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Evaluation of vascular changes in cattle relative to time-off endophyte-infected tall fescue

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THESIS

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The Graduate School

University of Kentucky

2012

EVALUATION OF VASCULAR CHANGES IN CATTLE RELATIVE

TO TIME-OFF ENDOPHYTE-INFECTED TALL FESCUE

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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2012

ABSTRACT OF THESIS

EVALUATION OF VASCULAR CHANGES IN CATTLE RELATIVE

TO TIME-OFF ENDOPHYTE-INFECTED TALL FESCUE

Twenty-four steers were grazed on endophyte (*Neotyphodium coenophialum*)-infected [[*Lolium arundinaceum* (Schreb.) Darbysh]; E+] tall fescue (TF) and exposed to ergot alkaloids for an 106-d grazing period. Cattle were removed from pasture, placed in dry lots, and fed a non-toxic diet to evaluate changes in vascular contraction relative to time-off E+ TF pasture. Lateral saphenous veins (SV) were biopsied from steers at 0-, 21-, 42-, and 63-d off TF pasture and from 6 control steers at 0- and 63-d off bermudagrass (BG) pasture. To evaluate contractile response, biopsied SV were exposed to increasing concentrations of ergotamine in a multimyograph. Cross-sectional scans of the caudal artery were taken using color Doppler ultrasonography on 0-, 8-, 15-, 21-, 29-, 36-, 42-, and 45-d to determine artery luminal area. Contractility of SV was less for TF than BG steers on d 0, but was similar between the two treatments by d 63. Luminal areas of the caudal arteries in E+ TF steers relaxed over time and were similar to BG steers by 36 d off pastures. Results indicated that alkaloid-induced vasoconstriction in cattle grazed on E+ TF can be relaxed in 5 to 6 weeks after they are placed on non-toxic diets.

KEYWORDS: Beef Cattle, Fescue Toxicosis, Ergot Alkaloids, Vasoconstriction, Bovine Vascular Changes

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September 7, 2012

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DEDICATION

This thesis is dedicated in memory of my late dear friend and colleague, Dr. Chris

Raines. He always challenged me to question conventional wisdoms and in the process

helped me to become a better person.

ACKNOWLEDGEMENTS

the passion that I have for agriculture. Also I acknowledge my undergraduate professors from Penn State University, Drs. Burt Staniar, Daniel Kniffen, Chris Raines, and Marvin Hall for their encouragement to pursue my graduate degree and always challenging me to better myself. I would not be who I am today without the help of the many supportive and loved family and friends I have.

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Chapter 1: Introduction

There is a reported 14 million ha of tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh] grown in the humid areas of the southeastern United States located in the upper transition zone, also known as the "Fescue Belt" (Bouton, 2011). The tall fescue variety 'Kentucky 31,' originally collected in 1931 on the farm of William Suitor in Menifee Co., Kentucky, was released in 1943. Use of this cool-season forage grass was soon widespread as it possessed many agronomic advantages. In the late 1970s scientists discovered that 'Kentucky 31' was infected with an endophyte (*Neotyphodium coenophialum*; E+) that imparted the agronomic advantages seen in this forage, but also the declines in animal performance that had been a concern of cattle producers (Ball, 1984). Ergot alkaloids, a class of compounds produced by the endophyte, were determined to be the cause of three different syndromes in animals that had consumed tall fescue. Of these syndromes, fescue foot and fat necrosis were only observed in isolated cases, while the third, fescue toxicosis attributes to colossal economic losses for the livestock industries. Hoveland (1993) cited annual economic losses to the cattle industry at greater than $600 million.

Symptoms of fescue toxicosis include poor weight gains, rough hair coat, reduced reproductive performance, fescue foot, lower milk yields, excessive salivation, and low serum prolactin levels (Hemken et. al, 1984; Stuedemann and Hoveland, 1988). Ergot alkaloids produced by the endophyte can alter cardiovascular function (Strickland et al., 2011) and induce constriction in vascular tissue of extremities of animals grazing tall

fescue (Strickland et al., 2009). Cattle consuming ergot alkaloids are unable to regulate body temperature, leading to a higher risk of heat stress in elevated temperatures (Aldrich et al., 1993). When fescue cattle are transported to feedyards, physiological stressors from toxicosis can be combined with the stress of transport to increase the risk of high morbidity and death loss (Aiken et al., 2001). An unknown factor in this interaction is whether vasculature recovers from ergot alkaloid exposure after animals are removed from E+ tall fescue pastures.

Work by Klotz et al. (2009) showed that the ergot alkaloid, ergovaline, has a very strong receptor affinity and disassociates very slowly, leading to bioaccumulation in vascular tissues. Realini et al. (2005) reported that ergot alkaloids ingested during grazing were deposited in adipose tissue. A portion of ergot alkaloids are rapidly metabolized and excreted in the urine of cattle grazing E+ tall fescue within 48-h after cattle are removed from E+ tall fescue pastures (Stuedemann et al., 1998; Hill et al., 2000). With this rapid excretion and clearance of ergot alkaloids it would be expected that cattle finished at a feedlot after coming off of E+ pastures would not exhibit carryover effects of fescue toxicosis during the finishing phase (Realini et al., 2005). However, Realini et al. (2005) reported unpublished data from S.K. Duckett that indicated fescue toxicosis symptoms were present in feedlot cattle that had previously grazed E+ tall fescue. This research suggests that alkaloids present in adipose tissues from cattle previously grazing E+ tall fescue may be released and metabolized during the feedlot phase, causing residual toxicosis symptoms and poor cattle performance.

To determine if fescue toxicosis symptoms are still present in cattle after removal from E+ tall fescue a pen study was conducted to evaluate vascular changes in steers

relative to time-off E+ tall fescue. It was hypothesized that initially vascular contractility would be reduced in cattle removed from E+ tall fescue pasture. As time-off pasture increased, vascular contractility indicative of recovery from ergot alkaloids would be seen in steers previously grazed on E+ tall fescue. To monitor these changes in vascular contraction and constriction over time, an in vitro method, a myograph, and an in vivo method, Doppler ultrasonography, will be compared and contrasted to determine their effectiveness as diagnostic tools of hemodynamic and vascular changes in cattle recovering from fescue toxicosis.

Chapter 2: Literature Review

Tall fescue is a cool-season perennial grass that is a wind-pollinated, highly self-infertile polyploidy that is extensively utilized for forage, runoff control, and turfgrass (Bacon, 1995). This cool-season grass was introduced from Europe into the United States during the 1800s where it was planted in test plots in several states (Buckner, 1979). However, its use did not become widespread until the 1930s. The discovery that led to the widespread dispersion of the forage grass occurred in 1931 on the farm of William Suiter in Menifee County, Kentucky. A unique biotype that was both persistent and productive was discovered on a steep hillside pasture. Dr. E. N. Fergus, an agronomy professor at the University of Kentucky was impressed with the forage and obtained seed from the biotype for trials. This led to the later foundation and commercial release of the popular forage cultivar 'Kentucky 31' in 1943 (Fergus, 1952).

