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EFFECT OF ERGOT ALKALOIDS ON BOVINE FOREGUT VASCULATURE,
NUTRIENT ABSORPTION, AND EPITHELIAL BARRIER FUNCTION

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements of the degree of Doctor of Philosophy in the
College of Agriculture at the University of Kentucky

By
Andrew P. Foote
Lexington, Kentucky

Director: Dr. D. L. Harmon, Professor of Animal Science
Lexington, Kentucky
2013

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ABSTRACT OF DISSERTATION

EFFECT OF ERGOT ALKALOIDS ON BOVINE FOREGUT VASCULATURE, NUTRIENT ABSORPTION, AND EPITHELIAL BARRIER FUNCTION

Ergot alkaloids present in endophyte-infected (E+) tall fescue are thought to be the causative agent of fescue toxicosis, a syndrome affecting cattle in the eastern United States. Many of the observed signs of fescue toxicosis are thought to be attributed to peripheral vasoconstriction; however, there are data indicating that ergot alkaloids can alter blood flow to the gut. An experiment was conducted using right ruminal artery and vein collected from heifers shortly after slaughter. Vessels were mounted in a multi-myograph to determine the vasoconstrictive potentials of ergot alkaloids present in E+ tall fescue. Results indicated ergot alkaloids have the potential to induce vasoconstriction of foregut vasculature. A second experiment was conducted to determine if the additional ergot alkaloids present in E+ tall fescue increase the vasoconstrictive response above that of ergovaline. Results indicated that ergovaline is the main alkaloid responsible for vasoconstriction in bovine vessels. A third study was performed to determine the effect of ergot alkaloids on ruminal epithelial blood flow in the washed rumen of steers exposed to E+ or endophyte-free (E-) tall fescue seed. Steers were dosed with seed followed by a washed rumen experiment with differing levels of ergovaline incubated in the rumen. Results indicated that E+ tall fescue seed treatment reduced ruminal epithelial blood flow. Additionally, incubating ergovaline in the rumen during the washed rumen further decreased epithelial blood flow and volatile fatty acid (VFA) absorption. A final study was conducted to determine the acute effects of ergot alkaloids on isolated rumen epithelial absorptive and barrier functions and the potential for ruminal ergovaline absorption. Results indicate that acute exposure to ergot alkaloids does not alter the absorptive or barrier function of rumen epithelium and ergovaline is absorbed from the rumen. Data from this series of experiments have shown that ergot alkaloids from E+ tall fescue can induce vasoconstriction of blood vessels in the foregut of cattle, reduce blood flow to the rumen epithelium, and decrease VFA absorption. The decrease in nutrient absorption could contribute to the observed symptoms of fescue toxicosis, including depressed growth rates and general unthriftiness.

Keywords: tall fescue, fescue toxicosis, vasoconstriction, epithelial blood flow, VFA absorption

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July 18, 2013
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EFFECT OF ERGOT ALKALOIDS ON BOVINE FOREGUT
VASCULATURE, NUTRIENT ABSORPTION, AND EPITHELIAL
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FREQUENTLY USED ABBREVIATIONS

ADF – Acid Detergent Fiber

CP – Crude Protein

d – Day

DMI – Dry Matter Intake

DM – Dry Matter

E+ – Endophyte-infected

E- – Endophyte-free

ERN – Ergonovine

ERO – Ergocornine

ERP – Ergocryptine

ERS – Ergocristine

ERT – Ergotamine

ERV – Ergovaline

LSA – Lysergic Acid

min – Minute

NDF – Neutral Detergent Fiber

OM – Organic Matter

VFA – Volatile Fatty Acid

CHAPTER 1. INTRODUCTION

Tall fescue (*Lolium arundinaceum*) is a common cool season forage grown in the eastern United States. The many positive agronomic qualities have made tall fescue very popular with beef cattle producers; although the negative effects on cattle are well documented (Hoveland et al., 1983; Schmidt et al., 1982; Strickland et al., 1993). The association of a fungal endophyte (*Neotyphodium coenophialum*) with tall fescue results in the production of numerous ergot alkaloids (Lyons et al., 1986), which are implicated in causing vasoconstriction and is thought to lead to many of the observed signs of fescue toxicosis (Strickland et al., 2011). Most research has focused on the constriction of peripheral vasculature such as the caudal artery (Aiken et al., 2007; Aiken et al., 2009), the dorsal pedal vein (Solomons et al., 1989), and the saphenous vein (Klotz et al., 2006; Klotz et al., 2008; Klotz et al., 2010). However, limited data have shown that ergot alkaloids from endophyte-infected (E+) tall fescue could decrease blood flow to portions of the gastrointestinal tract (Rhodes et al., 1991). Most studies have focused on ergovaline alone as the main causative agent of vasoconstriction; however other ergot alkaloids present in E+ tall fescue can induce vasoconstriction (Klotz et al., 2007; Klotz et al., 2010). The combination of ergot alkaloids present in E+ tall fescue could negatively impact the vasculature of the bovine foregut, reducing blood flow to the absorptive surface of an organ where approximately 45% of the digestible energy of cattle is absorbed (Kristensen et al., 2005). A reduction in blood flow to the rumen of cattle could potentially reduce nutrient absorption and could contribute to the signs of fescue toxicosis including reduced growth rate and general unthriftiness; but could be

dependent on the potential for ergovaline and other ergot alkaloids to cross the rumen epithelium. Additionally, ergot alkaloids could negatively impact the rumen epithelium directly by altering the absorptive and barrier functions of the epithelium.

CHAPTER 2. A REVIEW OF THE LITERATURE

Introduction

Forages are an important component of beef production in the United States as they are utilized in all segments of the industry from cow-calf production to finishing. Tall fescue (*Lolium arundinaceam*) plays an important role in the U.S. beef industry due to its popularity as a cool season grass in the eastern United States. Many factors compromise the productive potential of systems utilizing tall fescue, including reduced animal performance with endophyte-infected varieties and reduced forage production with endophyte-free varieties. The syndrome commonly referred to as fescue toxicosis is one of the greatest factors limiting the productivity and efficiency of beef production in the eastern half of the United States and the beef industry as a whole. Tall fescue has been extensively studied to determine the agronomic characteristics and the effects on cattle; however, few advances if any have been made in alleviating the syndrome in cattle.

Tall Fescue Agronomic Qualities

Tall fescue is a cool season grass that is prevalent in pastures of the eastern United States. One of the most popular cultivars, 'Kentucky 31' was released in 1942 (Fergus and Buckner, 1972) and has since been incorporated into about 14 million hectares (35 million acres) of pasture land (Buckner and Bush, 1979). This grass was preferred by farmers due to its positive agronomic characteristic compared to other forages (Thompson et al., 2001); however, it was quickly noticed that cattle grazing tall

fescue displayed poor performance (Stuedemann and Hoveland, 1988). The anomaly seen in cattle grazing tall fescue was termed fescue toxicosis and has since been associated with the presence of an endophytic fungus (*Neotyphodium coenophialum*) present in the intercellular spaces of fescue plants (Bacon et al., 1977). The endophyte was found in tall fescue pastures in Georgia on which cattle displayed symptoms of fescue toxicosis whereas the endophyte was not present or present at low levels (0 – 50% infection) in pastures housing cattle that showed no symptoms of fescue toxicosis (Bacon et al., 1977). The correlation of the endophyte presence and fescue toxicosis has also been observed in cattle fed endophyte-infected fescue hay or seed while steers fed non-infected hay or seed appeared normal (Schmidt et al., 1982).

The persistence and widespread use of endophyte-infected tall fescue is thought to be attributed to the positive benefits the endophyte provides for the plant. In a greenhouse study, Arachevaleta et al. (1989) showed that only 75% of endophyte-free tall fescue plants survived during severe drought stress while all endophyte-infected plants survived and displayed greater regrowth after harvest. It has also been shown that endophyte-infected tall fescue in plots had a smaller reduction in tiller density than endophyte-free plots under drought stress and recovered fully after irrigation (West et al., 1993). This increased density was also observed the following year which was reported to be a relatively wet year. In addition to drought tolerance, the endophyte has been shown to increase the reproductive capacity of tall fescue. Infected tall fescue was shown to have 44 % filled seed heads while uninfected tall fescue had only 19% filled seeds (Clay, 1987). It has been hypothesized that this could lead to a gradual shift from a mixed

population of endophyte-infected and endophyte-free stands to one that is predominantly infected over time (Pedersen et al., 1990).

The endophyte present in tall fescue has also been shown to provide insect resistance to the plant. Extracts of endophyte-infected tall fescue were shown to act as feeding deterrents for Argentine stem weevils (*Listronotus bonariensis* (Kuschel)) and seems to be similar to endophyte-infected ryegrass (Prestidge et al., 1985). Tall fescue infected with the endophyte also reduces larval weight and increases the length of the larval stage of fall armyworm (*Spodoptera frugiperda*) but survival was not affected (Clay et al., 1985). Additionally, the presence of the endophyte in tall fescue plants (or extracts of the plants) has been shown to reduce feeding by the oat bird cherry aphid (*Rhopalosiphum padi* L.), greenbug (*Schisaphis graminum* Rondani), and large milkweed bug (*Oncopeltus fasciatus* Dallas) in laboratory and greenhouse experiments (Siegel et al., 1985).

In addition to insect resistant properties, the endophyte presence in tall fescue is also related to nematode resistance. Pedersen et al. (1988) demonstrated that growing endophyte-infected tall fescue in nematode-containing soil in a greenhouse resulted in lower levels of spiral (*Helicotylenchus dihystera*) and stubby root (*Paratrichodorus minor*) nematodes in the soil. Soil populations of *Pratylenchus scriveri* and *Tylenchorhynchus acutus* were lower in plots containing endophyte-infected tall fescue than endophyte-free plots (West et al., 1988) in Arkansas. It is thought that resistance to nematodes could contribute to the drought tolerant properties of endophyte-infected tall fescue due to less predation on roots and greater root growth.

It is clear that the endophyte and the tall fescue plant display a positive mutualistic relationship that allows the plant and endophyte to thrive in good conditions and survive in poor conditions. However, along with the benefits of drought tolerance, insect resistance, and plant regrowth, negative effects on mammals consuming endophyte-infected tall fescue are costly to the animal and the producer.

Fescue Toxicosis Syndrome in Beef Cattle

Classic fescue toxicosis symptoms are generally categorized into two categories: 1) fescue foot and 2) summer slump, which are predominant at low and high ambient temperatures respectively. Fescue foot is described as a dry gangrene of mainly the tail and rear legs with associated discoloration and hair loss on the distal tail (Jacobson et al., 1963). Sloughing of the hoof, loss of the limb between the hoof and dewclaw, and necrosis of the ear tips has also been noted (Hemken et al., 1984; Strickland et al., 1993). It is likely that fescue foot is caused by alterations in the blood flow to the extremities and is exhibited in cold ambient temperatures. Summer slump or summer syndrome is characterized by depressed average daily gain, decreased feed intake, increased salivation, inability to tolerate heat, elevated body temperature, increased respiration rate, rough hair coats, and the animals seek shade or wet spots (Hemken et al., 1984; Hoveland et al., 1983). Summer syndrome is more problematic for cattle producers than fescue foot due to frequent and prolonged heat stress conditions in the southeast United States as compared to extended low ambient temperatures necessary to result in tissue necrosis associated with fescue foot. Other symptoms commonly associated with fescue toxicosis include reduced conception rate, lower milk production, and necrosis of mesenteric fat (Hoveland et al., 1983). It seems that many of the symptoms associated with fescue

toxicosis can be attributed to constriction of the peripheral vasculature and concomitant reduction in blood flow. Vasoconstriction would prevent proper heat dissipation resulting in increased core body temperatures and respiration rate. Improper blood flow to the extremities during cold stress could result in inadequate warming of the tissues resulting in the clinical signs associated with fescue foot.

One of the best indicators of fescue toxicosis is a reduction in serum prolactin. Depressed serum prolactin in cattle has been well documented (Brown et al., 2009; Hurley et al., 1980; Thompson et al., 1987). Rats treated with an extract of endophyte-infected seed also display lowered serum prolactin (Porter et al., 1985). Concentrations of prolactin have also been shown to be reduced in the pituitary of steers grazing endophyte-infected tall fescue (Schillo et al., 1988). This is likely synonymous with the inhibition of prolactin gene expression in rat prolactotrophs treated with ergocryptine (Maurer, 1981). It appears that ergot alkaloids bind to D₂ receptors that are negatively coupled to adenylate cyclase (Lamberts and Macleod, 1990) resulting in a decrease in cAMP. It has been shown that increasing cAMP increases prolactin gene expression and this is inhibited by ergocryptine (Maurer, 1981).

Causative Agent

Since the discovery of the negative effects of grazing tall fescue were documented, researchers have worked to find the causative agent(s). As discussed above, it is likely that the compounds produced by the endophyte, and the causative agents of fescue toxicosis, would cause vasoconstriction. Constriction of the peripheral vasculature could be accomplished by these compounds through binding to either adrenergic,

serotonergic, or dopaminergic receptors. Several different compounds have been studied in association with fescue toxicosis and include several types of alkaloids.

Three types of alkaloids are found in tall fescue: diazaphenanthrene, pyrrolizidine, and the ergot alkaloids (Bush and Burrus, 1988). The main alkaloid in the diazaphenanthrene group is perloine (Figure 2.1) which has been shown to decrease apparent cellulose digestibility and raise body temperatures of sheep (Boling et al., 1975). However, perloine production has been observed in fescue not infected with an endophyte (Strahan et al., 1987).

The most common pyrrolizidine alkaloids are N-acetylloine, and N-formylloine (Figure 2.2). The presence of these pyrrolizidine alkaloids have been shown to be associated with the presence of the endophyte in the fescue plant (Bush et al., 1982; Strahan et al., 1987). In an experiment using various extraction techniques, a methanol extract was shown to inhibit insect feeding and increase mortality of insects (Johnson et al., 1985). The methanol extract possessed a much higher concentration of N-acetyl- and N-formylloine than the hexane and ethyl acetate extracts also tested.

Most fescue toxicosis research conducted in the 1980's and 1990's measured the concentration of the loline alkaloids in the seed or grass consumed by the animals. Research conducted at the University of Kentucky using tall fescue seed with a high level of N-acetyl and N-formylloine and a control diet of orchardgrass hay showed that feed intake was lowest with the high level of alkaloids, highest with the orchardgrass, and intermediate with the low level of loline alkaloids (Jackson et al., 1984). Rectal temperature, respiration rate, and body weight change also seemed to follow the same

patterns seen for intake. The results from this study could indicate that loline alkaloids are the causative agent of many of the symptoms of fescue toxicosis; however this assumption is flawed due to the lack of data regarding other toxins that may have been present. The more correct interpretation may be that the level of endophyte infection is responsible for differences in observed fescue toxicosis symptoms. It is still unclear if loline alkaloids are partially or fully responsible for the fescue toxicosis syndrome. For example, in vitro data indicate that N-acetylloline has no vasoconstrictive activity in peripheral tissue (Klotz et al., 2008) indicating that the loline alkaloids are likely not responsible for the symptoms of fescue toxicosis caused by vasoconstriction of peripheral vasculature. Additionally, pyrrolizidine alkaloids were also shown to have no binding affinity for D₂ receptors (Larson et al., 1999).

Ergot alkaloids have been proposed to be the primary causative agents of fescue toxicosis because of similar symptoms seen in the human ailment ergotism, which is characterized as a loss of peripheral sensation, edema, loss of affected tissue, hallucinations, twitches, and spasms (Haarmann et al., 2009). It has been demonstrated that ergot alkaloids are produced by *N. coenophialum* in association with fescue (Lyons et al., 1986) when cultured in vitro (Porter et al., 1979). There are two classes of ergot alkaloids known to be present in endophyte infected tall fescue: ergopeptides (Figure 2.3a-e) and ergolines (Figure 2.3f-g).

Ergot alkaloids have been demonstrated to bind to D₂ receptors with varying degrees of affinity (Larson et al., 1999; Larson et al., 1995). In one study, inhibition of radioligand [³H]YM-09151-2 binding by dopamine and several ergot alkaloids was measured and K_I values were calculated to estimate the concentration at which 50% of

maximum binding occurs (Larson et al., 1999). Ergovaline, ergotamine, and ergocryptine displayed lower K_I values (<10 nM) than the two ergoline alkaloids ergonovine and ergine (366 and 748 nM respectively). The K_I for dopamine was 1,828 nM. A lower K_I indicates greater binding affinities of ergonovine and ergine for the D_2 receptor than dopamine.

No single alkaloid has been implicated as the causative agent for fescue toxicosis and it is likely that the observed symptoms are caused by an additive or synergistic mechanism of several alkaloids. However, data collected thus far provide strong evidence for the ergot alkaloids to be the main compounds responsible for fescue toxicosis.

Metabolism, Absorption, and Excretion of Ergot Alkaloids

Information regarding the metabolism of ergot alkaloids by ruminants is a vital component to determining the etiology of fescue toxicosis. The complexity of ruminant digestion exposes these compounds to a number of enzymes and digestive processes.

Several studies have been conducted to determine the ruminal contribution to ergot alkaloid metabolism. A rat bioassay was used to determine if tall fescue seed lost potency if incubated in rumen fluid (Westendorf et al., 1992). Incubating the endophyte-infected fescue seed in rumen fluid improved average daily gain of rats but not to the level of rats fed non-endophyte infected seed. Incubation also had no effect on final weight or average daily intake. The results suggested that the toxic compounds in tall fescue seed were not completely metabolized by rumen microbes.

More recent studies have shown that ergot alkaloids are fairly stable in the rumen environment. Stuedemann et al. (1998) showed that ergot alkaloid concentrations

increased in the supernatant of rumen fluid after in vitro incubation for 24 hours compared to incubation in autoclaved rumen fluid. Another study showed that greater amounts of ergovaline were released from fescue clippings incubated with viable rumen fluid compared to autoclaved rumen fluid (Ayers et al., 2009). The same study showed that lysergic acid concentrations also increased after 48 hours of incubation suggesting that it is also somewhat stable in the rumen environment or is produced as a metabolite of the breakdown of other ergot alkaloids. Ruminal ergovaline concentrations have been shown to increase from 0 to 3 days after commencing treatments in sheep fed tall fescue straw and seed (De Lorme et al., 2007). Ergovaline concentrations in the rumen fluid continued to increase as length of treatment increased to 28 days. Lysergic acid increased from day 0 to day 3 but rumen concentrations were not different at day 28 compared to day 3.

A study using sheep fed fescue straw and seed showed that only 35% of the ergovaline consumed was accounted for while 248% of the lysergic acid consumed was accounted for (De Lorme et al., 2007). This suggests that some ergot alkaloids are converted to lysergic acid. The low level of ergovaline excretion may not necessarily be due to metabolism or conversion to lysergic acid or other compounds. Ergovaline, but not lysergic acid, has been shown to accumulate in blood vessels with high affinity (Klotz et al., 2009) and ergot alkaloids have been found in subcutaneous fat of steers grazing endophyte infected tall fescue (Realini et al., 2005).

In vivo data regarding absorption of ergot alkaloids in ruminants is nonexistent. Studies using parabiotic chambers have shown that lysergic acid crosses the ruminal and omasal mucosa to a greater extent than other ergot alkaloids (Ayers et al., 2009; Hill et

al., 2001). Ergonovine displayed a similar transport potential to ergotamine and ergocryptine (Hill et al., 2001) while ergovaline was not transported across the ruminal or omasal mucosa of sheep (Ayers et al., 2009). However the concentration of ergovaline in the mucosal chamber was only between 1 and 2 ng/mL while ruminal ergovaline concentrations in vivo have been reported to be at least 4.6 ng/mL (De Lorme et al., 2007). It is likely that ergovaline would be absorbed similarly to the other ergopeptide alkaloids when present at physiological concentrations or at least concentrations similar to the other ergopeptides studied. In vitro measures have shown that ergovaline can cross Caco-2 cells (human intestinal cells) at a rate of about $7.5 \text{ ng/cm}^2 \cdot \text{min}$ (Shappell and Smith, 2005), which was similar to the rate of ergotamine flux across sheep omasum in a parabiotic chamber (Hill et al., 2001).

Through the inclusion of sodium azide to stop cellular metabolism and active transport in the parabiotic chamber, Hill et al. (2001) attempted to discern the active transport contribution of ergot alkaloid absorption from the rumen. Ergot alkaloid movement to the serosal chamber was inhibited only after 240 minutes of incubation. It is unclear from this experiment if active transport contributes to the absorption of ergot alkaloids. If there is an active transport mechanism for ergot alkaloid absorption, an adaptation period may increase the presence or activity of associated transporters resulting in an increased proportion of ergot alkaloid absorbed with extended consumption.

Absorption of ergotamine has been studied in humans due to the positive effects on the treatment of migraines. Ergotamine bioavailability is considered low and is apparently better absorbed when administered rectally (Tfelt-Hansen and Koehler, 2008).

The inclusion of caffeine with ergotamine also increases ergotamine bioavailability by increasing its solubility (Schmidt and Fanchamps, 1974).

The main excretory route for absorbed ergot alkaloids appears to be urine with some excreted via bile (Stuedemann et al., 1998). Ergot alkaloids have been observed in urine of cattle 12 hours after switching to an endophyte-infected pasture and were undetectable in urine 96 hours after switching from an endophyte-infected pasture to an uninfected pasture (Stuedemann et al., 1998). It was later reported in sheep that ergovaline was not excreted via urine and about 55% of the lysergic acid excreted was recovered in urine (De Lorme et al., 2007).

Fescue toxicosis is a complex syndrome affecting beef cattle. Multiple body systems are affected by the toxins produced by the endophyte-plant interaction. It seems likely that ergot alkaloids contribute significantly to the observed signs of fescue toxicosis in cattle. It is not abundantly clear which specific ergot alkaloid(s) is the causative agent of the syndrome.

Ergot Alkaloid Vasoactivity

Many of the fescue toxicosis symptoms have traditionally been thought to be caused by peripheral vasoconstriction and a concomitant reduction in blood flow to the skin and extremities. In vivo and in vitro demonstrations of vasoconstriction caused by ergot alkaloids common to endophyte-infected tall fescue are abundant.

Rhodes et al. (1991) used radiolabeled microspheres to show that blood flow to some core body tissues including kidney, duodenum and colon is decreased in steers consuming endophyte-infected tall fescue seed. Vasoconstriction was also observed using

Doppler ultrasonography in the caudal artery of heifers four hours after consuming endophyte-infected tall fescue seed (Aiken et al., 2007). Another study showed reduced caudal artery luminal area 27 or 51 hours after heifers began consuming either 0.39 or 0.79 μg ergovaline/kg BW (Aiken et al., 2009). Cattle consuming endophyte-infected tall fescue also display a 50% reduction in blood flow to the skin over the ribs (Rhodes et al., 1991) which is characteristic of the thermoregulatory symptoms associated with fescue toxicosis.

The use of in vitro techniques allows researchers to measure vasoactivity of specific compounds on isolated vasculature from both peripheral and core body tissues. Solomons et al. (1989) demonstrated that ergotamine, ergosine, and agroclavine induce a contractile response while a mixture of loline alkaloids did not induce vasoconstriction. Additionally, ergotamine induced a greater response than both ergosine and agroclavine. Another ergot alkaloid, lysergic acid amide was shown to induce a contractile response in bovine lateral saphenous vein and dorsal metatarsal artery in a myograph (Oliver et al., 1993). It was also shown that the artery and vein respond differently, as the lateral saphenous veins were more sensitive than the dorsal metatarsal artery. Dyer (1993) utilized uterine and umbilical arteries and showed that ergovaline can induce a vasoconstrictive response in core body vasculature. Ergovaline has also been shown to induce a large vasoconstrictive response in the bovine lateral saphenous vein and is more potent than lysergic acid (Klotz et al., 2007; Klotz et al., 2006). These results led to the hypothesis that ergopeptines (e.g. ergovaline, etc.) are more potent vasoconstrictors than the ergoline alkaloids (e.g. lysergic acid, etc.). However it was later proven that

ergonovine possesses a vasoconstrictive potential similar to ergocornine, another ergopeptine (Klotz et al., 2010).

