurely separates, and the placenta does not rupture as the fetus is expelled (Cross, 2011), and the blood-filled microcotyledon tufts give the placenta a red color. As such, red bag foals are often still encapsulated by the placenta after expulsion from the uterus (Cross, 2011). The resulting individuals have often been asphyxiated by the placenta and may succumb, only surviving with intensive human intervention. However, even with human intervention these foals are typically known to be unproductive.

Pregnant mares suffering from fescue toxicosis lack normal mammary gland development, and often exhibit decreased milk production – or a complete agalactia – post-parturition (Cross, 2011). Lack of mammary development before parturition is of principle concern to farm managers, as normal mares (*e.g.*, mares not grazing E+ tall fescue) will have noticeable mammary gland development three to six weeks before parturition (Ginther, 1992), indicating to the farm manager the need for increased observation. The lack of mammary development before parturition poses two problems to the farm manager. Of primary concern is the death of both mare and foal due to lack of proper observation at foaling. Indeed, it has been noted that most foals born to mares grazing E+ tall fescue will die (Putnam et al., 1991), and many of those parturition events will result in the death of the mare as well (Monroe et al., 1988; Putnam et al., 1991). However, if both mare and foal survive the parturition event, the next concern is of proper passive immunity passed to the foal through the mother's colostrum (Ginther, 1992). The same hormonal cascade that affects parturition (Ginther, 1992) also affects lactogenesis and galactopoesis (Freeman et al., 2000), and is interfered with when mares are grazing E+ tall fescue. The result of this agalactia is an insufficient supply of

colostrum, leaving the newborn foal with an increased susceptibility to disease due to lack of passive immunity transfer.

Fertility and Cyclicity

In livestock grazing E+ tall fescue, both fertility and early gestation have been problematic as well. Calving rates were decreased from 96% to 55% when cows grazed E+ tall fescue (Schmidt et al., 1986). A three year study revealed a similar observation, as cows grazing E- tall fescue exhibited a 94.6% calving rate vs. a 55.4% calving rate for cows grazing E+ tall fescue (Gay et al., 1988). Investigations into the cause of decreased pregnancy rates revealed that although heifers were cycling normally, corpus luteum (CL) function may have been impaired. Evaluation of corpora lutea from cycling heifers who had grazed E+ tall fescue pasture for 56 days revealed that large luteal cell number and the number of mitochondria within these cells were increased compared to corpora lutea from heifers grazing E- tall fescue (Ahmed et al., 1990). A further complication is involved when considering heat stress. Although heat stress alone may cause reduced diameter of the CL and reduced P4 when compared to thermoneutral conditions, the interaction of heat stress and E+ tall fescue compared to E- tall fescue significantly reduced P4 and the diameter of the dominant preovulatory follicle to a greater extent than heat stress alone (Burke et al., 2001b).

Horses may also have decreased fertility and early reproductive efficiency when grazing E+ tall fescue. Monroe et al. (1988) observed that gravid mares ingesting E+ tall fescue exhibited prolonged gestation, agalactia, dystocia, and the death of several foals. In addition, when the same mares were re-bred after parturition, mares still grazing E+

tall fescue pasture exhibited decreased pregnancy rates compared to mares still grazing E- tall fescue. However, Brendemuehl et al. (1994) found no differences in 60-day pregnancy rates between mares grazing E- and E+ tall fescue, although mares grazing E+ tall fescue exhibited increased interovulatory intervals, increased progesterone levels, and a tendency for increased early embryonic death. Subsequently, Arns et al. (1997) reported no differences between mares ingesting E+ and E- tall fescue seed, and suggested that one source of variability among the experiments may have been concentration of ergot alkaloid in treatment diets.

The effect of fescue toxicosis on early reproductive efficiency in horses is an area that may be of concern. As previously mentioned, many of the signs of fescue toxicosis may not be overtly severe, going undetected or being mistaken by producers, managers, and veterinarians (Strickland et al., 2011). However, with an economic impact of $102 billion dollars annually (American Horse Council Foundation, 2005), and with the many advances made in equine reproduction (McKinnon et al., 2011), any effect of fescue toxicosis on early reproductive efficiency in the horse could be costly to ignore. Current research in this area is not conclusive, which anecdotally allows many to still maintain that E+ tall fescue is a good forage for all horses except gravid mares.

Between-study Endophyte Variation: A Problem With A Solution

Before the commencement of a study, investigators should take care to identify sources of variation from previous studies. Fescue toxicosis trials can be challenging, as a collaboration among chemists, plant scientists, and animal scientists is often required. Thus the application of new findings – such as realizations of between-study variation or

advancements in methodology – may not be immediately applied to a new experiment. Between-study variations can include the obvious differences of species and breed, as well as the less obvious differences in method of delivery of ergot alkaloid, concentration of ergot alkaloids animals are exposed to, concentration of additional ergot alkaloids in the diet (if ergovaline is the primary ergot alkaloid), and differences between laboratory analyses of ergot alkaloid concentrations.

Within experiments, a homogenous population of animals may be desirable in elucidating the signs of fescue toxicosis that are not overtly severe, with special care taken to note that the concentration of ergot alkaloid required to reach toxicity is different for each species. Lambs have been shown to be susceptible to fescue toxicosis when ergovaline was present at a concentration of 640 μg ergovaline/kg total diet (Gadberry et al., 2003), while cows can be susceptible to fescue toxicosis when ergovaline is present 130-190 μg ergovaline/kg total diet (Mizinga et al., 1992; Burke et al., 2001b). A minimum concentration of alkaloids resulting in fescue toxicosis in horses has not been established, but gravid mares showed signs of fescue toxicosis when ergovaline was present at a concentration of 390 μg ergovaline/kg total diet (Putnam et al., 1991), while non-pregnant mares may exhibit altered interovulatory intervals and early embryonic death when ergovaline is present at a concentration of 1171 ng ergovaline/g fresh weight grass (Brendemuehl et al., 1994). Similarly, while breed data within species is not available for either lambs or horses, data in cattle suggests a difference in fescue toxicosis susceptibility between Angus and Romosinuano breeds (Burke et al., 2010).

With between species susceptibility to ergot alkaloids reaching orders of magnitude in difference, investigators must be certain that each treatment animal is

ingesting a precise amount of ergot alkaloid. Differences of method of delivery (injection, dry seed, or wet grass pasture) (Browning et al., 1998; Burke et al., 2001a; Burke et al., 2001b), amount of ergot alkaloid exposure (Brendemuehl et al., 1994; Arns et al., 1997), concentration of secondary ergot alkaloid in the diet (if ergovaline is considered to be the primary ergot alkaloid) (Klotz et al., 2008,2010; Foote et al., 2011), and differences between lab analyses of ergot alkaloid concentration (Yates et al., 1985; Rottinghaus et al., 1991; Craig et al., 1994) could result in experimental animals receiving unknown amounts of ergot alkaloids. Most of these parameters have the potential for wide variation, and should be as similar as possible within and between studies. Of particular concern are differences in laboratory analyses, which in some cases differ in orders of magnitude, ergot alkaloids isolated, and at times repeatability (K. J. McDowell, Personal Communication).

Given such tremendous sources of variation, a need for a standardized method of toxicity determination has arisen. Such a method would go beyond measurement of ergot alkaloid intake in treatment animals by providing real-time data on individual physiological responses to ergot alkaloid ingestion. Early reports observed that ergot alkaloids produced a vasoconstriction *in vitro* in equine vasculature (Abney et al., 1993; Klotz and McDowell, 2010); vasoconstriction has also been observed *in vivo* in cows using Doppler ultrasound (Aiken et al., 2007). Recently, a model published from this lab (Moore et al., 2008; McDowell et al., 2009) demonstrated that horses fed ground E+ tall fescue seed exhibited a marked decrease in diameter, area, and circumference of the left palmar artery, as measured by Doppler ultrasound. This model – dubbed the McDowell Model –may be able to provide investigators with a satisfactory bioassay for real-time

measurement of signs fescue toxicosis in an individual exposed to E+ tall fescue. Use of the McDowell Model may allow future researchers to establish standards for baseline toxicity in equine fescue toxicosis research.

Summary

Estrous cycle manipulation and tracking is an important facet of the equine breeding industry, with the incorrect prediction of an individual mare's ovulation costing the producer valuable time within the breeding season. Equine reproduction is an area of tremendous economic concern, but previous studies on the effect of E+ tall fescue on equine fertility and cyclicity have been inconclusive. An assessment of the literature reveals that current methods of determining fescue toxicosis in horses are insufficient, as even baseline levels of toxic intake have not been established. Attempts at conclusive investigation into the effect of E+ tall fescue on equine early reproductive efficiency must include satisfactory determinations of toxicity levels in treatment animals through a real-time bioassay. A recent approach to the establishment of a real-time measurement of the premonitory signs fescue toxicosis has been the McDowell Model, which measures the established vasoconstrictive response to E+ tall fescue using Doppler ultrasound. The objective of this thesis is to explore the effect of fescue toxicosis on certain parameters of the estrous cycle, including differences in interovulatory interval, serum concentrations of PRL, P4, and E2, and blood flow to the CL, while simultaneously using the McDowell Model to assess levels of toxicity in treatment mares.

CHAPTER THREE: Ingestion of Endophyte-Infected Tall Fescue Seed Induces Peripheral Vasoconstriction but Does Not Affect Cyclicity in Non-Pregnant Mares

Introduction

After its discovery in 1931on a Menifee Co., KY farm, tall fescue (*Lolium arundinaceum*) was adapted for use as a forage with the distinguishing characteristics of dependability, resistance to grazing stress, and resistance to insect feeding (Buckner et al., 1979). These characteristics allowed this cultivar of tall fescue, now called Kentucky 31 (KY31), to propagate very successfully across much of the Southeastern United States. Current estimates suggest that the KY31 cultivar of tall fescue occupies (or grows on) between 14 and 19 million hectares of land in the United States (Bacon, 1995; Bouton, 2007; Hannaway et al., 2009). The hardiness of KY31 is due to a symbiotic relationship with an endophytic fungus *Acremonium coenophialum* (now *Neotyphodium coenophialum*) and the alkaloids it produces (Glenn et al., 1996).

Although the symbiosis of *N. coenophialum* is beneficial to the tall fescue plant, the ergot alkaloids produced elicit detrimental health effects in horses and other livestock which ingest it. Late pregnant mares grazing endophyte infected (E+) tall fescue may suffer from prolonged gestation, altered hormone concentrations, agalactia and dystocia, and death of the mare and/or foal (Monroe et al., 1988; Redmond et al., 1991a). These detrimental health effects are reduced in severity – but not eliminated –with the administration of the dopaminergic D2 receptor (DRD2) antagonists (Redmond et al., 1991a; Redmond et al., 1994; Jones et al., 2003). Thus, the ergot alkaloid agonism of DRD2 is thought to play a significant role in equine fescue toxicosis.

Although a small number of studies have been performed with equine fescue toxicosis on cyclicity and early reproduction in mares, the results have differed. Brendemuhl et al. (1994) reported prolonged luteal phases and increased plasma progesterone (P4) levels in non-pregnant mares grazing E+ tall fescue (1171 ng ergovaline/g wet grass). However, Arns et al. (1997) observed that serum concentrations of P4, luteal phase duration, and interovulatory intervals were not changed, concluding that the concentration of ergovaline (the primary ergopeptine ergot alkaloid in E+ tall fescue) in treatment diets (up to 308 μg ergovaline/kg total diet) was insufficient to produce any detrimental effects. Studies in other species involving the effects of E+ tall fescue on fertility and cyclicity suggest that this issue warrants further study. Cows grazing E+ tall fescue exhibited decreased pregnancy rates (Gay et al., 1988; Brown et al., 1992), while interovulatory intervals were increased (Seals et al., 1996). Decreased concentrations of prolactin (PRL) (Porter and Thompson, 1992) and P4 (Mahmood et al., 1994) have also been reported in cattle grazing E+ tall fescue.

One potential aid in determining the effects of E+ tall fescue on animal health is the establishment of a satisfactory *in vivo* bioassay capable of determining early signs of fescue toxicosis. Recent reports from this lab (McDowell et al., 2009; Moore et al., 2008) indicate that ingestion of E+ tall fescue seed causes a significant constriction of the palmar artery (PA) which is readily observed by Doppler ultrasonography. Such reports are consistent with reports of vasoconstriction in cattle (Aiken et al., 2007). This model may be able to provide investigators with a satisfactory bioassay for determining the premonitory signs of fescue toxicosis in an individual exposed to E+ tall fescue.

The objective of this research study was to determine whether mares ingesting E+ tall fescue seed would exhibit altered interovulatory intervals, altered serum hormone concentrations, and altered blood flow to the corpus luteum (CL) compared to when the same mares were ingesting endophyte-free (E-) tall fescue seed. In addition, whether ingestion of E+ fescue seed caused constriction of the palmar vein (PV) as well as the PA was examined. The McDowell model of assessing vasoconstriction of the PA (Moore et al., 2008; McDowell et al., 2009) was used to observe the physiological signs of fescue toxicosis, allowing confirmation that the concentration of ergot alkaloids in the treatment diets was high enough to elicit a measurable biological response.

Materials and Methods

Animals

Animal protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee. This study was conducted from May through September, 2011, using 12 non-pregnant, normally cycling mares of mixed breed, ranging from age 4 to 20 years (with three older mares of unknown age used).

Diet

All mares were kept on low endophyte pasture (E+ tall fescue was 4% of available forage, with 19 μg ergovaline /kg available forage) and given *ad libitum* access

to water. Prior to the beginning of the experiment, mares were acclimated to the experimental diet over one interovulatory period. During this period, mares were acclimated to diet bulk with slowly increasing amounts of a grain mixture (Appendix A) until 0.3% of BW (average BW was 513 kg) was reached, and slowly increasing amounts of E- fescue seed (Kentucky 32 tall fescue seed) until 0.3% of BW was reached. Grain mixture and fescue seed were mixed together and top-dressed with molasses to increase palatability. During treatment periods, all mares were fed 0.3% of BW of either E+ or E- fescue seed per day, with E+ fescue seed (Kentucky 31 tall fescue seed) containing 6.5 mg total ergovaline (ergovaline + ergovalinine) per kg seed, and E- fescue seed containing undetectable levels of total ergovaline, as measured by high-performance liquid chromatography using the procedure of Yates (1988) as modified by Bush (Yates and Powell, 1988; Aiken et al., 2009). The E+ diet resulted in animals being fed 19.5 μg ergovaline + ergovalinine per kg BW, or an average of 10 mg per day. All seed was ground by passing it through a hammer mill prior to feeding.