Tall fescue is well adapted to the humid areas of the southeastern United States in the transition zone, also known as the "Fescue Belt." This zone exists between the temperate northeast and the subtropical southeast extending as far west as eastern Oklahoma and Kansas. Reports from surveys by Shelby and Dalrymple (1987) indicate that 95% of all United States tall fescue pastures are infected with the endophyte. This forage offers many agronomic advantages including wide environmental adaptation, persistence under a wide range of management systems, good forage yield, a long growing season, and excellent seed production (Ball, 1984). While tall fescue exhibits many desirable qualities, poor animal performance occurs in livestock grazing this forage. These anti-quality factors are attributed to the production of ergot alkaloids

produced by the fungal endophyte that exists in a purely mutualistic association with the plant residing intercellularly in leaf and stem tissues (White, 1988; Clay, 1988). Several alkaloids isolated from tall fescue have been proven to be biologically active; however, the major toxin produced in culture was ergovaline (Porter et al. 1979, 1981). This symbiotic relationship imparts the endophyte with a reliable source of nutrients, protection, and propagation while improving the plant's capacity to survive drought, insect pests and mammalian herbivory (Ball et. al, 2007; White, 1988). In 1976, a USDA-ARS scientist, Dr. Joe Robbins made the discovery of the fungal endophyte residing inside the tall fescue plant (Ball, 1984). This discovery led to the development of endophyte-free (E-) cultivars in the 1980s which initially proved superior to toxic tall fescue showing increased rates of gain, better reproductive performance, and increased milk production (Roberts and Andrae, 2004). However, as use of the new cultivars increased, it became apparent that E- varieties lacked the stand persistence, drought tolerance, and resistance to other environmental factors that its E+ counterparts possessed (West et al., 1993; Latch, 1993; Malinowski and Belesky, 2000). More recently cultivars containing non-ergot alkaloid producing endophytes have been developed. These new varieties increase plant persistence and resistance to diseases and pests without the added negative effects of ergot alkaloids on animal performance (Roberts and Andrae, 2004). Novel endophyte tall fescue cultivars provide a valuable tool for development of management strategies to mitigate the negative effects of fescue toxicosis in cattle.

Fescue Toxicosis Effects on the Cardiovascular System

Cattle grazing toxic E+ tall fescue suffer from fescue toxicosis which induces symptoms of reduced growth or milk production, decreased feed intake, rough hair coat, elevated body temperature, increased respiratory rate, lower serum prolactin levels, excessive salivation, and reduced reproductive performance (Hemken et. al, 1984; Strickland et. al, 2009; Stuedemann and Hoveland, 1988). Other symptoms exhibited include reduced grazing time and extended time in shade, mud holes, ponds or streams (Stickland et. al. 2009; 1993). Many of these symptoms can be explained by compromised cardiovascular function linked to the presence of ergot alkaloids in cattle consuming E+ tall fescue (Strickland et. al, 2011), which induces constriction of blood vessels in peripheral tissues. Early research by Jacobson et al. (1963) linked blood vessel dysfunction to fescue toxicosis reporting blood vessel congestion, perivascular hemorrhage, and gangrene in the tail of heifers consuming tall fescue. This altered cardiovascular function leads to an inability to regulate body temperature and consequently a higher risk of heat stress in high ambient temperatures (Aldrich et. al, 1993). Hemken et al. (1981) found the most detrimental effects occurred when ambient temperatures exceeded 31°C.

Diets containing E+ tall fescue seed (0.05 ppm to 1.18 ppm ergovaline) have been shown to cause reduced blood flow to the skin of wethers and steers (Rhodes et. al, 1991). This reduced blood flow to periphery tissues resulted in an increase in rectal temperatures. Rhodes et al (1991) also noted a reduction in blood flow to the duodenum and colon. These findings indicate that reduced blood flow to the gastrointestinal tract could impede the animal's ability to absorb nutrients, in turn causing the reduction in

growth and decreased DM intake seen in cattle suffering from fescue toxicosis (Stickland et. al, 1993). It has been suggested that for every 10% increase in infestation of endophyte in tall fescue pastures, a 0.045 kg decrease in steer average daily gain is possible (Garner et al., 1984; Crawford et al., 1989)

The ergot alkaloid class of chemical compounds is favored as the primary cause of fescue toxicosis and in particular, cardiovascular dysfunction (Strickland et. al, 2008). Vascular tissues contain receptors in the membranes of endothelial cells. These receptors act to moderate blood pressure and flow through the vessels. Ergot alkaloids' ability to interact with these receptors is attributed to their structural similarity to biogenic amines, like norepinephrine, dopamine, and serotonin, which causes impairment in vascular function via both agonistic and antagonistic activity (Berde, 1980). Ergovaline has been found to be the major toxin produced by the fungal endophyte of tall fescue (Porter et al. 1979, 1981). While evidence from Oliver et al. (1998) and Klotz et al. (2008) indicated the possibility of an additive alkaloid exposure effect on adrenergic receptors, compelling results indicate that $5\text{-}HT_2$ receptors and not adrenergic are involved in vasoconstriction (Dyer, 1993; Schoning et al., 2001; Klotz et al., 2007). Ergovaline is the primary vasoconstrictor, especially in peripheral vasculature, and differential responses occur in peripheral versus core vascular tissues (Foote et al., 2012). This differential response is the result of the population of receptor sites varying across tissues and organs in vivo.

Along with these effects on blood vessel morphology and blood flow, in vitro bioassays using vascular models have shown a sustained contractile response to ergovaline (Dyer, 1993; Schöning et al., 2001; Klotz et al., 2007). Dyer et al. (1993) suggested that ergovaline has a high, almost irreversible, binding affinity with vascular

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receptors. It was suggested that if this binding affinity were strong enough, it could possibly permit a post-absorptive accumulation of ergovaline through a delayed clearance caused by an initial buildup in animals grazing E+ tall fescue (Klotz et al., 2009). This bioaccumulative effect was proven in in vitro studies by Klotz et al. (2009) showing that repetitive editions of 1 x 10^{-7} M ergovaline caused an increasing contractile response and resulted in a significant increase in ergovaline extracted from increasingly exposed saphenous veins. Realini et al. (2005) reported that subcutaneous fat from E+ cattle contained higher concentrations of ergot alkaloids than that from cattle exposed to the novel endophyte tall fescue, AR542. The results indicated a deposition of ergot alkaloids in adipose tissue of cattle consuming toxic E+ tall fescue. Along with evidence from Klotz et al. (2009), these results support the assumption that ergot alkaloids bioaccumulate in vivo.

Evidence of the link between ergot alkaloids of toxic E+ tall fescue and structural changes in bovine vascular tissue was shown by Stickland et al. (1996) when they reported that ergopeptides, ergonovine, α-ergocrytptine, and ergovaline, stimulated growth of bovine smooth muscle tissue in vitro. Williams et al. (1975) reported thickened walls and small lumens of blood vessels in the feet of calves given an alcohol extract of tall fescue hay. This evidence supports the assumption that smooth muscle growth is a significant contributor to cardiovascular dysfunction in cattle exhibiting fescue toxicosis. Strickland et al. (2009) surmised that the effects of ergot alkaloids on vascular tissues could lead to changes in blood vessel luminal diameter and blood flow. Induced vasoconstriction and vascular smooth muscle cell hyperplasia (Oliver and Schutlze, 1997) substantially reduce blood flow to peripheral tissues, in turn leading to a decline in

efficiency of heat transfer from core tissues and surface dissipation. Aiken et al. (2007) further proved this assumption with Doppler ultrasonography showing that heifers consuming E+ tall fescue seed exhibited reduced caudal artery area and blood flow rates when compared to heifers receiving an E- seed diet.

