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EFFECTS OF FESCUE HERBICIDES PLATEAU® AND CIMARRON® ON PREGNANCY MAINTENANCE IN BROODMARES AND ON ALKALOID CONCENTRATIONS IN ENDOPHYTE INFECTED TALL FESCUE

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ABSTRACT OF THESIS

EFFECTS OF FESCUE HERBICIDES PLATEAU® AND CIMARRON® ON PREGNANCY MAINTENANCE IN BROODMARES AND ON ALKALOID CONCENTRATIONS IN ENDOPHYTE INFECTED TALL FESCUE

Ingestion of endophyte infected (E+) fescue by pregnant mares can cause significant reproductive problems. Plateau® and Cimarron® herbicides suppress fescue while leaving desired forages unharmed. To determine if these herbicides are harmful to pregnant mares, they were allowed to graze pastures treated with Plateau®, Cimarron®, or vehicle carrier. Pregnancies were monitored via ultrasonography, blood chemistry, and hematology. Of the components measured only creatinine differed among treatments over time (P=0.0003) and that increase was only significant in one of four studies.

Two additional experiments were conducted to determine the effect of the herbicides on alkaloids within E+ fescue. A greenhouse experiment utilizing 52 pots of E+ fescue treated with Plateau®, Cimarron®, or nothing was inconclusive, as some alkaloids increased while others decreased. These results indicated that UV light may be required for normal plant death. In a field experiment 12 plots of mixed vegetation were sprayed with the same treatments, and herbicides decreased ergovaline, N-formylloline, and lysergic acid content (P=0.0460, P=0.0324, P=0.0093 respectively). In conclusion, the herbicides did not alter blood components outside physiological norms, but the alkaloids were still present in dying E+ fescue. It may be safest to remove late gestation mares until E+ fescue is completely decayed.

KEYWORDS: mare, imazapic, metsulfuron methyl, fescue, *Neotyphodium coenophialum*

___Kathleen Scarlett Black, BS___

_____July 24, 2008_____

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THESIS

Kathleen Scarlett Black

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2008

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THESIS

A thesis submitted I partial fulfillment of the
requirements for the degree of Master of Science in the
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By

Kathleen Scarlett Black

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

Lexington, Kentucky

2008

I dedicate this work in honor of all of those
that have supported and encouraged me
in all I my endeavors.

Thank you to my
Family and Friends.

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CHAPTER ONE
TALL FESCUE TOXICOSIS
A LITERATURE REVIEW

Introduction

Fescue Toxicosis is the general term that encompasses all of the deleterious effects seen in livestock grazing tall fescue (*Festuca arundinacea* Schreb.) infected with the fungal endophyte *Neotyphodium coenophialum* (Glen *et al.*, 1996). Cattle, sheep, and horses can experience decreases in weight gain and changes in hormone concentrations and patterns while consuming endophyte infected tall fescue. Tissue necrosis can also occur in cattle and sheep, while pregnant mares suffer reproductive problems.

Tall Fescue History and Development

Tall fescue, a seed propagated, perennial, cool season, bunch grass, was imported into the United States from Europe in the 1800's (Buckner *et al.*, 1979). One of the most widely used endophyte infected tall fescue (E+ fescue) cultivars is Kentucky 31 (KY31). It was found in 1931 on a farm in Menifee County Kentucky owned by W.M. Suiter. This specific cultivar was released by the University of Kentucky in 1943 with the following distinguishing features of "1) dependability; 2) adaptability to a wide range of soils; 3) affording grazing during most of the year; [and] 4) palatability to livestock" (Buckner *et al.*, 1979). It was not, however, until 1972 that KY31 was registered as a specific ecotype of tall fescue (Fergus and Buckner, 1972). Over time, tall fescue has been adapted for use in nearly half of the United States (Figure 1.1). In 1940 it was estimated that tall fescue occupied approximately 40,000 acres (16,187 hectares), but by 1973 it occupied around 35 million acres (approximately 14 million hectares) (Buckner *et al.*, 1979) of which 5.5 millions acres (approximately 2.2 million hectares) are in Kentucky alone (Lacefield *et al.*, 2003). Additionally, it has been estimated that 85% of Kentucky pastures contain E+ fescue (Lacefield *et al.*, 1993). The spread of tall fescue is due to the use of KY31 and another cultivar out of Oregon called Alta for soil conservation purposes. Traits that have enabled E+ fescue to out compete other plant species include its ability to thrive in numerous soil and climate types, to endure water

logging, and to grow at lower temperatures than other cool season grasses (Buckner *et al.*, 1979; Burns and Chamblee, 1979).