Experimental Design

A 2x2 crossover experimental design with repeated measures was implemented, such that each period (P) of the 2x2 crossover was the duration of one estrous cycle (from ovulation, Day 0, to the next ovulation). A washout period was inserted between P1& P2, where Lutalyse (10mg) was given on Day 6 so that each mare's washout period lasted a minimum of 11 days (McCann et al., 1992). Mares were randomly assigned to a sequence of E+ E- or of E- E+ . Treatment E+ was defined as E+ fescue seed, while treatment E-

was defined as E- fescue seed, the control treatment. All mares were removed from treatment at the conclusion of P2 , and observed for 12 days before removal from the experiment.

Data Collection

For each mare in the study, the parameters of blood flow through the CL and blood vessel measurements were recorded. For PA and PV, diameter (mean of long and short diameters of the elliptical vessels), area, circumference and resistivity index (RI; calculated by (Peak Systolic Velocity - End Diastolic Velocity) / Peak Systolic Velocity) were measured. Ultrasound images of the CL were recorded every other day, except for once per day around the time of ovulation. During these exams, images of CL blood flow were obtained using the Doppler Power setting on the ultrasound machine (Titan ultrasound, SonoSite Inc, Bothwell, WA, with a L52/10-5 MHz linear array probe). Measurements of the PA and PV were assessed every fourth day, and on the day of ovulation, using the Pulsed Wave setting of the ultrasound machine (C11/8-5 MHz microconvex array probe). Blood samples were collected via jugular venipuncture every other day, except for once per day around the time of ovulation. Blood samples were collected in non-heparinized tubes, and serum was separated by centrifugation and stored at -20 ^{0}C for assay for PRL, P4, and estradiol (E2) (radioimmunoassays, courtesy of Dr. Don L. Thompson, Louisiana State University). Interovulatory interval was defined as the time interval (days) between observed ovulations, and each of these was recorded for future analysis of the effect of E+ tall fescue on interovulatory interval. Because not all

interovulatory intervals were the same, all data were truncated at day 16 after the first ovulation, then normalized to the second ovulation for analysis.

CL Blood Flow Image Analysis

Corpus luteum blood flow was assessed using images taken with the Titan ultrasound machine. During image capture, gray scale and color images were obtained on a vertical plane from the apex to the base of the CL, designated as the overall image, and area was assessed. After the animal work was completed, images were quantitatively analyzed using ImageJ, v 1.44, developed by the US National Institutes of Health (Rasband, 1997), to determine the area of the blood flow through the CL. Prior to image assessment, images were selected that contained no flash or color artifacts. If no such image was obtained, artifacts were removed from the image such that only color immediately surrounding the CL remained. After the image was loaded into ImageJ, the linear calibration was set for each image using the known distance scale given by the ultrasound. The color portion of the picture was then separated out by using the "HSU stack" option, stacking to images, and adjusting the threshold until only the region of interest was selected. Finally, blood flow area was reported using the "analyze particles" utility in ImageJ. A quantitative index of the relative amount of blood flow through the luteal tissue was created by taking the ratio of the colored area to a sectional plane at the maximum diameter of the CL, as previously described (Acosta et al., 2002). If two CLs were present, both ratios were added together such that the total area ratio was analyzed. Only total area ratios were included in the final analysis.

Statistical Analyses

Interovulatory interval, peak serum concentrations of P4 (averaged over days 4-12 of each period), and number of days which P4 concentration was > 1ng/ml (days in diestrus) were analyzed as a two-period, two-treatment crossover design (Model 3.1, Appendix B). Blood vessel measurements (mean diameter, circumference, area, and RI of both the PA and PV), CL blood flow, and blood hormone concentrations (PRL, P4, and E2) were analyzed as a two-period, two-treatment crossover design with repeated measures. To more accurately reflect treatment differences at specific times within the estrous cycle, serum concentrations of PRL, P4, and E2 were analyzed for each period separately, and were combined across periods if period effects were not significant. In addition, serum concentrations of P4 were analyzed when concentrations were >1ng/ml, and serum concentrations of E2 were analyzed when P4<1ng/ml. The repeated measure of time was each measurement day in a treatment period. This model (Model 3.2, Appendix B) includes an analysis of sequence effect (E+ E- vs. E- E+). Because of the design, assumptions of no carryover effect and no treatment-by-period effect must be made, as the two terms are aliased with the sequence effect (Wallenstein and Fisher, 1977). Therefore, a significant sequence effect will indicate a significant treatment-by-period and carryover effect as well. If sequence effects were significant, then only data from P1 was considered for statistical inferences. All data were analyzed using the MIXED procedure of SAS (SAS Institute, 2010) v. 9.2, where effects were analyzed for significance using Fishers exact test. Individual tests for within day differences of means were analyzed using a two-sided t test for differences in least square means.

Results

Two of the twelve mares did not ovulate during the last period of the experiment, one from each treatment group; therefore their data were dropped from the study. Outliers and leverage points were considered influential if their Cooks D values differed appreciably from the totality of the other values (Kuehl, 2000). Although influential observations may be physiologically normal, the frequency of data collection for this study is not in keeping with classical descriptive studies (Townson et al., 1989), thus the inclusion of influential observations in this data analysis may skew results. Three observations each were identified as influential leverage points and dropped from the analysis of serum PRL and serum E2. Results are summarized in Table 3.1 - Table 3.3. Palmar vein lumen diameter, circumference, and area had significant sequence effects. As such, only data from the analysis of P1 is presented for those parameters.

Table 3.1. Statistical analysis of the hormones prolactin (PRL), progesterone (P4), and estradiol (E2) for each period.[a]

Parameter	Effect	P-Value	Parameter	Effect	P-Value
PRL			*P4*		
	Treatment	0.0005		Treatment	0.1222
	Period	0.0025		Period	0.4929
	Sequence	0.2611		Sequence	0.1214
	Time	0.0451		Time	<0.0001
	Period x Time	0.3223		Period x Time	0.9991
	Sequence x Time	0.9795		Sequence x Time	0.9283
	Treatment x Time	0.6978		Treatment x Time	0.9477
E2			*P4 > 1 ng/ml*		
	Treatment	0.5603		Treatment	0.1409
	Period	0.2584		Period	0.3264
	Sequence	0.1045		Sequence	0.1180
	Time	<0.0001		Time	0.0004
	Period x Time	0.7599		Period x Time	0.9864
	Sequence x Time	0.4309		Sequence x Time	0.9145
	Treatment x Time	0.3443		Treatment x Time	0.7259
E2 (P4<1ng/ml)			*P4 Peak*		
	Treatment	0.9070		Treatment	0.0568
	Period	0.9436		Period	0.8345
	Sequence	0.1126		Sequence	0.0720
	Time	0.0302			
	Period x Time	0.6394			
	Sequence x Time	0.9262			
	Treatment x Time	0.5289			

[a] Blood hormone concentrations were additionally analyzed to more accurately reflect treatment differences at specific times within the estrous cycle, such that P4 was analyzed when P4 values were > 1 ng/ml, and E2 was analyzed when P4 values were < 1 ng/ml.

Table 3.2. Statistical analysis of the lumen diameter, circumference, area, and resistivity index (RI), for the palmar artery (PA), and palmar vein (PV).

Parameter	Effect	P-Value	Parameter	Effect	P-Value
PA Lumen Diameter			*PV Lumen Diameter*		
	Treatment	<0.0001		Treatment	<0.0001
	Period	0.2110		Time	0.1138
	Sequence	0.0830		Treatment x Time	0.0493
	Time	0.0352			
	Period x Time	0.8744	*PV Circumference*		
	Sequence x Time	0.5459		Treatment	<0.0001
	Treatment x Time	0.0063		Time	0.0723
				Treatment x Time	0.1600
PA Circumference					
	Treatment	<0.0001	*PV Area*		
	Period	0.3814		Treatment	<0.0001
	Sequence	0.0567		Time	0.1966
	Time	0.1647		Treatment x Time	0.3488
	Period x Time	0.8003			
	Sequence x Time	0.6495	*PV RI*		
	Treatment x Time	0.0081		Treatment	0.2850
				Period	0.3553
PA Area				Sequence	0.4838
	Treatment	<0.0001		Time	0.7879
	Period	0.2896		Period x Time	0.7298
	Sequence	0.0825		Sequence x Time	0.4757
	Time	0.1618		Treatment x Time	0.1082
	Period x Time	0.7646			
	Sequence x Time	0.6015			
	Treatment x Time	0.0117			
PA RI					
	Treatment	<0.0001			
	Period	0.1417			
	Sequence	0.7883			
	Time	0.0881			
	Period x Time	0.9210			
	Sequence x Time	0.7885			
	Treatment x Time	0.0087			

Table 3.3. Statistical analysis of the corpus luteum (CL) blood flow (relative to CL area), interovulatory inverval (IOI), and number of days when progesterone (P4) was > 1 ng/ml.

Parameter	**Effect**	**P-Value**
CL Blood Flow (Relative to CL Area)		
	Treatment	0.0350
	Period	0.1724
	Sequence	0.6044
	Time	<0.0001
	Period x Time	0.1529
	Sequence x Time	0.4995
	Treatment x Time	0.8103
IOI		
	Treatment	0.1142
	Period	0.876
	Sequence	0.4436
Days When P4 was > 1ng/ml		
	Treatment	0.2688
	Period	0.083
	Sequence	0.1518

Ingestion of E+ tall fescue seed caused significant decreases in PA and PV lumen diameters (Figures 3.1 and 3.2, respectively), and PA circumference, area, and RI over time, with only a tendency for significant sequence effects in these parameters. Once the sequence effect was taken into account, there were no significant treatment differences over time for the PV circumference, area, and RI. Treatment with E+ tall fescue seed decreased mean CL blood flow when compared to E- tall fescue seed, and CL blood flow was different by time (Table 3.3 and Figure 3.3). However, the treatment x time interaction indicated that there were no significant differences between treatments over time (Figure 3.4). In addition, when mares ingested E+ tall fescue seed, interovulatory intervals were not significantly altered (E+: 20. ± 0.4; E-: 19 ± 0.6) and the number of days when P4 was > 1 ng/ml were not significantly altered (E+: 12.6 ± 0.43; E-: 11.8 ± 0.61).

Analysis of serum concentrations of PRL treatment means showed that E- and E+ (6.09 ± 0.42 and 4.12 ± 0.31 ng/ml, respectively) treatment groups were different. Period and time effects were also significant when mares ingested E+ tall fescue seed. However, serum concentrations of PRL were not continually suppressed by treatment over time (Figure 3.5). Serum concentrations of P4 (Figure 3.6) and E2 (Figure 3.7) did not have significant treatment by time interactions, and means of P4 and E2 were not different between treatments. Additional analysis of serum concentrations of E2 when serum concentrations of P4 were < 1 ng/ml serum concentrations of P4 when concentrations of P4 were > 1 ng/ml revealed that there were no significant differences in treatment means, or between treatments over time. However, peak P4 values tended to increase when mares were fed E+ tall fescue. Review of the data suggested that this may have been

affected by mares who double ovulated during treatment periods. When mares that double ovulated were excluded from analysis, there was no effect of treatment on peak serum concentrations of P4 (Figure 3.7; $P = 0.5524$).

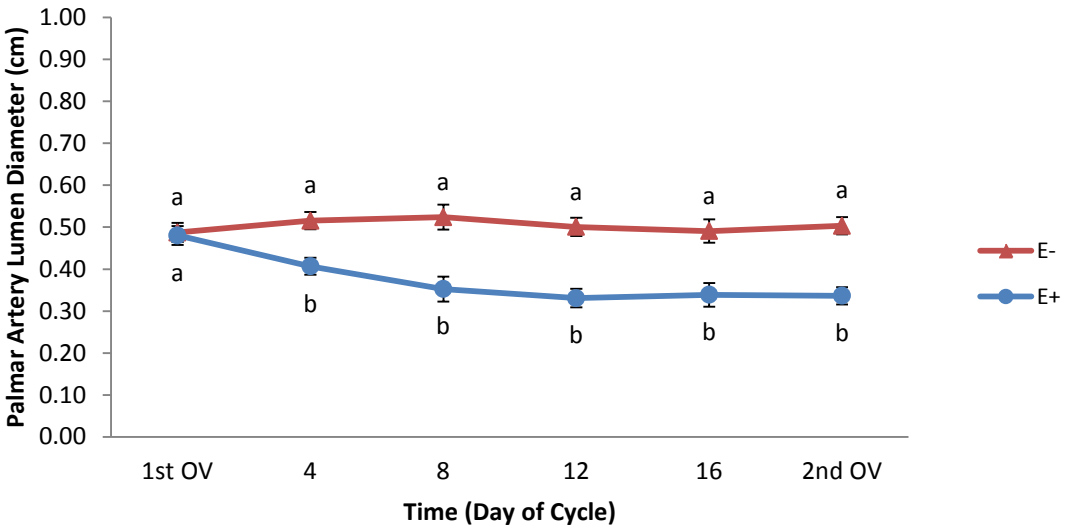

Figure 3.1. Comparison of palmar artery lumen diameters between treatments

Palmar artery (PA) lumen diameter (cm; lsmean ± SEM) measured over the days of the estrous cycle. Since there was no period effect, P1 & P2 were combined. Treatments within the same day that have different letters are different ($P < 0.05$).