Previous research has shown that 94% of ergot alkaloids are excreted in the urine of cattle grazing E+ tall fescue and that alkaloids are metabolized at a rapid rate and voided from urine within 48-h after cattle are removed from E+ tall fescue pastures (Stuedemann et al., 1998; Hill et al., 2000). Realini et al. (2005) speculated that with rapid excretion and clearance of ergot alkaloids it could be expected that cattle finished in the feedlot after coming off of E+ pasture would not exhibit any carryover effects of fescue toxicosis in the final finishing period. Realini et al. (2005) was able to show that ergot alkaloids ingested during grazing were deposited in adipose tissue. Unpublished data from S. K. Duckett showed feedlot cattle exhibited fescue toxicosis symptoms if they had previously grazed endophyte-infected tall fescue (Realini et al., 2005). Allen et al. (2001) reported similar results to Duckett showing depressed monocyte immune cell function and elevated rectal temperatures in steers that had previously grazed E+ tall fescue upon arrival at the feedlot. By d 14 after arrival no temperature difference was detected between body temperature of steers that had grazed E+ tall fescue compared to those that had grazed uninfected pastures (Allen et al., 2001). However, Allen et al. (2001) noted as time progressed temperature effects were reversed with steers that had grazed E+ tall fescue exhibiting lower rectal temperatures on d 112 after arrival at the feedlot. These results demonstrate a much longer-lasting effect of ergot alkaloids on bovine thermoregulatory mechanisms than previously known. The research suggests that

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alkaloids present in adipose tissues or other tissues in cattle previously grazing E+ tall fescue may cause residual toxicosis symptoms and poor cattle performance. Interestingly however, contrasting results of a study by Klotz et al. (2012) showed that the vasculature of cattle grazed on high E+ tall fescue pastures and placed on a non-toxic diet for 91 to 103 d before slaughter had recovered. Further research is required to determine at what time point fescue toxicosis symptoms are no longer observed and contractile differences are no longer significant in cattle that have been removed from toxic E+ tall fescue.

Current research has examined ergot alkaloid effects on blood vessels using various bioassay models located in peripheral tissues of the animal. This is mainly due to the fact that blood flow to extremities is reduced in animals suffering from fescue toxicosis. In addition, technical challenges of conducting research on blood flow in live animals, particularly in deeper tissues, limit applications and justify the need to examine primarily peripheral tissues. In the next sections, we will examine current research using both myograph and Doppler ultrasonography to examine vasoconstriction caused by fescue toxicosis.

Myograph Studies Related to Fescue Toxicosis

Various in vitro bioassay models have been used to examine blood vessels of cattle (Solomons et al., 1989; Oliver et al., 1993; Klotz et al., 2006, 2012, Dyer, 1993) using a myograph systems that allows for rapid screening of alkaloids and other compounds for vascular activity (Mulvany and Halpern, 1977; Nielsen-Kudsk et al., 1986; Oliver et al., 1993, 1998). Species such as the horse (Abney et al., 1993), guinea pig, and rat (Schöning et al., 2001) have also been studied using similar vascular bioassay models. This approach allows researchers to study the direct effects of individual ergot

alkaloids in pure form and combinations. This method also eliminates the risk of indirect variable effects on physiological functions that ergot alkaloids commonly induce in vivo. This methodology allows researchers to explore not only blood vessels from different parts of the body, but also to conduct detailed dose response studies, determine molecular target sites, and provide comparative toxicity information on various ergot alkaloids (Strickland et al., 2009).

Myograph studies involve dissecting segments of blood vessels from select areas of animals for use in in vitro bioassay experiments. These dissections use previously published protocols (Solomons et al., 1986, 1989; Oliver and Abney, 1989; Oliver et al., 1990, 1992; Klotz et al., 2006, 2008, 2011). Past studies have examined dorsal pedal vein (Solomons et al., 1989), dorsal metatarsal artery (Oliver et al., 1993), bovine uterine and umbilical arteries (Dyer, 1993), lateral saphenous vein (Oliver et al., 1993, 1998; Klotz et al., 2006, 2007, 2008, 2009, 2012), and more recently right ruminal artery and vein (Klotz et al., 2011; Foote et al., 2011, 2012).

Solomons et al. (1989) determined the vasoconstrictive responses of the bovine dorsal pedal veins to ergot alkaloids and a sample containing loline and its derivative alkaloids. The ergot alkaloids, ergotamine, ergosine, and agroclavine were found to cause vasoconstriction of this particular peripheral vein of cattle, with ergotamine giving the largest contractile response and therefore concluded the most toxic. While ergotamine was more potent than the α-adrenergic agonist, norepinephrine, none of the ergot alkaloids were as successful at reaching a significant maximal contractile force (Solomons et al., 1989). Loline and loline derivative alkaloids at 10^{-5} and 3×10^{-9} M concentrations were found to partially inhibit norepinephrine-elicited vascular

contraction. However this vasoconstrictive response was lesser when compared to that of ergopeptide alkaloids and appeared to be unrelated to α-adrenoceptor activity. Solomons et al. (1989) results support contentions that ergot alkaloids from E+ tall fescue act as partial receptor agonists and are responsible for peripheral vasoconstriction in animals suffering from fescue toxicosis (Aiken et al. 2007; Rhodes et al., 1991; Walls and Jacobson, 1970).

Research by Oliver et al. (1993) examined effects of the tall fescue ergot alkaloid, lysergamide (lysergic acid amide) on bovine lateral saphenous vein and dorsal metatarsal artery. Lysergamide induced vasoconstriction similar to that seen previously from ergot alkaloids ergonovine and ergotamine (Oliver et al., 1990, 1992). Partial inhibition of contractile response induced by the adrenergic antagonist, phenylephrine, was seen when tissue was preincubated in lysergimide, indicating a possibility of partial agonist or antagonist activity on receptors. Further evidence of receptor activity was seen when contractile response of bovine vessels preincubated in lysergimide were noticeably inhibited in the presence of serotonin, indicating possibility of activity on serotonergic receptors. Oliver et al. (1993) concluded that lysergimide alone possesses vasoconstrictor activity and could serve as a partial agonist or antagonists on both adrenergic and serotonergic receptors. Activity on serotonergic receptors was further shown in research by Dyer (1993) when activity of the potent vasoconstrictor, ergovaline, showed high affinity for 5-HT$_2$ receptors but not adrenergic receptors in bovine uterine and umbilical arteries. This sustained contractile response to ergovaline (Dyer, 1993) also reinforces Rhodes et al. (1991) findings that blood vessels in deeper tissues are also affected by ergot alkaloids. In later saphenous vein bioassays Oliver et al. (1998) showed greater

contractile responses to selective adrenergic receptor agonists for α_2- adrenergic receptors in cattle pastured on E+ tall fescue. Work by Schöning et al. (2001) agreed with that of Dyer (1993) when it was shown serotonergic agonist potency and maximum contractile response to ergovaline in rat tail artery were in the same range as bovine uterine and umbilical arteries.