Limited work has been conducted to determine the specific receptors involved in the vasoconstriction observed in peripheral and core body vasculature. Dyer (1993) demonstrated that vasoconstriction of uterine and umbilical arteries could be drastically reduced by ketanserin, a 5-HT_{2A} receptor antagonist. A study using rat and guinea pig arteries showed that ergovaline appears to induce most of its vasoactivity through 5-HT_{2A} and 5-HT_{1B/1D} receptors and potentially α_1 -adrenergic receptors (Schoning et al., 2001). The specific receptors involved in inducing vasoconstriction will likely vary depending on the tissue type and receptor population present on the plasma membrane. Klotz et al. (2012) used agonists of various 5-HT receptors as a measure of the contribution to vasoconstriction of the lateral saphenous vein in cattle. Greater responses were observed for 5-HT₂ and 5-HT_{2A} agonists than 5-HT_{2B}, 5-HT_{1B}, and 5-HT₇. Additionally, α_2 -adrenergic receptors are present in bovine lateral saphenous veins and when stimulated can induce a vasoconstrictive response (Oliver et al., 1998).

It is clear that ergot alkaloids from endophyte-infected tall fescue can induce vasoconstriction of peripheral and core vasculature and it is likely that this contributes significantly to the fescue toxicosis syndrome. The effect of reduced blood flow to certain tissues is likely variable. A reduction in blood flow to the skin will likely reduce heat dissipation and potentially make animals more prone to heat stress while a reduction in blood flow to the gastrointestinal tract could alter nutrient absorption and thereby reduce animal performance.

Ergot Alkaloids Effect on the Gastrointestinal Tract

The gastrointestinal tract of cattle is the first organ system to encounter the toxins present in endophyte-infected tall fescue, and could potentially be negatively affected by these toxins. The functions of the gastrointestinal tract including digestion, absorption, and immunological (barrier function), serve and protect every other body system. A disruption in any function of the gastrointestinal tract can have severe effects on the productivity and health of the animal.

The effects of the endophyte present in tall fescue on digestibility have been extensively studied with varied results. Many studies have shown that endophyte-infected tall fescue seed can reduce DM and OM digestibility in cattle (Aldrich et al., 1993b) as well as DM, OM, NDF, and cellulose digestibility in sheep (Hannah et al., 1990). Additional studies utilizing sheep and steers fed fescue hay with or without the endophyte have also shown decreases in DM digestibility (Aldrich et al., 1993a) and DM, OM, CP, and ADF digestibility (Matthews et al., 2005). Another study feeding endophyte-infected or endophyte-free fescue straw to sheep showed no differences in DM, ADF, or CP digestibility (De Lorme et al., 2007). Many of the differences observed in published data regarding digestibility is likely due to variation in toxin levels, species, and diet differences.

An alteration in digestibility by ergot alkaloids could be due to several factors including microbial fermentation inhibition and decreases in gastrointestinal tract motility. Direct studies on the effects of ergot alkaloids on microbial populations are limited; however, studies have shown indirectly that endophyte-infected tall fescue does not alter ruminal fermentation. Volatile fatty acid concentrations have been shown to

increase with the inclusion of endophyte-infected tall fescue seed (Hannah et al., 1990), but when the decrease in ruminal liquid volume is considered, it is likely that VFA production was unchanged. Ruminal pH and ammonia (Aldrich et al., 1993a; De Lorme et al., 2007) are not affected by the endophyte. Additionally, methane production was reported to be similar in cattle grazing endophyte-free and endophyte-infected tall fescue pastures (Pavao-Zuckerman et al., 1999). Taken together, it is unlikely that the toxins produced by endophyte-infected tall fescue significantly alter the microbial environment in the rumen.

Gastrointestinal tract motility could be altered by ergot alkaloids present in tall fescue. McLeay and Smith (2006) reported an inhibition of A and B cyclical contraction and an increase in the tonus (baseline) of the reticulorumen smooth muscle after intravenous injection of ergotamine and ergovaline in sheep. Another study showed that intravenous injection of ergotamine reduced the frequency of reticular contraction and increased the baseline in sheep (Poole et al., 2009). This study also showed that in vitro incubation of reticular wall strips with ergotamine or ergovaline increased tonic contractions.

Blood flow to the gastrointestinal tract is important for nutrient absorption and tissue health. Rhodes et al. (1991) using radiolabeled microspheres showed that cattle consuming endophyte-infected tall fescue seed and hay at a high ambient temperature had reduced blood flow to the duodenum and colon. However, Harmon et al. (1991) reported no effect of endophyte-infected tall fescue consumption on portal vein blood flow. These divergent results could be due to differences in ambient temperature or level of intoxication. Currently, there is a lack of data that explores the vasoconstrictive potential

of specific ergot alkaloids on gut vasculature, which could encounter higher alkaloid concentrations due to absorption, and their vasoactivity could alter nutrient absorption. Ergot alkaloid induced vasoconstriction of gut vasculature and a corresponding reduction in blood flow could cause a decrease in nutrient absorption and concomitantly cause the economically damaging symptom of depressed growth in cattle consuming tall fescue. Harmon et al. (1991) is the only report to date on the effect of fescue toxicosis on nutrient absorption and it was reported that endophyte-infected tall fescue consumption was related to a decrease in portal acetate flux.

Another major function of the gastrointestinal tract is to act as a barrier separating the outer environment from the blood and the rest of the body. The digestive tract including the rumen has been shown to contain harmful compounds such as pathogenic bacteria (Nagaraja et al., 2005; Narayanan et al., 2002), endotoxins (Nagaraja et al., 1978), lipopolysaccharides (Zebeli and Ametaj, 2009), and biogenic amines such as histamine (Irwin et al., 1979). These compounds are also associated with laminitis (Nocek, 1997), liver abscesses (Tadepalli et al., 2009), and acute interstitial pneumonia (Loneragan et al., 2001). A disruption in the barrier function of the gastrointestinal tract of cattle can lead to an increased incidence of these conditions, causing decreased productivity or death. While there is no direct indication that ergot alkaloids or other components of endophyte-infected tall fescue could alter the barrier function of the bovine gastrointestinal tract, several reports indicate that tight junction function in the gut could be altered. Ergocristine, an ergopeptine alkaloid present in tall fescue, has been shown to cause a decrease in transepithelial electrical resistance in cultured porcine brain endothelial cells (Mulac et al., 2012), indicating a disruption of the blood-brain barrier.

Additionally, steers grazing endophyte-infected tall fescue were shown to have a greater acute-phase protein response, including haptoglobin and TNF- α , to a lipopolysaccharide challenge than steers grazing uninfected-tall fescue (Filipov et al., 2000). While there was no difference in basal levels of haptoglobin and TNF- α , an increased responsiveness to a lipopolysaccharide challenge could indicate that steers have greater sensitivity due to prior exposure to inflammatory compounds passing through the gastrointestinal tract.

It is possible that ergot alkaloids present in endophyte-infected tall fescue could have significant effects on the gastrointestinal tract of cattle. Alterations in the function of the gastrointestinal tract could lead to several of the observed symptoms of fescue toxicosis including depressed growth and intake. Additionally, the health of these animals could be greatly compromised by reductions in the barrier function of the gastrointestinal tract. More research is need to determine the specific effects of ergot alkaloids on the function of the gastrointestinal tract under controlled experimental conditions with a standardized fescue treatment protocol.

In Vitro Measures of Nutrient Flux and Epithelial Permeability

In vitro measures of nutrient flux and epithelial permeability provide many advantages over in vivo methods, including removal of microbial metabolism and inter-conversion of nutrients, decreased expense, and high throughput. Using in vitro methods also allows the manipulation of conditions, such as chemical concentrations, that is difficult in live animals. It should also be noted that in vitro methods only provide partial information and do not always directly apply to live animal physiology. There are also in vivo experimental models such as the washed reticulorumen to study absorption of

nutrients. These methods will likely provide a more accurate measure of true nutrient flux in contrast to the in vitro Ussing chamber model.

One of the most commonly used in vitro tools for measuring in vitro nutrient flux and epithelium permeability is the Ussing chamber. This chamber was originally designed to study ion transport across the frog skin (Lindemann, 2001; Ussing, 1949). The use of the chamber and isolated tissue allows the relatively easy use of radioactive isotopes of ions. The unique capability of the Ussing chamber is the ability to set the electrical difference across the epithelium. When the potential difference across the epithelium is set to zero there is no electrical driving force for the movement of ions across the epithelium, and movement of ion is solely due to active transport.

The Ussing chamber has been modified to be used with a multitude of epithelial tissues including small intestine (Clarke, 2009), large intestine (Polentarutti et al., 1999), rumen (Sehested et al., 1996a), cell cultures (Grasset et al., 1984), lung epithelium (Fischer and Clauss, 1990), nasal mucosa (Wheatley et al., 1988) and many other tissues from many species.

Epithelial permeability or barrier function can be measured in many ways. Flux of macromolecules that are not actively absorbed by the epithelium of interest provides one measure of gut permeability. Flux of molecules that are not metabolized or actively transported such as mannitol (Penner et al., 2010), Cr-EDTA (Schweigel et al., 2005), and inulin (Wheatley et al., 1988) can be measured easily by using radioactive isotopes or fluorescent molecules such as fluorescein 5(6)-isocyanate (Klevenhusen et al., 2013). When an increase in macromolecule flux across isolated epithelium is observed, it is

likely that the barrier function, and specifically tight junctions, is weakened. Another measure of barrier function is electrical conductance or resistance of the epithelium (Wilson et al., 2012). Conductance is the ease with which electrical current passes through the tissue or simply passive ion transport across the epithelium. The passive movement of ions (mainly Na^+) is related to the tightness of cell to cell junctions similarly to macromolecule movement.

In vitro measures of gastrointestinal tract function can provide valuable information in a setting that allows for the control of conditions that is not always possible in the living animal. The use of these in vitro methods in combination with in vivo techniques can lead to valuable data and information on the physiology of the gastrointestinal tract of animals under specific treatments.

Fescue toxicosis is a complex syndrome in beef cattle that has not yet been completely characterized. This syndrome results in a large annual loss in productivity in the beef industry of the United States. The effect of the toxins present in tall fescue potential alters the function of the bovine gastrointestinal tract leading to several of the observed symptoms of fescue toxicosis. The objectives of the research presented here were: 1) to determine the effect of ergot alkaloids on bovine ruminal vasculature; 2) to determine if ergot alkaloids alter blood flow to the absorptive surface of the bovine reticulorumen and nutrient absorption from the washed reticulorumen; 3) to determine if ergot alkaloids have a direct on effect on the isolated ruminal epithelium absorptive and barrier functions; and 4) determine if ergovaline, one of the main causative agents of fescue toxicosis, can be transported across the rumen epithelium.

Figure2.1. Chemical structure of perloine, a diazaphenanthrene alkaloid

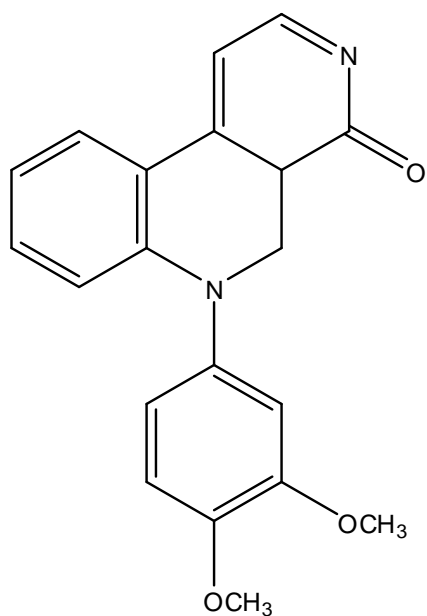


Figure2.2. Chemical structure of pyrrolizidine alkaloids loline (a), N-formylloline (b), and N-acetylloline.

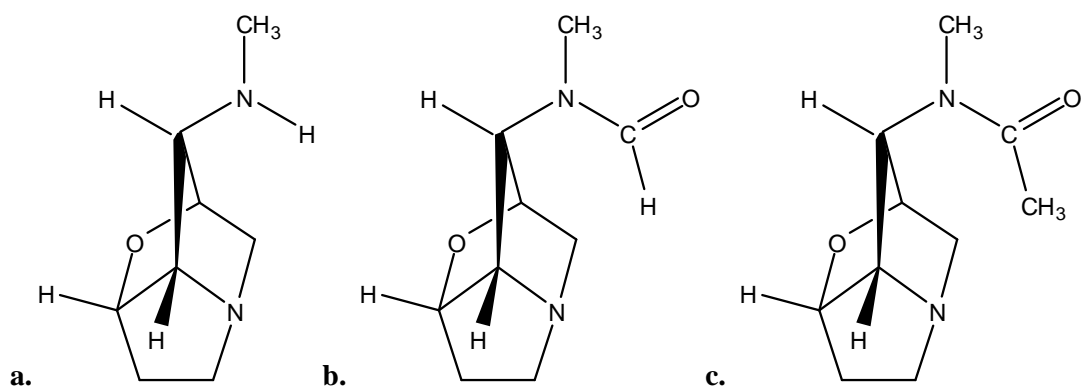
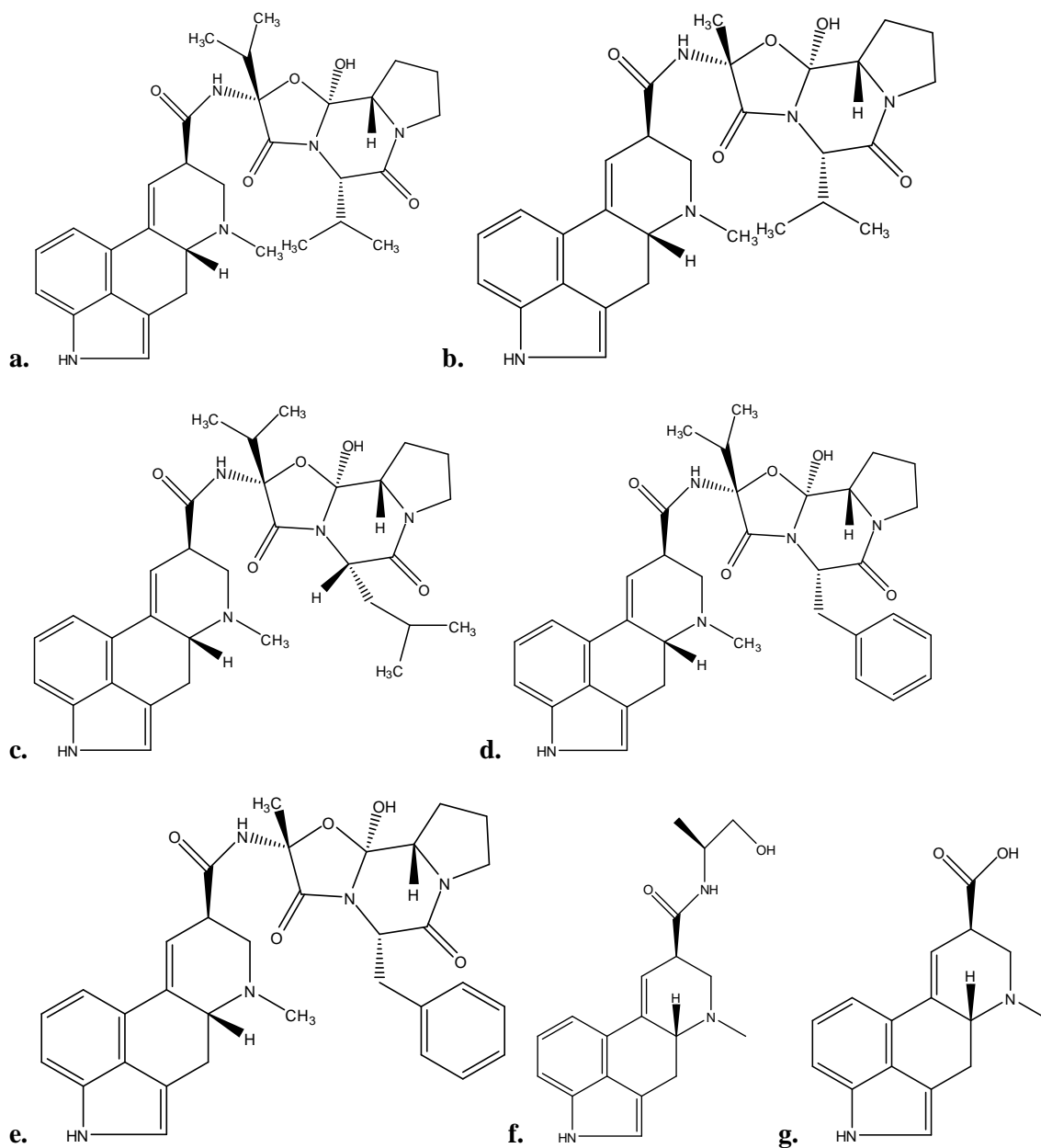


Figure 2.3. Chemical structures of ergot alkaloids a) ergocornine, b) ergovaline, c) ergocryptine, d) ergocristine, e) ergotamine, f) ergonovine, and g) lysergic acid.



CHAPTER 3: EFFECT OF ERGOT ALKALOIDS ON CONTRACTILITY OF BOVINE RIGHT RUMINAL ARTERY AND VEIN¹

Introduction

The association of *Neotyphodium coenophialum* with tall fescue (*Lolium arundinaceum*) results in the production of numerous ergot alkaloids (Lyons et al., 1986). Ergot alkaloids have been implicated in causing vasoconstriction, which is a source of clinical symptoms of the fescue toxicosis syndrome (Strickland et al., 2011). Prior research has mainly focused on constriction of peripheral vasculature like the caudal artery (Aiken et al., 2007), saphenous vein (Klotz et al., 2006) and the dorsal pedal vein (Solomons et al., 1989). There is a paucity of data addressing the effects of ergot alkaloids on vasculature supporting core body tissues. Rhodes et al. (1991) is the only study that has addressed this. Using labeled microspheres, they reported a reduction in blood flow to the duodenum, colon, and kidney of cattle consuming endophyte-infected tall fescue at high ambient temperatures.

Westendorf et al. (1992), Moyer et al. (1993), and De Lorme et al. (2007) all assert that the rumen is the primary site of alkaloid liberation, degradation and possibly absorption. Bovine ruminal tissue has been shown to have a capacity to absorb ergot alkaloids (Ayers et al., 2009). Using sheep, Westendorf et al. (1993) demonstrated that only 50 to 60% of ingested alkaloids are recovered in abomasal contents. The fraction of alkaloids disappearing in the reticulorumen likely represents both microbial degradation and absorption. It is hypothesized that absorption of ergot alkaloids combined with the

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tendency of ergopeptine alkaloids to bioaccumulate in vascular tissue (Klotz et al., 2009) could result in vasoconstriction and a concomitant reduction in blood flow. Klotz et al. (2011) recently developed a ruminal artery and vein bioassay designed to evaluate the response different toxicants elicit. Therefore, the objective of this study was to determine if ergoline and ergopeptine alkaloids (Figure 3.1) would elicit a vasoactive response in bovine ruminal artery and vein preparations in vitro.

Materials and Methods

Methods used with live animals in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals and Tissues

Predominantly Angus-bred heifers (n = 10; BW = 498 ± 9 kg) were utilized in this study. Heifers were fed a basal diet consisting of cotton seed hulls, soybean hulls, corn, and soybean meal (41.9, 31.4, 21.45, and 5.25% of diet on an as-fed basis, respectively). The nutrient composition of the diet was 9.9% CP and 58.0% NDF on a DM basis. Heifers were fed this diet for a minimum of 232 days.

Sections of right ruminal artery and vein were collected from the ventral coronary groove of the rumen shortly after each animal was stunned by captive bolt, exsanguinated, and eviscerated at the University of Kentucky abattoir. Tissues were immersed in a modified Krebs-Henseleit oxygenated buffer (95% O₂ + 5% CO₂; pH = 7.4; mM composition = D-glucose, 11.1; MgSO₄, 1.2; KH₂PO₄, 1.2; KCl, 4.7; NaCl, 118.1; CaCl₂, 3.4; and NaHCO₃, 24.9; Sigma Chemical Co., St. Louis, MO) and placed on ice for transport to the laboratory.

Artery and vein were separated, cleaned of excessive fat and connective tissue, sliced into 2- to 3-mm cross sections, and examined under a dissecting microscope (Stemi 2000-C, Carl Zeiss Inc., Oberkochen, Germany) at 12.5× magnification to ensure structural integrity of the vessel. Dimensions of the blood vessels were recorded (Axiovision, version 20, Carl Zeiss Inc.) to ensure the segment size was consistent. Vessel segments were then mounted on luminal supports in a tissue bath (DMT610M Multichamber myograph, Danish Myo Technologies, Atlanta, GA.) containing 5 mL of continuously gassed (95% O₂ + 5% CO₂) modified Krebs-Henseleit buffer at 37°C. The buffer used for transport was further modified for tissue incubation by adding desipramine ($3 \times 10^{-5} M$; D3900 Sigma Chemical Co.) and propranolol ($1 \times 10^{-6} M$; P0844, Sigma Chemical Co.) to inactivate neuronal reuptake of catecholamines and to block β -adrenergic receptors, respectively. Tissue samples were equilibrated to a resting tension of 1.0 g for ruminal artery and 0.5g for ruminal vein for 90 min with buffer replacement at 15 min intervals, as validated by Klotz et al. (2011). Vessels were then exposed to KCl (120 mM) to ensure tissue viability and to provide a reference for normalization of contractile responses.

Concentration Responses of Ergot Alkaloids

Stock standards of ergovaline tartrate ($\geq 93\%$ purity, supplied by F. T. Smith, Auburn University, Auburn, AL), ergotamine D-tartrate ($\geq 97\%$ purity, #45510, Fluka, Sigma Chemical Co.), α -ergocryptine (99% purity, E5625, Sigma Chemical Co.), and ergocristine (05-9034-17, Research Plus, Inc., Barnegat, NJ) were prepared in 100% methanol. D-lysergic acid hydrate (95% purity, Acros Organics, Geel, Belgium) was prepared in 80% (vol/vol) methanol that contained $1.2 \times 10^{-4} M$ acetic acid. Ergonovine

maleate (100% purity, E6500, Sigma Chemical Co.) was prepared in H₂O. Ergocornine (>95% purity, E131, Sigma Chemical Co.) was prepared in dimethyl sulfoxide. Standards were prepared in a concentration of $2 \times 10^{-2} M$ and added in 25- μ L aliquots to achieve a concentration of $1 \times 10^{-4} M$ in the tissue bath. This kept concentration of vehicle below 0.5% in the incubation buffer. Serial dilutions of $2 \times 10^{-2} M$ stock alkaloid standards were prepared to achieve the desired treatment concentrations ranging from $1 \times 10^{-11} M$ to $1 \times 10^{-4} M$ in the myograph baths, except for ergotamine, which was prepared at $2 \times 10^{-3} M$ with a maximum concentration in the incubation buffer of $1 \times 10^{-5} M$.

Data Collection and Analysis

Isometric contractile responses to KCl, lysergic acid, ergonovine, ergocristine, ergocryptine, ergocornine, ergotamine, or ergovaline were digitized and recorded as grams of tension using a Powerlab/8sp and Chart software (version 7.1; ADInstruments, Colorado Springs, CO). The maximum tension observed for the 9-min incubation period after the addition of a treatment was recorded and corrected for baseline tension measured just before the addition of the 120 mM KCl reference addition. Values were normalized as a percentage of the contractile response induced by the KCl reference addition to compensate for differing responses due to tissue size and animal variation. Contractile response data are presented as percentage means \pm SEM of the maximum contractile effect induced by 120 mM KCl. Alkaloid potency was calculated from the concentration response data using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) and is presented as the molar concentration of the alkaloids producing 50% of the maximum response (**EC₅₀**). This analysis utilized a sigmoidal concentration response curve that used a three parameter equation:

$$y = bottom + \frac{(top - bottom)}{(1 + 10^{(\log EC_{50} - x)})}$$

where top and bottom are the plateaus in the units of percentage of 120 mM KCl contractile response. The maximum contractile response observed for each alkaloid is defined as E_{max} . Contractile responses were only considered valid responses if the mean for a given concentration was greater than the lowest contractile response.

Statistical Analysis

Contractile response data of ruminal artery and veins exposed to ergot alkaloids were analyzed as a completely randomized design using the mixed model procedure (SAS Inst. Inc., Cary, NC). Terms of the model included alkaloid, concentration, vessel, and all interactions. For the comparison of E_{max} and EC_{50} data, the interaction of alkaloid \times vessel and effect of vessel were not significant ($P = 0.18$ and 0.23 , respectively); therefore, these terms were removed from the model and the E_{max} and EC_{50} data were analyzed separately for artery and vein. Following analysis of variance, pair-wise comparisons of least square means (\pm SEM) were conducted using LSD if the probability of a greater F -statistic was significant for the tested effect.