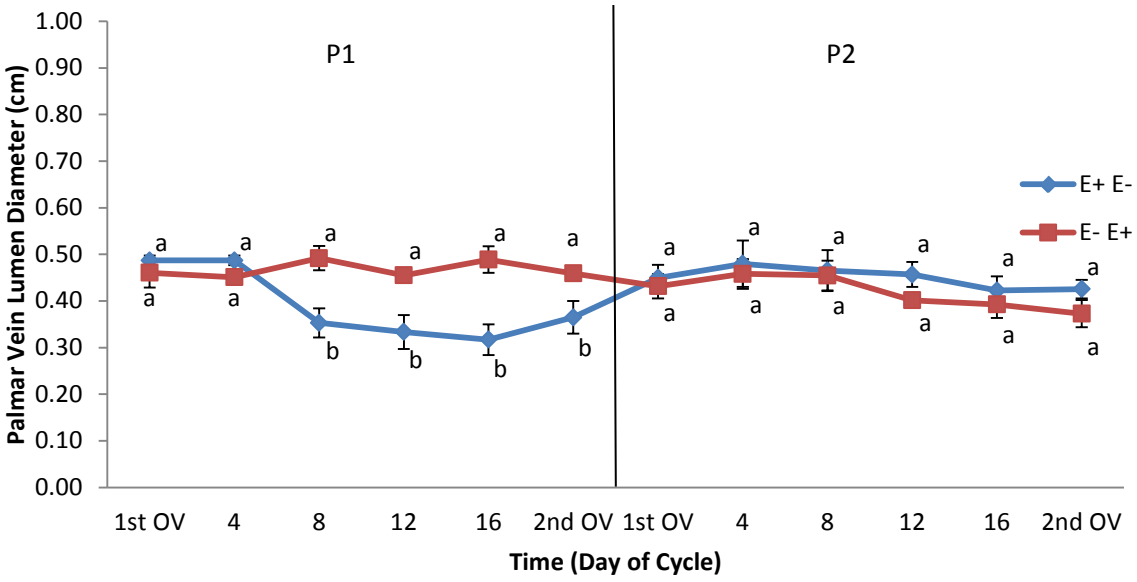

Figure 3.2. Comparison of palmar vein lumen diameters between treatments by period

Palmar vein (PV) lumen diameter (cm; lsmean ± SEM) measured over the days of the estrous cycle. Since there was a significant period effect, and a significant sequence effect, P1 & P2 are displayed separately. Treatments within the same day that have different letters are different ($P < 0.05$).

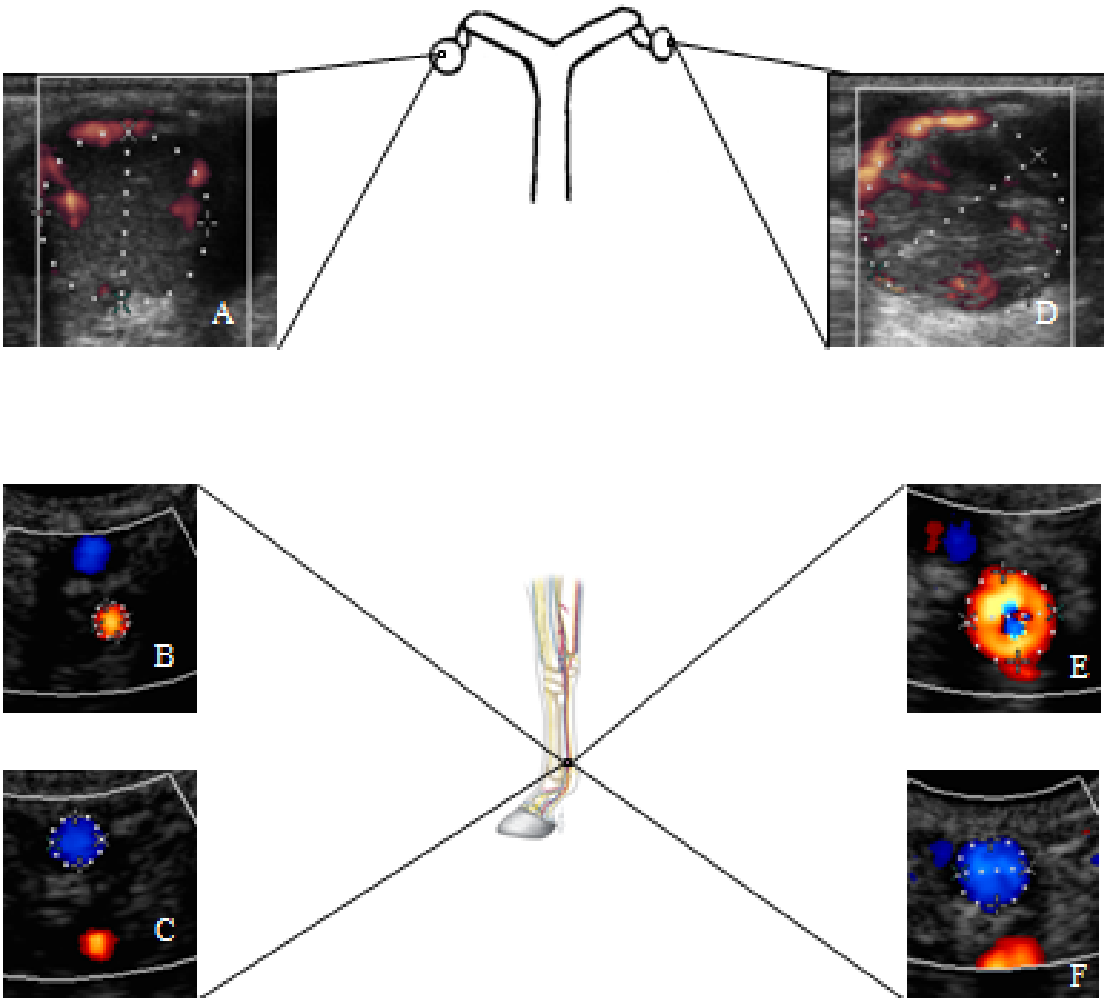

Figure 3.3. Schematic of blood flow differences within one non-pregnant mare when ingesting endophyte-infected (E+) vs. endophyte-free (E-) tall fescue seed

Frames A, B, and C represent the corpus luteum (CL) blood flow, palmar artery (PA) lumen diameter, and plamar vein (PV) lumen diameter (respectively) observed in one mare on Day 8 of her estrous cycle who had been ingesting E+ tall fescue seed since the beginning of that estrous cycle. Frames D, E, and F represent the CL blood flow, PA lumen diameter, and PV lumen diameter (respectively) observed within that same mare on Day 8 of her estrous cycle who had been ingesting E- tall fescue seed since the beginning of that estrous cycle. Total area ratios in frames A and D are 3.78 and 6.26, respectively. Short and long diameters for PA and PV were averaged together, and in frames B, C, E, and F, are 0.33 cm, 0.35 cm, 0.58 cm, and 0.45 cm, respectively.

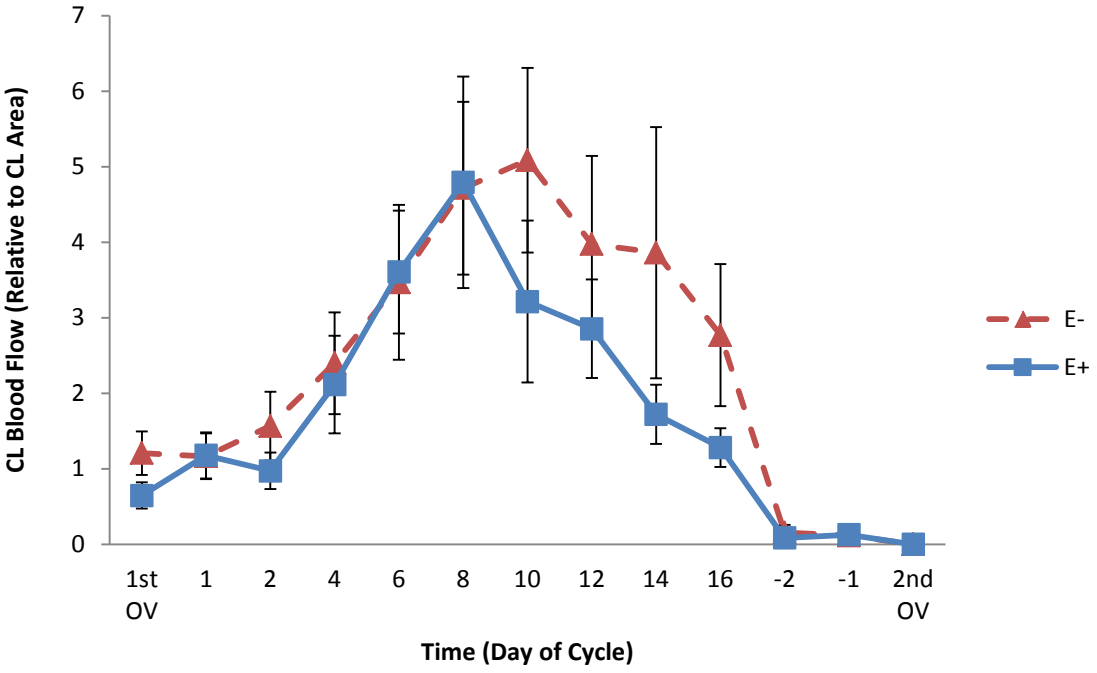

Figure 3.4. Comparison of CL blood flow between treatments

Corpus luteum (CL) blood flow (relative to CL area) (lsmean ± SEM) measured over the days of the estrous cycle. Since there was no period effect, P1 & P2 were combined. There was no treatment x time interaction, so no within-day comparisons were made.

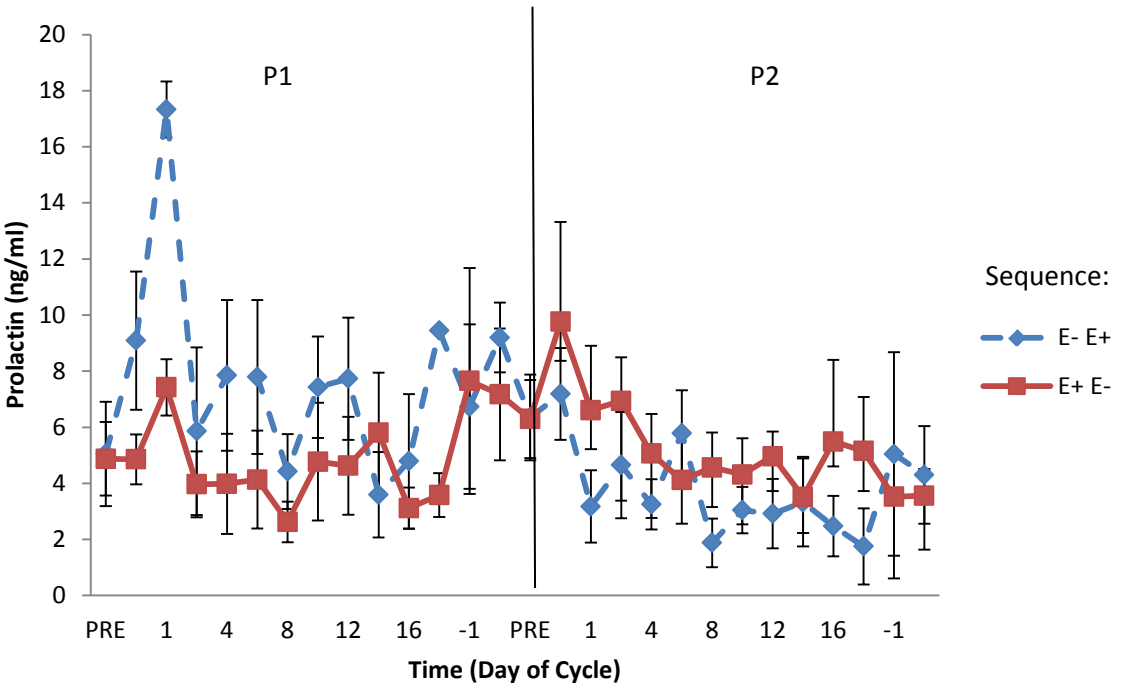

Figure 3.5. Comparison of serum concentrations of prolactin between treatments by period

Serum concentrations of prolactin (PRL; ng/ml; lsmean ± SEM) measured over the days of the estrous cycle. Data collected during the acclimation period was averaged together and is shown as "PRE" on the graph. Since there was a significant period effect, P1 & P2 are displayed separately. There was no treatment x time interaction, so no within-day comparisons were made.

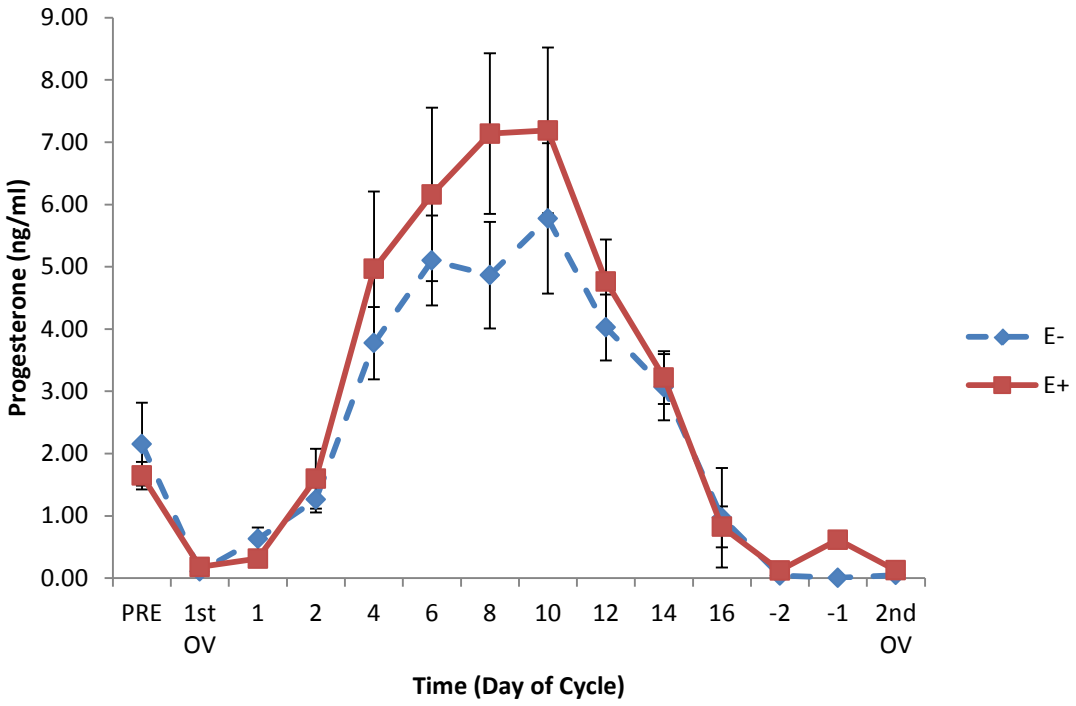

Figure 3.6. Comparison of serum concentrations of progesterone between treatments.