More recent research by Klotz et al. (2006) validated a bioassay using bovine lateral saphenous vein similar to that previously conducted by Oliver et al. (1990, 1992, 1993). Using bovine lateral saphenous vein on a multimyograph, Klotz et al. (2006) validation was achieved using a dose-response of norephinephrine to evaluate effects of limb of origin (left vs. right) and overnight storage of vessels. Klotz et al. (2006) also examined the vasoconstrictive effects of lysergic acid during this experiment. Results determined there was no significant difference in contractile responses of vascular tissue in reference to limb of origin or between tissue tested the day of dissection and that tested 24 hours later. Lysergic acid was found to only trigger appreciable contractile response in extremely high concentrations (1×10^{-4} M) indicating its role as a causative agent in fescue toxicosis is likely minor. Klotz et al. (2007) further examined the vasoconstrictive potentials of ergovaline and two more documented ergopeptides, ergopeptine and ergotamine. Klotz et al. (2007) found that contractile responses for ergovaline and ergotamine were similar at 1×10^{-8} M concentrations and increased with increasing alkaloid concentrations. However, ergovaline induced contraction could not be reversed by repeated buffer changes over an extended period. This confirmed previous findings by Dyer (1993) that ergovaline is a potent and persistent vasoconstrictor. These findings indicated that ergot alkaloids may bioaccumulate in the bovine vasculature. Klotz et al.

(2009) provided strong support of this bioaccumulation theory by reporting that vascular tissue repeatedly exposed to ergovaline in vitro caused increases in the contractile response along with a measurable bioaccumulation of alkaloids extracted from the tissue.

Additional research from Klotz et al. (2008) examined vasoconstrictive potentials of increasing doses of ergovaline, lysergic acid, and N-acetylloline individually or in paired combinations. As was seen in previous research (Klotz et al., 2006), lysergic acid evaluated individually did not produce appreciable contractile response until the addition of $1x10^{-4}$ M lysergic acid. Results for individual evaluations were similar to that of Klotz et al. (2007). N-acetylloline by itself did not produce significant contractile response; however, when evaluated in combination with the other alkaloids, there was an increase in contractile response. Klotz et al. (2012) also showed a noticeable difference in contractile responses in cattle grazing low versus high levels of E+ tall fescue pastures. Extended grazing on high E+ pastures induced functional alterations of blood vessels seen as reduced contractile capacity and altered serotonergic receptor activity. Steers on low E+ pastures exhibited much higher contractile responses compared to those on high E+ pastures. In addition, Klotz et al. (2012) showed that while grazing high E+ pastures alters vascular responses, these alterations can be resolved by removing cattle from E+ pastures and placing cattle on a non-toxic finishing diet.

To date, the bovine right ruminal artery and vein is the most recent bioassay validated for use in studying bovine vascular tissue (Klotz et al., 2011). Foote et al. (2011) used this bioassay to profile the dose response of 7 different ergot alkaloids and a E+ tall fescue extract. Results indicated that ergot alkaloids associated with E+ tall fescue are indeed vasoactive and can potentially alter arterial blood supply and venous

drainage from the bovine foregut. In a separate study, Foote et al. (2012) evaluated the vasoconstrictive effects of ergovaline and a toxic tall fescue extract on bovine right ruminal artery and vein tissues. While tall fescue extract appeared to induce greater contractile response when compared to pure ergovaline there were no significant differences in the responses. While ergovaline is still considered a primary vasoconstrictor, these results indicate a possibility of non-ergovaline ergot alkaloids playing an important role in vasoconstriction of core vasculature. These data support Rhodes et al. (1991) findings that ergot alkaloids affect core vasculature but indicate a clear differential response of peripheral and core vasculature to ergot alkaloids.

While the myograph is the only method available to study direct effects of ergot alkaloids in pure form and in combination on blood vessels and eliminates much of the risk of variable effects of physiological functions induced in vivo, it ultimately does not provide real-time results indicative of what actually occurs in vivo. In vitro studies can provide valuable fundamental information, but there is a need for an evaluation method where effects of ergot alkaloids on vasculature can be studied in vivo. With this information researchers will be able to develop management protocols that can alleviate the adverse effects of ergot alkaloids on bovine blood flow.

Doppler Ultrasonography Studies Related to Fescue Toxicosis

Color Doppler ultrasonography was introduced to the medical field in the 1980s and in the 1990s was combined with B-mode (two dimensional, gray scale) scans to produce duplex images (Aiken and Strickland, 2012). This combination of scans provided a useful diagnostic and research tool to examine the hemodynamics, characteristics, and physiology of blood flow and the cardiovascular system. Doppler

ultrasonography has been successfully used in human medicine to evaluate arterial thickness (Raninen, 1997) and investigate peripheral vascular disease (Whelan et al., 1992). Poulin and Robbins (1996) evaluated the hemodynamics and cross-sectional luminal areas of the middle cerebral artery in humans during hypoxia and hypercapnia. Peterson et al. (1997) found a correlation between the severity of renal disease and median resistive and pulsatility indices by using pulsed-wave Doppler measurements of downstream renal artery resistance. Primary use of this technology in large animal veterinary medicine to assess vasculature has been primarily with horses (Hoffman et al., 2001; Mario et al., 1997; Raisis et al., 2000). Use in cattle has been far less prevalent, but what research has been done has proven ultrasound imaging to be an efficient and non-invasive means of examining vascular changes in peripheral blood vessels (Raisis et al., 2000; King, 2006; Aiken et al., 2007, 2009).

A field technique using Doppler ultrasonography to detect changes in blood flow characteristics in the medial caudal artery of cattle has been successfully developed and has proven especially useful in evaluation of vascular function in cattle suffering from fescue toxicosis (Kirch et al. 2008; Aiken et al., 2007, 2009b). Using a pulse-wave transducer, cross-sectional color Doppler images can be used to trace luminal areas of vessels and combined with blood flow velocities derived from Doppler spectra from a longitudinal image of the vessel to calculate blood flow rate (Aiken and Strickland, 2012). Luminal areas derived from cross-sectional scans can also be used to evaluate contractile responses of smooth muscle within artery and vein walls to environmental conditions, pharmaceuticals, or toxicants (Aiken et al., 2007; 2009; 2011). This

technology allows for in vivo vascular assessment, unlike the multimyograph, proving to be a useful diagnostic and research tool that is non-invasive, repeatable, and objective.

In research involving the use of toxicants, Doppler ultrasonography determinations of constriction and blood flow in small arteries or veins are possible if comparisons are made between with groups of animals and without toxicant treatment or between baseline measures and after animals are exposed to the toxicant (Aiken and Strickland, 2012). Kirch et al. (2008) demonstrated the ability of Doppler imaging to detect vascular changes and blood flow rates in opposing environmental conditions of hot (32.7°C) and cold (8.1°C) temperatures. A constrictive response to ergot alkaloids was determined from cross-sectional images of the medial caudal artery in beef heifers (Aiken et al., 2007, 2009; Kirch et al., 2008). For sheep, the caudal artery is unreliable due to tail docking; therefore cross-sectional images of the posterior auricular artery that supplies blood to the ear were used to assess vasoconstriction (Aiken et al., 2011). A comparison to determine differences in the variation of lumen area measurements of the caudal artery between tracing the interface between the tunic adventitia and media layers or the outer boundary of the color Doppler flow signal indicated that lumen area of the caudal artery can be measured with similar precision with either tracing method (Aiken et al., 2008). While research conducted with Doppler ultrasonography to evaluate ergot alkaloid toxicities in livestock is limited, it has shown to be an effective, quick, and non-invasive method to visualize and evaluate the complex hemodynamic and vascular changes in cattle that are subjected to fescue toxicosis.

Chapter 3: Materials and Methods

Experimental Site

A pen experiment was conducted for 63 d in 2011 at the University of Kentucky C. Oran Little Research Center in Woodford County. The experimental protocol was approved by the Institutional Animal Care and Use Committee at UK (2011-0791).