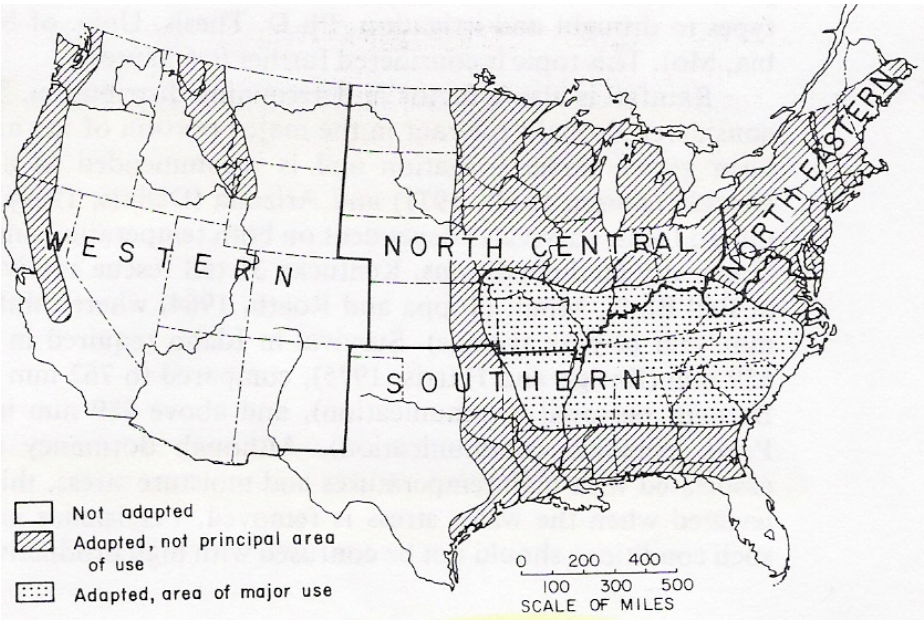


Figure 1.1 Tall fescue has been adapted for use in nearly half of the United States, with the transitional zone in the eastern states being the primary adaptation zone (Burns and Chamblee, 1979 with permission).

Tall Fescue Characteristics

Tall fescue can reach heights of 2 to 4 feet (0.6 to 1.2m) in the flowering stage (Ball *et al.*, 2002). It has a round culm and its 4 to 24 inches (10.16 to 60.96cm) long leaves have a dull upper surface and shinier lower surface (Figure 1.2 c). The leaves also have prominent veins giving them a rough texture (Figure 1.2 b). Tall fescue is seed propagated and has a panicle inflorescence (seed head) (Figure 1.3), containing spikelets of 5 to 9 flowers each (Roberts, 2006). Once the seed is in the ground it can start germinating within a few hours with ideal conditions of temperatures of 20 to 25ºC for 8 hours followed by 10 to 15 ºC for 16 hours (Boyce *et al.*, 1976). Germination will then last 14 days (Taylor *et al.*, 1979), followed by a growing season in Kentucky from March through November. Peak growth times are in late April-early May and again in late October-early November (Ball *et al.*, 2002) resulting in an estimated annual yield of 2 to 4 tons (1.81 to 3.63 metric tons) of dry matter a year (Lacefield *et al.*, 2003).

(a)

(b)

(c)

Figure 1.2. Tall fescue is a bunch grass (a) with long, wide leaves with prominent veins (b) that have a dull appearing upper surface and shinier lower surface (c).

Figure 1.3. The inflorescence of flowering tall fescue is a panicle, which has a rachis with subdivision where each spikelet contains florets.