Results and Discussion

Exposure of ruminal artery and vein segments to ergot alkaloids resulted in an interaction between alkaloid, concentration, and vessel type (artery or vein) for the contractile response data ($P < 0.0001$). Concentration response curves for the ruminal artery are shown in Figure 3.2. Contractile responses were first observed for ergovaline ($P = 0.016$) and ergotamine ($P = 0.019$) at 10^{-6} M concentration. Ergonovine ($P = 0.04$),

ergocornine ($P = 0.002$), and ergocryptine ($P = 0.014$) did not cause an arterial contraction until exposure to the 10^{-5} M dose, and ergocristine did not elicit a response until the 10^{-4} M addition ($P = 0.042$). Studies measuring the effect of ergot alkaloids on bovine arteries have been limited; however, *in vitro* contractile responses have been reported for lysergamide in bovine metatarsal artery (Oliver et al., 1993) and ergovaline in bovine uterine artery (Dyer, 1993). Both studies demonstrated that the ergot alkaloids tested caused significant vasoconstriction. Consumption of ergot alkaloids from endophyte-infected tall fescue was also shown to induce *in vivo* vasoconstriction of the caudal artery in heifers within either 4 h (Aiken et al., 2007) or 27 h (Aiken et al., 2009) of initial consumption.

The EC_{50} for the ruminal artery (Table 3.1) were similar for all alkaloids except ergotamine, which was greater ($P = 0.0107$), indicating that ergotamine is least potent at stimulating ruminal artery contraction. However, ergotamine exposure may still be a significant risk to foregut blood flow as the other ergot alkaloids tested here if bioaccumulation (Klotz et al., 2009) or metabolism affect target tissue concentrations *in vivo*. The E_{max} (Table 3.1) was greatest for ergovaline and least for ergocristine. Previous studies have led to the theory that ergopeptines (i.e. ergovaline) have a greater vasoconstrictive activity than the ergoline alkaloid lysergic acid (Klotz et al., 2007; Klotz et al., 2006). However more recent studies have reported that the ergoline alkaloid, ergonovine, has an E_{max} equal to or greater than some ergopeptine alkaloids (Klotz et al., 2010). The ruminal artery data from the present study indicate a similar trend where ergonovine possessed an E_{max} similar to ergotamine, ergocornine, and ergocryptine, and a greater response than ergocristine (Table 3.1).

Concentration response curves for ruminal vein are shown in Figure 3.3.

Exposure of the ruminal vein to ergovaline, ergotamine, ergocryptine, and ergocristine induced contractile responses, whereas ergonovine ($P = 0.094$) only produced a slight response at the 10^{-4} concentration. Ergocornine ($P = 0.156$) and lysergic acid ($P = 0.5692$) did not produce a contractile response. The smallest alkaloid concentration where a response was observed was 10^{-6} M for ergovaline ($P = 0.009$) and ergotamine ($P = 0.017$), 10^{-5} M for ergocryptine ($P = 0.029$), 10^{-4} M and ergocristine ($P = 0.005$). The EC_{50} for alkaloids tested in the ruminal vein (Table 3.1) were similar ($P = 0.205$) for alkaloids eliciting a contractile response. Ergovaline had the greatest E_{max} for the ruminal vein ($P < 0.0001$; Table 3.1) but did not differ from ergotamine ($P = 0.205$). Ergotamine also did not differ from ergocryptine ($P = 0.133$) or ergocristine ($P = 0.249$). Similar alkaloid profiling studies using the bovine lateral saphenous vein model indicated that ergocryptine and ergocristine had similar E_{max} values (Klotz et al., 2010) and venous data presented in the current study follows the same pattern of the previous report with similar contractile responses to ergocryptine and ergocristine. Responses of the ruminal vein to ergotamine and ergovaline indicates (similar E_{max} values) another similarity of the ruminal vein to the peripheral vasculature model (Klotz et al., 2007). The lack of a response from lysergic acid, ergonovine, and ergocornine has not been previously reported and differs from what was reported for peripheral vasculature (Klotz et al., 2006; Klotz et al., 2010). Ergonovine has been shown to have a greater E_{max} than ergocryptine and ergocristine in peripheral vasculature (Klotz et al., 2010). This may be a result of the difference in functions of tissue that peripheral and core vasculature serve.

Ergovaline induced a greater contractile response in the ruminal artery than in the ruminal vein ($P < 0.0001$) but ergocryptine ($P = 0.218$), ergocristine ($P = 0.425$), and ergotamine ($P = 0.162$) produced similar responses in both vessel types. In contrast, a previous study comparing the response of bovine lateral saphenous vein and dorsal metatarsal artery with lysergamide showed that the vein was more sensitive to the ergot alkaloid than the artery (Oliver et al., 1993). These opposing results could be due to differences in the alkaloids tested or a difference in peripheral versus core vasculature.

Earlier reports have led to the development of a hypothesis that ergopeptines that are similar at the R_1 position (Figure 3.1C) but differ at the R_2 position will result in a similar contractile responses in the multimyograph bioassay (Klotz et al., 2010). This hypothesis was supported by findings that ergopeptines that share an R_1 methyl group (ergovaline and ergotamine) resulted in similar contractile responses in the lateral saphenous vein (Klotz et al., 2007). The hypothesis was further substantiated when ergopeptines with an isopropyl R_1 group resulted in similar contractile responses in the lateral saphenous vein (Klotz et al., 2010). Results in the current study for ergot alkaloids that have a methyl R_1 group (ergovaline and ergotamine) produced similar contractile responses in ruminal vein, but not in the ruminal artery. Similarities were also seen between ergocristine and ergocryptine (isopropyl R_1) in the ruminal vein, but the contractile response was different from ergocornine, which also has an isopropyl R_1 . The difference between the methyl and isopropyl R_1 also wasn't observed in the ruminal vein indicated by similar contractile responses for ergocristine, ergocryptine and ergotamine. These apparent differences in responses between ruminal and peripheral vessels could be the result of different receptor populations present on the various blood vessels combined

with the fairly promiscuous binding of ergot alkaloids with the different biogenic amine receptors (Weber, 1980).

In the current study, lysergic acid failed to induce a significant contractile response in the ruminal artery or vein. Lysergic acid has been previously reported to cause a contractile response in lateral saphenous vein but only at the 10^{-4} M level, which represents a supra-physiological concentration (Klotz et al., 2006) that a grazing animal would likely never encounter. The previous report from Klotz et al. (2006) combined with data presented in the current study demonstrating a lack of response in ruminal vasculature indicate that lysergic acid does not exert a direct vasoconstrictive effect on bovine tissues.

The data presented in this study show that ergot alkaloids have the potential to induce vasoconstriction of bovine right ruminal artery and vein. Constriction of foregut vasculature could significantly reduce blood flow to or from the rumen of cattle consuming endophyte-infected tall fescue. A reduction in blood flow could result in decreased absorption rates of vital nutrients and fermentative end products. Diminished nutrient absorption could contribute to the reduced growth rates and poor performance observed in cattle grazing endophyte-infected tall fescue. Knowing this, it is critical that future research be directed at determining the primary site(s) and mechanisms of ergot alkaloid absorption.

Table 3.1. The EC₅₀ (*M*) and E_{max} (% of KCl maximum) means and SE for ergot alkaloids in the right ruminal artery and vein bioassay^{1,2,3}

Alkaloid	Ruminal Artery		Ruminal Vein		
	EC ₅₀	E _{max}	EC ₅₀	E _{max}	
Lysergic Acid	n/a ⁴	n/a	n/a	n/a	
Ergonovine	1.1 × 10 ^{-5b} ± 4.4 × 10 ⁻⁶ (10)	20.8 ^{cd} ± 2.7	n/a	n/a	
Ergocornine	1.2 × 10 ^{-5b} ± 4.4 × 10 ⁻⁶ (10)	24.8 ^{bc} ± 2.7	n/a	n/a	
Ergocryptine	8.6 × 10 ^{-6b} ± 4.4 × 10 ⁻⁶ (10)	22.0 ^{bcd} ± 2.7	1.7 × 10 ^{-5a} ± 3.9 × 10 ⁻⁵ (7)	17.2 ^b ± 2.7	
Ergocristine	1.2 × 10 ^{-5b} ± 4.9 × 10 ⁻⁶ (8)	15.5 ^d ± 2.7	1.2 × 10 ^{-4a} ± 4.7 × 10 ⁻⁵ (5)	18.6 ^b ± 2.7	
Ergotamine ⁵	3.0 × 10 ^{-5a} ± 4.6 × 10 ⁻⁶ (9)	28.7 ^b ± 2.7	2.0 × 10 ^{-5a} ± 5.2 × 10 ⁻⁵ (4)	23.2 ^{ab} ± 2.9	
Ergovaline	5.6 × 10 ^{-6b} ± 4.4 × 10 ⁻⁶ (10)	70.1 ^a ± 2.7	2.0 × 10 ^{-6a} ± 3.5 × 10 ⁻⁵ (9)	29.3 ^a ± 2.7	

^{a-d}Means within column containing different superscripts differ ($P < 0.05$)

¹EC₅₀ = measure of the potency of an alkaloid, expressed as the molar concentration required to produce 50% of the maximum contractile response. E_{max} = maximal contractile response observed, expressed as a percentage of the 120 mM KCl reference addition response.

²Some data did not fit a sigmoidal curve preventing the calculation of EC₅₀ values for some data. This resulted in variable numbers of experimental replicates which are denoted in parenthetical values following each SEM for EC₅₀ values.

³For E_{max} values n = 10 except ergotamine (n = 9) and lysergic acid (n = 9)

⁴n/a = no contractile response was measured for these vessels in response to these ergot alkaloids ($P > 0.05$)

⁵Ergotamine was only soluble at 2 × 10⁻³ M resulting in a maximum concentration in the incubation buffer of 1 × 10⁻⁵ M.

Figure 3.1. Chemical structure of ergot alkaloids: A) D-lysergic acid and B) ergonovine which are both examples of ergoline alkaloids present in *Neotyphodium coenophialum* infected tall fescue (*Lolium arundinaceum*). C) Generalized structure of ergopeptine alkaloids indicating the variable R₁ and R₂ sites. Common R₁ groups are methyl (ergotamine, ergovaline, and α -ergosine) and isopropyl (ergocristine, ergocornine, and α -ergocryptine). Common R₂ groups are isopropyl (ergovaline and ergocornine), isobutyl (α -ergosine and α -ergocryptine), and methyl benzyl (ergotamine and ergocristine).

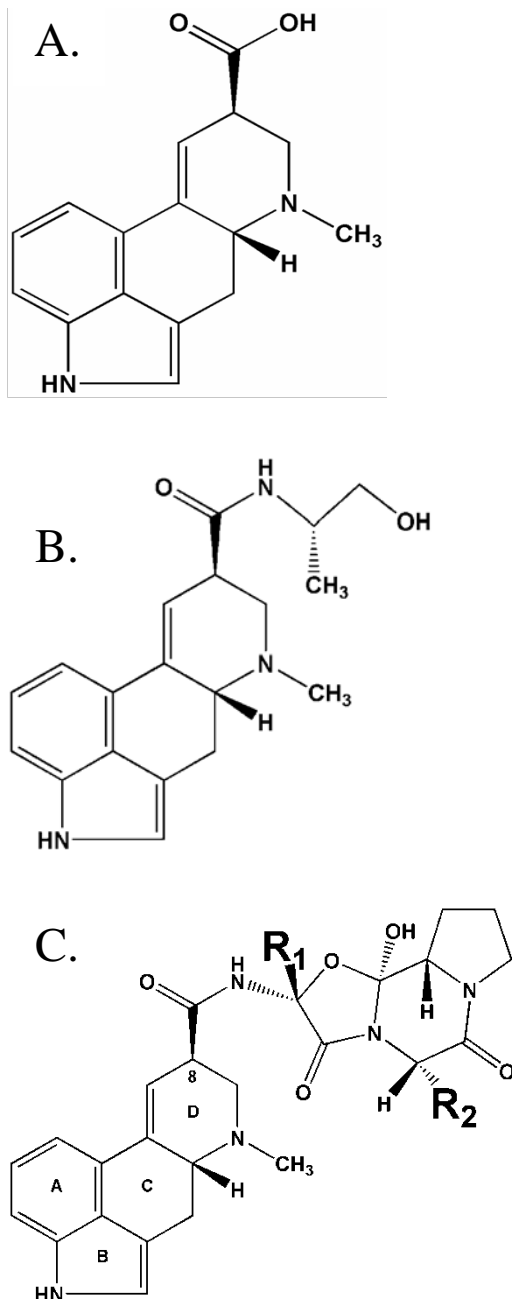


Figure 3.2. Contractile responses of right ruminal artery preparations to A) increasing concentrations of ergocornine (ERO), ergocryptine (ERP), and ergocristine (ERS); B) ergotamine (ERT) and ergovaline (ERV); and C) ergonovine (ERN) and lysergic acid (LSA); (n = 10 each). Effects of alkaloid, concentration, and alkaloid × concentration were significant ($P < 0.05$); however, LSA failed to induce a contractile response.

Regression lines represent the fitting of data to a sigmoidal concentration response curve with the following 3-parameter equation: $y = \text{bottom} + [(\text{top} - \text{bottom}) / (1 + 10^{(\text{LogEC}_{50} - x)})]$, where top and bottom are the plateaus in the units of percent of 120 mM KCl contractile response and EC_{50} is the molar concentration of the alkaloid producing 50% of the maximum response. Missing regression lines indicate data could not be fit to the concentration response model.

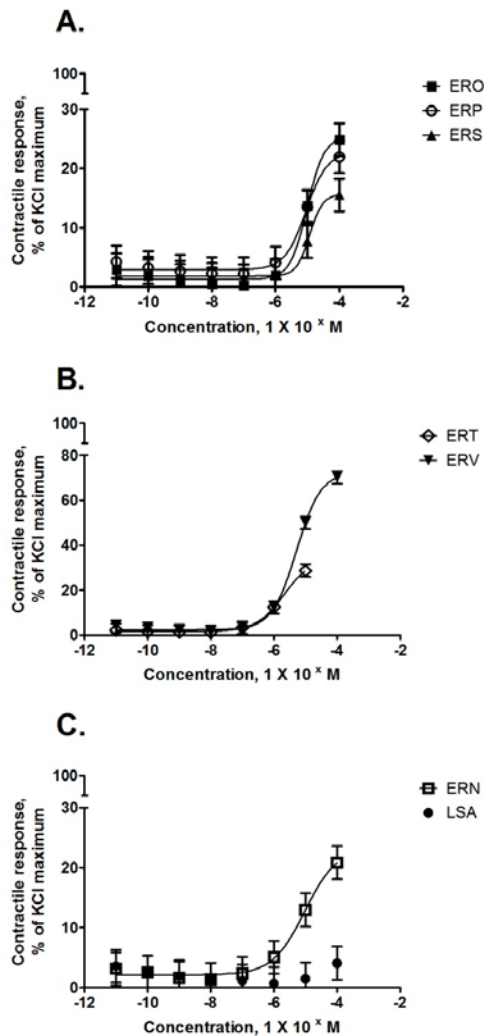
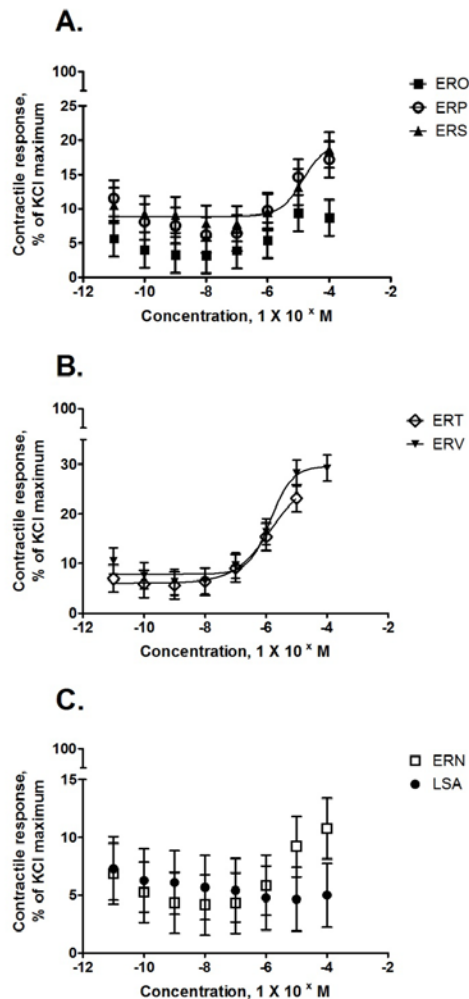


Figure 3.3. Contractile responses of right ruminal vein preparations to A) increasing concentrations of ergocornine (ERO), ergocryptine (ERP), and ergocristine (ERS); B) ergotamine (ERT) and ergovaline (ERV); and C) ergonovine (ERN) and lysergic acid (LSA; n = 10 each except ERT and LSA n = 9 each). Effects of alkaloid, concentration, and alkaloid × concentration were significant ($P < 0.05$), however ERO and LSA failed to induce a contractile response. ERN induced only a slight response ($P = 0.094$). Regression lines represent the fitting of data to a sigmoidal concentration response curve with the following 3-parameter equation: $y = \text{bottom} + [(\text{top} - \text{bottom}) / (1 + 10^{(\text{LogEC}_{50} - x)})]$, where top and bottom are the plateaus in the units of percent of 120 mM KCl contractile response and EC_{50} is the molar concentration of the alkaloid producing 50% of the maximum response. Missing regression lines indicate data could not be fit to the concentration response model.



CHAPTER 4: CONSTRICTION OF BOVINE VASCULATURE CAUSED BY ENDOPHYTE-INFECTED TALL FESCUE SEED EXTRACT IS SIMILAR TO PURE ERGOVALINE¹

Introduction

The association of endophyte-infected tall fescue consumption with vasoconstriction is well documented. Aiken et al. (2007) using color Doppler ultrasonography, demonstrated that heifers consuming endophyte-infected tall fescue seed displayed a smaller caudal artery area. Also, decreased blood flow to the skin as well as some core body tissues such as the duodenum (Rhodes et al., 1991) has been observed using labeled microspheres. Ergot alkaloids have long been implicated as the causative agents of vasoconstriction and likely contribute to most of the observed symptoms of the fescue toxicosis syndrome (Strickland et al., 2011). Most studies related to fescue toxicosis and vasoconstriction have focused on ergovaline alone; however, in vitro studies have shown that ergot alkaloids other than ergovaline including ergonovine, ergotamine, ergocristine, ergocryptine, and ergocornine can induce contractile responses in bovine lateral saphenous vein (Klotz et al., 2007; 2010). Preliminary results (Figure 4.1) showed that an extract of endophyte-infected tall fescue seed (**E+EXT**) serially diluted based on ergovaline concentration induced a greater contractile response in ruminal artery and vein preparations in vitro compared with pure ergovaline. Findings from this experiment led to the development of a hypothesis that the presence of ergot alkaloids other than ergovaline in the extract are responsible for the increased contractile response. The objective of the current

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Foote, A.P., D.L. Harmon, K.R. Brown, J.R. Strickland, K.R. McLeod, L.P. Bush, and J.L. Klotz. 2012 Constriction of bovine vasculature caused by endophyte-infected tall fescue seed extract is similar to pure ergovaline. J. Anim. Sci. 90: 1603-1609.

experiment was to determine if the greater contractility produced by the extract is attributed to the presence of the other ergot alkaloids. This was accomplished by using the bovine lateral saphenous vein bioassay to represent peripheral vasculature and the right ruminal artery and vein bioassay to represent core vasculature, to compare E+EXT with an endophyte-free tall fescue seed extract (**E-EXT**), ergovaline alone, and a mixture of commercially available ergot alkaloids (**ALK**) mixed to mimic the E+EXT alkaloid concentrations.

Materials and Methods

Animals and Tissues

Animal protocols in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Exp. 1. Angus-cross open heifers (n = 10; BW = 498 ± 9 kg) were utilized in an initial test of E+EXT vasoactivity. Handling of heifers prior to slaughter was described previously by Foote et al. (2011). Heifers were fed a basal diet consisting of cotton seed hulls, soybean hulls, corn, and soybean meal (41.9, 31.4, 21.45, and 5.25% of diet on an as-fed basis, respectively). Sections of right ruminal artery and vein were collected from the ventral coronary groove of the rumen shortly after each animal was stunned by captive bolt, exsanguinated, and eviscerated at the University of Kentucky abattoir as described by Klotz et al. (2011). All tissues were placed in a modified Krebs-Henseleit oxygenated buffer (95% O₂ + 5% CO₂; pH = 7.4; mM composition = D-glucose, 11.1; MgSO₄, 1.2; KH₂PO₄, 1.2; KCl, 4.7; NaCl, 118.1; CaCl₂, 3.4; and NaHCO₃, 24.9; Sigma Chemical Co., St. Louis, MO) and placed on ice for transport to the laboratory.

Vessels were cleaned of excess connective tissue and fat, cut into 2- to 3-mm segments and examined under a dissecting microscope (Stemi 2000-C, Carl Zeiss Inc., Oberkochen,

Germany) at 12.5× magnification to ensure structural integrity of the vessel. Dimensions of blood vessel segments were recorded (Axiovision, version 20, Carl Zeiss Inc.) to ensure that size was consistent. Vessel segments were mounted on luminal supports in a tissue bath (DMT610M Multichamber myograph, Danish Myo Technologies, Atlanta, GA.) containing 5 mL of continuously gassed (95% O₂ + 5% CO₂) modified Krebs-Henseleit buffer at 37°C. The buffer used for transport was further modified for tissue incubation by adding desipramine (3×10^{-5} M; D3900 Sigma Chemical Co.) and propranolol (1×10^{-6} M; P0844, Sigma Chemical Co.) to inactivate neuronal reuptake of catecholamines and to block β-adrenergic receptors, respectively. Ruminal veins and arteries were equilibrated to 0.5 and 1.0 g of resting tension, respectively, for 90 min with buffer replaced every 15 min. Ruminal arteries and veins were then exposed to the reference compound, 120 mM KCl before beginning the addition of treatments.

Exp. 2. Holstein steers (n = 6; BW = 498 ± 28 kg) were fed a mixed-grain diet for a minimum of 30 d before slaughter to minimize the influence of endophyte-infected tall fescue alkaloids in the diet. In addition to ruminal vessels, sections of the cranial branch of the lateral saphenous veins (2 to 3 cm) were collected as described by Klotz et al. (2006). Ruminal vessels were handled as described above for Exp. 1, and the saphenous veins were equilibrated to 1.0 g of resting tension for 90 min with buffer replaced every 15 min. and 0.1 mM norepinephrine for saphenous vein before beginning the addition of treatments.

Preparation of Extracts

For preparation of the crude fescue seed extracts, ground endophyte-free or endophyte-infected tall fescue seed was packed in columns and the void volume was filled with 80% ethanol. Seed was allowed to steep for 12 h after the solvent front cleared the seed, followed by elution of the column with 80% ethanol. Ethanol was evaporated from the eluate and the

remaining residue was freeze-dried and ground under liquid N. To further purify the extract, 300 g of extract was suspended in 150 mL of H₂O and shaken for 5 min. A hexane liquid-liquid extraction was performed a total of 6 times by shaking 900 mL of hexane for 5 min with the hexane phase discarded. Chloroform (900 mL) was then added to the aqueous phase and shaken for 5 min a total of 6 times discarding the aqueous phase each time. Chloroform was removed by rotary evaporator under vacuum (Yamato Scientific America Inc., Santa Clara, CA). The residue was solubilized in 50 mL of 80% methanol to generate final stock extracts. Three separate purified extracts were used in these experiments: an E+EXT for Exp.1, an E+EXT for Exp. 2, and an E-EXT for Exp. 2. The concentrations of all ergot alkaloids were not quantified but were identified in the E+EXT for Exp.1.