Progesterone (ng/ml; lsmean ± SEM) measured over the days of the estrous cycle. Since there was no period effect, P1 & P2 were combined. Data collected during the acclimation period was averaged together and is shown as "PRE" on the graph. There was no treatment x time interaction, so no within-day comparisons were made.

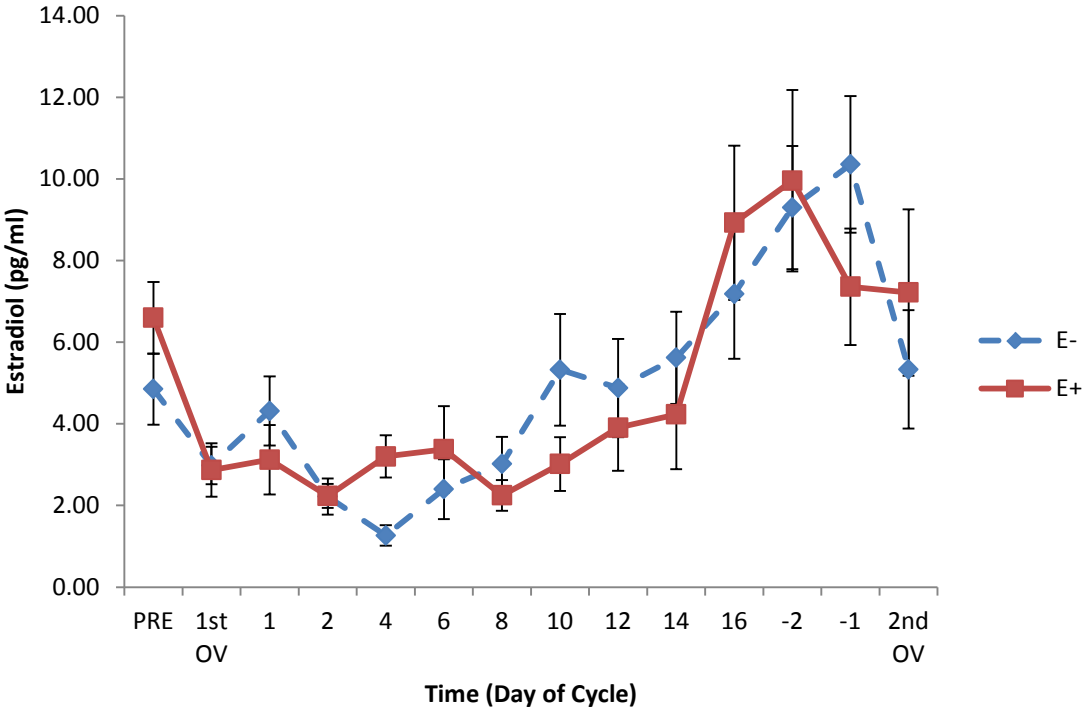

Figure 3.7. Comparison of serum concentrations of estradiol between treatments.

Estradiol (pg/ml; lsmean ± SEM) measured over the days of the estrous cycle. Since there was no period effect, P1 & P2 were combined. Data collected during the acclimation period was averaged together and is shown as "PRE" on the graph. There was no treatment x time interaction, so no within-day comparisons were made.

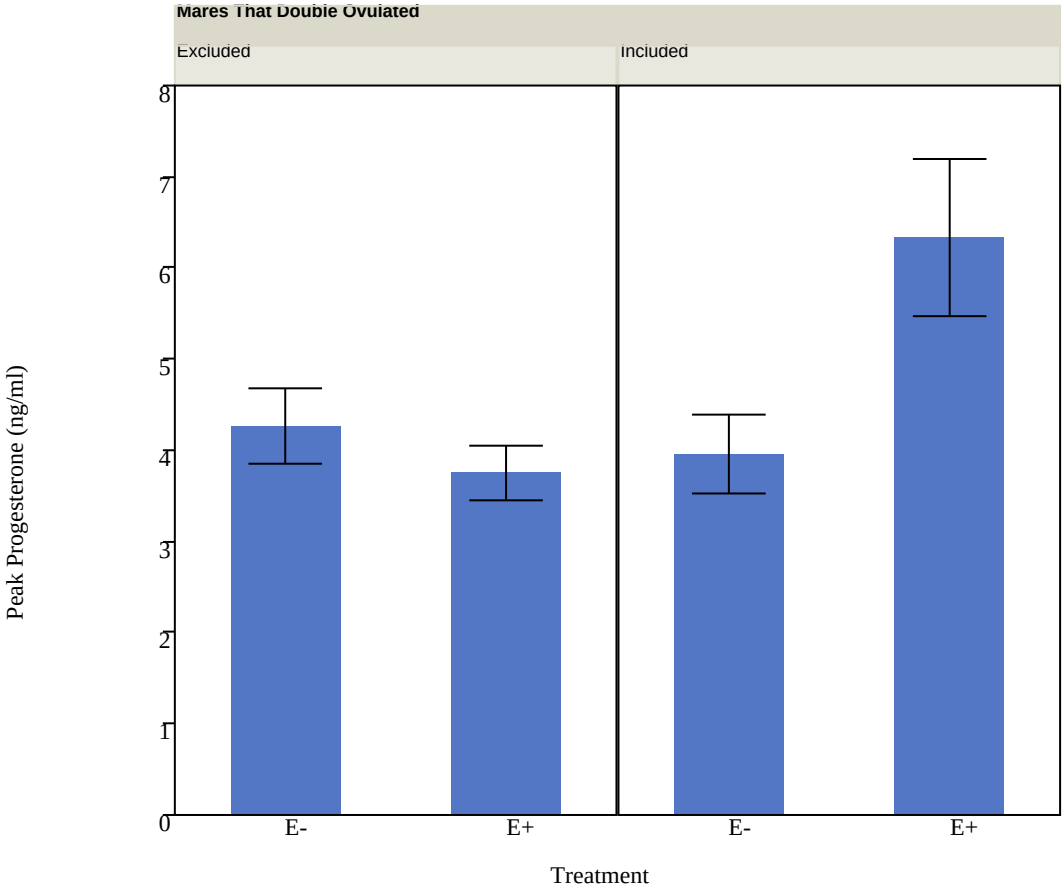

Figure 3.8. Peak serum progesterone (ng/ml; lsmean ± SEM) concentrations when mares that double ovulated were included or excluded.

When peak serum progesterone concentrations included mares who double ovulated, treatment means for mares who consumed E+ tall fescue seed tended (P = 0.0568) to be increased compared to treatment means for mares who consumed E- tall fescue seed. However, when peak serum progesterone concentrations excluded mares who double ovulated, there was no difference in treatment means (P = 0.5524).

Discussion

Effects of E+ tall fescue on non-pregnant mares have been hypothesized to include increased interovulatory interval, increased serum concentrations of P4, and decreased serum concentrations of PRL. These effects, however, are inconsistent, and are represented by conflicting reports in the literature (Monroe et al., 1988; Brendemuehl et al., 1994; Arns et al., 1997). While some suggest that the interovulatory interval, and therefore serum concentration of P4, is increased in mares grazing E+ tall fescue (Brendemuehl et al., 1994), others have failed to confirm this finding (Arns et al., 1997). A variety of deleterious early reproductive effects are observed when cattle graze E+ tall fescue (Bond and Bolt, 1986; Mahmood et al., 1994), including decreased pregnancy rates and increased interovulatory intervals (Seals et al., 1996). Although the 14 day viable pregnancy rates tended to be lower and early embryonic death rates tended to be higher in mares grazing E+ tall fescue, pregnancy rates after 60 day are not significantly different when comparing mares grazing E+ and E- tall fescue (Brendemuehl et al., 1994). The objective of this current study was to elucidate the changes related to the estrous cycle in the non-pregnant mare ingesting E+ tall fescue.

Mares ingesting E+ tall fescue seed did not exhibit decreased serum concentrations of PRL over time when compared to mares ingesting E- tall fescue seed. Additionally, when mares ingested E- tall fescue seed, mean serum concentrations of PRL for the non-pregnant mares in this study (6.09 ± 0.42 ng/ml) are similar to previously reported serum concentrations of PRL (7.0 ± 1.0 ng/ml) (Thompson et al., 1986). There was considerable within and between animal variability, as evidenced by the large standard errors associated with the PRL data. In addition, PRL concentrations

in non-pregnant mares are considerably lower than in non-pregnant cattle (typically < 10 ng/ml for mares (Johnson, 1986; King et al., 2008) and 100-200 ng/ml for cattle (Aiken et al., 2007; Aiken et al., 2009), and these lower values may make it more difficult to demonstrate a reduced concentration of PRL due to E+ fescue seed. Finally, serum PRL differs somewhat over the estrous cycle and with season in non-pregnant mares (Johnson, 1986; King et al., 2008), and in these experiments not all mares consistently ate all of their ration, both of which might have added to the variability of PRL in these studies.

Mean serum concentrations of PRL for non-pregnant mares may be different from serum concentrations of PRL in late pregnant mares grazing both E- (51.6 ± 4.50 ng/ml) and E+ (16.1 ± 3.81 ng/ml) tall fescue pastures (Monroe et al., 1988). When Monroe et al. (1988) reported PRL concentrations from pregnant mares in mid-gestation grazing E+ vs. E- pastures, the values reported for the E+ mares were similar to those reported here. This suggests that, in pregnant mares, instead of decreasing serum concentrations of PRL, the ingestion of E+ tall fescue suppresses the increase in serum concentrations of PRL that occurs in late gestation. Thus, a suppression of serum concentrations of PRL over time below normal serum concentrations in non-pregnant mares may be difficult. Other studies investigating the effects of E+ tall fescue on serum concentrations of PRL in non-pregnant mares (Arns et al., 1997) found significant differences in serum concentrations of PRL between treatments. However, the differences in PRL observed by Arns et al. (1997) were not analyzed over time, as were the treatment differences in this study. This suggests that the differences reported by Arns et al. (1997) may have ignored the subtle daily changes of PRL through the estrous cycle. This hypothesis is supported by the

observation that, in this study, mean serum concentration of PRL did not differ over time, even though the overall means of the two treatments were different.

Serum concentrations of E2 and P4 differed significantly over time ($P < 0.0001$ and $P < 0.0001$, respectively), yet the cyclic nature of these hormones during the estrous cycle suggests that this is to be expected. Thus, the treatment x time interaction effect was used to reveal that the ingestion of E+ tall fescue seed did not significantly alter serum concentrations of P4 or E2. Still, the analysis of treatment effects suggested that the ingestion of E+ tall fescue seed tended to increase serum concentrations of P4 across each of the three methods of analyzing P4. However, the removal of mares who double ovulated nullified this tendency. These results differ from those reported by Brendemuehl et al. (1994), but agree with those reported by Arns et al. (1997). Data reported by Brendeuehl et al. (1994) suggested that the increase in serum concentrations of P4 were due to increased cycle lengths. Data reported by Arns et al. (1997), however, suggested that serum concentrations of P4 in mares grazing E+ tall fescue seed were not different from previously reported serum concentrations of P4 in mares grazing E- tall fescue. However, the mention of mares who double ovulated was not included by either of these studies. In this study, the removal of mares who double ovulated resulted in treatment effects which were not different. This reveals the dynamic nature of hormones during the estrous cycle, and should be taken into account in future studies.

Interovulatory intervals were unchanged over two cycles in mares ingesting E+ tall fescue seed. Thus, the lack of changes in P4 and interovulatory intervals do not agree with the results reported from Brendemuehl et al. (1994). This discrepancy could result from differences in ergot alkaloid concentration within treatment diets. Brendemuehl et

al. (1994) reported an ergovaline concentration of 1171 ppb in E+ tall fescue pastures, while Arns et al. (1997) reported a maximum of 308 ppb ergovaline concentration in their diets. Arns et al. (1997) also estimated that mares in the that Brendemuehl study may have consumed close to 30 mg of ergovaline/day, while their own mares consumed only up to 2.8 mg ergovaline/day. In grazing studies, however, it is not possible to know the precise amount of forage consumed, and the alkaloid concentrations in the forages may change over time.

Mares fed the treatment diet in this study were fed E+ tall fescue seed with a concentration of 6500 ppb total ergovaline (ergovaline + ergovalinine), giving an average consumption of 10 mg ergovaline/day. This amount is five times higher than that reported by Arns et al. (1997), but only one-third the amount potentially consumed in the Brendemuehl et al. (1994) study. The primary implication of this observation is that the 1171 ng/g ergovaline in the pastures that are 90% endophyte infected in the Brendemuehl study may not represent typical pasture conditions on commercial horse farms. This concentration is over ten-fold higher than the concentrations fed by Arns et al. (1997) and three times higher than this study. Thus, it is possible that the concentration of ergovaline fed to treatment mares in this study was not high enough to elicit changes similar to those seen by Brendemuehl et al. (1994).

Peripheral vasoconstriction in non-pregnant mares was significantly increased over time by the ingestion of E+ tall fescue seed, as measured by the PA and PV lumen diameter, and PA area, circumference, and RI. This agrees with and expands upon previous reports from this lab (Moore et al., 2008; McDowell et al., 2009), which only noted decreases in PA lumen diameter, area, and circumference when mares were fed E+

tall fescue seed. As of yet, the vasoconstrictive effects of E+ tall fescue in horses have not been shown to produce the same deleterious effects seen in cattle (Porter and Thompson, 1992; Strickland et al., 2011). However, the anatomical and physiological differences between horses and other species may allow the horse to overcome some of the detrimental effects of vasoconstriction induced by the ingestion of E+ tall fescue.