Tall fescue is classified as a C_3 plant, meaning that it assimilates CO_2 by the Calvin cycle with 3-phosphoglyceric acid as the primary product (Wolf *et al.*, 1979). As a C_3 plant and displaying some characteristics of photorespiration, tall fescue becomes light saturated at only 25 to 50% full sunlight (Wolf *et al.*, 1979), unlike C_4 plants, e.g. bermuda grass, that have a higher photosynthetic potential using nearly full sunlight (Ball *et al.*, 2002), making tall fescue less efficient than other grasses. The excess energy resulting from photosynthesis, after respiration and growth, is stored as carbohydrates in the stem bases of tall fescue (Ball *et al.*, 2002). These nonstructural carbohydrates are the primary source of energy that fuels the biochemical processes of the plant (Wolf *et al.*, 1979) and are mobilized when needed, such as after a drought.

The Endophyte Infecting Tall Fescue

The endophyte originally suggested as the causative agent of fescue toxicosis by Charles Bacon and coworkers (1977) was named *Epichloe typhina* (Sampson, 1933). This classification was later challenged and changed to *Acremonium coenophialum* (Morgan-Jones and Gams, 1982), and in 1996 the classification was again challenged and

changed to its current one of *Neotyphodium coenophialum* (Glen *et al.*, 1996). This endophyte is located intercellularly (Bacon *et al.*, 1977; Christensen and Voisey, 2007; Figure 1.4).

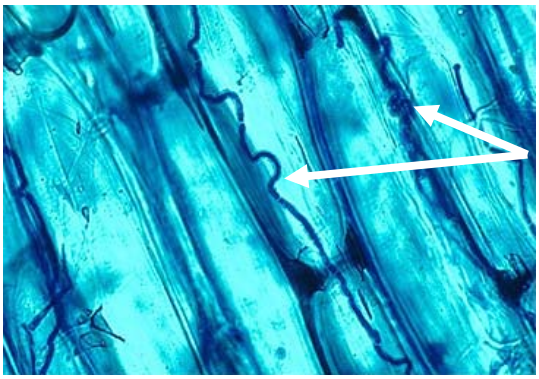


Figure 1.4. The endophyte *Neotyphodium coenophialum* is located intercellularly primarily in the leaf sheath, indicated by arrow. (Roberts and Andrae, 2004 with permission).

The *N. coenophialum* endophyte has enzymes that are able to dissolve the middle lamellae as the endophyte and plant grow, and because of this location the endophyte is able to take up nutrients from the plant (Belesky *et al.*, 1988). The endophyte receives all of its nutrients from the living tissue of the tall fescue plant, such that when the plant has rapid growth, for example in the spring, the endophyte also has a period of rapid growth resulting in an increase alkaloid content (Hinton and Bacon, 1985). The symbiotic relationship between the tall fescue plant and the endophyte is based on defensive mutualism (Clay, 1988). Charles Bacon (1994) summarized that together they are able to have "enhanced drought tolerance, increased tillering and growth, and increased resistance to herbivory from mammals and insects."

N. coenophialum is found in the leaf sheath, meristematic areas, and inflorescence, with the highest concentration in the seed (Bacon, 1994); (Figure 1.5).

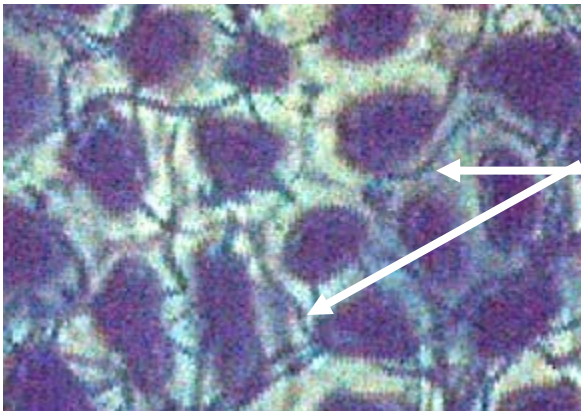


Figure 1.5. The endophyte is at the highest concentration in the seed of the plant. (Roberts and Andrae, 2004 with permission).