Analysis of Treatments

Quantitative determination of ergovaline concentration in E+EXT for Exp. 1 was conducted using HPLC with fluorescence detection as described in Aiken et al. (2009) with the modifications described in Koontz et al. (2012). Quantitative determination of ergot alkaloid levels in the E+EXT, E-EXT, and ALK for Exp. 2 was conducted as follows. Stock solutions of test samples were diluted in 80:20 methanol:water at 1:100,000 for ergovaline and ergovalinine, 1:1,000 for ergotamine and ergotaminine, and lysergic acid and its epimer, and 1:30 for ergonovine, ergocornine and ergocorninine, α -ergocryptine and α -ergocryptinine, and ergocristine and ergocristinine for ultra-performance liquid chromatography/tandem mass spectrometry quantitative analysis using an Acquity UPLC-TQD (Waters Inc., Milford, MA). Five microliters of diluted test sample was injected (full loop mode) onto an Acquity UPLC BEH column (C18, 1.7 mm particle size, 2.1 x 100 mm; Waters Inc.) for reverse-phase separation of the ergot alkaloids. Separation was accomplished with a linear binary gradient using water with

0.04% NH₄OH (eluent A) and acetonitrile with 0.04% NH₄OH (eluent B) and a constant flow of 0.5 mL per minute. Gradient program conditions were as follows: initial to 0.6 min, 100% eluent A; at 6.0 min, 10% eluent A/90% eluent B; at 6.1 to 8.5 min, 100% eluent B; at 8.6 to 10 min, 100% eluent A. Detection was accomplished by running the triple-quad mass detector (Waters Inc.) in the MS-MS mode following positive electrospray ionization (see Table 4.1 for parameters). Concentrations of each alkaloid were determined using an external calibration curve with 6 points and an internal standard (methysergide, 5.0 fmol on column). The calibration curves were linear ($R^2 > 0.97$) within a range of 5 to 250 fmol. Area under the curve values for both the “ine” and “inine” epimers were summed for quantitation because interconversion of the epimers readily occurs in solution. Five femtomoles on column was established as the lower limit of quantitation for the ergot alkaloids. Concentrations of the measured ergot alkaloids are presented in Table 4.2.

Preparation of Treatments

Stock standards of ergovaline tartrate ($\geq 93\%$ purity, supplied by F. T. Smith, Auburn University, Auburn, AL) for the ergovaline treatment were prepared in 100% methanol. The ALK treatment was prepared by dissolving ergovaline, ergotamine D-tartrate ($\geq 97\%$ purity, #45510, Fluka, Sigma Chemical Co.), ergocornine ($>95\%$ purity, E131, Sigma Chemical Co.), α -ergocryptine (99% purity, E5625, Sigma Chemical Co.), ergocristine (05-9034-17, Research Plus, Inc., Barnegat, NJ), D-lysergic acid hydrate (95% purity, Acros Organics, Geel, Belgium), and ergonovine maleate (100% purity, E6500, Sigma Chemical Co.) in 80% methanol. The alkaloid concentrations of the ALK treatment were based on what was quantified in the E+EXT before to the start of the myograph experiments and was made to have as similar of an ergot alkaloid profile as the E+EXT as possible (Table 4.2). Working treatments were added in 25- μ L

aliquots to the tissue bath, keeping the organic solvent concentration less than 0.5% in the incubation buffer. Serial dilutions of the stocks were performed to achieve the desired treatment concentrations, ranging from $1 \times 10^{-11} M$ ergovaline to $1 \times 10^{-4} M$ for ergovaline alone, $5.89 \times 10^{-11} M$ to $5.89 \times 10^{-6} M$ ergovaline for the ALK treatment and $3.24 \times 10^{-11} M$ to $3.24 \times 10^{-6} M$ ergovaline for the E+EXT treatment in the myograph baths. The E-EXT was serially diluted in a manner equivalent to the E+EXT dilutions (1:10; vol/vol) with the only difference being the extract, thus controlling for and allowing the evaluation of the potential vasoactivity from the non-ergot alkaloid components of the extract.

Data collection and Analysis

Grams of tension of induced isometric contractions were recorded and digitized using a Powerlab/8sp and Chart software (version 7.1, ADInstruments, Colorado Springs, CO). The maximum observed tension for the 9-min incubation period after treatment addition was recorded and corrected for baseline tension. Baseline tension was measured immediately before the addition of the reference compound. Contractile responses for the treatments were normalized to the contractile response induced by a reference compound (120 mM KCl for the ruminal artery and vein and 0.1 mM norepinephrine for the saphenous vein) and are presented as percentage means \pm SEM. To measure potency of treatments, an EC_{50} was calculated for the saphenous vein data using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). This analysis utilized a sigmoidal concentration response curve with a variable slope:

$$y = B + \frac{(B - T)}{1 + 10^{\log EC_{50} - x} \times S}$$

where B is the bottom plateau, T is the top plateau (E_{\max}), and S is the Hill slope. Analysis of ruminal artery and vein data used a 3-parameter equation similar to the one above, but with the Hill slope set to 1.

Statistical Analysis

For both experiments, the experimental unit was the animal with 2 duplicates from each animal analyzed for each treatment. Data for Exp. 1 were analyzed as a completely randomized design using a mixed model (SAS Inst. Inc., Cary, NC). Fixed variables in the model included treatment, concentration, and treatment \times concentration. Unlike Exp. 1, concentrations of ergovaline between treatments for Exp. 2 varied; therefore, the individual concentration response points were not compared. Parameters from the lines describing the data for the ALK, E+EXT, and ergovaline treatments (EC_{50} , Hill slope, E_{\max}) and the data for E-EXT were analyzed as a completely randomized design as described for Exp. 1. An ANOVA was conducted followed by pair-wise comparisons of least square means using LSD when the probability of a greater F -statistic was significant ($P < 0.05$) for the tested effect.

Results and Discussion

Exp. 1

Experiment 1 was conducted to determine if the extract generated for use in in vivo experiments produced an expected contractile response associated with ergot alkaloids. Results using a right ruminal artery and vein bioassay are shown in Figure 4.1. There was an interaction of ergovaline and E+EXT treatments and concentration of treatments ($P = 0.045$) for the ruminal artery. For the ruminal vein, there was an effect of concentration ($P < 0.0001$) but no effect of treatment ($P = 0.14$) or interaction ($P = 0.16$). The E+EXT induced a greater response than

ergovaline (47.9% versus 30.6% of KCl maximum response) in the ruminal artery at 10^{-6} M ergovaline ($P = 0.018$). The extract appeared to produce a greater response than ergovaline treatment for the ruminal vein at the 10^{-7} M ergovaline level (17.5% versus 9.6% of KCl maximum response), although there was not an interaction of treatment and concentration. The 10^{-6} M ergovaline E+EXT failed to produce a ruminal vein response. The inclusion of a 120-mM KCl reference compound after the 10^{-6} M addition of E+EXT produced a contractile response, indicating that the vessels were viable and E+EXT was not lethal to the ruminal vein. Failure to respond to a greater concentration of agonist could indicate a tachyphylactic response to ergot alkaloids by the ruminal vein. The results from Exp. 1 with the bovine right ruminal artery indicated that either 1) the chemical nature of the extract in general contributes to a greater observed contractile response compared with identical concentrations of pure ergovaline or 2) that other alkaloids are present in the extract and contribute to the overall contractile response. The latter explanation is supported by previous studies using in vitro bioassays that have shown that other ergot alkaloids produced by the endophyte are vasoactive at varying potencies and efficacies (Foote et al., 2011; Klotz et al., 2010).

Exp. 2

The E+EXT had a lower calculated E_{\max} compared with ergovaline ($P < 0.0001$) and ALK was intermediate to the other treatments (Table 4.3) for the saphenous vein. The lower E_{\max} for the E+EXT resulted in a reduced EC_{50} for E+EXT compared with ALK or ergovaline ($P = 0.008$). Although the Hill slopes for the E+EXT were greater ($P = 0.006$) than the ALK and ergovaline slopes, this difference likely has no physiological relevance. Looking at the graphical representation of the data (Figure 4.2A) the observed contractile responses all follow the same basic line, regardless of treatment. Even though the extrapolated E_{\max} is different for these

treatments, the data suggest that ergovaline is mostly responsible for the local vasoconstriction of peripheral vasculature.

The E+EXT treatment also displayed a similar trend for a decreased EC_{50} compared with ALK and ergovaline in the ruminal vein bioassays (Table 4.3); however it should be noted that only 1 or 2 concentrations of the treatments tested resulted in a response in the ruminal artery and vein bioassays, and therefore interpretation of the EC_{50} data for the ruminal vessels is tenuous. Graphical representation of the ruminal artery (Figure 4.2B) and ruminal vein (Figure 4.2C) data shows that there is likely no difference in the response to increasing concentrations for these 3 treatments. The results from this experiment differ from the results of Exp. 1 (Figure 4.1). Differences in the results could be related to the ruminal arteries being less responsive in Exp. 2 compared with Exp. 1. For Exp. 2, the maximum contractile response of the saphenous vein to ergovaline (91.2% of norepinephrine response; Figure 4.2A) was greater than a previous report where the maximal response was about 70% of the maximum norepinephrine contractile response (Klotz et al., 2007). Conversely, ergovaline induced a smaller response by the ruminal artery (30.6 % of KCl response; Figure 4.2B) than a previous study (Foote et al., 2011). Maximum contractile response to E+EXT in the ruminal artery was slightly greater for Exp. 1 (29.2 % of KCl response) than Exp. 2 (20.6 % of KCl response). The results for the ruminal vein in response to the addition of 1×10^{-6} M ergovaline (24.4 % of KCl; Figure 4.2C) were also similar to a previous report (29.3 % of KCl response; Foote et al., 2011). Results were also similar for response of the ruminal vein to E+EXT in both Exp. 1 (17.5 % of KCl response at 1×10^{-7} M ergovaline) and Exp. 2 (18.5 % of KCl response).

The observed differences in responsiveness could result from inherent differences in the cattle used for the 2 experiments; Exp. 1 used Angus-cross heifers, where Exp. 2 utilized

Holstein steers. Previous studies have shown that breed can influence susceptibility to fescue toxicosis. Browning (2004) showed that Hereford steers grazing endophyte-infected tall fescue pasture gained less than Senepol steers on the same pasture, and studies with mice have shown that animals can be selected for resistance or susceptibility to fescue toxicosis (Hohenboken and Blodgett, 1997). Heifers from Exp. 1 also had been fed a finishing diet consisting of predominantly cottonseed hulls, soybean hulls and soybean meal (Foote et al., 2011), whereas steers in Exp. 2 had been previously used for several experiments and consumed fescue hay, fescue pasture, as well as a corn based diet. It is also possible that vessels from heifers could respond differently to ergot alkaloids than steers. In vivo data in rat mesenteric arterioles indicate that females are more sensitive to certain catecholamines than males (Altura, 1972) and it is possible that ergot alkaloids, which bind some of the same receptors as catecholamines (Larson et al., 1999; Schoning et al., 2001), could also induce variable contractile responses between steers and heifers.

To test the possibility that the extraction process included a compound that is vasoactive in this bioassay and not one of the measured ergot alkaloids, an extract of endophyte-free tall fescue seed was titrated in the saphenous vein and ruminal artery and vein bioassays (Figure 4.3). The E-EXT failed to induce a contractile response in the saphenous vein ($P > 0.11$), ruminal artery ($P > 0.3$), and the ruminal vein ($P > 0.3$).

Klotz et al. (2008) has shown that there is no effect of the combination of lysergic acid and ergovaline and only the effect of ergovaline was observed in lateral saphenous veins of heifers. Additionally, lysergic acid has also been shown to have no vasoactive effect on ruminal artery or vein in vitro (Foote et al., 2011) and only a slight effect at 10^{-4} M lysergic acid in the saphenous vein bioassay (Klotz et al., 2006), which is a super-physiological concentration. Other

alkaloids produced by the endophyte and tall fescue symbiont have varying degrees of effectiveness and potency on bovine vasculature in vitro. Vasoconstrictive response to ergonovine is greatest in the saphenous vein (Klotz et al., 2010), moderate in the ruminal artery, and almost nonexistent in the ruminal vein (Foote et al., 2011). In contrast, other ergot alkaloids, such as ergotamine, ergocristine, and ergocryptine, have also been shown to induce contraction in all 3 vessel types (Foote et al., 2011; Klotz et al., 2010). The combined concentration of ergot alkaloids other than ergovaline is $9.6 \times 10^{-8} M$ and $1.6 \times 10^{-7} M$ for the E+EXT and ALK respectively. Previous work with the saphenous vein bioassay showed a slight response to ergocryptine, ergocristine, and ergonovine at $1.0 \times 10^{-7} M$ (Klotz et al., 2010); however, the ruminal artery and vein bioassays required a greater concentration to elicit a contractile response. Foote et al. (2011) showed that the ruminal vein would respond to ergotamine at $1.0 \times 10^{-6} M$ and to ergocryptine, ergocornine, ergonovine, and ergocristine at $1.0 \times 10^{-5} M$. Results of this study indicate that combinations of alkaloids similar to those present in tall fescue may not intensify the contractile response induced by ergovaline at the local level.

These data indicate that there is a differential response of peripheral vasculature and core vasculature to ergot alkaloids. Data from the ruminal artery and vein in Exp. 2 showed similarities to the results in Exp. 1, with the response to E+EXT appearing to be greater than the contractile response to ergovaline; however, statistical differences could not be assessed. The saphenous vein data in Exp. 2 indicates that the addition of ergot alkaloids other than ergovaline has no additional vasoconstrictive effect above that of ergovaline. It appears that the additional non-ergovaline ergot alkaloids could be more important with respect to vasoconstriction of core vasculature.

Conclusions

Data from these experiments indicate that an extract of endophyte-infected tall fescue seed is capable of inducing a contractile response similar to a mixture of ergot alkaloids and ergovaline alone. The results support ergovaline as being primarily responsible for vasoconstriction, especially in the peripheral vasculature.

Table 4.1. Parameters for tandem mass spectroscopy quantitative analysis of ergot alkaloids.¹

Alkaloid	Retention time, min ²	Transition, <i>m/z</i>	Cone voltage, <i>v</i>	Collision energy, <i>v</i>	Dwell time, s
Ergovaline	3.93; 4.67	534.35 > 223.12	38.0	34.0	0.036
Ergotamine	4.24; 5.02	582.29 > 223.11	30.0	34.0	0.036
Ergocornine	4.56; 5.23	562.29 > 223.12	30.0	34.0	0.036
α -Ergocryptine	4.72; 5.41	576.29 > 223.12	32.0	38.0	0.036
Lysergic Acid	1.85; 2.05	269.16 > 43.94	38.0	24.0	0.328
Ergocristine	4.77; 5.48	610.29 > 223.12	30.0	34.0	0.036
Ergonovine ³	2.95	326.16 > 223.08	38.0	28.0	0.036

¹Additional transitions used to verify peak identity (transitions were used for both epimers). Ergovaline = 534.35 > 268.14, 223.12, 207.98, 43.93; Ergotamine = 582.29 > 223.11, 208.04, 191.74, 180.11, 43.93; Ergocornine = 562.29 > 305.15, 268.13, 223.11, 208.04, 43.93; α -Ergocryptine = 576.29 > 268.14, 223.12, 208.05, 180.18, 43.93; Lysergic Acid = 269.16 > 254.08, 207.95, 197.04, 181.98, 43.93; Ergocristine = 610.29 > 268.13, 223.12, 208.05, 180.12, 43.93; Ergonovine = 223.08, 208.01, 197.03, 180.20, 44.00.

²First value = “ine” form; Second value = “inine” form (epimer).

³No epimer form found.

Table 4.2. Concentration of ergot alkaloids in the endophyte-infected tall fescue seed extract (E+EXT) and ergot alkaloid mixture (ALK) used in Exp. 2 as measured by ultra-performance liquid chromatography / tandem mass spectrometry.¹

Alkaloid	Concentration, <i>M</i>		
	E+EXT	ALK	E-EXT
Ergovaline	3.2×10^{-6}	5.9×10^{-6}	ND ²
Ergotamine	4.3×10^{-8}	1.1×10^{-7}	7.5×10^{-11}
Ergocornine	4.0×10^{-8}	1.0×10^{-9}	1.3×10^{-13}
α -Ergocryptine	2.0×10^{-9}	1.0×10^{-8}	2.6×10^{-12}
Lysergic Acid	9.0×10^{-9}	1.1×10^{-8}	ND
Ergocristine	2.0×10^{-9}	2.8×10^{-8}	6.5×10^{-12}
Ergonovine	1.5×10^{-10}	1.4×10^{-10}	ND

¹Values represent the working concentrations of the ergot alkaloids present in the myograph chamber at the greatest treatment concentration.

²ND = Not detectible

Table 4.3. Experiment 2: the EC₅₀, Hill slope, and E_{max} least square means (\pm SEM) for an ergot alkaloid mixture (ALK), endophyte-infected tall fescue seed extract (E+EXT), and pure ergovaline in bovine lateral saphenous vein and EC₅₀ for the treatments in the right ruminal vein and artery.¹

Treatment	Saphenous vein			Ruminal vein	Ruminal artery
	log EC ₅₀	Hill slope	E _{max}	log EC ₅₀	log EC ₅₀
ALK	-5.91 \pm 0.17 ^a	0.51 \pm 0.04 ^b	86.14 \pm 4.02 ^b	-5.87 \pm 0.15 ^a	-5.17 \pm 0.30 ^a
E+EXT	-6.48 \pm 0.17 ^b	0.69 \pm 0.04 ^a	61.51 \pm 4.02 ^c	-6.99 \pm 0.15 ^b	-5.69 \pm 0.19 ^a
Ergovaline	-5.62 \pm 0.17 ^a	0.51 \pm 0.04 ^b	105.70 \pm 4.02 ^a	-6.22 \pm 0.15 ^a	-5.37 \pm 0.18 ^a

^{a, b} means within column with differing superscripts differ ($P < 0.01$)

¹log EC₅₀ = measure of potency of a treatment, expressed as the log of the molar concentration of ergovaline required to induce 50% of the maximal contractile response for each treatment; E_{max} = Maximal contractile response extrapolated by the model used to fit the data, expressed as percentage of 0.1 mM norepinephrine contractile response

Figure 4.1. Experiment 1: contractile response of bovine right ruminal artery (A) and vein (B) to increasing concentrations of ergovaline and an endophyte-infected tall fescue seed extract (E+EXT; n = 10 for each treatment) standardized to ergovaline concentration. The contractile response was dependent upon both treatment and concentration ($P = 0.0045$) for the right ruminal artery; however for the ruminal vein, the effect of concentration was significant ($P < 0.0001$) but effect of treatment ($P = 0.139$) and the interaction ($P = 0.16$) were not significant. Nonlinear regression lines represent the fitting of data to a sigmoidal concentration response curve. Missing regression lines indicate data could not be fit to the concentration response model.

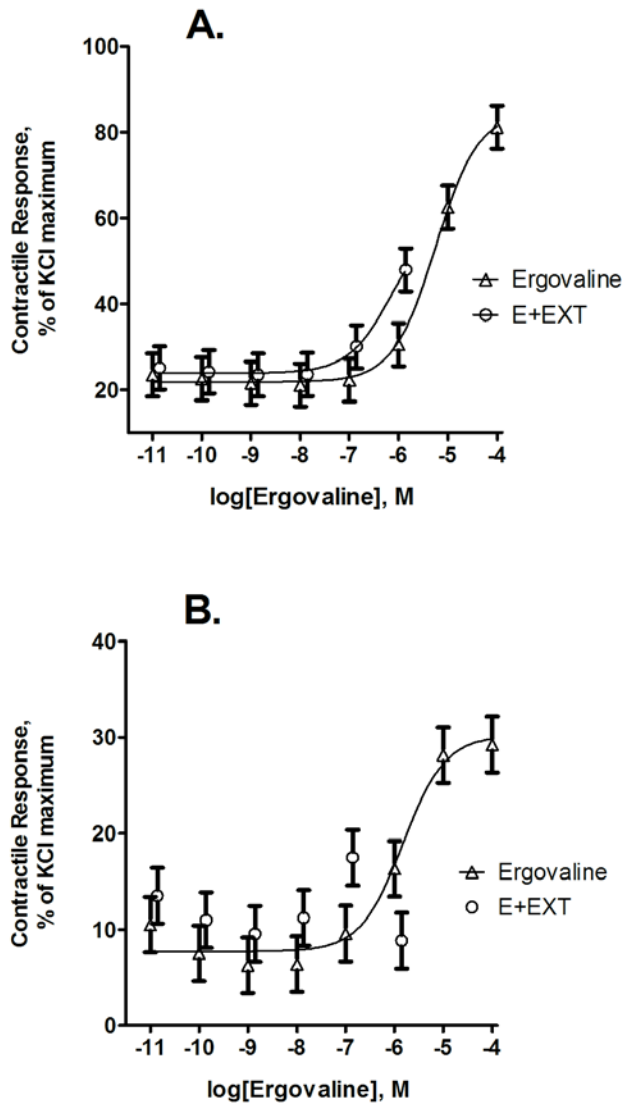


Figure 4.2. Experiment 2: contractile response of bovine lateral saphenous vein (A), right ruminal artery (B), and right ruminal vein (C) to ergovaline, an endophyte-infected tall fescue seed extract (E+EXT) standardized to ergovaline concentration, and a mixture of ergot alkaloids (ALK; n = 6 each) that reflects the alkaloid profile of E+EXT. Regression lines represent the fitting of data to a sigmoidal concentration response curve.

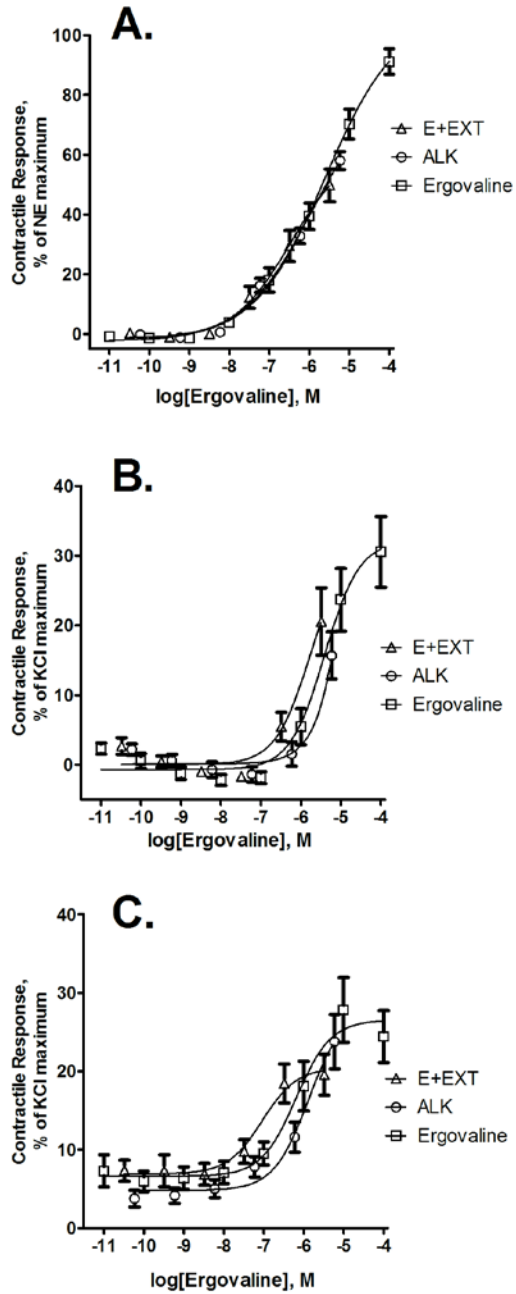
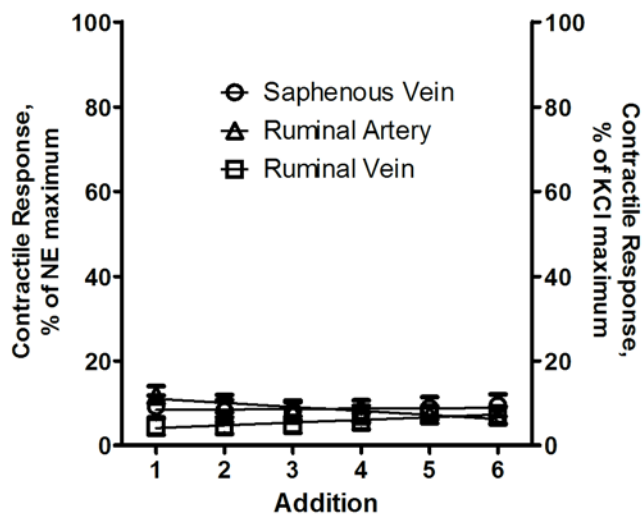


Figure 4.3. Experiment 2: contractile response of bovine lateral saphenous vein, ruminal artery, and ruminal vein to an endophyte-free tall fescue seed extract (E-EXT). The E-EXT was serially diluted from the stock concentration and additions of the E-EXT were added in order of increasing concentration (stock is addition 6). Saphenous vein data is normalized to the contractile response to 0.1 mM norepinephrine and the ruminal artery and vein are normalized to the contractile response to 120 mM KCl. The E-EXT failed to induce a contractile response in any of vessels used in these bioassays ($P > 0.11$).