Animal Management

Twenty-four predominately Angus steers grazed E+ tall fescue pastures (1.0 ha) for 88 days during a separate grazing study. Upon completion of the grazing study on July 14, steers grazed on E+ were moved to holding pastures (3.0 ha) also containing E+ tall fescue until the beginning of the biopsy trial. Six steers grazed on 'Wrangler' bermudagrass [*Cynodon dactylon* (L). Pers.] pasture (3.0 ha) were used as an non-toxic control. Steers grazing E+ tall fescue and bermudagrass pastures had mean BW of 361.5 ± 4 kg and 370 ± 18 kg, respectively. Cattle were removed from pasture and penned on August 2 to evaluate changes in vascular contraction and constriction as time-off of E+ pasture increased. Steers were fed a non-toxic diet of corn silage ad libitum and a mixture of soybean hulls, soybean meal, and mineral premix mixture at 2.3 kg/steer/day (as fed). Cattle were treated as needed at the beginning of the study with moxidectin dewormer (1 mL/10 kg BW pour-on) (Cydectin®, Fort Dodge Animal Health, Fort Dodge, IA). Vascular responses of steers were monitored over this time period. Contractility was used as the factor to indicate vascular recovery from ergot alkaloid exposure.

Experimental Design and Animal Measures

Steers were removed from pastures in two groups of 15 staggered by two days. Twelve steers from the E+ pastures were randomly assigned to four pens (3 steers per pen). Two days later, the remaining twelve E+ steers were removed from pasture and randomly assigned to the four pens. Six steers were removed from bermudagrass pasture on the day between the two days that the two groups of steers were removed from E+ tall fescue. The six steers removed from bermudagrass were placed into one pen.

Saphenous Vein Biopsy

Cranial branches of lateral saphenous veins were biopsied on d 0, 21, 42, and 63 from steers that had previously grazed on E+ tall fescue (n = 24) or E- bermudagrass (n = 6) pastures. A maximum of 3 steers were biopsied on a given day relative to removal from pasture.

Lateral saphenous veins used for experiments were biopsied from steers using methods reported by Klotz et al. (2008). Before each biopsy, steers were placed in a left lateral recumbency using a tilting hoof trimming table (Spring-O-Matic Inc., Marion, KS). The biopsy site was clipped free of hair, cleaned with povidine-iodine soap solution, disinfected with 70% ethyl alcohol, and locally anesthetized with lidocaine (2% injectable; The Butler Co., Dublin, OH). An incision of approximately 10-cm was made through the skin in the tarsal region slightly above and parallel to the cranial branch of the lateral saphenous vein. Upon identification of the vessel, ligatures were placed after the division of the lateral saphenous vein in cranial and caudal branches and before the cranial branch merged with a branch of the cranial tibial vein.

Isolated venous tissue was excised in a 5-cm section, placed in a modified Krebs-Henseleit oxygenated buffer solution (95% O_2/5% CO_2; pH = 7.4; mM composition glucose, 11.1; $MgSO_4$, 1.2; KH_2PO_4, 1.2; KCl, 4.7; NaCl, 118.1; $CaCl_2$, 3.4; and $NaHCO_3$, 24.9; Sigma Chemical Co., St. Louis, MO) for transport, and kept on ice until processed. Immediately after the biopsy surgery, steers received penicillin (Procaine G, 6,600 U/kg of BW; Norbrook Inc., Kansas City, MO) and flunixin meglumine (Flunixiject, 1.1 mg/kg of BW; IVX Animal Health Inc., St. Joseph, MO) and were returned to the pens. Administration of flunixin meglumine was continued for 3 d post-operatively.

Doppler Ultrasonography

Steers in the last groups to be biopsied (n = 6) and all control steers (n = 6) were used to monitor vasoconstriction/relaxation of the caudal artery using color Doppler ultrasonography. Ultrasound scanning sessions began at 1300 h and ended at approximately 1400 h. Ultrasound measures were taken during the experimental period at 0, 8, 15, 21, 29, 36, 42, and 45 days after being placed in the pens and fed the nontoxic diet. Day 0 was the day that steers were placed in the pens and therefore represents baseline measures. Ultrasound scans of the caudal artery at the 4th coccygeal (Cd4) vertebrae were measured using an Aloka 3500 Ultrasound Unit (Aloka, Inc., Wallingford, CT) with a UST-5542 (13 MHz) linear array transducer set to a 2-cm depth. The scanning protocol followed those described by Aiken et al. (2007) with the exception that five B-mode scans were taken at a frequency of 6.0 MHz to determine cross-sectional area of the caudal artery. Following freezing of an individual scan, frames stored in the cine memory of the unit were searched to store the image exhibiting the maximum flow

signal and assumed to be at peak systolic phase. The flow signal was traced to estimate lumen area (Aiken et al., 2008).

Laboratory Analyses

Biopsied blood vessels were used in vitro to evaluate changes in contractile response to increasing concentrations of the ergot alkaloid, ergotamine. Experiments were run the same day as biopsy. Tissue processing for all vein segments followed methods validated by Klotz et al. (2006) and Solomons et al. (1989). Tissue preparation for experiments consisted of removal of excess fat and connective tissue. Cleaned segments were sliced into 2- to 3-mm cross sections. Cross-sections were examined under a dissecting microscope (Stemi 2000-C, Carl Zeiss Inc., Oberkochen, Germany) at $12.5\times$ magnification to measure dimensions for assurance of consistent segment size and to verify physical integrity of tissue. Cross-sections were suspended horizontally in a 5-mL tissue bath (DMT610M Multichamber myograph, Danish Myo Technologies, Atlanta, GA) containing continuously oxygenated modified-Krebs Henseleit buffer (95% O_2/5% CO_2; pH = 7.4; 37°C), with 3 x 10^{-5} M desipramine and 1 x 10^{-6} M propanolol (Sigma Chemical Co.) to inactivate catecholamine-neuronal uptake and β-adrenergic receptors, respectively. After equilibration to 1 g of tension (~1.5 h), tissues were exposed to the α-adrenergic agonist norepinephrine (1 x 10^{-4} M; Sigma Chemical Co.) to verify tissue viability and as a reference for normalization of the corresponding contractile responses.

Cross-sections of lateral saphenous veins were run in duplicate from each animal (n=30) for each alkaloid experiment. After recovery from the 1 x 10^{-4} M norepinephrine addition (45 to 60 min) and the reestablishment of the 1-g baseline tension, alkaloid

additions occurred in 15-min intervals. Each 15-min interval consisted of a 9-min incubation period, followed by a washout period during which duplicate aliquots of buffer minus the alkaloid were incubated with the vein segment for two 2.5-min periods, followed by a final buffer replacement and 1-min recovery before the next addition. The cumulative alkaloid addition experiments were 8 consecutive additions every 15 min of ergotamine at fixed concentrations ranging from 1 x 10^{-11} to 1 x 10^{-4} M.

Data Collection and Statistical Analyses

Myograph Experiments

Isometric contractions of lateral saphenous vein were recorded as grams of tension in response to exposure to norepinephrine and ergotamine. The data were digitally recorded using a Powerlab/8sp (ADInstruments, Colorado Springs, CO) and Chart software (Version 7.2, ADInstruments). The contractile response was recorded as the greatest contractile response, in grams, within the 9 min after a treatment addition and corrected by the baseline tension recorded just before the addition of 1 x 10^{-4} M norepinephrine. The response data were normalized as a percentage of the contraction produced by norepinephrine (1 x 10^{-4} M). Normalization compensated for variation of the tissue responsiveness due to differences in tissue size or individual cattle. To construct the concentration-response curve for the ergotamine experiments, the normalized data were averaged within dose, and SE were calculated. The mean response and SE were then plotted to illustrate the response of the bovine lateral saphenous vein.