Even at a lower concentration of ergovaline consumed by mares in this study (compared to those fed by Brendemuehl et al. (1994)), vasoconstriction still occurred. Thus, peripheral vasoconstriction may serve as a more sensitive indicator of a non-pregnant mare's exposure to E+ tall fescue than serum concentrations of PRL. Furthermore, vasoconstriction of the PA may be a more sensitive indicator of a non-pregnant mare's exposure to E+ tall fescue than vasoconstriction of the PV. The sequence effects in the PV lumen diameter, area, and circumference indicate that, rather than an insufficient washout period, mares ingesting E+ tall fescue seed during P2 did not respond in the same vasoconstrictive manner as mares ingesting E+ tall fescue seed during P1. In addition, the significant vasoconstrictive response observed in the PA indicates that the PV response is not due to an insufficient intake of E+ tall fescue seed, but could be related to other parameters not measured in this study. Thus, since sequence effects were not observed in any of the blood hormone, CL blood flow, or PA parameters measured, the response of the PV may need to be evaluated separately from other parameters during future *in vivo* research in order to account for and further describe this between subject variability.

Further observations indicate that while non-pregnant mares may exhibit a decreased PA and PV lumen diameter when ingesting E+ tall fescue seed, those

vasoconstrictive effects may not extend internally to decrease CL blood flow over time. Still, studies investigating the vasculature of the CL in cows have observed that in the mid-cycle CL, an induction of luteolysis with $PGF_{2\alpha}$ results in an immediate increase in blood flow followed by a decrease, an effect not seen in the early CL (Acosta et al., 2002). Thus, the lack of vascular response seen in the early CL may contribute to its ability to be resistant to $PGF_{2\alpha}$. This may represent the mechanism by which mares in previous studies exhibited increased interovulatory intervals (Brendemuehl et al., 1994), as decreased blood flow to the CL in mares ingesting E+ tall fescue could result in a failure of the CL vasculature to respond appropriately to $PGF_{2\alpha}$. However, results from this study could not confirm this hypothesis. This indicates that the dramatic results observed by Brendemuehl et al. (1994) may have been due to the extremely high concentration of ergot alkaloid in treatment pastures which were mostly (90%) infested with E+ tall fescue.

The results from this study suggest that, the lumen diameter of the PA and PV, and the PA RI, area, and circumference, are significantly decreased in non-pregnant mares ingesting E+ tall fescue seed. Meanwhile, interovulatory interval, number of days of diestrus, CL blood flow, serum concentrations of PRL, P4 and E2, and PV RI are not significantly affected by the ingestion of E+ tall fescue seed when mares are fed up to 10 mg of total ergovaline per day. The vasoconstriction observed in the PA and PV suggests that Doppler ultrasound may be used as a tool to diagnose exposure to E+ tall fescue. Future studies are needed to further elucidate whether the differences in vasoconstriction between the PA and PV are directly correlated, and with an increased sample size further

elucidate the apparent between subject variability in the vasoconstrictive potential of the PV.

CHAPTER FOUR: A Population of Biogenic Amine Receptors With Vasoconstrictive Properties Exists in Isolated Equine Palmar Artery, Palmar Vein, and Uterine Artery Samples

Introduction

After its discovery on a 1931 Menifee Co., KY farm, tall fescue (*Lolium arundinaceum*) was promoted as a forage with the distinguishing characteristics of dependability, resistance to grazing stress, and resistance to insect feeding (Buckner et al., 1979). These characteristics allowed the KY31 cultivar of tall fescue to propagate very successfully across much of the Southeastern United States. Current estimates suggest that between 14 and 19 million hectares of land in the United States grow the KY31 cultivar of tall fescue (Bacon, 1995; Hannaway et al., 2009). Later research discovered the source of the hardiness of KY31 tall fescue to be the endophytic fungus *Acremonium coenophialum* (now *Neotyphodium coenophialum;* Glenn et al., 1996).

Although the symbiosis with *N. coenophialum* is advantageous to tall fescue, the ergot alkaloids produced by the fungus cause a symptomology in the animals that consume it referred to as fescue toxicosis. Detrimental reproductive signs are seen in pregnant mares grazing endophyte-infected (E+) tall fescue, which can include death of both mare and foal (Monroe et al., 1988; Stidham et al., 1982). These signs are thought to be brought about in part by the binding of ergot alkaloids to dopaminergic receptors. This is supported by the alleviation – but not elimination – of negative reproductive effects in horses by the administration of type-2 dopaminergic receptor (DRD2) antagonists (Redmond et al., 1994; Jones et al., 2003).

Signs in other animal models – such as increased rectal temperatures, increased shade seeking, and sloughing of the hooves, tips of ears, and tips of tails – are the result of increased peripheral vasoconstriction due to the agonistic binding of ergot alkaloids to α-adrenergic and serotonergic receptors (Dyer, 1993; Oliver et al., 1998; Schöning et al., 2001; Klotz et al., 2012). Similar signs caused by vasoconstriction have not been reported in horses grazing E+ tall fescue. Increased sweating in horses grazing E+ tall fescue has been observed and has been explained as a possible compensatory mechanism which allows the horse to overcome some of the detrimental vasoconstrictive effects of fescue toxicosis during periods of heat stress (Cross et al., 1995; Bony et al., 2001).

Investigations have confirmed that ergot alkaloids found in E+ tall fescue will elicit a vasoconstrictive response in equine vasculature. In an *in vitro* study, Abney et al. (1993) used isolated equine lateral saphenous vein and dorsal metatarsal artery to compare the vasoconstrictive effects of various ergot alkaloids found in E+ tall fescue (ergotamine, ergonovine, and N-acetyl loline) to various biogenic amine compounds (norepinephrine, phenylephrine, BHT-920, and serotonin). This study demonstrated for the first time that ergot alkaloids were vasoactive in equine vasculature, although not as potent as the biogenic amine compounds, and was later confirmed by Klotz and McDowell (2010). Recently, an *in vivo* model of measuring vasoconstriction in the horse using Doppler ultrasound has been established (Moore et al., 2008; McDowell et al., 2009). Horses ingesting E+ tall fescue seed exhibited a decreased diameter of the palmar artery compared to horses ingesting endophyte-free (E-) tall fescue seed, as measured by Doppler ultrasound (McDowell et al., 2009). Research currently underway also suggests that E+ tall fescue may elicit a vasoconstrictive response in uterine arteries, as pregnant

mares ingesting E+ tall fescue seed exhibited a vasoconstriction similar to that seen in the peripheral vasculature (McDowell et al., 2012).

Although these studies provide strong evidence that E+ tall fescue elicits a vasoconstrictive response in equine vasculature, the specific mechanism of action of ergot alkaloids in the horse is still unknown. The agonistic action of ergot alkaloids on α-adrenergic and serotonergic receptors produced a marked vasoconstriction described in species other than the equine, such as the bovine (Dyer, 1993; Klotz et al., 2010) and the ovine (Gadberry et al., 2003). While a constrictive effect has been observed in equine vasculature in response to ergot alkaloids, the exact profile of receptors which might be responsible for this response has not yet been determined. Knowledge of what receptors are present would help target future research in determining what receptors specifically interact with tall fescue alkaloids. Therefore, the purpose of this study was to profile the genes for dopaminergic, serotonergic, and α-adrenergic receptor types and subtypes, as expressed within the equine medial palmar artery (PA), medial palmar vein (PV), and uterine artery (UA) transcriptomes.

Materials and Methods

Animals and Tissue Collection

Immediately after euthanasia, PA, PV, and UA were collected from 14 mixed breed mares in total. Forty vessels were collected. Additionally, heart, kidney, hypothalamus, and pituitary tissues were collected from one individual and pooled for

use as positive tissue control. Mares were euthanized over an 8 month period of time, and represent a cull population from the University of Kentucky Veterinary Science breeding population. After dissection, each vessel was cleaned of adipose and connective tissue, placed in an aluminum pouch, and snap frozen in liquid nitrogen. Blood vessels were stored in a -80 °C freezer until RNA isolation. The experimental protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee.

Sample Preparation

Isolation of RNA was completed using the TRIzol reagent (Invitrogen, Cat. No. 15596-018) isolation protocol (Appendix C), and the RNeasy RNA cleanup kit (QIAGEN, Cat. No. 74104) protocol (Appendix D) was used to remove any carbohydrate and protein contamination from the RNA samples. Concentration of RNA was verified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.), and quality was evaluated using an Experion RNA StdSens analysis kit (Bio-Rad laboratories Inc., Cat. No. 700-7106) procedure (Appendix E). Any RNA samples that were found to have DNA contamination were treated using the Ambion TURBO DNA-*free* DNase treatment and removal reagents (Applied Biosystems, Part No AM1907; Appendix F). The final purified RNA samples were used to make cDNA using the AccuScript High Fidelity 1st strand cDNA synthesis kit (Agilent Technologies, Cat. No. 200820) for reverse transcriptase-mediated polymerase chain reaction (Appendix G).

Primers

Genes for specific subtypes of α-adrenergic, serotonergic, and dopaminergic receptors were chosen based on previous reports of expression in vascular tissue within *The Sigma-RBI Handbook of Receptor Classification and Signal Transduction* (SIGMA-RBI, 2006). Equine-specific nucleotide sequences were obtained from previously published sequences in Genbank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD), or by performing the Basic Local Alignment Search Tool (BLAST) of predicted equine sequence available in Genbank (Zhang et al., 2000). Sequences used for primer sets in Experiment 1 are shown in Table 4.1; sequences used for primer sets in Experiment 2 are shown in Table 4.2. Primers were constructed using PrimerQuest (Integrated DNA Technologies).

Table 4.1. List of primers used to amplify equine-specific nucleotide sequences within isolated palmar artery, palmar vein, and uterine artery vessels for Experiment 1.

Receptor	Gene ID #	Accession Number	Forward Primer	Reverse Primer	Location	Annealing Temperature	PCR Product (bp)
Adrenergic							
α-1A	100054461	XM_001494586.2	5'- AGT GTC TCC GCA GAA AGC AGT CTT -3'	5'- TCG GTC TTT GGG CAC TGT AAT CCT -3'	1451-1668	60.1-59.9	218
α-1B	100071055	XR_044650.1	5'- AAC CAG ACC TCG AGC AAT TCC ACA -3'	5'- GCC AAC ACA AGA TGA ACA TGC CGA -3'	205-1042	60.3-60.0	838
α-1D	100066003	XR_044537.1	5'- ACC TGC AGA CGG TCA CCA ACT ATT -3'	5'- TCC GTC ATG ATG GCA GGG TAC TTT -3'	641-893	60.0-60.0	253
α-2B	100051684	NM_001164012.1	5'- ACC CTG CCT CAT CAT GAT CCT TGT -3	5'- ACC CAG GCT GTA GCT GAA GAA GAA -3'	549-1194	59.9-59.7	646
Serotonergic							
5-HTR$_{1B}$	100009680	NM_001081779.1	5'- AAG CCG AGG AAG AAG TGT TGG ACT -3'	5'- GCC AAC ACA CAA TAA ACG CTC CCA -3'	572-982	60.0-60.0	411
5-HTR$_{1D}$	100071630	XM_001501440.1	5'- CAA CGC CTT TGT GCT TAC CAC CAT -3	5'- AAT GAC ACA GAG GTG CAG GAT GGA -3'	165-396	60.0-60.0	232
5-HTR$_{2A}$	100009685	NM_001081784.1	5'- ACT CCA GAA CCA GGG CAT TTC TGA -3'	5'- AAG AAG CTA ATT TGG CCC GTG TGC -3'	560-841	60.0-60.1	282
5-HTR$_{2B}$	100057052	XM_001498084.1	5'- ACT GGC TGC CTT CTT TAC ACC TCT -3'	5'- TTG TGA GAA CCA TCC AGC ATT GCC -3'	703-903	59.9-59.7	201
5-HTR$_{4}$	100034053	NM_001163982.1	5'- CCC TTT GGT GCC ATT GAG TTG GTT -3'	5'- AGC GTT CAT CAT CAC AGC AGA GGA -3'	264-1041	60.0-60.0	778
5-HTR$_{7}$	100009688	XM_001501255.2	5'- AAG TTG TGA TCG GCT CCA TCC TGA -3	5'- GTT TGC ATA GCC CAG CCA CAG AAA -3'	494-1383	59.9-59.9	890
Dopaminergic							
DRD1	100058846	XM_001916862.1	5'- ACA TCA TGT GCT CCA CTG CGT CTA -3'	5'- AGG AGC TTT CGG GTT GAG AAC ACT -3'	308-778	60.0-60.0	471
DRD2	100062195	XM_001501996.2	5'- ACA CAG ACC AGA ACG AGT GCA TCA -3'	5'- TGG GCA TGG ACT GGA TCT CAA AGA -3'	530-1051	60.1-59.8	522
DRD3	100061160	XM_001917412.1	5'- TCA CTA CCA ACA TGG CAC TGG ACA -3'	5'- TCG ACA ACC TGC CGT TAC TGA GTT -3'	408-922	60.1-60.0	515
DRD4	100147552	EF012228.1	5'- AGG AAA GCT GCT TAG GTC TCG GTT -3'	5'- TCA GCC TGT TAA CCT GTC ACT GCT -3'	829-1255	59.9-60.0	427
DRD5	100069604	XM_001917813.1	5'- TCA TGA TCG TAA CCT ACA CGC GCA -3'	5'- ACG GGA CCA TGC AGT TAA GGA TGA -3'	713-955	60.0-60.0	243

Table 4.1 (continued)

Receptor	Gene ID #	Accession Number	Forward Primer	Reverse Primer	Location	Annealing Temperature	PCR Product (bp)
Other							
PGR	100033883	AF053141	5'- ACT ACG TTG TGG AGA ATG GGC TGT -3'	5'- ACA GGT GTT GAA TTT GGC TGG ACC -3'	164-586	60.0-59.7	423
ER α	791249	AF007799.1	5'- TTC TGG AAT GTG CCT GGC TAG AGA -3'	5'- AGA CTT CAG GGT GCT GGA CAG AAA -3'	19-290	59.5-59.9	272
ER β	100033964	AJ439894.1	5'- TGC TGA TGG TGG GCC TGA TAT G -3'	5'- AGG ATC ATG GCC TTG ACA CAG AGA -3'	62-263	59.1-59.6	202
Control							
GAPDH	100033897	AF083897.1	5'- ATC ATC CCT GCT TCT ACT GGT GCT -3'	5'- ACA AAG TGG TCG TTG AGG GCA ATG -3'	71-378	59.6-59.8	308
AFP	100034185	U28947.1	5'- TTA CTG GCA CGC AAG AAG GAC TCT -3'	5'- TCC TAA TTT CTG GAA GAG GCC GCA -3'	399-1295	60.0-60.0	897

Table 4.2. List of primers used to amplify equine-specific nucleotide sequences within isolated palmar artery, palmar vein, and uterine artery vessels for Experiment 2.