CHAPTER 5: ERGOT ALKALOIDS FROM ENDOPHYTE-INFECTED TALL FESCUE DECREASE RETICULORUMINAL EPITHELIAL BLOOD FLOW AND VOLATILE FATTY ACID ABSORPTION FROM THE WASHED RETICULORUMEN¹

Introduction

Ergot alkaloids produced by endophyte-infected (*Neotyphodium coenophialum*) tall fescue (E+; *Lolium arundinaceum*) are known to induce vasoconstriction of peripheral vasculature both in vitro (Klotz et al., 2007; Klotz et al., 2010) and in vivo (Aiken et al., 2007; Aiken et al., 2009). It has also been shown that blood flow to internal organs including portions of the gut could be reduced in cattle consuming E+ seed (Rhodes et al., 1991). Additionally, heat stress can reduce gut blood flow (Bell et al., 1983) and exacerbate fescue toxicosis signs. Ergot alkaloids have been shown to induce vasoconstriction of ruminal vessels in vitro (Foote et al., 2011), and ergovaline is thought to be the main causative agent of the vasoconstriction in these vessels (Foote et al., 2012). Blood flow to the absorptive surface of the gut is required for absorption of nutrients and a decrease in the blood flow reduces the absorption of nutrients by reducing the trans-epithelial gradient (Dobson, 1984). It is possible that ergot alkaloids could alter blood flow to the absorptive surface of the gut of cattle and therefore decrease VFA absorption, contributing to some of the signs of fescue toxicosis including decreased growth rate (Strickland et al., 2011) due to the significant contribution of VFA to the caloric requirements of cattle (Bergman, 1990). The effect of ergot alkaloids on gut physiology has not been extensively studied. Feeding E+ hay has been shown to have little effect on

¹This chapter has been submitted and is in review with the Journal of Animal Science

portal nutrient flux (Harmon et al., 1991), although the steers were likely not receiving adequate levels of ergot alkaloids to induce toxicosis. The purpose of this experiment was to determine the effect of ergot alkaloids on reticuloruminal epithelial blood flow and VFA absorption from the washed reticulorumen of steers receiving E+ or endophyte-free (E-) tall fescue seed in the presence of increasing levels of ergot alkaloids in steers housed at thermoneutral (TN) and heat stress (HS) conditions.

Materials and Methods

All protocols in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Preparation of Tall Fescue Seed Extract

Ergovaline and ergovalinine were extracted from 'Kentucky 31' tall fescue seed as described previously (Foote et al., 2012). A crude extract was prepared using 80% ethanol. The residue of the eluent was dried and then reconstituted in water and mixed (600 g of extract and 300 mL of H₂O). The crude extract residue was mixed with hexane (1.8 L) for 10 min. The aqueous and hexane fractions were allowed to separate for 2 h followed by separation of the fractions. The aqueous fraction was re-extracted with hexane 2 additional times (3 total), and the aqueous fractions were combined. The ergot alkaloids remained in the aqueous fraction. The aqueous fraction was then mixed with chloroform (1.8 L) for 10 min and allowed to separate for 2 h. The ergot alkaloids were extracted into the chloroform phase (performed a total of 3 times). The chloroform fractions were combined and evaporated using a rotary evaporator (Yamato Scientific America Inc., Santa Clara, CA). The residue was solubilized in 80% methanol. The final extract was analyzed by HPLC with fluorescence detection as previously described

(Aiken et al., 2009; Koontz et al., 2012). The ergovaline concentration used in this experiment is the combined concentration of ergovaline and ergovalinine.

Animals and Treatments

Experiment 1. Eight ruminally cannulated Holstein steers (*Bos taurus*; BW = 255 ± 1.3 kg) were used in a randomized complete block incomplete-crossover design experiment. Steers were paired by weight and housed in individual pens in a climate controlled barn. During the experiment steers were pair-fed alfalfa cubes (*Medicago sativa*; 19.1 % CP, 36.6 % ADF, 46.4 % NDF on a DM basis) once daily at 0800 to control for any effect of feed intake on response variables in this experiment. Intake was limited to $1.5 \times NE_m$ (NRC, 2000), and steers were given ad libitum access to water. The ambient temperature was controlled to provide either a TN (22°C) or HS (32°C) environment. These temperatures have been previously shown to stress Holstein steers as indicated by increased respiration rate, core temperature, and skin temperature (Koontz et al., 2012).

Treatments were applied in a split plot design with seed as the whole plot treatment and buffer applied during the washed rumen portion of the experiment as the subplot treatment. One steer from each pair was assigned to receive an E+ seed treatment ('Kentucky 31' tall fescue seed; 4.45 ppm ergovaline/ergovalinine) and the other steer was assigned to receive an E- seed treatment ('Kentucky 32' tall fescue seed; 0.0 ppm ergovaline/ergovalinine). Seed treatments began one day earlier for the E+ steers than the E- steers due to pair feeding. Seed was ground to pass a 2-mm screen and dosed ruminally 2× daily at $15 \mu\text{g of ergovaline} \cdot \text{kg BW}^{-1} \cdot \text{d}^{-1}$ for 7 days at ambient TN and at

HS conditions. Temperature treatments were run in consecutive periods as the TN and HS could not be conducted simultaneously.

On day 8, a washed reticulorumen experiment was conducted. At 0700 steers were weighed, and a jugular catheter was placed. A serum sample was collected for prolactin analysis, and a plasma sample was collected for background deuterium oxide (D_2O) concentrations. Rumen contents were removed through the cannula with the assistance of a vacuum, weighed, sampled for DM analysis (AOAC, 1990), and the container was covered with straw and placed in a 40°C water bath or a 40°C forced-draft oven. The rumen was washed with 10 kg of warm tap water followed by 3 rinses of warm saline (0.9 % NaCl; 10 kg saline/rinse). Buffers (15 kg, 40°C) were incubated in the rumen in a sequence of 3 buffer treatments (Figure 5.1) that were each separated into equilibration and sampling buffers. The 3 buffer treatments were control buffer containing only the extract vehicle (**CON**; methanol), **1× EXT** (15 µg ergovaline · kg BW^{-1}), and a **3× EXT** (45 µg ergovaline · kg BW^{-1}). The buffer sequence was held constant so that the CON treatment would always be incubated before the extract-containing buffer and be affected by potential carry-over effects of the extract incubation. A separate experiment was conducted to determine the effect of time on changes in blood flow and nutrient absorption. The buffer composition has been previously described (Kristensen and Harmon, 2004) and is shown in Table 5.1. The sampling buffer was identical to the equilibration buffer except for the addition of Cr-EDTA and D_2O (3, 6 and 9 g D_2O for CON, 1× EXT and 3× EXT buffers respectively). All buffers were mixed by infusing a gas mixture (75% CO_2 :25% N_2) continuously into the ventral rumen during incubation. A sampling catheter was also placed in the ventral rumen to allow

buffer sample collection. Between buffer treatments the rumen was rinsed with 10 kg of warm 0.9% saline. All buffers and saline rinses were weighed before and after incubation in the rumen. At the completion of a washed rumen experiment, rumen contents were replaced and steers were fed.

Rumen buffer samples (~17 mL) were collected at 0, 5, 10, 15, 20 and 30 min during incubation of a sampling buffer. Blood samples (~15 mL) were collected via jugular catheter at 5, 15, and 30 min into heparinized syringes during incubation of each sampling buffer. All samples were immediately placed on ice (~1°C). At the conclusion of the experiment, blood was centrifuged at $5,000 \times g$ at 4°C for 15 min, and buffer samples were centrifuged at $4,000 \times g$ at 20°C for 10 min. Plasma was collected and stored at -20°C. An aliquot of ruminal buffer supernatant (4 mL) was stored at 4°C for Cr-EDTA analysis by atomic absorption spectroscopy (AAAnalyst 200, Perkin Elmer Inc., Waltham, MA). For VFA analysis, 0.2 mL of the internal standard 2-ethyl butyrate (Acros Organics, Geel, Belgium) was added to 2 mL of buffer supernatant, vortexed, and combined with 0.2 mL of a 50% (w/v) meta-phosphoric acid solution. The sample was thoroughly mixed and frozen at -20°C. At analysis, samples were thawed and centrifuged at $21,000 \times g$ for 20-min and, VFA concentrations were analyzed by GC with a flame ionization detector (Agilent HP6890 Plus GC with Agilent 7683 Series Injector and Auto Sampler; Agilent Technologies, Santa Clara, CA) using a Supelco 25326 Nukol Fused Silica Capillary Column (15 m \times 0.53 mm \times 0.5 μ M film thickness; Sigma/Supelco, Bellefonte, PA).

Ruminal buffer sample aliquots (0.5 mL) and plasma sample aliquots (0.5 mL) were filtered through 10 kDa spin filters (Spin-X UF, Corning Inc., Corning, NY) at

10,000 × g at 32°C for 20 min. The D₂O was quantified by high temperature conversion elemental analysis-isotope ratio mass spectrometry (Finnigan Delta V Plus, Thermo Scientific, Bremen, Germany; Storm, et al., 2011). Serum samples were collected after spinning blood samples at 3,000 × g for 15 min. Serum bovine prolactin was measured by RIA using previously described methods (Bernard et al., 1993).

Experiment 2. Four ruminally cannulated Holstein steers (BW = 294 ± 7.4 kg) from Exp. 1 were housed as described in Exp. 1. Experiment 2 was conducted exactly as Exp. 1 with the exception of the washed rumen portion of the experiment. Briefly, steers were paired by BW and assigned to either the E+ or E- seed treatment and pair-fed alfalfa cubes at 1.5× NE_m for 7 d at TN and HS conditions followed by a washed rumen experiment on day 8 of each period. Seed treatments were then switched and the experiment was repeated. The CON buffer was incubated in place of the 1× EXT and 3× EXT buffer treatments to test the possibility of time confounding the effect of buffer treatment on the measured parameters during the washed rumen experiment.

Model Derivation and Calculations

A model developed by Storm et al. (2011) was used to calculate ruminal liquid passage rate, physiological water influx into the rumen, and residual water in the rumen prior to introduction of the buffer. Calculation of these parameters was conducted as described in Storm et al. (2011) except that the Solver add-in of Excel (Microsoft Corp., Redmond, WA) was utilized. These parameters were estimated for each individual buffer incubation (n = 72 for Exp. 1 and n = 48 for Exp. 2). A local solution was found for every data set. The model was set to minimize the residual sum of squares and was also visually assessed using a residual plot.

Ruminal liquid volume, pool of metabolites, ruminal flux of metabolites, and ruminal clearance were calculated as described in Storm et al. (2011). Briefly, the volume of liquid in the rumen was calculated at each sampling time point, and using the measured concentration of VFA and D₂O at the corresponding time point, a ruminal pool of VFA and D₂O was calculated at each time point. Ruminal flux of VFA and D₂O were determined by calculating the change in pool size and correcting for physiological influx and passage out of the rumen in the liquid as:

$$\text{Ruminal Flux mmol/h} = \frac{Q_{t-1} - Q_t - F_{Liq, \Delta t} + F_{Sal, \Delta t}}{\Delta t},$$

where Q is pool size (mmol), t is sampling time point, F_{Liq,Δt} is liquid flow rate out of the rumen (L/h), and F_{Sal,Δt} is flow of physiological water into the rumen. Ruminal clearance of D₂O was calculated as the ruminal flux of D₂O relative to the concentration difference of the ruminal liquid and blood as:

$$\text{Ruminal Clearance L/h} = \frac{\text{Ruminal Flux}}{\left(\frac{C_{Ru,t-1} + C_{Ru,t}}{2}\right) - \left(\frac{C_{A,t-1} + C_{A,t}}{2}\right)},$$

where C_{ru} is concentration of metabolite in the ruminal buffer (mmol/L) and C_A is concentration of metabolite in arterial blood. Additionally, to determine the association of blood flow to the absorptive surface of the reticulorumen and VFA absorption, efficiency of VFA absorption was calculated as VFA flux (mmol/h) / Ruminal epithelial blood flow (L/h).

Statistical Analysis

Data for feed intake were analyzed as a randomized complete block design using the mixed model procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). The model included fixed effects of day, temperature, and day \times temperature. Because steers were pair-fed, only DMI data from the steers receiving E+ seed treatment were analyzed as intake was equal for steers receiving E- seed. Data from the TN and HS portions of the experiment for serum prolactin, rumen content analysis, reticuloruminal epithelial blood flow, and VFA flux were analyzed separately due to unequal variances as tested using Bartlett's test ($P < 0.01$). Serum prolactin data were analyzed as a randomized complete block design using mixed model procedure of SAS. Fixed effects included day, seed, and day \times seed and random effects included crossover period. Data from the TN and HS portions of the experiment were analyzed separately for the washed rumen portion of the experiment. Data from the washed rumen portion of the experiment including blood flow and VFA flux were analyzed as a randomized complete block split plot with a whole plot factorial using the mixed model procedure of SAS. The whole plot factors included seed treatment and crossover period and the sub-plot factor was the buffer treatment. Analysis of variance was conducted followed by pair-wise comparisons of least square means using LSD when the probability of a greater F -statistic was significant ($P < 0.05$).

Results and Discussion

Induction of Fescue Toxicosis

Endophyte-infected tall fescue seed was dosed so that steers received 0.015 mg of ergovaline \cdot kg BW⁻¹ \cdot d⁻¹. This dose of ergovaline provided as ground seed introduced via a ruminal cannula has been previously reported to sufficiently induce fescue toxicosis

based on reduced intake, greater respiration rates at HS conditions, and reduced heart rates compared to steers consuming E- seed (Koontz et al., 2012).

Alfalfa cubes were offered at $1.5 \times NE_m$, and the steers receiving the E- seed treatment were fed the same amount as the paired E+ steers (Figure 5.2). Under TN conditions, DMI did not decline over the 7-d feeding period, although there was a numerical decrease from about 1.5 to $1.2 \times NE_m$. When steers were housed in heat stress conditions (approximately 30°C), voluntary feed intake was reduced to $0.7 \times NE_m$ ($P = 0.018$ for temperature \times seed treatment). Given the pair-fed design of this experiment, it is not possible to determine that the E+ tall fescue seed caused a decrease in feed DMI. However, depressed intake was one of the first signs of fescue toxicosis to be described and was thought to contribute to the decreased growth rate of cattle on tall fescue pastures (Strickland et al., 1993). The impact of toxic tall fescue on intake can be variable depending on the ambient temperature, as steers receiving an E+ seed treatment displayed decreased intake compared to steers receiving E- seed when housed at 32°C , but not when steers were housed at 22°C (Koontz et al., 2012). It is unlikely that any factors other than the E+ resulted in the large reduction in intake observed in this study.

The wet weight of ruminal contents on a BW basis (Table 5.2) tended to be greater for E+ than E- steers ($P = 0.12$) at TN conditions. Dry matter percentage ($P = 0.03$) and the dry ruminal contents on a BW basis ($P < 0.01$) were also greater in E+ steers at TN. Steers housed in HS conditions and receiving E+ seed had greater wet weights (Table 5.2; $P = 0.03$), greater dry ruminal content weight ($P < 0.01$), and a 50% greater DM percentage ($P < 0.01$). Steers receiving E- seed were pair fed to receive the same amount of feed daily as steers receiving E+ seed, but the rate of DMI (kg/h) was not

controlled as steers were fed once daily. It is likely that E- steers had a greater rate of DMI as has been previously reported in steers at TN conditions (Koontz et al., 2012). These data could indicate a difference in the particulate or liquid passage rates of steers consuming toxic tall fescue compared to steers receiving E- seed. Data from the model used to estimate liquid passage rate during the washed rumen experiment indicated there was no difference in liquid passage rate between steers receiving E+ and E- at either TN or HS ($P > 0.40$, data not shown); however, this estimate is not necessarily consistent with measures in the fed state. Ergot alkaloids have been shown to reduce cyclical contractions of reticuloruminal smooth muscle in sheep (McLeay and Smith, 2006; Poole et al., 2009), which could lead to a decrease in passage rate and DMI. It is also possible that a difference in water intake could exist between the 2 groups of cattle, as water intake was not measured in the current experiment. Previous research has shown that water intake is not affected by tall fescue seed consumption in steers (Aldrich et al., 1993b) or sheep (Hannah et al., 1990).

At TN, serum prolactin concentrations were affected by seed treatment ($P = 0.002$; Figure 5.3A). There was also an effect of day ($P = 0.03$) but no interaction of seed treatment \times day ($P = 0.52$) on serum prolactin. Steers receiving E+ seed had lower prolactin concentrations on d-0 and d-8 than E- steers; however, prolactin concentration decreased by 46% in E+ steers from day 0 to day 8 and by 18 % in E- steers. At HS conditions, there was an interaction of seed treatment \times day ($P = 0.02$) on serum prolactin concentrations, indicating that steers receiving the E+ seed treatment had a greater reduction (92%) in serum prolactin concentration than steers receiving the E- seed treatment (66%). Prolactin concentration is a common indicator of fescue toxicosis, as

ergot alkaloids are potent dopamine receptor (D₂) agonists (Larson et al., 1999; Larson et al., 1995; Strickland et al., 2011). Binding of an agonist to the D₂ receptors on the surface of the lactotrophs in the pituitary causes a decrease in prolactin production and release by several mechanisms (Lamberts and Macleod, 1990). High ambient temperatures have been shown to increase prolactin concentrations in Holstein steers by both increasing secretion rates and decreasing metabolic clearance rates (Smith et al., 1977). Feed restriction of heifers housed at TN conditions has been shown to have no effect on circulating prolactin concentrations (Ronchi et al., 2001). Another study has reported no difference in prolactin concentrations of Holstein steers fed E- seed at TN and HS conditions (Aldrich et al., 1993b). Taken together, DMI and prolactin data presented here indicate that the steers receiving the E+ seed treatment were suffering from the fescue toxicosis syndrome.

Washed Rumen Model Variables

The rate constant for liquid passage out of the rumen (Table 5.3) was constrained to be greater than or equal to 0.0 %/h. The liquid passage rate range was slightly larger than that reported by Storm et al. (2011). Due to the large variation and range of estimates, these parameters were estimated individually for each combination of steer, buffer, seed treatment, and temperature. The influx of physiological water flowing into the rumen was never negative, indicating that there was a net movement of water into the rumen through saliva production or movement across the rumen epithelium. The range of predicted physiological water influx (Table 5.3) was smaller than that reported by Storm et al. (2011), but the values were within the range previously reported (Storm et al.,

2011). The volume of water present in the rumen prior to introduction of the buffer, or residual water, was similar to the values reported by Storm et al. (2011).

Reticuloruminal Epithelial Blood Flow

Reticuloruminal clearance of D₂O was used as an estimate of reticuloruminal epithelial blood flow. At TN (Figure 5.4A), steers receiving E+ seed had lower reticuloruminal epithelial blood flow than steers receiving E- seed (20.0 L/h versus 28.4 L/h; $P < 0.038$ for the interaction) during the incubation of CON buffer treatment. This decrease in blood flow appeared similar at HS conditions (Figure 5.4B; 24.0 L/h for E+ steers versus 31.2 L/h for E- steers, SEM = 3.9), however, these values were not different ($P = 0.21$) largely because of increased variability during heat stress. When ergot alkaloids were introduced into the washed rumen of steers at TN (Figure 5.4A) at either the 1× EXT or the 3× EXT level, there was a large decrease in reticuloruminal epithelial blood flow ($P < 0.038$) to approximately 10 L/h for both E+ and E- steers. The same effect was seen at HS (Figure 5.4B). The lack of a further decrease in ruminal epithelial blood flow at the 3x EXT level could indicate that the maximum effect was achieved with the lower ergot level present in the 1× EXT incubation.

The baseline blood flow measurement of 28.4 L/h for E- TN steers in this experiment is lower than that reported in lactating Holstein cows (Storm et al., 2011) where reticuloruminal epithelial blood flow was reported to range from 48 to 92 L/h depending on the diet and buffer treatment. The use of D₂O to estimate epithelial blood flow is unique in that it only estimates blood flow to the epithelium that is in contact with the buffer and directly involved in absorption at the time of measurement. Storm et al. (2011) used 30 kg of buffer where the current experiment used 15 kg of buffer. It is likely

that the surface area of the reticulorumen exposed to the buffer is greater with the greater mass of buffer. Additionally, Storm et al. (2011) utilized 565-kg lactating cows, where 255-kg steers were used in the current experiment. When reticuloruminal epithelial blood flow is expressed per kg BW, the results are similar (0.11 and 0.08 L of blood flow / kg BW for steers and cows, respectively). Portal blood flow calculated by dilution of para-aminohippurate has been shown to be much greater in lactating cows than either heifers or steers (Huntington et al., 1989). Gut blood flow appears to be linked to level of intake and not sex or production state.

Ergot alkaloids, including ergovaline, as well as an endophyte-infected tall fescue seed extract have been shown to induce vasoconstriction of ruminal arteries and veins in vitro (Foote et al., 2012; Foote et al., 2011). Rhodes et al. (1991) also showed that blood flow to some gut tissues including the duodenum could be reduced by exposure to ergot alkaloids using labeled microspheres. Constriction and dilation of blood vessels is the most important factor in regulation of blood flow (Levick, 2010). The concentration of ergovaline in the ruminally incubated buffer was approximately $4.7 \times 10^{-7} M (\pm 6.8 \times 10^{-9})$ for the 1× EXT and $1.4 \times 10^{-6} M (\pm 1.6 \times 10^{-8})$ for the 3× EXT incubation. The least concentration of ergovaline shown to induce vasoconstriction in ruminal arteries and veins in vitro was $10^{-6} M$ ergovaline (Foote et al., 2011), which is essentially equal to the initial concentration of ergovaline in the ruminal buffer during the 3× EXT treatment but greater than the 1× EXT initial buffer concentration. In ruminal veins, a tall fescue seed extract was shown in one study to induce vasoconstriction at $10^{-7} M$ (normalized to ergovaline concentration), whereas in another study vasoconstriction was not observed until a $10^{-6} M$ ergovaline (Foote et al., 2012). From the data presented here it is not

possible to infer that ergovaline is absorbed from the washed rumen and induces a local vasoconstriction, however, it is noteworthy that a concentration of ergovaline that is on the low end of concentrations known to induce vasoconstriction of ruminal vessels in vitro can alter blood flow in vivo (Foote et al., 2012; Foote et al., 2011).

VFA Flux

Steers receiving E+ seed under TN conditions in Exp. 1 (Table 5.4) tended to have lower rates of total VFA flux ($P = 0.10$), acetate flux ($P = 0.13$), butyrate flux ($P = 0.07$), and isovalerate flux ($P = 0.05$). This follows the trend seen with blood flow that steers receiving E+ seed had lower blood flow than steers receiving E- seed. Seed treatment had no effect on VFA flux when steers were housed in HS conditions ($P > 0.40$; Table 5.4). When ergot alkaloids were included in the ruminally incubated buffer at TN, flux of total VFA, acetate, propionate, and butyrate were decreased ($P < 0.01$), and isovalerate tended to be less ($P = 0.13$); however, valerate flux was not affected by the addition of the extract to the buffer compared with the CON treatment ($P = 0.17$). Under HS conditions, addition of ergot alkaloids at both the 1× EXT and the 3× EXT concentrations caused a decrease in total VFA, acetate, propionate, butyrate, and valerate ($P < 0.01$) and tended to decrease isovalerate flux ($P = 0.08$).

The flux values observed in the current study are similar to the values reported for net portal flux in a similar experiment using lactating cows (Storm et al., 2011) when adjusted for BW. The flux rates reported here were also less than the absorption rates from a washed reticulorumen of Holstein steers (Kristensen and Harmon, 2004); however, the absorption rate did not account for passage out of the rumen with liquid as was done in the data presented here. When looking at portal blood flux of VFA the data

from Kristensen and Harmon (2004) was similar to the data presented here; although the portal flux data was slightly greater, which could be due to the contribution of omasal and hindgut absorption in the portal flux measurement.