Concentration responses of ergotamine were compared between those of saphenous veins from cattle that had previously grazed E+ tall fescue and bermudagrass

pastures. Data for ergotamine were analyzed as a completely randomized design using mixed models of SAS (version 9.2; SAS Inst. Inc., Cary, NC) for main effects of d-off pasture, concentration of ergotamine addition, and d-off pasture x ergotamine addition concentration with steer as the experimental unit. Contractile response as a percent of the norepinephrine response served as the dependent variable. Analysis of variance (ANOVA) was conducted and mean separation using least significant difference (LSD) was used only if ANOVA was significant for the interaction of the two class variables. Separate analyses were run for d 0 and d 63 comparisons replacing the class variable of d off pasture with pasture to compare contractile responses of veins from cattle that had previously grazed tall fescue versus bermudagrass pastures.

Doppler Ultrasonography

Caudal artery luminal areas were analyzed using mixed models of SAS. Steers were used in the statistical analysis as the experimental unit. Days on non-toxic diet (DNTD) was analyzed as a continuous variable (linear and quadratic regression coefficients), and pasture treatment as a discrete variable (Freund and Littell, 1981). Models containing only interactions between treatments and regression coefficients were analyzed to determine significant ($P \leq 0.10$) linear and quadratic regression coefficients for each pasture treatment (Littel et al., 1996). In the presence of a treatment x DNTD interaction ($P \leq 0.10$), least square means for treatments were compared at 7-d intervals using the PDIFF option of SAS.

Chapter 4: Results and Discussion

Tall fescue pastures were sampled on June 17 for vegetative and reproductive tillers as part of a previous seedhead suppression grazing trial conducted as part of Ben Goff's dissertation research (personal communication). Results indicated vegetative and reproductive tillers contained a mean of 0.15 ± 0.50 ppm ergovaline and ergovalinine. Pasture tiller samples taken on August 1 from holding pastures prior to saphenous vein biopsies the following day showed higher levels of ergovaline and ergovalinine with a mean of 0.57 ppm.

Myograph Experiments

Maximal contractile responses of bovine lateral saphenous veins biopsied on d 0, 21, 42, and 63 to increasing concentrations of ergotamine were 31.3, 46.7, 55.1, and 73.9% of the norepinephrine-induced maximum (NE max), respectively (at 1×10^{-4} M; Figure 1). The onset of a contractile response was observed between 1×10^{-8} to 1×10^{-6} M ergotamine across all time points ($P < 0.05$). Contractile responses were slightly higher than those previously reported for ergotamine (42.9% of NE max, 1×10^{-6} M ergotamine) by Solomons et al. (1989) using bovine dorsal pedal vein. Days 0 and 63 showed significant contractile responses between each other and compared to all other time points ($P < 0.05$). No difference was seen between contractile responses on d 21 and d 42 ($P < 0.05$). A concentration effect became significant at 1×10^{-6} M ($P < 0.05$) for all days. Effect of d × concentration also showed significant contractile responses at all time points at either 10^{-6} or 10^{-7} M ergotamine ($P < 0.05$). Results demonstrated that lateral saphenous vein tissue was increasingly responsive to the ergot alkaloid, ergotamine, as

time-off E+ tall fescue increased. Comparison of contractile responses of saphenous veins from tall fescue steers to that of bermudagrass steers on d 0 yielded maximal responses of 31.3 and 55.1%, respectively. Steers at 0-d off tall fescue pasture had a much lower ($P <$ 0.05) contractile response to ergotamine compared to veins biopsied from steers 0-d off bermudagrass pasture (Figure 2). However, by d 63 contractile responses of tall fescue steers were similar to those of bermudagrass steers resulting in maximal contractile responses of 73.9% for tall fescue steers and 74.5% for bermudagrass steers (Figure 3). Similar results were published by Klotz et al. (2012) who demonstrated that vascular alterations induced by fescue toxicosis were ameliorated in steers from ergot alkaloid exposure during a 91 – 103 d finishing period on a non-toxic diet.

This study's results support those of Klotz et al. (2012) by providing further evidence that ergot alkaloid-induced vascular changes in cattle are reversible and are an indication of recovery from ergot alkaloid exposure over time. While results for ergot alkaloid concentrations in vascular tissue were not obtained in this particular study, in vitro bioaccumulation of ergot alkaloids (Klotz et al., 2009; Mulac and Humpf, 2011) and persistence of these compounds in inducing contractile response (Solomons et al., 1989; Dyer, 1993; Schöning et al., 2001; Klotz et al., 2007) have been shown in previous studies. As was hypothesized, contractile responses of saphenous veins from tall fescue cattle were much lower compared to those of control steers that had previously grazed bermudagrass pasture on 0 d off pasture. Chronic alkaloid exposure over time affects adrenergic and serotonergic receptors (Oliver et al. 1993, 1998). Once removed from E+ tall fescue pastures, dissipation of ergot alkaloids from cellular binding sites on receptors or a gradual turnover of alkaloid-receptor complexes may have lead to increased

contractility of vascular tissues as seen on d 63 with saphenous veins from tall fescue steers exhibiting contractile responses similar to those of bermudagrass steers. While multimyograph studies continue to provide an effective method of testing direct effects of specific compounds on vascular tissue in a laboratory setting, the need exists for an evaluation method with more practical in-field application.

Doppler Ultrasonography

Luminal areas of the caudal arteries of steers previously grazed on E+ tall fescue increased linearly over time (Figure 4). A curvilinear increase was found for luminal areas of the caudal arteries in steers that had previously grazed bermudagrass. This curvilinearity seen in the luminal areas in bermudagrass steers can be explained by the initially high ambient temperatures experienced at the beginning of the study in early August. As the study progressed through September and into early October, ambient temperatures became cooler. Greater variation in caudal artery lumen areas would be expected to be seen in cattle on the non-toxic diet as their ability to respond to environmental conditions is unabated (Aiken et al., 2007) unlike the steers previously grazed on E+ tall fescue who may still be experiencing residual vasoconstrictive effects from ergot alkaloids present in their vascular tissues. At d 0 luminal areas showed no significant differences (P = 0.297) of least squares means between tall fescue and bermudagrass treatments. This may be due to the stress of placement into an unfamiliar environment when steers were taken off pasture and placed into pens. By d 8 significant differences could be seen in lumen areas between tall fescue and bermudagrass steers (P = 0.003) with tall fescue steers exhibiting lower lumen areas compared to bermudagrass

steers. Lumen areas of tall fescue steers had relaxed and were similar to steers that had grazed bermudagrass by d 36 on the non-toxic diet ($P = 0.10$).

Initial vasoconstriction seen in steers that had previously grazed E+ tall fescue was consistent with that seen in heifers fed E+ tall fescue seed in pen studies by Aiken et al. (2007, 2009). The relaxation of caudal arteries of tall fescue steers over time and similarity to that of lumen areas of bermudagrass steers by d 36 agrees with detection of the increasing contractile responses of saphenous veins of tall fescue steers and similarity to contractile responses of saphenous veins of bermudagrass steers by d 63 as seen in the multimyograph studies. These combined results provide strong evidence that these signs are indicative of vascular changes seen in cattle recovering from fescue toxicosis. While it was not determined if complete recovery from ergot alkaloid exposure was achieved, the results show that time to recovery was in 5 to 6 weeks, which extends past the 8 to 10 d period previously determined for prolactin concentrations to return to baseline measures by Aiken et al. (2001, 2006). Doppler ultrasonography proves to be an accurate tool for determination of vascular sensitivities and responses to toxicants such as ergot alkaloids. In the future, color Doppler ultrasonography may prove to be a sufficient non-invasive technique to replace the myograph in studies evaluating vasoconstriction and vascular changes in peripheral tissues of livestock suffering from fescue toxicosis.