Receptor	Gene ID #	Accession Number	Forward Primer	Reverse Primer	Location	Annealing Temperature	PCR Product (bp)
Serotonergic							
5-HTR_{1B}	100009680	NM_001081779.1	5'- ACG TGA ACC AAG TCA AAG TGC GAG -3'	5'- AGG GCA GCC AAC ACA CAA TAA ACG -3'	857-988	59.5-60.0	132
5-HTR_{1D}	100071630	XM_001501440.1	5'- TGG CCA CGA CTG ACC TCT TAG TTT -3'	5'- AAT GAC ACA GAG GTG CAG GAT GGA -3'	239-396	59.8-60.0	158
5-HTR_{2A}	100009685	NM_001081784.1	5'- ACT CCA GAA CCA GGG CAT TTC TGA -3'	5'- ACC TTG GAA TCA TCC TGT AGC CCA -3'	560-662	60.0-59.7	103
5-HTR_{2B}	100057052	XM_001498084.1	5'- ACT GGC TGC CTT CTT TAC ACC TCT -3'	5'- CGT TAG GCG TTG AGG TGG TTT GTT -3'	703-817	59.9-59.6	115
5-HTR_{7}	100009688	XM_001501255.2	5'- ATA TGC CTT CTT CAA CCG GGA CCT -3'	5'- TCT CAG CAA GCT TCA GAG CCT CAT -3'	1404-1525	59.9-59.9	122
Control							
GAPDH	100033897	AF083897.1	5'- ATC AAG AAG GTG GTG AAG CAG GCA -3'	5'- ACA AAG TGG TCG TTG AGG GCA ATG -3'	227-378	60.6-59.8	152

Experiment 1

To minimize the evaluation of gene expression for unexpressed receptor genes, Experiment 1 determined which of the genes were present in the transcriptome of the isolated vessels. Four controls were used throughout this experiment: a negative primer control, a positive primer control, a positive tissue control, and a no template control. Alpha-fetoprotein (AFP) was used as a negative primer control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive primer control (reference gene) against a pooled sample homogenate. A pooled sample of heart, kidney, hypothalamus, and pituitary tissues were collected and used as a positive tissue control. Gene amplification was performed using the Taq polymerase (5prime, Cat. No 2200310) amplification protocol (Appendix H) and cDNA obtained from the sample vessels. Upon removal from the thermal cycler (Eppendorf Mastercycler ep Gradient S), samples were transferred to an electrophoresis gel (100 ml 1x TAE, 1.5g agarose, 2µl ethidium bromide), allowed to run for 20 minutes at 100 volts, and PCR product bands were visualized using a Kodak Gel Logic 6000 PRO image system.

Experiment 2

Experiment 2 was a semi-quantitative expression analysis of serotonin (5-hydroxytryptamine, 5-HT) receptors subtypes 1B, 1D, 2A, 2B, and 7, within the transcriptome of the isolated vessels (Table 2). Relative abundance of serotonergic receptor mRNA and GAPDH mRNA were determined using semi-quantitative real-time PCR performed on an ABI 7000 Sequence Detection System (Applied Biosystems,

Roster City, CA). Gene amplification was completed using SYBR Green (Applied Biosystems, Cat. No. 4309155) amplification protocol (Appendix I). Quantitation of serotonergic mRNA expression was conducted using the relative standard curve method (Applied Biosystems Inc., 2008) with expression normalized to GAPDH mRNA expression. The standard curve for each gene was generated from a pooled homogenate of all experimental tissues, which was serially diluted 1-fold, 2-fold, 4-fold, 8-fold, 1000-fold, and 10,000-fold.

Statistical Analyses

Expression data generated in Experiment 2 were analyzed using Mixed Models of SAS, version 9.2 (SAS Institute, 2010). Fixed effects were vessel, receptor, and the interaction of vessel*receptor. These effects were evaluated as a completely randomized design, with the model statement shown in Model 4.1 (Appendix J). Significance of main effects and the interaction were determined using the F test within ANOVA. Normal distribution of the data was evaluated using the Shapiro-Wilk's test in the Univariate procedure of SAS. Mean separations were conducted on significant interactions using the LSD feature in SAS and comparisons with a P-value less than 0.05 were considered significant.

Results

Experiment 1

Among all the different genes evaluated, only DRD2, DRD3, and 5-HTR_4 were determined to be absent across all vessels measured (Table 4.3). Of the genes chosen for inclusion in Experiment 1, the equine-specific nucleotide sequences for ADRα-1D and ADRα-2B – which were predicted using BLAST – produced primers which yielded unreliable results. Attempts to reconcile this led to the discovery that following the initiation of this experiment the ADRα1D and ADRα2B published equine-specific gene sequences (used to construct primers for Experiment 1) had been removed from the NCBI database. This resulted in the removal of these two genes from the study. Presence of a gene within the transcriptome was defined as the ability of the primer for that gene to amplify at least 75% of the samples that were tested against it (Figure 4.1). Results from Experiment 1 revealed a large group of serontonergic receptors expressed within the transcriptome of equine PA, PV, and UA. Because the group of serotonin receptors was sequenced and expressed more completely, they were chosen for quantitation in Experiment 2.

Table 4.3. Genes detected, not detected, or removed in Experiment 1.

Detected	**Not Detected**	**Removed**
Adrenergic	*Dopaminergic*	*Adrenergic*
α-1A	DRD2	α-1D
α-1B	DRD3	α-2B
Serotonergic	*Serotonergic*	
5-HTR_{1B}	5-HTR_4	
5-HTR_{1D}		
5-HTR_{2A}		
5-HTR_{2B}		
5-HTR_7		
Dopaminergic		
DRD1		
DRD4		
DRD5		
Other		
PGR		
ER α		
ER β		

Experiment 2

The main effect of vessel type was significantly different in relative quantity of total serotonergic receptor mRNA ($P < 0.0001$). The main effect of receptor was significant as well ($P < 0.0001$). All serotonergic receptor subtype mRNA's were detected, but only receptor 5-HT_{2B} had a greater quantity of mRNA from all others tested ($P < 0.0001$), while receptor 5-HT_{1D} tended to be greater than receptor 5-HT_{2A} ($P = 0.0556$). The interaction of vessel*receptor was significant ($P < 0.0001$), indicating that expression of receptor type differed across vessel type. Within the PA (Figure 4.2), the 5-HTR2B gene was expressed in the greatest relative quantity ($P = 0.0388$), while all other receptors were not different. Within the PV (Figure 4.2), receptor 5-HT_{2A} had a greater quantity mRNA than receptor 5-HT_{1B} ($P = 0.0104$), and tended to be greater than receptors 5-HT_{1D} ($P= 0.0816$) and 5-HT_7 ($P = 0.0809$). Within the UA (Figure 4.2), receptor 5-HT_{2B} had the greatest quantity of mRNA ($P < 0.0001$), while receptor 5-HT_{2A} had a lower quantity of mRNA than 5-HT_{2B} ($P < 0.0001$) and 5-HT_{1D} ($P = 0.0003$), yet only tended to be lower than 5-HT_{1B} ($P = 0.0640$) and 5-HT_7 ($P = 0.0849$).

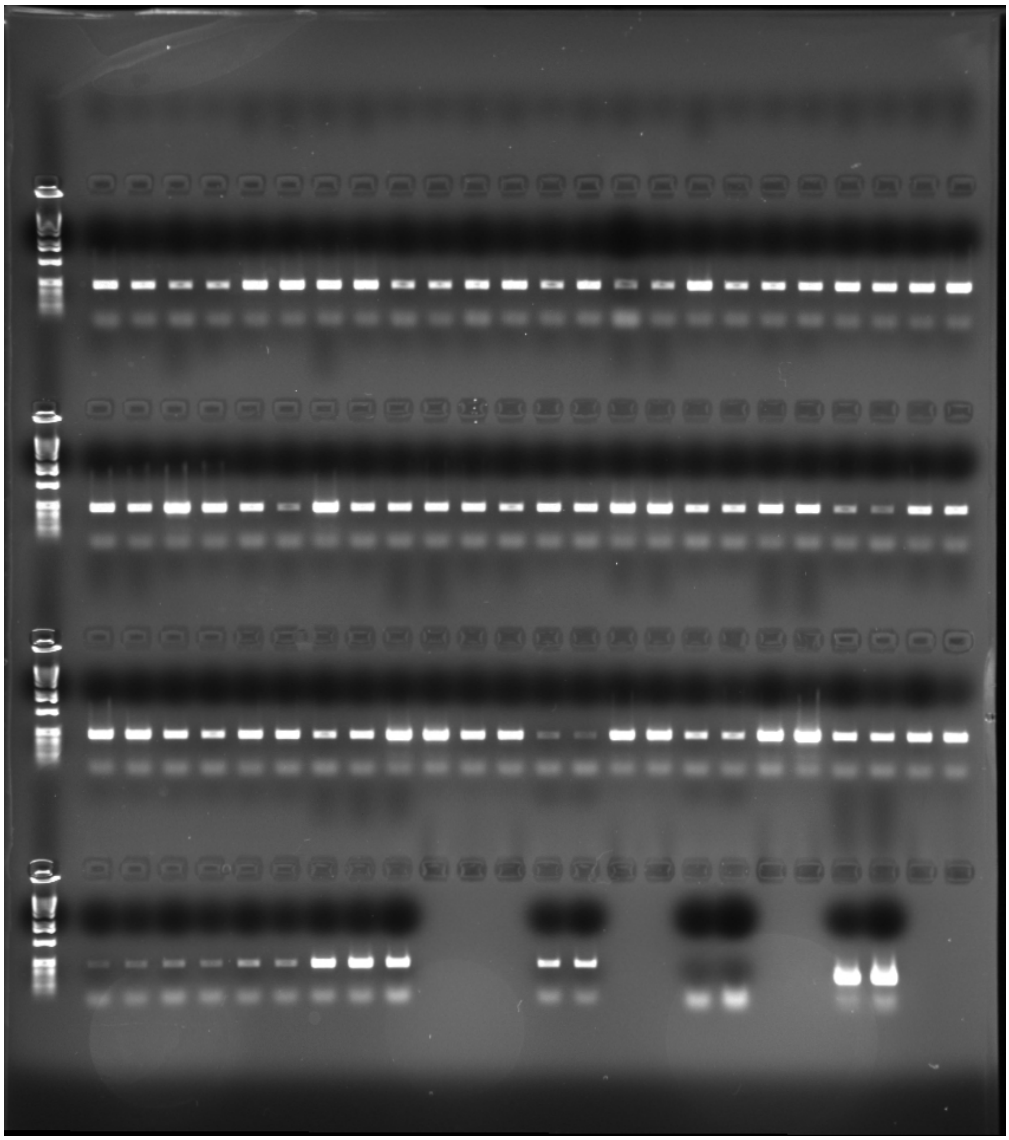

Figure 4.1. Example of positive results from a single biogenic amine receptor, DRD1.

An example gel from Experiment 1 where a single biogenic amine receptor was evaluated (DRD1 shown). Wells 1-80 are duplicates of all 40 samples; the last three well duplicates are (from left to right) positive tissue control (a heart, kidney, hypothalamus, and pituitary tissue homogenate), no template control (no cDNA in the reaction well), and positive primer control (GAPDH).

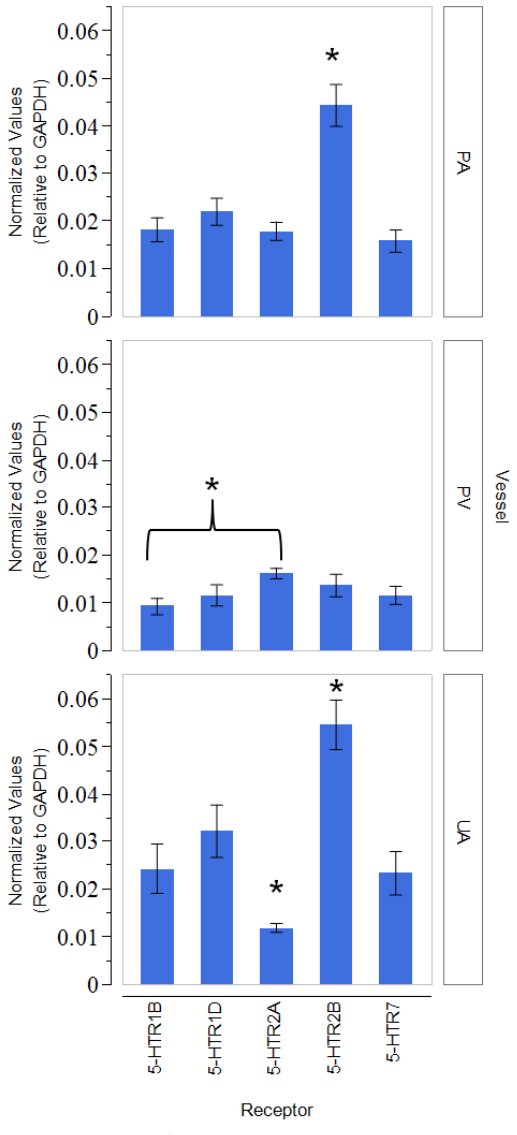

Figure 4.2. Expression of serotonergic (5HT) receptors by vessel.

Expression of 5HT receptors was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the standard curve method. Within the PA, the 5-HTR2B gene had the greatest relative quantity mRNA (P =0.0388). Within the PV, the 5-HTR2A gene had a relative quantity of mRNA greater than 5-HTR1B only (P =0.0104). Within the UA, receptor 5-HT_{2B} has the greatest relative quantity mRNA (P <0.0001), while 5-HT_{2A} had the lowest relative quantity mRNA (P <0.0001).