The effect of fescue toxicosis and ergot alkaloids on nutrient absorption has been largely unstudied. The one study that attempted to look at endophyte-infected tall fescue and nutrient absorption showed that other than a slight decrease in net portal acetate flux in steers receiving E+ hay; there was no effect on nutrient absorption (Harmon et al., 1991). The tall fescue hay used by Harmon et al. (1991) had a measured ergovaline concentration of 134 ppb, and the steers consumed an average of 5.26 kg DM/d, resulting in a total intake of approximately 0.7 mg of ergovaline/d. This dose is much less than the dose used in the experiment presented here and by Koontz et al. (2012), which is about 4 mg ergovaline/d. Additionally, there was no reported difference in prolactin concentration between the E+ and E- groups by Harmon et al. (1991), indicating that those steers might not have been experiencing fescue toxicosis.

A disruption in absorption of VFA could have large negative impacts on production of cattle. It is estimated that the three predominant VFA, acetate, propionate, and butyrate account for 45% of DE intake in ruminants (Kristensen et al., 2005), most of which are absorbed from the rumen. Propionate is thought to provide 43 to 77% of the glucose carbon in cattle (McLeod et al., 2006). It is clear that VFA contribute significantly to the energy supply as well as providing most of the carbon needed for production in ruminants. A decrease in the absorption of VFA from the rumen could severely depress cattle performance. One of the most common signs of fescue toxicosis is depressed growth of cattle, and this could potentially be attributed in part to a decrease in

ruminal VFA absorption. Decreasing VFA absorption in the rumen could lead to greater interconversions of the VFA by microbes and a greater loss of carbon through methane production, which could further contribute to inefficiency of production. It is possible that the observed decrease in VFA absorption in response to ergot alkaloid exposure could be an important cause of clinical signs the fescue toxicosis syndrome.

Relationship Between Epithelial Blood Flow and VFA Flux

Absorption of nutrients from the gut is limited by several factors, including mixing of the contents allowing presentation of the nutrient to the absorptive surface, permeability of the nutrient, and removal with blood from the serosal side of the epithelium (Dobson, 1984). Storm et al. (2011) reported a correlation coefficient for ruminal disappearance of propionate and epithelial blood flow of 0.56, indicating that blood flow to the absorptive surface of the rumen has a large effect on VFA absorption. Storm et al. (2011) showed that reticuloruminal epithelium and ruminal vein blood flow are dynamic and responsive to nutrients (specifically fermentative end products) present in the lumen of the rumen.

In the current study, efficiency of VFA absorption was calculated by expressing VFA absorption (mmol/h) relative to epithelial blood flow (L/h) resulting in the units of mmol of VFA absorbed/L of epithelial blood flow. Steers receiving E+ seed under TN conditions tended to have a greater amount of propionate absorbed per unit of blood flow (Table 5.5; $P = 0.08$). Including ergot alkaloids from an E+ tall fescue seed extract in the ruminal buffer increased the efficiency of butyrate absorption at TN ($P = 0.02$) as well as isovalerate ($P < 0.01$). There was a seed treatment \times buffer interaction ($P = 0.03$) for valerate absorption efficiency under TN conditions. This indicates that steers receiving E-

seed, including the 1× EXT in the ruminally incubated buffer increased valerate absorption efficiency compared to CON and the 3× measuring intermediate. For the steers receiving E+ seed, inclusion of the 1× EXT and the 3× EXT increased valerate absorption efficiency, but the 1× EXT and the 3× EXT were not different ($P = 0.32$).

When steers were housed under HS conditions, propionate absorption efficiency was increased with the inclusion of the 1× EXT ($P = 0.04$). The increase was greater in steers receiving the E- seed treatment ($P = 0.07$ for the interaction). Butyrate absorption efficiency in steers housed in HS conditions was higher during the incubation of the 1× EXT treatment compared to the CON incubation and the 3× EXT was intermediate ($P = 0.02$). The tendency for an interaction of seed treatment × buffer treatment for butyrate absorption efficiency at HS ($P = 0.10$) indicates that the effect of the extract was more evident in steers receiving E- seed. There was a seed treatment × buffer treatment interaction for valerate absorption efficiency at HS ($P = 0.03$). Steers receiving E- seed had greater valerate absorption efficiency during the 1× EXT and the 3× EXT incubation than the CON incubation, but the 1× EXT and 3× EXT values were not different. Steer receiving E+ seed showed increased valerate absorption efficiency during the 1× EXT incubation, but it returned to values similar to CON during the 3× EXT incubation (Seed × Buffer $P = 0.03$). Additionally, the increase in valerate absorption efficiency in response to ergot alkaloids incubated ruminally was greater in steers receiving E- seed (Seed × Buffer $P = 0.03$). Isovalerate absorption efficiency in steers housed in HS conditions followed a similar trend to that of valerate absorption efficiency with a seed treatment × buffer treatment interaction ($P = 0.01$). For the HS portion of the experiment, it is apparent that VFA absorption efficiency is more responsive to ergot alkaloids in

steers receiving E- seed than steers receiving E+ seed, which could indicate an acute response to ergot alkaloids.

The effects of ergot alkaloids on VFA absorption efficiency observed in this experiment were only observed for VFA that have the potential to be metabolized by ruminal epithelium (propionate, butyrate, valerate, and isovalerate). These VFA are also larger, more lipophilic molecules than acetate. This increase in absorption efficiency could be due to either an alteration in epithelial metabolism, an alteration in the barrier function of the ruminal epithelium, or potentially through mechanisms related to transporters involved in VFA absorption. It is likely that the ergot alkaloids are increasing the epithelial metabolism of VFA, which leads to a greater concentration gradient across the rumen epithelium. This would lead to a greater amount of VFA removed from the rumen per unit of blood flow. It is also possible that the decrease in blood flow reduces the oxygen flowing to the epithelium causing a decrease in metabolic activity of the epithelial cells, and this decrease in epithelial metabolism of VFA could result in increased VFA absorption efficiency. However, it is likely that if there was a decrease in epithelial metabolism, there would be a decrease in the concentration gradient leading to a decrease in flux. Additionally, ergocristine has been shown to alter barrier function of porcine brain endothelial cells (Mulac et al., 2012). This effect was not seen with ergometrine (also known as ergonovine) or ergotamine and took more than 4 h of incubation for the effect to be observed. It is possible that ergot alkaloids present in E+ tall fescue seed extract could alter the barrier function of the rumen epithelium, which could in turn increase permeability of VFA. However, it is likely that if barrier function was greatly altered that the increase in VFA absorption efficiency would be more evident

for acetate, which has the lowest permeability. Additionally, a disruption of the ruminal barrier function could allow the translocation of microbes and endotoxins into the ruminal tissue and portal blood. Microbes entering the portal circulation have been shown to cause liver abscesses (Tadepalli et al., 2009), which is related to decreased growth and feed efficiency (Brink et al., 1990). Ergot alkaloids present in E+ tall fescue appear to alter VFA epithelial metabolism or disrupt the barrier function of the gut, which could contribute to the observed signs of fescue toxicosis such as depressed growth.

Experiment 2

Experiment 2 was conducted because the order of buffer treatment administration could not be randomized in Exp. 1, and it was necessary to rule out the possibility that blood flow and VFA flux change over the duration of the washed rumen portion of the experiment. Blood flow values were less for steers receiving the E- seed in Exp. 2 (Figure 5.5) than Exp. 1; however, blood flow measures for steers receiving the E+ seed treatment were similar in both experiments. It is not clear why these measures were less for only the E- steers during Exp. 2, but it is not likely that the buffer treatments are responsible for this difference. There was a statistical interaction for seed treatment \times buffer ($P = 0.045$) at TN (Figure 5.5A), although this was due to an increase in blood flow for E- steers during the third buffer incubation and was not the severe decrease in blood flow that was observed in Exp. 1 with the addition of ergot alkaloids in the buffer. Reticuloruminal epithelial blood flow in steers housed in HS conditions did not change during the three CON buffer sequences (effect of buffer $P = 0.18$). The data from Exp. 2 indicate that the effect of the buffer treatment observed in Exp. 1 was due to the addition of the ergot alkaloids from the E+ tall fescue seed and not due to time.

Flux of VFA from the washed rumen in Exp. 2 (Table 5.6) was lower for E- steers than in Exp. 1, which was similar to the blood flow measures for Exp. 2. Overall there was no effect of buffer on VFA flux at TN conditions with the exception of valerate flux ($P < 0.03$), which indicates an increase in valerate flux during the second and third buffer sequences. There was a seed \times buffer interaction ($P = 0.03$) for isovalerate flux at TN conditions, due to an increase in isovalerate flux for E+ treated steers during buffer sequence 2 compared to buffer 1 and 3 of the E+ treated steers. Additionally, there was no difference in VFA flux between the different buffers in steers housed at HS conditions ($P > 0.11$). There was an increase in the SEM in Exp. 2 compared to Exp. 1 for VFA flux values, which was likely due to the reduction in the number of animals used. This increase in variation could reduce to power to detect differences in flux measurements; however, flux of VFA did not follow the same trends as observed in Exp. 1. In Exp. 1 buffer treatment decreased VFA flux by 30 to 50%. There was no effect of buffer treatment on VFA flux in Exp. 2 ($P > 0.11$) with the exception of an increase in valerate flux at TN ($P = 0.03$), which indicates the effects of the buffer treatments observed in Exp. 1 are due solely to the inclusion of the tall fescue seed extract. At HS conditions there was an effect of seed treatment on total VFA, acetate, propionate, and butyrate flux ($P < 0.03$) and a tendency for isovalerate flux ($P = 0.05$) indicating that E- treated steers had greater flux rates than steers receiving E+ seed. This was not seen in Exp. 1 or in the TN portions of the experiment, but was likely due to E+ steers having lower flux rates than observed in Exp. 1 for HS conditions and greater variation in the data.

It is clear from these data that ergot alkaloids present in E+ seed and E+ seed extract are responsible for the reduction in epithelial blood flow and VFA absorption. It is

likely that ergot alkaloids incubated in the rumen cause a local vasoconstriction and decrease blood flow to the absorptive surface of the bovine rumen; however, ruminal absorption of ergot alkaloids, especially ergopeptine alkaloids such as ergovaline is not definitive and not extensively studied. Lysergic acid, lysergol, ergonovine, ergotamine, and ergocryptine were shown to cross ruminal epithelium in a parabiotic chamber (Hill et al., 2001). Another study incubated ergovaline on the mucosal side of a parabiotic chamber with ruminal epithelium mounted and showed that ergovaline could not be measured on the serosal side of the chamber after 4 h of incubation (Ayers et al., 2009). This study incubated ergovaline in the mucosal buffer at a concentration of about 2 ng/mL. The interpretation of these data is limited because it is likely that if ergovaline crossed the epithelium, then it would be well below the limit of detection. In Hill et al. (2001), only 2% of ergotamine and 1.5% of ergocryptine was accounted for in serosal buffer. If ergovaline, which is similar in structure to ergotamine and ergocryptine, has a similar transport capacity there would only be a concentration of about 0.04 ng ergovaline/mL in the serosal side of the parabiotic chamber, which is well below the detection limit of more sensitive HPLC-MS based analytical methods (Smith et al., 2009). Due to the relatively rapid onset of physiological response observed in the experiment present here, it is likely that ergovaline is crossing the ruminal epithelium in the washed rumen model, inducing local vasoconstriction, and in turn reducing blood flow to the absorptive surface of the rumen. It is also possible that the ergot alkaloids could bind receptors on the ruminal epithelial cells and induce signaling pathways that alter blood flow and other effects on the gut.

Conclusions

Ergot alkaloids from tall fescue seed and a tall fescue seed extract induce at least a 50% reduction in blood flow to the absorptive surface of the reticulorumen of cattle housed in thermoneutral and heat stress conditions. Additionally, ergot alkaloids cause a reduction in VFA absorption from a washed reticulorumen that is likely related to the reduction in blood flow to the absorptive surface of the foregut. The depression of nutrient absorption related to toxic tall fescue exposure could contribute to the common signs of fescue toxicosis such as decreased growth and performance.

Table 5.1. Chemical composition of the buffer incubated in the washed reticulorumen¹ (mmol/kg).

Item	Content
NaHCO ₃	24.0
NaOH	95.0
KHCO ₃	30.0
K ₂ HPO ₄	2.0
CaCl ₂	1.5
MgCl ₂	1.5
Acetic Acid	72.0
Propionic Acid	30.0
Butyric Acid	12.0
Isovaleric Acid	2.0
Valeric Acid	1.3

¹The buffer was mixed and agitated by gas (75% CO₂:25%N₂).

Table 5.2. Ruminant content weight and DM of steers that have received endophyte-infected (E+) or endophyte-free (E-) tall fescue seed for 7 d at thermoneutral (22°C) and heat stress (30°C).

Item	Seed Treatment		SEM (n = 6)	P-value
	E+ ¹	E- ¹		
Thermoneutral				
Rumen Content, kg/100 kg BW	13.52	11.85	0.76	0.12
DM, %	14.73	10.38	1.60	0.03
Dry Contents, kg/100 kg BW	2.01	1.24	0.16	<0.01
Heat Stress				
Rumen Content, kg/100 kg BW	15.39	11.95	1.35	0.03
DM, %	14.33	7.23	1.40	<0.01
Dry Contents, kg/100 kg BW	2.21	0.80	0.37	<0.01

¹Steers receiving E+ seed treatment received 15 µg of ergovaline · kg BW⁻¹·d⁻¹ from ‘Kentucky 31’ tall fescue seed and the E- seed treatment received an equal amount of ‘Kentucky 32’ tall fescue seed with 0.0 ppm ergovaline.

Table 5.3. Variables estimated by the model utilizing Cr-EDTA concentrations and buffer weights using the methods described by Storm et al. (2011).

Parameter	Estimate		SEM
	Minimum	Maximum	
Experiment 1 (n = 72)			
Rate constant for passage, %/h	0.00	41.98	1.33
Physiological water influx, L/h	0.12	5.44	0.16
Ruminal water residues, L	0.01	3.39	0.09
Experiment 2 (n = 48)			
Rate constant for passage, %/h	0.00	29.54	1.17
Physiological water influx, L/h	0.10	4.81	0.17
Ruminal water residues, L	0.03	2.05	0.09

Table 5.4. Experiment 1. Flux of VFA (mmol/h) from the washed reticulorumen of steers receiving endophyte-infected (E+) or endophyte-free (E-) tall fescue seed twice daily housed at thermoneutral (22°C) and heat stress (30°C) conditions for 7 d. Volatile fatty acid flux was measured using a sequential buffer sequence with a control buffer (CON), a buffer containing 15 µg ergovaline/kg BW (1× EXT), and a buffer containing 45 µg ergovaline/kg BW (3× EXT).

Item	E ⁻¹			E ⁺¹			SEM (n = 6)	P-value		
	Control	1× EXT	3× EXT	Control	1× EXT	3× EXT		Seed	Buffer	Seed × Buffer
Thermoneutral										
Total VFA	565.8	370.4	344.8	526.0	254.5	239.1	65.02	0.10	<0.01	0.81
Acetate	320.6	201.7	221.4	314.8	128.2	134.9	42.19	0.13	<0.01	0.61
Propionate	177.3	88.6	75.4	152.2	86.6	67.4	19.42	0.47	<0.01	0.83
Butyrate	57.5	39.8	37.4	48.2	32.0	28.4	5.60	0.07	<0.01	0.99
Valerate	5.9	5.5	5.6	6.1	4.0	4.8	0.58	0.18	0.17	0.40
Isovalerate	6.5	4.6	5.0	4.8	3.7	3.9	0.74	0.05	0.13	0.85
Heat Stress										
Total VFA	473.9	304.5	307.7	441.2	297.4	227.9	69.05	0.63	<0.01	0.76
Acetate	263.7	162.5	179.6	264.2	178.9	122.5	40.90	0.77	<0.01	0.50
Propionate	141.2	95.9	82.0	122.6	78.3	64.6	21.47	0.50	<0.01	0.99
Butyrate	54.4	35.8	34.9	42.1	31.0	30.1	7.04	0.40	<0.01	0.67
Valerate	8.6	5.5	6.0	6.6	5.5	5.8	0.98	0.59	<0.01	0.13
Isovalerate	6.1	4.8	5.2	5.7	3.8	4.8	0.97	0.62	0.08	0.87

¹Steers receiving E+ seed treatment received 15 µg of ergovaline · kg BW⁻¹ · d⁻¹ from ‘Kentucky 31’ tall fescue seed and the E- seed treatment received an equal amount of ‘Kentucky 32’ tall fescue seed with 0.0 ppm ergovaline.

Table 5.5. Efficiency of VFA absorption (mmol VFA absorbed / L blood flow) from the washed reticulorumen of steers receiving endophyte-infected (E+) or endophyte-free (E-) tall fescue seed twice daily housed at thermoneutral (22°C) and heat stress (30°C) conditions for 7 d.¹

Item	E ⁻²			E ⁺²			SEM (n = 6)	P-value		
	Control ³	1× EXT	3× EXT	Control	1× EXT	3× EXT		Seed	Buffer	Seed × Buffer
Thermoneutral										
Total VFA	19.5	37.6	25.9	27.4	30.5	26.5	4.81	0.90	0.11	0.33
Acetate	11.1	20.6	16.6	16.5	15.6	14.9	3.39	0.88	0.46	0.32
Propionate	6.1	5.7	5.5	7.9	8.1	7.5	1.61	0.08	0.93	0.98
Butyrate	2.0	3.3	2.9	2.5	3.7	3.2	0.42	0.26	0.02	0.95
Valerate	0.2	0.6	0.5	0.3	0.5	0.5	0.05	0.68	<0.01	0.03
Isovalerate	0.2	0.5	0.4	0.3	0.5	0.4	0.06	0.90	<0.01	0.84
Heat Stress										
Total VFA	15.3	29.6	26.4	21.1	23.2	14.0	5.40	0.49	0.14	0.10
Acetate	8.5	15.5	15.6	12.6	13.8	7.9	3.35	0.63	0.34	0.13
Propionate	4.5	9.6	7.0	5.9	6.4	3.9	1.61	0.42	0.04	0.07
Butyrate	1.8	3.5	2.9	2.0	2.3	1.7	0.50	0.24	0.02	0.10
Valerate	0.3 ^{CD}	0.5 ^A	0.5 ^{AB}	0.3 ^D	0.4 ^{BC}	0.3 ^D	0.04	0.07	<0.01	0.03
Isovalerate	0.2 ^C	0.5 ^A	0.4 ^{AB}	0.3 ^{BC}	0.2 ^C	0.3 ^C	0.05	0.08	0.03	0.01

¹VFA absorption efficiency (mmol VFA absorbed / L blood flow) = VFA absorption rate (mmol/h) / epithelial blood flow (L/h)

²Steers receiving E+ seed treatment received 15 µg of ergovaline · kg BW⁻¹·d⁻¹ from ‘Kentucky 31’ tall fescue seed and the E- seed treatment received an equal amount of ‘Kentucky 32’ tall fescue seed with 0.0 ppm ergovaline.

³Blood flow and VFA flux was measured using a sequential buffer sequence with a control buffer, a buffer containing 15 µg ergovaline/kg BW (1× EXT), and a buffer containing 45 µg ergovaline/kg BW (3× EXT).

^AMeans within row with uncommon superscript differ ($P < 0.05$)

Table 5.6. Experiment 2. Flux of VFA (mmol/h) from the washed reticulorumen of steers receiving endophyte-infected (E+) or endophyte-free (E-) tall fescue seed twice daily housed at thermoneutral (22°C) and heat stress (30°C) conditions for 7 d.

Item	E ⁻¹			E ⁺¹			SEM (n = 4)	P-value		
	Buffer 1 ²	Buffer 2	Buffer 3	Buffer 1	Buffer 2	Buffer 3		Seed	Buffer	Seed × Buffer
Thermoneutral										
Total VFA	464.1	335.8	501.4	531.5	624.0	347.0	127.68	0.62	0.80	0.20
Acetate	278.8	181.9	300.4	320.2	378.5	199.1	85.60	0.58	0.83	0.25
Propionate	127.8	99.8	136.8	148.9	166.0	96.2	32.31	0.69	0.65	0.14
Butyrate	47.5	43.2	50.5	51.0	63.9	39.6	10.61	0.73	0.58	0.19
Valerate	5.0	6.5	7.4	6.1	8.1	6.7	0.85	0.54	0.03	0.15
Isovalerate	5.0 ^{AB}	4.4 ^{AB}	6.2 ^{AB}	5.3 ^B	7.6 ^A	5.5 ^B	0.97	0.47	0.41	0.03
Heat Stress										
Total VFA	460.1	508.2	569.3	338.7	370.2	270.8	86.33	0.62	0.11	0.54
Acetate	267.2	302.0	327.1	204.0	207.5	159.2	55.48	0.03	0.94	0.64
Propionate	132.9	134.3	164.8	90.9	110.0	71.8	21.63	0.01	0.89	0.29
Butyrate	48.3	56.8	61.6	34.1	41.0	29.2	9.13	0.02	0.71	0.56
Valerate	5.5	8.1	7.3	5.7	6.6	6.0	1.12	0.34	0.32	0.72
Isovalerate	6.2	7.0	8.5	4.1	5.1	4.6	1.46	0.05	0.62	0.76

^AMeans within row with uncommon superscript differ ($P < 0.05$)

¹Steers receiving E+ seed treatment received 15 µg of ergovaline · kg BW⁻¹·d⁻¹ from ‘Kentucky 31’ tall fescue seed and the E- seed treatment received an equal amount of ‘Kentucky 32’ tall fescue seed with 0.0 ppm ergovaline.

²Buffers incubated contained only the excipient. Buffer 1 was the first incubation time followed by Buffer 2 and then Buffer 3.

Figure 5.1. Experimental design and sampling timeline of the washed rumen experiment conducted on d 8 of Exp. 1. Experiment 2 was identical to Exp. 1 with the exception of incubating 3 sequences of the control buffer. The control buffer contained only the excipient. The 1× EXT contained 15 µg ergovaline/kg BW and the 3× EXT contained 15 µg ergovaline/kg BW. Ergovaline was supplied as an extract of endophyte-infected tall fescue seed.

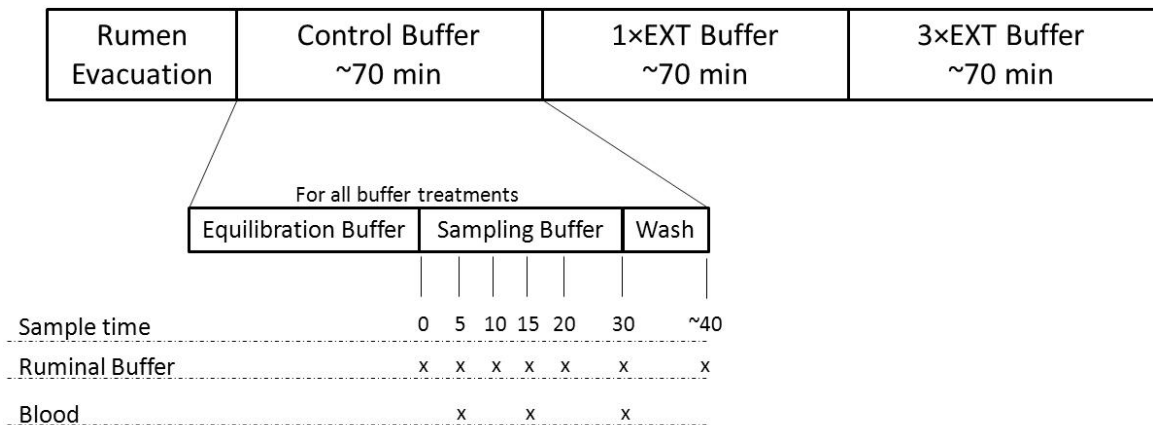


Figure 5.2. Feed intake of steers on d 1 and d 7 of receiving endophyte-infected (E+) tall fescue seed twice daily ($15 \mu\text{g}$ ergovaline/kg BW total) when housed at thermoneutral (TN; 22°C) and heat stress (HS; 30°C) conditions. Intake was restricted to $1.5 \times \text{NE}_m$. Bars with differing letters differ ($P = 0.018$ for temperature \times day interaction).