Figure 1. Responses to ergotamine concentration for lateral saphenous veins biopsied (n = 6 steers) at 4 time points relative to removal from E+ tall fescue pasture ($P < 0.05$)

Figure 2. Comparison of contractility responses to ergotamine concentrations for the lateral saphenous vein of E+ tall fescue (n = 6) vs. bermudagrass (n = 3) steers at day 0 ($P < 0.05$)
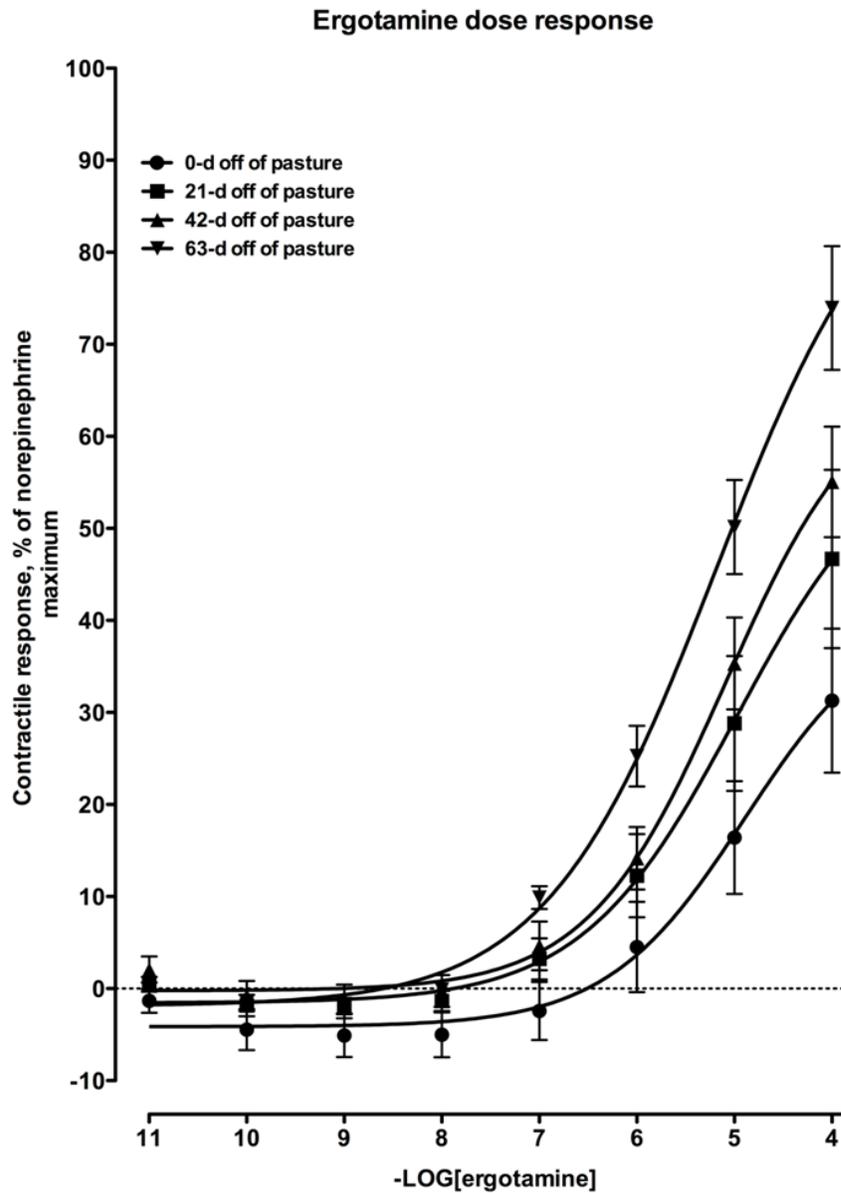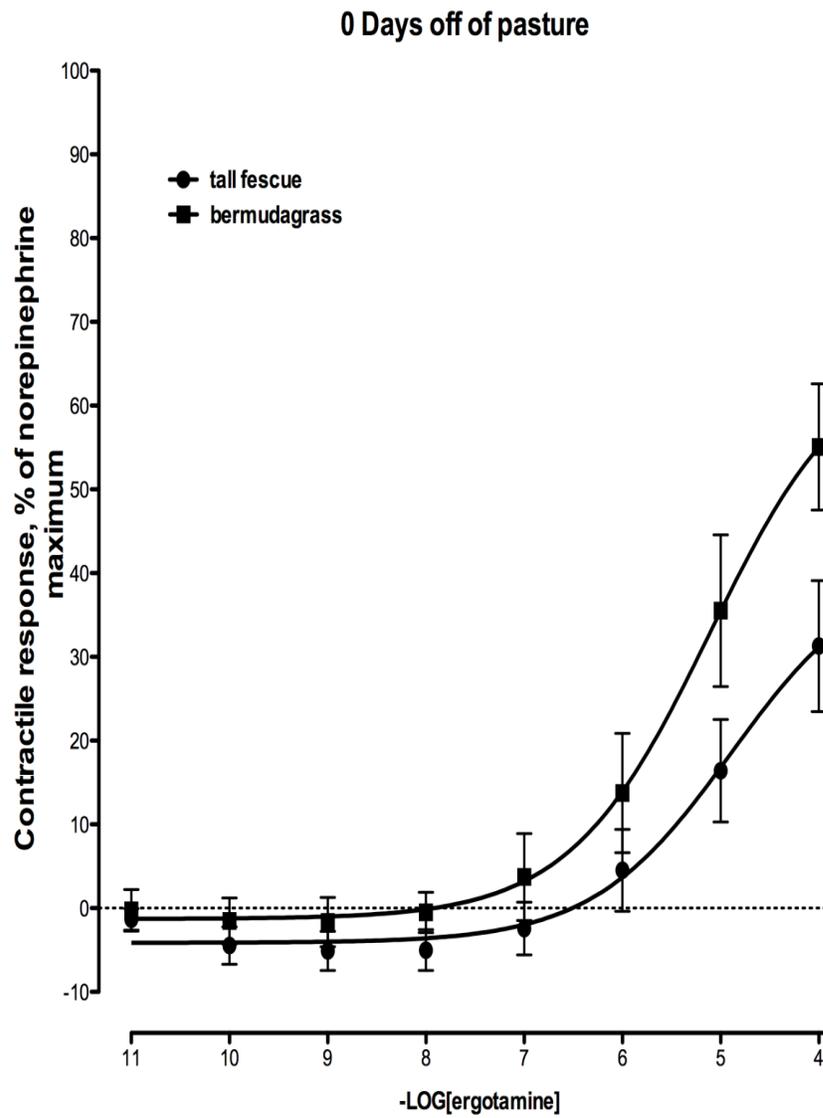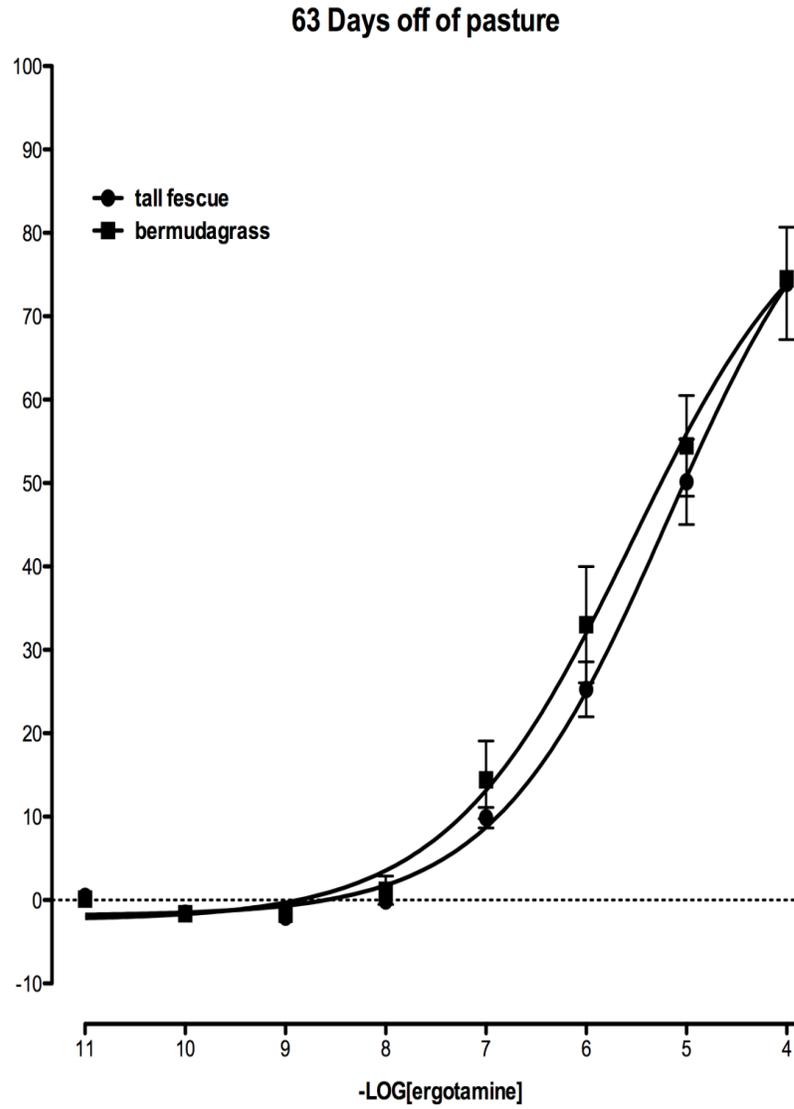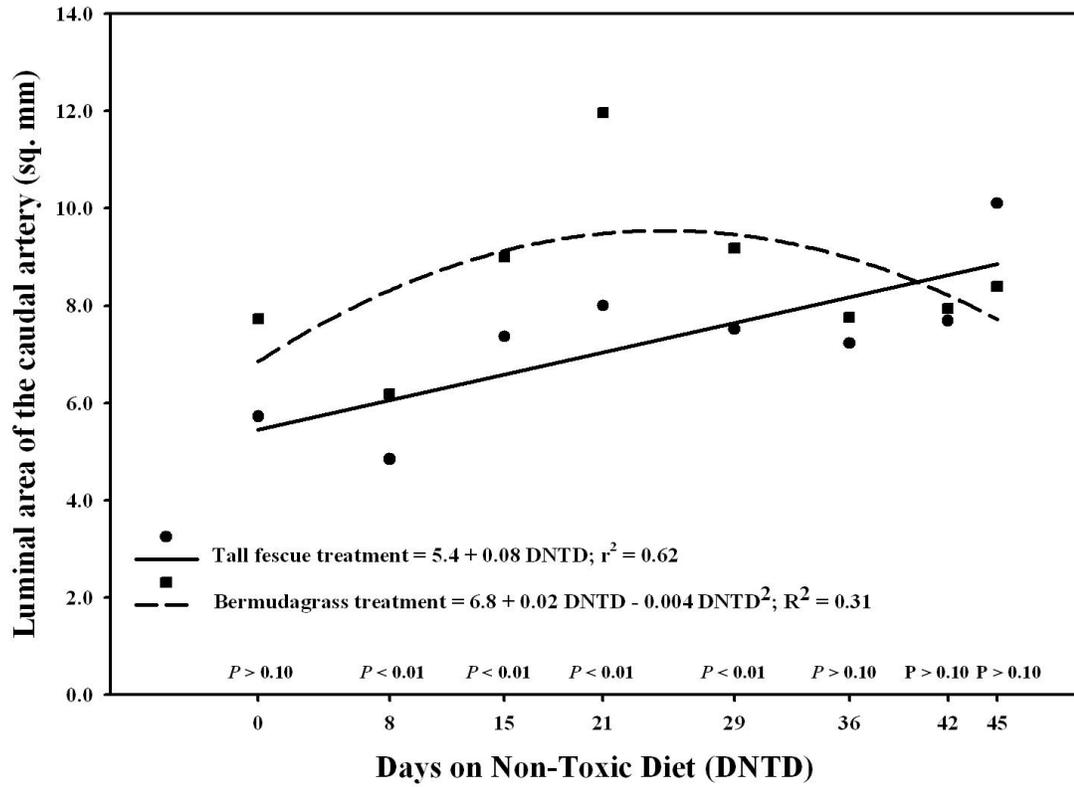