Discussion

Alpha adrenoreceptors – and not beta adrenoreceptors – were the focus of Experiment 1 because they are known to modulate vasoconstriction. Several alpha adrenoreceptors – including ADRα-1D, -2A, -2B, -2C, and -2D – were not profiled during this experiment due to an inability to obtain reliable corresponding equine-specific nucleotide sequences. However, the presence of the ADRα-1A and ADRα-1B receptors indicates that those alpha adrenoreceptors are present in equine vasculature, and that the profile of adrenoreceptor subtypes within equine vasculature will need to be more completely explored at a later time as these genes are sequenced. Future studies involving all subtypes missing from this study, as well as beta adrenoreceptors, will provide a complete profile of all adrenoreceptors within equine vasculature.

The presence of the DRD1-like receptors DRD1and DRD5 concurs with reports from other species (Jose et al., 2003; Zeng et al., 2004). However, the presence of the DRD2-like receptor DRD4 within equine vasculature has not previously been reported. Dopamine receptors have a wide distribution in the brain, and have a density 10-100 times that of the DRD2-like receptors (Hurley and Jenner, 2006). Although it is difficult to extrapolate from the brain to the extremities, it is interesting to note that within the human brain DRD4 does not behave like the other DRD2-like receptors, and instead has a distribution similar to the DRD1-like receptors (Hurley and Jenner, 2006). If the distribution of DRD4 throughout the rest of the body is similar to its distribution within the brain, then it is reasonable to conclude that DRD4 will be present alongside DRD1-like receptors in tissue where DRD1-like receptors are found.

Serotonin receptors 1B, 1D, 2A, 2B, and 7 were present in the tested equine vasculature, which concurs with a previous report (Weller et al., 1994). Similarly, 5-HTR_4 was not present in the evaluated equine vasculature, which agrees with previous reports of the distribution of this receptor (Hegde and Eglen, 1996). While it is not necessary to profile receptors which are not currently thought to be present in the vasculature, including such receptors validates this presence/absence study. In addition to demonstrating the presence of receptor subtypes already known to be present in the vasculature of other species (Weller et al., 1994; Rang and Dale, 2007), we have demonstrated the absence of receptor subtypes known to be absent in the vasculature of other species.

Combining the results from Experiment 1 with Experiment 2 yields a profile within equine vasculature of the 5-HT receptor subtypes, which are known to play a role in equine peripheral vasoconstriction (Rang and Dale, 2007). The interaction of receptor and vessel may indicate the important receptor subtype within each vessel type. Within the equine PA and UA, receptor subtype 5-HT_{2B} had the greatest expression level of mRNA ($P < 0.0001$). Serotonergic receptor 2B in mice has been shown to be important in regulating vascular structure and function (Nebigil et al., 2001). There is also evidence in mice that 5-HT_{2B} is important in controlling plasma serotonin levels.

Serotonergic receptor 2A is often mentioned alongside 5-HT_{2B} as being widely distributed throughout vascular epithelium and smooth muscle, where they induce vasoconstriction (Oyama and Levy, 2010). Interestingly, although relative quantities of 5-HT_{2B} were significantly different among vessel types, with 5-HT_{2B} relative quantities in the PV being the lowest, relative quantities of 5-HT_{2A} were not significantly different

between vessels. Thus, it seems that while 5-HT_{2A} is expressed at lower quantities in other vessels (relative to other receptors), it is slightly increased in the PV. In addition to its known vasoconstrictive effects, 5-HT_{2A} has known effects in the brain, including the mediation of elevated plasma levels of oxytocin, ACTH, and corticosterone (Zhang et al., 2002). In the peripheral vasculature, 5-HT_{2A} is also known to play a role in suppressing TNF-α-mediated inflammation (Yu et al., 2008).

The differences in relative quantity of receptor subtypes across vessels may be readily explained by understanding their physiological roles in response to environmental stimuli. Uterine arteries, for instance, must be responsive to normal physiological changes which may come about due to pregnancy or normal cyclicity. When the uterine arteries of non-pregnant mares were observed across the estrous cycle and at first detectable pregnancy using Doppler ultrasound, the time-averaged mean velocity and resistivity index significantly changed at first detectable pregnancy (Bollwein et al., 2003), and at different days of the estrous cycle (Bollwein et al., 1998). Vessels in the lower limb, in addition to perfusing peripheral tissues, must aid in overall thermoregulation, as contractility will be altered in response to changes in ambient temperature (Pollitt, 1992). Recent studies further indicate that vascular reactivity is enhanced by reduced ambient temperatures (Zerpa et al., 2010). Thus, although an environmental stimulus (such as a fight or flight response) would invoke a vasoconstrictive response in these vessel types (Lees et al., 2004; Bernard and Barr, 2011), the differences in relative quantity of receptor subtypes across vessels may emphasize the different physiological roles of those vessel types under normal circumstances.

The pharmacological differences of 5-HT, α-adrenergic, and dopaminergic receptors between the PA, PV, and UA components of the equine vasculature may be significant not only in explaining the pathogenesis of the equine vasoconstrictive response to fescue toxicosis, but also in explaining the lack of observed detrimental effects in response to this vasoconstriction. Until now, information regarding the profile of these receptors in the equine vasculature has been difficult to obtain. Epinephrine, norepinephrine, and 5-HT are potent vasoconstrictors within the equine digital vasculature (Elliott, 1997; Bailey and Elliott, 1998), but the precise receptor subtypes which are responsible for the vasoconstrictive effect of equine fescue toxicosis are still unknown. Furthermore, the impact of the presence of the dopaminergic receptors and the possible additive effect of the 5-HT and α-adrenergic receptors within the vasculature will need to be elucidated further. These data may aid in the identification of the biogenic amine receptor subtypes most potent in the vasoconstrictive effect of equine fescue toxicosis, and future experiments may be able to use these data to develop treatments or preventions for fescue toxicosis.

In conclusion, Experiments 1 and 2 describe a profile of 5-HT, dopaminergic, and adrenergic receptors within the equine PA, PV, and UA transcriptomes that are known to play roles in equine peripheral vasoconstriction. These receptors may also play roles in the vasoconstrictive response of equine fescue toxicosis, a hypothesis which is supported by recent observations *in vivo* of a vasoconstrictive response to E+ tall fescue (Moore et al., 2008; McDowell et al., 2009). Still, this profile of receptors with constrictive properties is far from conclusive, and immunohistochemical analysis would aid in the identification of functional biogenic amine receptors in the equine vasculature.

Knowledge of a profile of biogenic amine receptors in the equine vasculature would aid future research in determining what receptors specifically interact with the ergot alkaloids produced by tall fescue.

CHAPTER FIVE: Discussion & Summary

Losses attributable to fescue toxicosis likely exceed $1 billion annually, when economic impacts on ruminant, small ruminant, and equine industries are combined (Strickland, 2011). However, producers often fail to recognize problems with fescue toxicosis as many of the signs are not overtly severe and have the potential to go undetected. This is especially true for horses, as many fescue toxicosis signs not associated with the pregnant mare are not consistently reported. Signs such as heat intolerance, decreased growth rates, and decreased fertility are perhaps inconsistently reported because of the toxin load variability between studies coupled with an unknown baseline toxicity level at which harmful signs begin to be observed in animals ingesting endophyte-infected (E+) tall fescue. Therefore, an effort to improve repeatability must include a method of assessing a baseline toxicity level in animals ingesting E+ tall fescue.

Possible methods of assessing a baseline toxicity level in animals ingesting E+ tall fescue include measurements of blood concentrations of ergot alkaloids, measurements of ergot alkaloid metabolites in excrement, and measurement of ergot alkaloid concentration intake. Such measurements are not without error (see literature review), however, and investigators risk performing whole experiments without reaching a baseline toxicity level in treatment animals. Recent reports from this lab (Moore et al., 2008; McDowell et al., 2009) provide a model for a real-time measurement of the signs of fescue toxicosis in an animal exposed to E+ tall fescue by way of Doppler ultrasonography of the palmar artery (PA), dubbed the McDowell Model. This model substantiates and expands upon earlier reports (Abney et al., 1993) of the constrictive potential of ergot alkaloids in equine vasculature, and the possibility of measuring that

constriction *in vivo* using Doppler ultrasound (Aiken et al., 2007). Therefore, the McDowell Model may be able to provide investigators with a satisfactory real-time bioassay for determining the premonitory signs of fescue toxicosis in an individual exposed to E+ tall fescue.

Experiments from Chapters 3 & 4 were performed with the overall objective of exploring the effect of fescue toxicosis on certain parameters of the estrous cycle, including differences in interovulatory interval, serum concentrations of PRL, P4, and E2, and blood flow to the corpus luteum (CL). This thesis also aimed to strengthen, substantiate, and expand upon the previous reports which used Doppler ultrasound to assess vasoconstriction in the PA of treatment animals (Moore et al., 2008; McDowell et al., 2009). Taken together, Chapters 3 & 4 explored the effect that vasoconstriction plays in the equine response to fescue toxicosis.

Implications from Chapter 3 suggest that the effects of E+ tall fescue on parameters of cyclicity, serum concentrations of progesterone (P4), and blood flow may not differ when mares consume an average of 10 mg ergovaline + ergovalinine/day. Use of the McDowell Model to assess vasoconstriction in the PA and PV revealed that vasoconstriction was significantly increased in mares ingesting E+ tall fescue, as measured by the PA and PV lumen diameter, and the PA area, circumference, and RI. This indicates that the model is successful in providing a real-time bioassay of exposure to E+ tall fescue in horses, and as a biomarker of fescue toxicosis in non-pregnant mares is perhaps even more sensitive than serum concentrations of PRL. Of interest, however, is that alterations in interovulatory interval in mares ingesting E+ tall fescue seed did not agree with those of Brendemuehl et al (1994). This suggests that while a treatment effect

was reached in treatment mares, as confirmed by Doppler ultrasonography of the PA and PV, a maximal toxic effect may not have been reached. Thus, future studies employing the McDowell Model may wish to explore a scale in which toxicity is graded based on vasoconstriction. This again demands the establishment of a baseline level of toxicity. Experiments to determine a minimal level of ergot alkaloid exposure needed to cause PA vasoconstriction are underway (K. J. McDowell, Personal Communication).

Results from Chapter 4 suggest that the vasoconstriction observed in the PA and PV when horses ingest E+ tall fescue may be due to the agonistic action of ergot alkaloids on serotinergic and α-adrenergic receptors. Also, results from Chapter 4 reported that 5-HT 1B, 1D, 2A, 2B, and 7, dopamine receptors 1, 4, and 5, and α-adrenergic receptors 1A and 1B were present in the equine PA, PV, and UA, while only dopamine receptors D2 and D3, and 5-HT_4 were absent from the equine PA, PV, and UA. The presence of the type-1 like dopamine receptors will need to be explored further.

In Experiment 2 from Chapter 4, results from real time-PCR indicate that serotinergic receptors are present in the uterine artery in greater relative abundance than in either the PA or PV. Such a presence of receptors with vasoconstrictive potential could indicate an as of yet unobserved vasoconstriction in the equine response to fescue toxicosis. Research is underway which incorporates an investigation of the vasoconstriction of the uterine artery when elucidating the equine response to fescue toxicosis (McDowell et al., 2012).

Combined results from Chapters 3 & 4 indicate that the increased sensitivity of the PA relative to the PV may be due to the greater relative abundance of serotonergic receptors in the PA as compared to the PV. This combination of experiments may aid

future research in determining what receptors specifically interact with tall fescue alkaloids. In future whole animal research, this information may be relevant when comparing responses in the PA vs. PV, and may have a pertinent bearing on the development of Doppler ultrasonography as a tool for the diagnosis of an individual's exposure to E+ tall fescue. Further research is needed, however, to fully quantify the disparities in vasoconstriction between the PA and PV.

In Summary, observations from Chapters 3 & 4 indicate that there is no alteration of interovulatory intervals in mares ingesting E+ tall fescue seed. Additional observations of the PA and PV indicate that horses have a significant vasoconstrictive response to E+ tall fescue. This response is confirmed by the presence of dopaminergic, serotinergic, and α-adrenergic receptors as confirmed by PCR. Future studies may wish to further characterize the relationship between the profile of biogenic amine receptor subtypes reported in Chapter 4 and the *in vivo* vasoconstrictive response observed in Chapter 3. Measurement of vasoconstriction using the McDowell Model may be of use as a bioassay of fescue toxicity in non-pregnant mares, and may also be used to establish baseline and maximal levels of toxicity in the horse. In addition, a full profile of dopaminergic, serotinergic, and α-adrenergic receptors should include the full range of serotinergic and α-adrenergic receptors as better genetic materials become available, and immunohistochemical staining may be preferable in order to confirm that mRNA is being converted to protein.

Appendices

Appendix A: Composition of feed concentrate used in Chapter 3

Feed Type	**Manufacturer**	**Part in Mixture**
Sweet Feed, Performance 13	Woodford Feed	1/3
Pellet, UK Custom Mix	Farmers Feed Mill, Inc.	1/3
Race Horse Oats	Farmers Feed Mill, Inc.	1/3

Appendix B: Model statements used for statistical analysis of data from Chapter 3

Model 3.1:

$Y_{ijkl} = \mu + \delta_l + \beta_{i(l)} + \alpha_j + \gamma_k + \lambda_{r(lk)} + \varepsilon_{ijkl}$

We assume the following:

1. Y_{ijkl} is the response due to subject i, treatment j, and period k, and sequence l.
2. μ is an overall mean.
3. δ_l is a fixed effect due to sequence l; $\Sigma\, \delta_l = 0$, l=1,2.
4. $\beta_{i(l)}$ is a random effect due to subject i nested within sequence l; assume $\beta_{i(l)}$ iid, ~ N(0, σ^2_β), i=1,...12.
5. α_j is a fixed effect due to treatment j; $\Sigma\, \alpha_j = 0$, j=1,2.
6. γ_k is a fixed effect due to period k; $\Sigma\, \gamma_k = 0$, k=1,2.
7. ε_{ijkl} is the random error; assume ε_{ijkl} iid, ~ N(0, σ^2_ε).