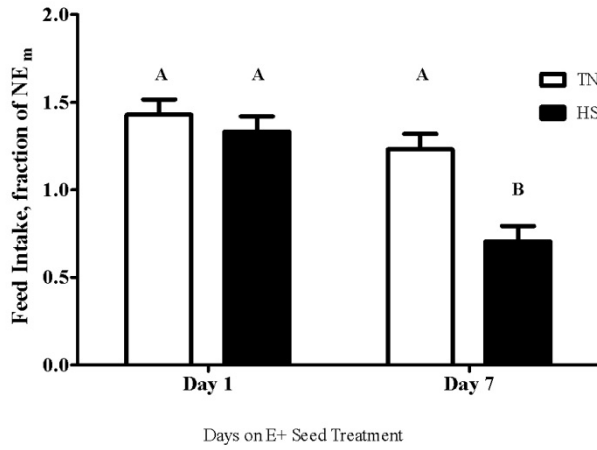


Figure 5.3. Serum prolactin concentration in steers on d 0 and d 8 of receiving endophyte-infected (E+) tall fescue seed twice daily (15 μ g ergovaline/kg BW total) when housed at A) thermoneutral (TN; 22°C) and B) heat stress (HS; 30°C) conditions. Effect of day ($P = 0.03$ at TN); Effect of seed treatment ($P = 0.002$ for TN); Day \times seed treatment ($P = 0.52$ for TN);). Bars with differing letters differ $P = 0.02$ for day \times seed treatment at HS.

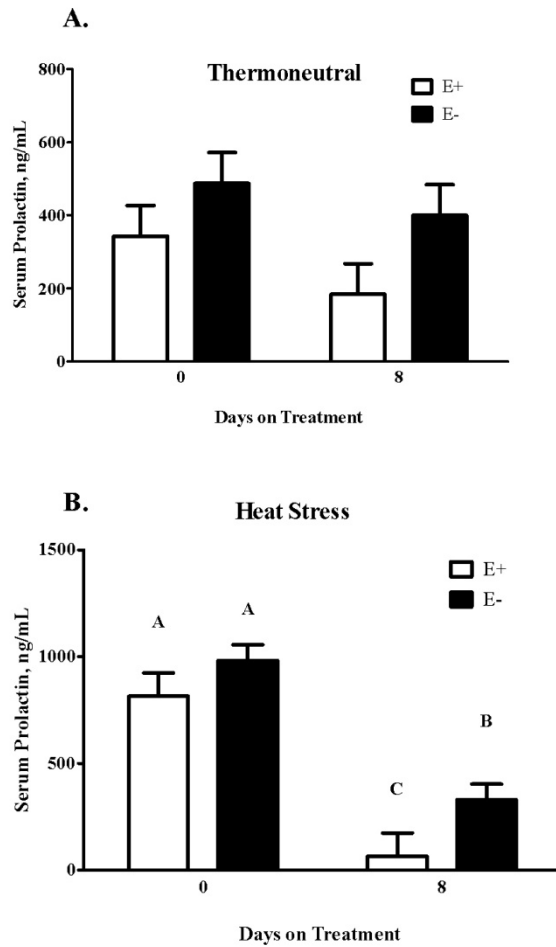


Figure 5.4. Experiment 1. Reticuloruminal epithelial blood flow measure by clearance of deuterium oxide from the washed reticulorumen of steers receiving endophyte-infected (E+) tall fescue seed twice daily (15 μg ergovaline/kg BW total) when housed at A) thermoneutral (TN; 22°C) and B) heat stress (HS; 30°C) conditions for 7 d. Blood flow was observed in the presence of a control buffer (Extract Level 0), a buffer containing 15 μg ergovaline/kg BW (Extract Level 1), and a buffer containing 45 μg ergovaline/kg BW (Extract Level 3). Bars without shared letters differ ($P = 0.038$ for TN; $P = 0.005$ for HS)

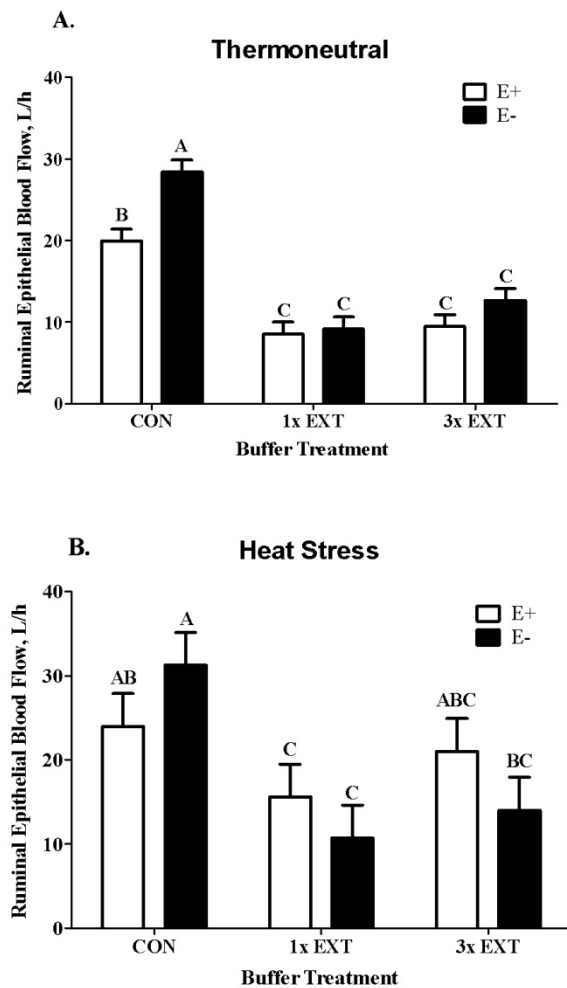
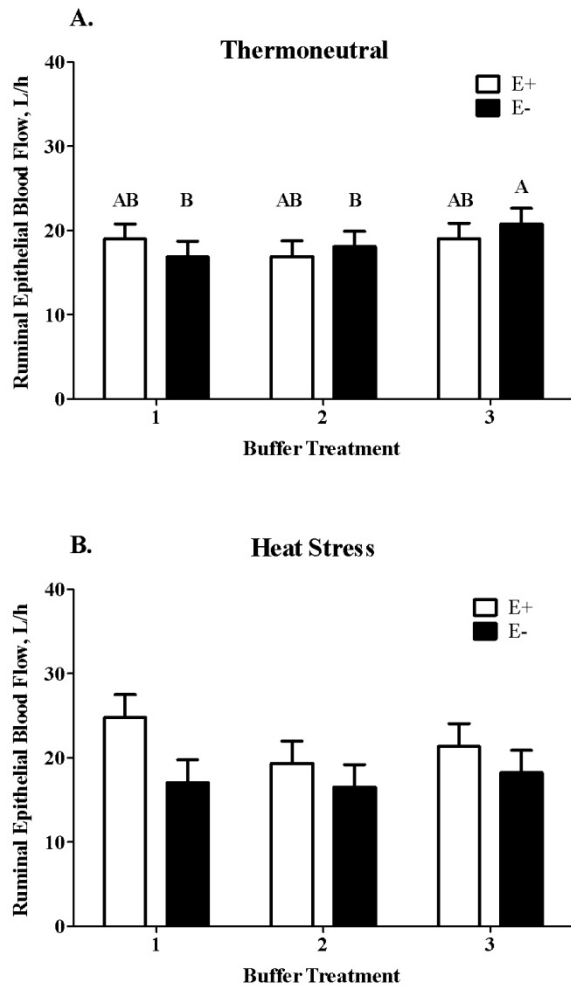


Figure 5.5. Experiment 2. Reticuloruminal epithelial blood flow measure by clearance of deuterium oxide from the washed reticulorumen of steers receiving endophyte-infected (E+) tall fescue seed twice daily (15 μg ergovaline/kg BW total) when housed at A) thermoneutral (TN; 22°C) and B) heat stress (HS; 30°C) conditions for 7 d. Blood flow was observed in the presence of three consecutive incubations of a control buffer. The control buffer contained only the excipient and is identical to the control buffer used in Exp. 1. Bars without shared letters differ (A. Seed treatment \times buffer treatment $P = 0.045$). For HS (B.) effect of seed treatment $P = 0.25$, effect of buffer treatment $P = 0.18$, seed treatment \times buffer treatment $P = 0.23$.



CHAPTER 6: ACUTE EXPOSURE TO ERGOT ALKALOIDS FROM ENDOPHYTE-INFECTED TALL FESCUE DOES NOT ALTER ABSORPTIVE OR BARRIER FUNCTION OF THE ISOLATED RUMINAL EPITHELIUM

Introduction

Ergot alkaloids such as ergovaline are synthesized by the endophyte (*Neotyphodium coenophialum*) present in tall fescue (*Lolium arundinaceum*) and are thought to be the main causative agents of fescue toxicosis in beef cattle (Strickland et al., 2011). Rhodes et al. (1991) used radio-labeled microspheres and reported that consumption of endophyte-infected tall fescue reduced blood flow to the gut of cattle. Supporting the findings of Rhodes et al. (1991), endophyte-infected tall fescue seed extract (**EXT**) has been reported to cause constriction of the ruminal artery and veins (Foote et al., 2012) and a follow up experiment reported a large reduction in epithelial blood flow to the washed reticulorumen of steers (Foote et al., submitted). However, variability in the response to endophyte infected tall fescue is evident as Harmon et al. (1991) showed a minor effect of fescue toxicosis on nutrient flux across or blood flow to splanchnic tissues.

Along with the reduced epithelial blood flow, a marked reduction in VFA flux across the washed rumen was observed (Foote et al., submitted). Additionally, a greater quantity of propionate, butyrate, and valerate were absorbed across the washed reticulo-rumen wall per unit of blood flowing to the reticulo-rumen. This indicates that ergot alkaloids may have a direct effect on the rumen epithelial cells that causes an increase in VFA absorption per unit of blood flow. The objectives of this experiment were to 1)

determine the effect of acute exposure to ergot alkaloids on total, facilitated, and passive VFA flux across the isolated bovine rumen epithelium, 2) determine the effect of acute ergot alkaloid exposure on isolated rumen epithelial barrier function, and 3) determine the flux of ergovaline, a principal alkaloid present in the tall fescue seed extract, across the rumen epithelium.

Materials and Methods

Methods used with live animals were reviewed and approved by the University of Saskatchewan Animal Research Ethics Board prior to beginning the study (protocol no. 20100021).

Preparation and Analysis of Tall Fescue Seed Extract

At the University of Kentucky, an extract of '3rd Millennium' tall fescue seed was isolated as previously described (Foote et al., submitted). The tall fescue seed extract was analyzed using a Waters Acquity UPLC-TQD (Waters, Inc., Milford, MA) with a sample manager. The sample manager was maintained at 4°C. An Acquity UPLC BEH (C18, 2.1 × 150 mm, 1.7 µm, Waters Inc.) column was used to perform chromatographic separation. Chromatographic separation was conducted at 75°C and the mobile phase was delivered at a constant flow rate of 0.5 mL/min using a 2-µL full loop injection. The binary mobile phase consisted of 5 mM ammonium carbonate (pH = 10.12, adjusted with ammonium hydroxide; mobile phase A) and acetonitrile (mobile phase B). The tall fescue seed extract was injected (2 µL) into the initial gradient conditions of 100% mobile phase A and 0% mobile phase B, which were maintained for 2 min following injection. Mobile phase B was increased at a linear rate to 95% over the next 6.4 min to

chromatographically resolve all of the ergot alkaloids. After the 6.4-min separation run, mobile phase B was held constant for an additional 3.1 min to flush the LC column. Mobile phase A increased at a linear rate to 100% over the next 0.05 min and held constant for an additional 1.45 min to allow for column re-equilibration in preparation for the next analysis. The total run time from one injection to the subsequent injection was 13 min, while data acquisition occurred from 2 to 8.4 min post injection. A tandem-quadrupole MS (Quattro Premier equipped with a Z-spray ion source, Waters Inc.) was coupled to the UPLC system. The mass spectrometer atmospheric pressure ionization was operated in positive electrospray ionization. The capillary voltage was 0.50 kV, and the source and desolvation temperatures were set at 150 and 400°C, respectively. The cone and desolvation gas flows were set at 125 and 800 L/h, respectively. Collision gas (argon) flow and pressure were set at 0.15 mL/min and 3.50×10^{-3} mbar, respectively. The MS tune was run in multiple reaction-monitoring mode with an automated dwell time. Six different scan functions were acquired simultaneously, corresponding to each of the molecular ions for the analytes. Chromatograms from selected reaction monitoring were used for quantification of the ergot alkaloids.

The extract contained the following ergot alkaloids (mg/mL): ergovaline (3.934), lysergic acid (0.084), ergocornine (0.056), ergotamine (0.040), ergocryptine (0.011), and ergocristine (0.008). The extract was diluted with 80% methanol into 2 concentrations (10 and 50 µg ergovaline/mL), and aliquots were stored in silanized amber vials at -20°C. The diluted extract was then shipped on dry ice to the University of Saskatchewan and stored at -20°C until it was used.

Animals and Tissue Collection

Six Holstein steers (BW = 107.9 ± 7.3 kg) were fed a mixed ration (Table 6.1) ad libitum for 23 to 30 d prior to slaughter. One steer a day was killed to collect tissue for Ussing chamber experiments. Steers were killed by captive bolt stunning, pithing, and exsanguination. The abdominal cavity was opened and a 100-cm² section of the caudal dorsal sac of the rumen was removed. The submucosal tissues were carefully removed and the epithelium was placed in an oxygenated buffer (pH 7.4 at 38°C) for transport to the laboratory (Table 6.2).

The epithelium was sectioned and mounted between two halves of an Ussing chamber with an exposed surface area of 3.14 cm² (used for acetate, butyrate, and ergovaline flux experiments; University of Leipzig, Germany) or 1.43 cm² (used in inulin flux measurements; Physiologic Instruments, San Diego, CA). A silicon rubber ring was placed on both sides of the epithelium section to protect the edge of the tissue from damage. For acetate and butyrate flux experiments, the tissues mounted in the chambers were bathed on each side with 15 mL of buffer (Table 6.2) that was mixed using a gas-lift system using 95% O₂/5% CO₂, except for the bicarbonate-free buffer which was gassed with 100% O₂. For inulin flux experiments, the tissues were bathed on each side with 10 mL of buffer that was mixed by a gas-lift system using 95% O₂/5% CO₂. For ergovaline flux experiments, the tissues mounted in the chambers were bathed on each side with 11 mL of buffer that was mixed by a gas-lift system using 95% O₂/5% CO₂. All buffers were maintained at 38.5°C. The chemical composition of the buffers (Table 6.2) were designed to determine total VFA flux (**Total**) without inhibition (i.e. the buffer contained HCO₃⁻ and did not contain Cl⁻ or NO₃⁻) and bicarbonate-independent nitrate insensitive VFA

flux (**Passive**) by inducing maximal inhibition (i.e. addition of Cl^- and NO_3^- and absence of HCO_3^-) (Aschenbach et al., 2009; Penner et al., 2009; Sehested et al., 1996b).

Facilitated VFA flux was calculated as the difference between Total and Passive flux. In all cases, the pH of the mucosal (6.2) and serosal (7.4) buffers were set to mimic the pH of rumen contents and blood, respectively.

Electrophysiology

Tissues were incubated under short-circuit conditions by passing current through the tissue to clamp the potential difference to 0 millivolt (**mV**). Potential difference and short-circuit current were measured using a computer-controlled voltage clamp device (VCC MC6 Multichannel Voltage/Current Clamp, Physiologic Instruments, San Diego, CA was used for inulin flux) connected to electrodes (Physiologic Instruments) via agar bridges (3% agar in 3 M KCl). The current required to clamp the transepithelial potential difference to 0 mV is equivalent to the short-circuit current (I_{sc}) but in the opposite direction. Pulses of current were applied every 10 s for the measurement of transepithelial conductance (G_t). For tissues used to measure VFA and ergovaline flux, the procedure for short-circuit conditions were the same except a different voltage clamp device (Ing. – Büro für Mess- und Datentechnik, Aachen, Germany) was used and different electrodes voltage-sensing (Argenthal electrodes for voltage measurements; Mettler Toledo, Urdorf, Switzerland), and current passing electrodes (Pt-PtCl electrodes (Free University of Berlin, Germany) connected via NaCl bridges for passing current).

Acetate and Butyrate Flux Experiments

After mounting, tissues were provided 30 min to allow tissue conductance (G_t) and short-circuit current (I_{sc}) to stabilize. After at least 10 min of stabilization, tissues

were blocked based on G_i . Within block, chambers were randomly assigned to a treatment so that each treatment was present in each block and each treatment was duplicated within buffer type. Treatments included control (**Con**; 75 μ L of 80% methanol), **low EXT** (75 μ L of a 10 μ g ergovaline/mL to yield a final concentration of 50 ng ergovaline/mL), and **high EXT** (75 μ L of a 50 μ g ergovaline/mL to yield a final concentration of 250 ng/mL). The volume of the treatment addition was designed to keep the volume of organic solvent at 0.5% of the total volume. The High EXT treatment concentration was equal to that used by Foote et al. (Submitted) and showed reduced epithelial blood flow and VFA absorption from the washed reticulorumen of steers. The low EXT treatment was meant to provide a more physiological concentration of ergovaline. Treatments were added to the mucosal buffer after the 30-min stabilization period and the chambers were allowed to equilibrate for 15 min prior to the addition of radioisotopes. A stock solution with 720 mM acetate and 720 mM butyrate was spiked with ^3H -acetate (37 kBq/ μ L; Perkin Elmer, Waltham, MA, NET003005) and ^{14}C -butyrate (3.7 kBq/mL; Moravek Biochemicals and Radiochemicals, Brea, CA, # MC-319) and 523 μ L was added to the mucosal buffer to achieve a final concentration of 25 mM acetate and 25 mM butyrate with 100 kBq ^3H and 74 kBq of ^{14}C . The radioisotopes were allowed to equilibrate for 45 min (Sehested et al., 1996a). Mucosal buffer samples (100 μ L) were collected at the end of the equilibration period and at the end of the experiment. Serosal buffer samples (500 μ L) were taken at the end of the equilibration period and every 30 min for 2 h resulting in 4 flux periods. Samples were placed in scintillation vials with 5 mL of scintillation cocktail and read on a Multi-Purpose Scintillation Counter

(model LS6500, Beckman Coulter, Mississauga, ON, Canada). Flux was calculated as described previously (Clarke, 2009; Wilson et al., 2012), and is as follows:

$$\text{Flux} = V \times \frac{S2 - S1 \times \text{dilution}}{\text{specific activity} \times \text{surface area} \times \text{time}}$$

where V is the volume of the buffer (mL), S is dpm/mL of buffer samples taken at the beginning (S1) and end (S2) of the flux period, dilution is the dilution factor after replacing the sample volume, specific activity is the activity of the isotope (dpm/ μ mole) on the mucosal side, surface area is the exposed area of the rumen epithelium (cm²), and time is the length of duration of the flux period (h).

Inulin and Ergovaline Flux Experiments

Tissues were equilibrated, blocked, and assigned to treatments as described above. For the inulin flux experiment, treatments included control (50 μ L of 80% methanol), Low EXT (50 μ L of a 10 μ g ergovaline/mL to yield a final concentration of 50 ng ergovaline/mL), and High EXT (50 μ L of a 50 μ g ergovaline/mL to yield a final concentration of 250 ng/mL). For the ergovaline flux experiments, treatments included Con (55 μ L of 80% methanol), Low EXT (55 μ L of a 10 μ g ergovaline/mL to yield a final concentration of 50 ng ergovaline/mL), and High EXT (55 μ L of a 50 μ g ergovaline/mL to yield a final concentration of 250 ng/mL). Treatments were added to the mucosal buffer after the 30 min equilibration period and the chambers were allowed to equilibrate for 15 min prior to the addition of ³H-inulin (74 kBq; Perkin Elmer) to the mucosal buffer and allowed to equilibrate for 45 min. No radioactivity was added to chambers designated for the ergovaline flux experiments. For the inulin flux experiments, mucosal buffer samples (100 μ L) were collected at the end of the equilibration period and

at the end of the experiment. Serosal buffer samples (500 μL) were taken at the end of the equilibration period and every 1 h for 3 h resulting in 3 flux periods. Samples were placed in scintillation vials with 5 mL of scintillation cocktail and read on a Multi-Purpose Scintillation Counter (model LS6500, Beckman Coulter). Inulin flux was calculated as described above. Samples were collected from the mucosal and serosal (500 μL from each) buffers for the ergovaline flux chambers at the same time and placed in silanized amber vials and stored at -20°C . Buffer samples designated for ergovaline analysis were shipped on dry ice to the University of Kentucky for analysis at the USDA-ARS, Forage-Animal Production Research Unit laboratory.

Buffer samples for ergovaline analysis were transferred to a 4-mL clear disposable borosilicate glass tube (12×75 mm; Fisher Scientific) and evaporated for 90 min at 60°C using a CentriVap Console (LABCONCO, Kansas City, MO). The residue was reconstituted in 500 μL of 80:20 MeOH:H₂O containing the analytical internal standard methysergide (200 fmol or 70.7 pg on column) and vortexed for 30 s. Each tube was centrifuged at $2000 \times g$ with a CR422 swinging bucket rotor (Jouan, Inc., Winchester, VA, USA) for 10 min at 4°C . The supernatant (350 μL) was transferred to deactivated amber screw-top autosampler vial (Waters Inc.). A subsample of the supernatant was used for a dilution; 100 μL of the supernatant was combined with 900 μL of the 80:20 MeOH:H₂O containing the analytical internal standard methysergide for a 1:10 dilution. Ergovaline was analyzed as described above for the tall fescue seed extract. Ergovaline flux was calculated using the method described above with the substitution of concentration of ergovaline on the mucosal side for specific activity on the mucosal side.

Statistical Analysis

Data were analyzed as a completely randomized design using mixed procedure (SAS 9.3; SAS Institute Inc., Cary, NC). The model included the fixed effects of treatment, flux period, and treatment \times flux period. Flux period was included as a repeated measure using an autoregressive covariance structure. When the F -test was significant ($P < 0.05$), pair-wise comparisons were conducted using LSD. Data are presented as least square means \pm SEM.

Results and Discussion

Volatile Fatty Acid Flux

There were no EXT treatment \times flux period interactions for total, passive, or facilitated acetate flux ($P = 0.25, 0.79, \text{ and } 0.33$, respectively) or total, passive, and facilitated butyrate flux ($P = 0.82, 0.99, \text{ and } 0.87$, respectively). Therefore, only data for main effects of treatment and flux period are presented (Tables 6.3 and 6.4).

Total, passive, and facilitated acetate flux across the isolated bovine rumen epithelium was not affected by acute exposure to a tall fescue seed extract ($P = 0.69, 0.85, \text{ and } 0.82$, respectively; Table 6.3). Butyrate flux across the isolated rumen epithelium was not affected by acute exposure to ergot alkaloids ($P = 0.96, 0.51, \text{ and } 0.63$, for total, passive, and facilitated, respectively; Table 6.3).

Flux period had no effect on total or passive acetate flux ($P = 0.27 \text{ and } 0.46$, respectively; Table 6.4). There was a tendency ($P = 0.08$) for higher facilitated acetate flux during the first flux period. Total and passive butyrate flux decreased after the second period ($P < 0.01$) and total flux decreased further during the fourth period.

Consumption of ergot alkaloids from wild-type endophyte-infected tall fescue has previously been shown to decrease portal flux of acetate in steers (Harmon et al., 1991) even though portal blood flow was not affected. Additionally, a slight reduction in total VFA, acetate, butyrate, and isovalerate flux from a washed reticulorumen of steers consuming endophyte-infected tall fescue seed housed at thermoneutral conditions has been reported (Foote et al., submitted). Acute exposure to ergot alkaloids by incubating an extract of endophyte-infected tall fescue seed in a washed reticulorumen caused a large reduction in VFA flux as well as blood flow to the epithelium and efficiency of VFA absorption (mmol VFA absorbed per unit of blood flow to the absorptive surface) from the washed reticulorumen was reported to increase with acute exposure to ergot alkaloids (Foote et al., submitted). This effect of ergot alkaloids was more pronounced with longer chain VFA such as butyrate. It was therefore thought that several factors could be contributing to the increase in VFA flux efficiency in response to ergot alkaloids including 1) an increase in specific absorption pathways (facilitated or passive VFA flux) or 2) an increase in epithelial metabolism. Results presented in this current experiment clearly show that acute exposure of rumen epithelium to ergot alkaloids has no effect on the absorptive pathways, either facilitated or passive, of bovine rumen epithelium. It is likely that the results previously observed for VFA absorption and absorption efficiency are due to alterations in blood flow and epithelial metabolism of VFA.