0 Days off of pasture

Figure 3. Comparison of contractility responses to ergotamine concentrations for the lateral saphenous vein of E+ tall fescue (n = 6) vs. bermudagrass (n = 3) steers at day 63 ($P < 0.05$)

63 Days off of pasture

Figure 4. Ultrasonic measures of luminal area of the caudal artery in E+ tall fescue (n = 6) vs. bermudagrass (n = 6) steers regressed over time

Chapter 5: Conclusion

Results indicated that cattle should be removed from E+ tall fescue pastures for a minimum of 4 to 5 weeks to obtain adequate recovery from ergot alkaloid exposure in their vasculature to facilitate a reduction in vasoconstriction and corresponding susceptibility to heat stress. Data from each diagnostic method supported both as effective tools for monitoring vascular changes in cattle recovering from fescue toxicosis as time-off E+ tall fescue pastures increases. While the myograph is still the best choice for testing direct ergot alkaloid effects on specific tissues, the agreement of the data sets indicate both as viable methods of evaluation.

Because clearance of ergot alkaloids was not determined in this study, there is a need for further research in this area. Furthermore, recovery from ergot alkaloid exposure could take longer for cattle with greater bioaccumulation of ergot alkaloids in their vasculature compared to that of steers used in the current study. Future research will require investigation of factors influencing recovery time from ergot alkaloid exposure such as concentration of ergot alkaloids in vascular tissues, varying endophyte infection levels in pastures, and nutritional planes (low vs. high) of cattle affected.

Color Doppler ultrasonography has the repeatability to provide objective in vivo measures of vasoconstriction in cattle induced by ergot alkaloids comparable to that of multimyograph experiments. In addition, the non-invasive manner of Doppler ultrasonography allows for practical application of this method in field research, lessening both stresses on livestock, labor, and resources needed to study vascular

changes in peripheral tissues of the animal. Future technological advancements in Doppler ultrasonography and continued research in the application of this technology will provide this as a useful tool for researchers and veterinarians alike as a means to detect severity of symptoms in cattle suffering from fescue toxicosis.

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Vita

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