Model 3.2:

$Y_{ijklm} = \mu + \delta_l + \beta_{i(l)} + \alpha_j + \gamma_k + \tau_m + \tau\alpha_{mj} + \tau\gamma_{mk} + \tau\delta_{ml} + \varepsilon_{ijklm}$

We assume the following:

1. Y_{ijkl} is the response due to subject i, treatment j, and period k, and sequence l.
2. μ is an overall mean.
3. δ_l is a fixed effect due to sequence l; $\Sigma\, \delta_l = 0$, l=1,2.
4. $\beta_{i(l)}$ is a random effect due to subject i nested within sequence l; assume $\beta_{i(l)}$ iid, ~ N(0, σ^2_β), i=1,...12.
5. α_j is a fixed effect due to treatment j; $\Sigma\, \alpha_j = 0$, j=1,2.
6. γ_k is a fixed effect due to period k; $\Sigma\, \gamma_k = 0$, k=1,2.
7. τ_m is a fixed effect due to time l; $\Sigma\, \tau_m = 0$.
8. $\tau\alpha_{mj}$ is a fixed interaction effect due to time m and treatment j; $\Sigma\, \alpha\tau_{jm} = 0$.
9. $\tau\gamma_{mk}$ is a fixed interaction effect due to time m and period k; $\Sigma\, \alpha\gamma_{jk} = 0$
10. $\tau\delta_{ml}$ is a fixed interaction effect due to time m and sequence l; $\Sigma\, \tau\delta_{ml} = 0$
11. ε_{ijklm} is the random error; assume ε_{jkm} iid, ~ N(0, σ^2_ε).

Appendix C:TRIzol ® Reagent (Invitrogen, Cat. No. 15596-018) isolation protocol

1. Homogenize tissue samples in 1ml TRIzol ® Reagent per 50-100 mg tissue. The sample volume should not exceed 10% of the bolume of TRIzol ® Reagent used for homogenization
2. Incubate the homogenized samples for 5 minutes at 15-30 °C to permit the complete dissociation of nucleoprotein complexes
    a. Add 0.2 ml of chloroform per 1 ml of TRIzol ® Reagent
    b. Cap samples, and shake vigorously by hand for 15 seconds
    c. Incubate at 15-30 °C for 2-3 minutes, then centrifuge the samples at no more than 12,000xg for 15 minutes at 2-8 °C
    d. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase
3. Transfer the aqueous phase to a fresh tube, discarding the old tube
    a. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5ml isopropyl alcohol per 1ml TRIzol ® Reagent used.
    b. Incubate samples at 15-30 °C for 10 minutes, then centrifuge at no more than 12,000xg for 10 minutes at 2-8°C.
    c. The RNA precipitate forms a gel-like pellet on the side and bottom of tube
4. Remove the supernatant.
    a. Wash the pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml TRIzol® Reagent used for the initial homogenization
    b. Mix the sample by vortexing, then centrifuge at no more than 7,500xg for 5 minutes at 2-8°C
5. At the end of the procedure, remove the supernatant, leaving only the pellet.
    a. Briefly dry the RNA pellet (air- or vacuum-dry for 5-10 minutes)
    b. It is important not to let the RNA pellet dry completely, as this decreases solubility greatly
    c. Dissolve RNA pellet in 50 µl of RNase-free water
    d. Store at -80°C for further use

Appendix D: RNeasy RNA Cleanup Kit (QIAGEN, Cat. No. 74104) protocol

1. Adjust the sample to a volume of 100 µl with RNase-free water. Add 350 µl of Buffer RLT, and mix well
2. Add 250 µl ethanol (96-100%) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer the sample (700 µl) to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000xg. Discard the flow-through.
4. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000xg to wash the spin column membrane. Discard the flow-through.
5. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000xg to wash the spin column membrane
    a. Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube to that the column does no contact the flow-though. Otherwise, carryover of ethanol will occur
6. Optional: place the RNeasy spin column in a new 2ml collection tube, and discard the old collection tube with the flow through. Close the lid gently, and centrifuge at full speed for 1 minute.
7. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30-50 µlRNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 minute at ≥8000xg to elute the RNA.
8. If the expected RNA yield is >30 µg, repeat step 7 using another 30-50 µl RNase-free water, or using the eluate from step 7.

Appendix E: Experion RNA StdSens Analysis Kit (Bio-Rad laboratories Inc., Cat. No. 700-7106) procedure

1. Preparing the gel stain
    a. Remove the RNA gel, RNA stain, and loading buffer from storage and allow them to equilibrate to room temperature for ~15 minutes. Keep the RNA stain protected from light.
    b. Briefly vortex the RNA stain (blue cap) and spin down in a microcentrifuge for 3-5 seconds. Make sure the DMSO in the stain is completely thawed.
    c. Pipet 600 µl RNA gel (green cap) into a spin filter tube.
    d. Centrifuge the gel at 1,500xg for 10 minutes. Confirm that all of the get has passed through the filter, and then discard the filter.
    e. Pipet 65 µl filtered gel into an RNase-free microcentrifuge tube. Add 1 µl RNA stain to the tube. Briefly vortex the solution. Keep the gel-stain (GS) solution protected from light.
    f. Cap the RNA stain tightly, since the DMSO is highly hygroscopic, and store it in the dark. Gel-stain solution should be used within the week.
2. Preparing the samples and RNA ladder
    a. Remove the RNA ladder (red cap) from storage and allow it to thaw on ice.
    b. Determine the amount of RNA ladder required. A total of 1 µl RNA ladder is required for each chip. Add 2 µl RNA ladder to account for evaporation during the heat denaturation step
    c. Pipet a minimum of 3 µl RNA ladder into an RNase-free microcentrifuge tube.
    d. Prepare all samples by pipetting at least 3 µl sample into an RNase-free microcentrifuge tube.
    e. Denature the ladder and samples for 2 minutes at 70°C.
    f. Cool the denatured ladder and samples by immediately placing the tubes on ice for 5 minutes.
    g. Spin down the ladder and samples in a microcentrifuge for 3-5 seconds. Store on ice until used.
3. Priming the chip
    a. Open the Experion priming station by pressing down on the front lever.
    b. Remove an Experion RNA StdSens chip out of its packaging and place it on the chip platform, matching the arrow on the chip with the alignment arrow on the chip platform. A post on the chip prevents insertion in the wrong position. Do not force the chip into position.
    c. Pipet 9 µl filtered get-stain solution into the well labeled **GS**. Insert the tip of the pipet vertically and to the bottom of the well when dispensing. Do not expel air at the end of the pipetting step.
    d. Carefully close the priming station by gently pressing down on the front lever. The lid should snap completely closed.

e. Set the pressure setting to **B** and the time setting to 1, as specified by the alphanumeric code on the chip.
f. Press the start button. The "Priming" message will illuminate on the LCD screen, the priming station will pressurize, and the timer will count down. Complete priming requires approx. 30 seconds. An audible signal indicates that priming is complete, and a "Ready" message will be displayed. Open the priming station by pressing down on the release lever.
g. Turn the chip over and inspect the microchannels for bubbles or evidence of incomplete priming. The glass chip will appear opaque and the microchannels will be difficult to see if they are primed properly.
h. Place the chip on a clean surface for loading samples.

4. Loading the samples and the RNA ladder into the chip
    a. Pipett 9 µl of the gel-stain solution into the other well labeled **GS**.
    b. Pipet 9 µl of filtered gel into the well labeled **G**.
    c. Vortex the loading buffer (yellow cap) before use.
    d. Pipet 5 µl of the loading buffer in each sample well (1-12) and the ladder well, labeled **L**.
        i. Make sure that the pipet tip is centered and positioned vertically all the way to the bottom of the well. This will aid in avoiding introducing bubbles into the sample wells.
        ii. All wells should be filled with the loading buffer, even when fewer than 12 samples are run.
        iii. Use a new pipet tip for each delivery to prevent contamination of the loading buffer stock.
    e. Pipet 1 µl denatured RNA ladder into the well lableled **L**.
        i. Every chip must have the RNa ladder loaded into the ladder well for accurate quantitation of samples.
    f. Pipet 1 µl sample into each of the 12 sample wells.
    g. If running fewer than 12 samples, add 1 µl loading buffer, TE buffer, or DEPC-treated water to the unused sample well(s).
    h. Place the chip in the Experion vortexing station.
    i. Turn on the vortexer, which will operate for 60 seconds and then automatically shut off. Remove the chip when the vortexer stops.
    j. Start the run immediately to prevent excessive evaporation and poor results or a chip performance error.
5. Running the RNA analysis
    a. Turn on the power to the Experion electrophoresis station by pushing the green button in the center of the front panel. The steady green LED above the button indicates that the unit is on.
    b. Launch the Experion software.
    c. Open the lid of the electrophoresis station. Place the primed chip, which has been loaded with samples, on the chip platform. Ensure that the chip is seared properly and then carefully close the lid.
    d. Select new run. Select the RNA StdSens protocol.
    e. Click the start button to begin the chip run.

f. After a run has started, the green LED in the center of the front panel on the electrophoresis station will begin blinking.
g. When the chip run is complete, a “Run Complete” message will be displayed. Remove the chip from the electrophoresis station and dispose of it properly. To prevent contamination of the electrodes, do not leave the chip in the electrophoresis station for an extended period of time.

Appendix F: TURBO DNA-free ™ DNase Treatment (Applied Biosystems, Part No AM1907) protocol

1. Add 0.1 volume 10X TURBO DNase Buffer and 1 µl TURBO DNase to the RNA, and mix gently
    a. For routine DNase treatment (≤200 µl nucleic acid per ml), use 1 µl TURBO DNase (2 U) for up to 1 µg of RNA in a 50 µl reaction.
2. Incubate at 37° C for 20-30 minutes
3. Add resuspended DNase Inactivation Reagent (typically 0.1 volume) and mix well
    a. For routine DNase treatment, use 2 µl or 0.1 volume DNase Inactivation Reagent, whichever is greater.
4. Incubate 5 minutes at room temperature, mixing occasionally
5. Centrifuge at 10,000xg for 1.5 minutes and transfer the RNA to a fresh tube
    a. RNA will be in the supernatant. Centrifugation pellets the DNase Inactivation Reagent. Avoid introducing the DNase Inactivation reagent into the solution.

Appendix G: AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Cat. No. 200820) protocol for Reverse Transcriptase-Mediated Polymerase Chain Reaction

1. Prepare the cDNA synthesis reaction by adding the following components to a microcentrifuge tube *in order*:
    a. RNase-free water to total volume 16.5 µl
    b. 2.0 µl of AccuScript RT Buffer (10x)
    c. 1.0 µl of gene-specific primer (0.1 µg/µl) OR oligo(dT) primer(0.5 µg/µl) OR 3 µl of random primers (0.1 µg/µl)
    d. 0.8 µl of dNTP mix (25 mM each dNTP)
    e. x µl of RNA. The quantity of RNA depends on the RNA purity, message abundance, and size of the target:
        i. if total RNA, target < 2kb, then 10-100 ng
        ii. if total RNA, target > 2kb, then 200-5000 ng
        iii. if mRNA, (all targets), then 0.1-100 ng
2. Incubate the reaction at 65 °C for 5 minutes.
3. Cool the reaction at room temperature to allow the primers to anneal to the RNA (approximately 5 minutes).
4. Add the following components to the reaction, in order, for a final reaction volume of 20 µl:
    a. 2 µl of 100 mM DTT
    b. 1 µl of Accuscript RT
    c. 0.5 µl of RNase Block ribonuclease inhibitor (40 U/µl)
5. If using random primers, incubate the raction at 25 °C for 10 minutes to extend the primers prior to the 42 °C synthesis step. If using oligo(dT) or gene-specific primers, proceed to step 6.
6. Place the tube in a temperature controlled thermal block at 42 °C and incubate the reaction for 60 minutes
7. Terminate cDNA synthesis by incubating the reaction at 70 °C for 15 minutes
8. Place the completed first-strand cDNA synthesis reaction on ice for use in downstream applications. If performing RT-PCR, proceed to the PCR amplification protocol.
9. For long term storage, place the reaction at -20 °C.

Appendix H: Taq Polymerase (5prime, Cat. No 2200310) amplification protocol

1. Prepare the reaction by adding the following components to sterile thin-walled PCR tubes *in order*:
    a. 13.75 µl nuclease-free water
    b. 0.5 µl forward primer (0.1ng/µl)
    c. 0.5 µl reverse primer (0.1ng/µl)
    d. 2.5 µl 10x Mg Buffer
    e. 2.5 µl dNTP mix (25mM each dNTP)
    f. 0.25 µl Taq polymerase
    g. 5.0 µl cDNA
2. Place the reaction into a thermocycler at the following program:
    a. 94 °C for 2.00 minutes
    b. 94 °C for 0.2 minutes, then 56 °C for 0.1. minutes, then 65 °C for 0.35 minutes
    c. Repeat above 35 times
    d. 65 °C for 1.00 minutes
    e. 4 °C for holding temperature

Appendix I: SYBR ® Green (Applied Biosystems, Cat. No. 4309155) real time-PCR protocol

1. Prepare the reaction by adding the following components to an optical 96-well plate *in order*:
    a. 7.0 µl nuclease-free water
    b. 1.5 µl forward primer (5 µM)
    c. 1.5 µl reverse primer (5 µM)
    d. 12.5 µl SYBR ® Green Master Mix
3. Place the reaction into a thermocycler at the following program:
    a. 94 °C for 2.00 minutes
    b. 94 °C for 0.2 minutes, then 56 °C for 0.1. minutes, then 65 °C for 0.35 minutes
    c. Repeat above 35 times
    d. 65 °C for 1.00 minutes
    e. 4 °C for holding temperature

Appendix J: Model statement for the statistical analysis of data in Experiment 2 of Chapter 4

Model 4.1:

$Y_{ij} = \mu + V_i + R_j + V*R_{ij} + \varepsilon_{ij}$

Where:

1. Y_{ij} is the relative quantity of receptor j within vessel i
2. μ is the overall mean
3. V_i is the fixed effect due to vessel; i=1,...40.
4. R_j is the fixed effect due to receptor; j=1,...5.
5. $V*R_{ij}$ is the interaction of vessel and receptor.
6. ε_{ij} is the random error term; assume ε_{ij} iid, $\sim N(0, \sigma^2_{\varepsilon})$.

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