Barrier Function and Electrophysiology

Rumen epithelium barrier function was assessed by the mucosal to serosal flux of inulin and G_t . The barrier function of rumen epithelium is thought to be attributed to the tight junctions in the stratum granulosum (Graham and Simmons, 2005). Tissue

conductance and the movement of hydrophilic macromolecules that are not transported transcellularly can be used as measures of barrier function. There was no treatment \times flux period interaction for inulin flux ($P = 0.84$, data not shown); therefore only the data for main effects are presented (Table 6.5). The mucosal to serosal flux of inulin was not affected by the addition of alkaloids ($P = 0.16$) or flux period ($P = 0.74$). Flux of inulin in this experiment was much lower than mannitol flux across isolated sheep rumen (Penner et al., 2010) but similar to Cr-EDTA flux across isolated sheep rumen (Schweigel et al., 2005), likely due to similar molecular weights of inulin and Cr-EDTA (approximately 375 and 340 Da, respectively) which is higher than mannitol (approximately 180 Da).

Tissue conductance was analyzed separately for each experiment. There were no treatment \times flux period interactions for G_t in the inulin flux experiment ($P = 0.31$) or ergovaline flux experiment ($P = 0.61$); therefore only data for the main effects are presented (Tables 6.5 and 6.6). The average G_t ranged from 1.63 to 3.36 mS/cm² and there was no effect of ergovaline treatment on G_t ($P > 0.17$; Tables 6.5 and 6.6). In all experiments there was an increase in G_t over time ($P < 0.01$; Tables 6.5 and 6.6). As there were no treatment \times flux period interactions, this was not an effect of the treatments, but simply a natural occurrence in the in vitro system.

Ergocristine has been shown to reduce barrier function of an in vitro blood-brain barrier model with an apical concentration of at least 5 μM (compared to the 0.09 and 0.47 μM concentrations used in the current study), as measured by electrical resistance and sucrose permeability (Mulac et al., 2012). The structure of ergocristine is similar to ergovaline with the same basic tricyclic peptide group that only differs at two points (Foote et al., 2011; Klotz et al., 2010) and has similar inhibition constants for dopamine

receptors (Larson et al., 1995; Lew et al., 1977). Based on in vitro studies (Foote et al., 2011; Klotz et al., 2010), it seems that ergocristine and ergovaline differ in their ability to elicit a vasoconstrictive response which is likely indicative of differences in membrane receptor binding; therefore, effects induced by ergocristine might not be representative of the potential of ergovaline to induce responses.

There were no treatment \times flux period interactions for I_{sc} in the inulin flux experiment ($P > 0.99$; Table 6.5) or ergovaline flux experiment ($P = 0.67$; Table 6.6); therefore only data for the main effects are presented. There was no effect of in vitro treatment on I_{sc} (Tables 6.5 and 6.6; $P > 0.09$). Short-circuit current increased after the first flux period in the ergovaline flux experiment ($P < 0.01$). While I_{sc} plateaued after the first flux period in the total VFA flux experiment, it continued to increase in the ergovaline flux experiment. Short-circuit current decreased after the first flux period in the inulin flux experiment ($P = 0.01$).

Ergovaline Flux

Ergovaline was not detected in the serosal buffer of the Low EXT treatment at any time point. Ergovaline was detected, but not quantifiable, in the serosal buffer of the High EXT treatment at the end of the second flux period (1 out of 12 chambers), the end of the third flux period (7 out of 12 chambers), and the end of the fourth flux period (3 out of 12 chambers). Ergovaline was never detected in the serosal buffer of the High EXT treatment for one chamber. Using the values for the lower limit of detection (0.6117 ng/mL) and the lower limit of quantification (1.0572 ng/mL) of ergovaline, a minimum and maximum mucosal to serosal flux rate for ergovaline was estimated. Minimum ergovaline flux is estimated as 0.25 ± 0.10 ng/(cm² · h) and the maximum ergovaline flux

rate is estimated to be $0.44 \pm 0.17 \text{ ng}/(\text{cm}^2 \cdot \text{h})$. This is equivalent to 0.47 and 0.83 $\text{pmol}/(\text{cm}^2 \cdot \text{h})$. Using the liquid volume (35 L) from the rumen contents of steers receiving endophyte-infected tall fescue seed (Foote et al., submitted), an approximation of the surface area exposed to rumen fluid containing ergovaline (calculated as half the surface area of a sphere with a volume of 35 L) is 2600 cm^2 . This would translate to a potential ruminal absorptive capacity of 15 to 27 μg of ergovaline a day (29 to 52 nmol of ergovaline). This represents less than 1% of the ergovaline consumed (approximately 4.4 mg) by steers in a previous study (Foote et al., In Press).

This is the first known report to show that ergovaline crosses the bovine rumen epithelium. Ayers et al. (2009) showed that ergovaline did not cross bovine rumen epithelium isolated in a parabiotic chamber; however, the concentration used in their experiment may have resulted in serosal concentrations below the limit of detection. The estimation of flux rates presented here are significantly lower than those reported for ergotamine transport across ovine rumen and omasum tissue (Hill et al., 2001) as well as ergovaline movement across Caco-2 cells (Shappell and Smith, 2005). The differences could be due to the much higher concentrations used in the previous studies (30.5 mM ergotamine and 22 μM ergovaline) compared to the more physiological concentrations (0.47 μM ergovaline) in the current study.

Data presented here show that acute exposure to ergovaline from wild-type endophyte-infected tall fescue has no effect on the absorptive function or the barrier function of the isolated bovine rumen epithelium. Previous reports displaying reduced VFA absorption from the washed rumen in response to acute ergot alkaloid exposure is likely due to reduced blood flow to the absorptive surface of the rumen. Results also

show that ergovaline can potentially be absorbed across the rumen epithelium, although at a very slow rate.

Table 6.1. Composition and nutrient content of diet fed to steers prior to in vitro experiments.

Item	% DM
Diet Composition, % DM	
Barley Silage	49.6
Barley Grain	42.0
Vitamin - Mineral Pellet ¹	8.4
Nutrient Composition, % DM	
DM (%)	47.3
Ash (%)	3.2
CP	13.6
ME (Mcal/kg)	2.4
NDF	33.9
ADF	20.6
Starch	31.7
EE	3.0

¹Contains (% of DM) Soybean Meal (57.15), ground barley grain (23.73), Dynamate (10.73; Mosaic Feed Ingredients, South Riverview, FL), ground limestone (5.51), trace mineral salt (2.47), Na-selenite (0.18), Vitamin ADE premix (0.08), Potassium chloride (0.07), MgO (0.04), MnO₂ (0.04).

Table 6.2. Chemical composition of the Ussing chamber buffers.

Item	Inulin Flux Buffer ¹		Bicarbonate Containing		Bicarbonate-Free	
	Serosal	Mucosal	Serosal	Mucosal	Serosal	Mucosal
CaCl ₂	1.0	1.0	-	-	-	-
Ca-gluconate	-	-	1.0	1.0	1.0	1.0
MgCl ₂	1.3	1.3	-	-	-	-
Mg-gluconate	-	-	1.3	1.3	1.3	1.3
NaCl	15.6	15.6	-	-	-	-
Na-gluconate	-	-	50.6	50.6	69.6	34.6
KCl	5.5	5.5	-	-	-	-
K-gluconate	-	-	5.5	5.5	5.5	5.5
NaH ₂ PO ₄	0.6	0.6	0.6	0.6	0.6	0.6
Na ₂ HPO ₄	2.4	2.4	2.4	2.4	2.4	2.4
Acetic Acid	10.0	10.0	-	-	-	-
Na-Acetate	12.1	12.1	-	-	-	-
L-Glutamine	1.0	1.0	1.0	1.0	1.0	1.0
HEPES-free acid	10.0	10.0	10.0	10.0	10.0	10.0
Na-Propionate	9.1	9.1	-	-	-	-
Na-Butyrate	3.9	3.9	-	-	-	-
NaOH	10.0	10.0	-	-	-	-
NaHCO ₃	24.0	24.0	24.0	24.0	-	-
Glucose	-	-	10.0	10.0	10.0	10.0
Acetazolamide	-	-	-	-	0.1	0.1
Na-nitrate	-	-	-	-	-	40.0
Gluconic Acid	-	20.0	-	20.0	-	1.0
Inulin	-	1.0	-	-	-	-
Mannitol	120.0	99.0	110.0	90.0	120.0	109.0
Penicillin G, mg/L	60.0	60.0	60.0	60.0	60.0	60.0
Kanamycin, mg/L	100.0	100.0	100.0	100.0	100.0	100.0
Fluorocytosine, mg/L	50.0	50.0	50.0	50.0	50.0	50.0
pH	7.4	6.2	7.4	6.2	7.4	6.2

¹The inulin flux buffers were also used for the ergovaline flux experiments. The inulin flux serosal buffer, without antibiotics was used as the buffer for transporting the tissue to the laboratory.

Table 6.3. Effect of acute exposure to ergot alkaloids on total, passive, and facilitated acetate and butyrate flux ($\mu\text{mol}/(\text{cm}^2 \cdot \text{h})$) across the isolated bovine rumen epithelium.

Item	Treatment ¹			SEM ²	P-value ³
	Control	Low EXT	High EXT		
Acetate Flux					
Total	1.37	1.28	1.35	0.08	0.69
Passive	0.48	0.48	0.53	0.09	0.85
Facilitated	0.79	0.72	0.76	0.09	0.82
Butyrate Flux					
Total	2.53	2.49	2.55	0.17	0.96
Passive	1.54	1.46	1.34	0.12	0.51
Facilitated	0.98	1.03	1.22	0.18	0.63

¹Control = 80% methanol (0.5% of the buffer volume), Low EXT = 50 ng ergovaline/mL, High EXT = 250 ng ergovaline/mL

²Standard Error of the Mean, n = 48

³Probability of a greater *F* statistic for the main effect of treatment

Table 6.4. Effect of time on total, passive, and facilitated acetate and butyrate flux ($\mu\text{mol}/(\text{cm}^2 \cdot \text{h})$) across the isolated bovine rumen epithelium.

Item	Flux Period ¹				SEM ²	P-value ³
	1	2	3	4		
Acetate Flux						
Total	1.40	1.27	1.31	1.34	0.07	0.27
Passive	0.45	0.49	0.55	0.49	0.07	0.46
Facilitated	0.89	0.75	0.64	0.74	0.08	0.08
Butyrate Flux						
Total	2.76	2.66	2.46	2.21	0.12	<0.01
Passive	1.61	1.52	1.38	1.27	0.08	<0.01
Facilitated	1.16	1.14	1.08	0.93	0.13	0.41

¹Flux Period is the 30 min blocks of time in which flux of acetate and butyrate were measured

²Standard Error of the Mean, n = 36

³Probability of a greater *F* statistic for the main effect of flux period

Table 6.5. Effect of acute exposure to ergot alkaloids and time of incubation on inulin flux (nmol/(cm² · h)), tissue conductance (G_t , mS/cm²), short-circuit current (I_{sc} ; μ Eq/(cm² · h) of isolated bovine rumen epithelium.

Item	Treatment ¹			SEM ²	P-value	Flux Period ³			SEM ⁴	P-value
	Control	Low EXT	High EXT			1	2	3		
Inulin Flux	6.27	5.80	6.97	0.43	0.16	6.32	6.16	6.57	0.40	0.74
G_t	2.41	2.67	2.27	0.42	0.79	2.09	2.45	2.81	0.25	<0.01
I_{sc}	-0.017	-0.017	-0.020	0.008	0.94	-0.015	-0.022	-0.017	0.005	0.01

¹Control = 80% methanol (0.5% of the buffer volume), Low EXT = 50 ng ergovaline/mL, High EXT = 250 ng ergovaline/mL

²Standard Error of the Mean, n = 36

³Flux Period is the 1 h blocks of time in which flux of inulin was measured

⁴Standard Error of the Mean, n = 36

Table 6.6. Effect of acute exposure to ergot alkaloids on the tissue conductance (G_t , mS/cm²) and short-circuit current (I_{sc} ; $\mu\text{Eq}/(\text{cm}^2 \cdot \text{h})$) of isolated bovine rumen epithelium during the ergovaline flux experiments.

Item	Treatment ¹			Flux Period ³						
	Control	Low EXT	High EXT	SEM ²	P-value	1	2	3	SEM ⁴	P-value
G_t	1.63	1.68	1.82	0.44	0.89	1.53	1.71	1.90	0.25	<0.01
I_{sc}	0.34	0.25	0.15	0.06	0.09	0.20	0.24	0.30	0.04	<0.01

¹Control = 80% methanol (0.5% of the buffer volume), Low EXT = 50 ng ergovaline/mL, High EXT = 250 ng ergovaline/mL

²Standard Error of the Mean, n = 33

³Flux Period is the 1 h blocks of time in which electrophysiology was measured

⁴Standard Error of the Mean, n = 35

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CHAPTER 7: SUMMARY AND CONCLUSIONS

Tall fescue is a cool season perennial grass that is well suited to grow in the transition zone of the eastern United States and is very popular with livestock producers. Cool season forages, tall fescue specifically, provides a timely flush of forage for cow-calf operations that utilize either a fall or spring calving system. Tall fescue can also be easily utilized for a backgrounding operation where additional body weight is added to calves before they are shipped to western feedlots. The main limiting factor for beef operations in the eastern United States that utilize tall fescue is the presence of the wild-type endophytic fungus that produces ergot alkaloids, which are thought to be responsible for the poor performance of cattle grazed on tall fescue pastures, particularly in hot weather.

For decades, it has been thought that the source of many of the observed signs of fescue toxicosis was peripheral vasoconstriction. There is limited published data showing that core body vasculature, including gastrointestinal vasculature, is also affected by the toxins present in tall fescue. This observation has led to the hypothesis that ergot alkaloids could have negative effects on foregut vasculature and result in a concomitant reduction in nutrient absorption, contributing to the reduced growth observed in cattle consuming tall fescue.

The first part of this series of experiments was designed to characterize the effect of a variety of ergot alkaloids, including ergoline and ergopeptine alkaloids, on the bovine right ruminal artery and vein. Vessels were isolated shortly after slaughter and a

multimyograph system was used to characterize the response of ruminal vasculature to increasing concentrations of ergot alkaloids. Results from this experiment showed that most ergot alkaloids induce a vasoconstrictive response in ruminal arteries and veins. Ergovaline induced the greatest response in the arteries, and the response to ergovaline and ergotamine was the greatest in the ruminal veins. Data from this first experiment indicates that ergot alkaloids, especially ergopeptine alkaloids, can induce vasoconstriction of bovine foregut vasculature indicating these toxins can potentially affect nutrient absorption.

The second portion of this series of experiments consisted of two experiments; the first showed that a tall fescue seed extract induced a greater vasoconstrictive response than pure ergovaline. It was therefore hypothesized that the additional ergot alkaloids present in the tall fescue seed extract could contribute to the increased vasoactivity compared to pure ergovaline. A follow-up experiment was conducted using ruminal artery and vein, as well as lateral saphenous veins (a peripheral vasculature model). Treatments included pure ergovaline, a tall fescue seed extract, a mixture of commercially available ergot alkaloids that mimicked the extract, and an extract of endophyte-free tall fescue seed. Results showed that there was no difference in the response of the saphenous vein, ruminal artery, or ruminal vein to the endophyte-infected tall fescue seed, pure ergovaline, or a mixture of ergot alkaloids. These data were contradictory to the previous experiment that showed an increased response to the seed extract compared to pure ergovaline. However, the data from the ruminal vessels appeared to trend toward a greater response to the extract than ergovaline alone, but the overall maximum response was smaller than the previous experiment. This reduced maximum response could have

resulted from the prior exposure of the animals to toxic tall fescue. The reduced response could have hindered the ability to distinguish true differences in the vasoactivity of the treatments. Nonetheless, it was clear from these studies that the main agent for local vasoconstriction is ergovaline.

Having clearly demonstrated that ergot alkaloids induce vasoconstriction of bovine foregut vasculature, the next step of this series of experiments was to determine if the observed *in vitro* vasoconstriction results in reduced blood flow and nutrient absorption *in vivo*. A washed reticulorumen experiment was conducted in steers exposed to either E+ or E- seed and reticuloruminal epithelial blood flow and VFA absorption were measured in the presence of increasing ergot alkaloids. When ergot alkaloids were introduced into the washed rumen, there was a large decrease in blood flow and VFA absorption. One of the most interesting findings was that at thermoneutral temperatures, steers receiving the endophyte-infected seed had reduced epithelial blood flow but there was no difference in VFA absorption. Exploring this further, VFA absorption efficiency was calculated (mmol VFA absorbed per unit of blood flow). This showed that ergot alkaloids present in the buffer caused an increase in VFA absorption efficiency, especially butyrate and valerate. This finding could be due to direct effect of ergot alkaloids on the rumen epithelium function. The increase absorptive efficiency could be the result of alterations in VFA absorptive pathways or epithelial metabolism.

The final experiment in this series was designed to determine the effect of ergot alkaloids on the absorptive and barrier functions of the isolated rumen epithelium as well as flux of ergovaline across the isolated rumen epithelium. Utilizing rumen epithelium mounted in Ussing chambers, this experiment showed that there was no effect of acute

exposure to ergot alkaloids on the flux of acetate and butyrate. Additionally, there was no effect on barrier function measured by inulin flux or tissue conductance. It was also shown that ergovaline can cross the rumen epithelium, albeit at a slow rate. It was estimated that this slow flux rate would result in about 1% of the ergovaline consumed being absorbed from the rumen.

It is clear from this series of experiments that ergot alkaloids could have severe negative effects on bovine foregut function. It is possible that if only 1% of the consumed ergovaline is absorbed from the rumen then other absorption sites probably make greater contributions to alkaloid absorption. However, the primary site(s) for alkaloid absorption and the vascular concentrations of alkaloids that are achieved remains elusive. Long term exposure to ergot alkaloids, specifically ergovaline, could result in the accumulation of ergot alkaloids in the vasculature tissue and eventually result in concentrations high enough to induce vasoconstriction. A small vasoconstrictive response can result in a large reduction in blood flow and nutrient absorption, as vessel diameter is exponentially related to blood flow (by a factor of 4).

It is also possible that ergot alkaloids present in the lumen of the reticulorumen could have effects on blood flow without being absorbed. Ergot alkaloids could bind to receptors present on the apical membrane of epithelial cells and initiate a signaling cascade that causes alterations in vasculature.

Based on the in vivo data from the washed reticulorumen experiment, it is apparent that ergot alkaloids present in the ruminal lumen can induce changes in blood flow to the absorptive surface of the rumen, which results in reduced VFA absorption.

One of the most severe fescue toxicosis symptoms is the reduced growth rate of cattle. A reduction in growth results in a reduced profit margin for producers. It is apparent based on the results presented herein that the toxins present in endophyte-infected tall fescue can alter nutrient absorption from the foregut of cattle, which likely contributes to the reduced growth rate in cattle suffering from fescue toxicosis. This series of experiments contributes to the body of literature that describes the toxicosis syndrome associated with tall fescue; a necessary first step in alleviating the syndrome and in turn increasing the productivity of the beef industry in the United States.

APPENDIX A: PERMISSION TO USE PUBLISHED MATERIAL

May 15, 2013
Dr. Steven Zinn
Editor-in-Chief of the Journal of Animal Science

Dear Dr. Zinn,

In partially fulfillment of the requirements for my doctoral dissertation at the University of Kentucky, I have published the following papers in the Journal of Animal Science:

Foote, A. P., D. L. Harmon, J. R. Strickland, L. P. Bush, and J. L. Klotz. 2011. Effect of ergot alkaloids on contractility of bovine right ruminal artery and vein. *J. Anim. Sci.* 89: 2944-2949.

Foote, A. P., D. L. Harmon, K. R. Brown, J. R. Strickland, K. R. McLeod, L. P. Bush, and J. L. Klotz. 2012. Constriction of bovine vasculature caused by endophyte-infected tall fescue seed extract is similar to pure ergovaline. *J. Anim. Sci.* 90: 1603-1609.

I am requesting permission to include these papers, with proper notation of copyright, in my dissertation.

Thank you for your assistance,

Andrew P. Foote

May 15, 2013

That is fine.

Good luck.

Steve

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VITA

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Teaching Experiences

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2008 – 2009	Tarleton State University - ANSC 406 Animal Nutrition

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- 2013 Dissertation Enhancement Award – University of Kentucky \$3,000 for travel to University of Saskatchewan
- 2012 Joseph P. Fontenot Travel Scholarship – American Society of Animal Science
- 2012 1st Place Poster Presentation - University of Kentucky Graduate Student Association Symposium
- 2008 2nd Place Master's level Agriculture Poster - Texas A&M System Research Symposium
- 2008 2nd Place Poster Presentation - Tarleton State University Research Symposium
- 2008 Honorable Mention - Texas AgriLife Conference Research Symposium
- 2007 Outstanding Graduate - Department of Animal Science, Tarleton State University
- 2006 & 2007 1st Place Undergraduate Oral Research Presentation – Tarleton State University Research Symposium
- 2005 First Team All-American Meat Judging Team

Publications

Peer-reviewed Publications

- Foote, A.P.,** N. B. Kristensen, J. L. Klotz, D. H. Kim, A. F. Koontz, K. R. McLeod, L. P. Bush, F. N. Schrick, and D. L. Harmon. Ergot alkaloids from endophyte-infected tall fescue decrease reticuloruminal epithelial blood flow and volatile fatty acid absorption from a washed reticulorumen. *J. Anim. Sci.* *Accepted* May 2013.
- Foote, A. P.,** B. D. Lambert, J. A. Brady, M. S. Brown, C. Ponce, J. C. MacDonald, and N. A. Cole. Phosphate transporter expression in beef cattle fed differing levels of wet distiller's grains. *Animal.* *Accepted.*

- Koontz, A. F., **A. P. Foote**, D. H. Kim, L. P. Bush, J. L. Klotz, K. R. McLeod, and D. L. Harmon. Alteration of fasting heat production during fescue toxicosis in Holstein steers. *J. Anim. Sci. Accepted May*, 2013.
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Abstracts

- Foote, A.P.**, N.B. Kristensen, J.L. Klotz, K.R. McLeod, L.P. Bush, and D.L. Harmon. 2013. Emerging Young Scholar Award: Effect of ergot alkaloids from endophyte-infected tall fescue on ruminal vasculature, blood flow, and nutrient absorption. Southern Section American Society of Animal Science Conference, Orlando, FL.
- Koontz, A.F., D.H. Kim, **A.P. Foote**, L.P. Bush, J.L. Klotz, K.R. McLeod, and D.L. Harmon. 2013. Effect of fescue toxicosis on nitrogen and energy balance in Holstein steers. International Symposium on Energy and Protein Metabolism and Nutrition.
- Kim, D.H., K.R. McLeod, J.L. Klotz, A.F. Koontz, **A.P. Foote**, and D.L. Harmon. 2013. Application of washed rumen technique for rapid determination of fasting heat

production in steers. International Symposium on Energy and Protein Metabolism and Nutrition.

- Foote, A.P.**, N.B. Kristensen, J.L. Klotz, D.H. Kim, A.F. Koontz, K.R. McLeod, L.P. Bush, and D.L. Harmon. 2012. Ergot alkaloids decrease rumen epithelial blood flow. *J. Anim. Sci.* 90 (E-Suppl. 3):431-432.
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- Koontz, A.F., **A.P. Foote**, D. H. Kim, L.P. Bush, J. L. Klotz, K.R. McLeod, D.L. Harmon. 2012. Alteration of Fasting Heat Production during Fescue Toxicosis in Holstein Steers. *J. Anim. Sci.* 90 (E-Suppl. 3): 622.
- Kim, D.H., K. R. McLeod, J. L. Klotz, A. F. Koontz, **A. P. Foote**, and D. L. Harmon. 2012. Evaluation of a Rapid Determination of Heat Production and Respiratory Quotient in Holstein Steers using the Washed Rumen Technique. *J. Anim. Sci.* 90 (E-Suppl. 3): 99.
- Klotz, J.L., J.R. Bussard, G.E. Aiken, **A.P. Foote**, D.L. Harmon, K.R. Brown, B.M. Goff, and J.R. Strickland. 2012. Lateral saphenous vein responses to serotonergic and α -adrenergic receptor agonists increase with time off endophyte-infected tall fescue. *J. Anim. Sci.* 90 (E-Suppl. 3): 34.
- Bussard, J.R., G.E. Aiken, J.R. Strickland, K.R. Brown, B.M. Goff, **A.P. Foote**, and J.L. Klotz. 2012. Changes in bovine vascular contraction and constriction relative to time off endophyte-infected tall fescue. *J. Anim. Sci.* 90 (E-Suppl. 3): 34.
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