The endophyte itself is not able to reproduce, so it uses the seeds of the tall fescue plant to propagate (Cheeke, 1998). As the tall fescue seed germinates, the fungal endophyte grows and invades the seedling within 2 days of germination and can be detected at the first internode of the emerging shoot (Bacon and Siegel, 1988). Once mature the E+ fescue plant can out compete non-infected tall fescue (E- fescue) and other grass species (Marks *et al.*, 1991). A possible explanation for the competitiveness of E+ fescue is the water content of the leaf sheaths. Leaf sheaths of the E+ fescue have a higher fractional water content than those of E- fescue, which enables E+ fescue to survive longer during drought conditions (Elbersen and West, 1997). Howard and coworkers (1992) observed that E+ fescue pastures with high infection levels had greater forage mass than those at lower levels of infection, further suggesting that the endophyte presence offers a competitive advantage to the plant during stress situations. Even though pastures of low endophyte infection and those of high endophyte infection have similar chemical compositions (Howard *et al.*, 1992), E+ fescue plant population levels increased with high stocking rates of cattle (Gwinn *et al.*, 1998) and horses (Singer *et al.*, 2001), implying yet another competitive advantage of E+ fescue.

Fescue Toxicosis in Livestock

The deleterious effects observed in livestock, due to consumption of E+ fescue, overshadow the benefits of the symbiotic relationship between the fungal endophyte and

tall fescue for plant survival. Fescue toxicosis has been estimated to cause over $600 million in economic losses annually to the cattle industry alone (Jones *et al.*, 2003), with no estimations to the losses in the horse or sheep industries. Researchers in Georgia were the first to report a correlation of endophyte infected tall fescue and the signs of fescue toxicosis in cattle (Bacon *et al.*, 1977). Fescue toxicosis in cattle manifests itself with both hypothermic and hyperthermic effects. "Fescue foot," a hypothermic effect, occurs when blood flow to extremities is decreased, such as in the winter, while the hyperthermic effect, "summer slump," primarily occurs in the summer. In both cases, vasoconstriction caused by alkaloids, to be discussed later, may be the primary cause.

Fescue foot can occur in both cattle and sheep with signs of dull hair coat, lameness, gangrenous tissue, and tip of tail and/or hoof loss. Lameness occurs in as little as 18 days in cattle (Jensen *et al.*, 1956) and 21 days in sheep (Tor-Agbidye *et al.*, 2001) after introduction into a E+ fescue pasture during the colder weather. Lesions can form just above the coronary band of cattle in as little as 6 weeks of eating E+ fescue hay during the late fall-early winter season (Jensen *et al.*, 1956). This line separates the healthy tissue from the affected tissue. Eventually, the tissue below the lesions becomes gangrenous and ultimately sloughs off. Blood vessels of the hoof are affected with thrombosis, congested blood vessels, thickened blood vessel walls, and constricted lumens (Aiken *et al.*, 2007; Corley *et al.*, 1973; Jensen *et al.*, 1956; Williams *et al.*, 1975). Other signs with gangrenous ergotism are arterial spasms, anoxemia, and capillary endothelial degeneration (Burfening, 1973). These signs are similar to those seen in animals grazing perennial ryegrass (*Lolium perenne*) infected with the endophyte *Neotyphodium lolii.* Unlike cattle and sheep, horses do not appear to develop fescue foot, however Rohrbach and coworkers (1995) found a correlation between areas of known E+ fescue presence and diagnosed cases of laminitis in horses.

In addition to problems in livestock associated with ingesting E+ fescue during the winter months, it causes problems in the summer months known as "summer slump." Summer slump is associated with increases in rectal temperatures in cattle (Chestnut *et al.*, 1991; Schmidt *et al.*, 1982) and in core temperatures in sheep (Aldrich *et al.*, 1993b); affected animals seek shade and watering holes to cool themselves during the day. Horses do not seem to suffer this increase in body temperature. Putman and coworkers

(1991) suggested that this could be due to increased heat dissipation from sweating in horses. Increased sweating was observed in geldings injected intravenously with an alkaloid produced by *N. coenophialum* (Bony *et al.*, 2001). In ruminants the inability to cool themselves predispose livestock to decreased daytime grazing, feed intake, and average daily gains (Gadberry *et al.*, 2003; Gallagher *et al.*, 1966; Hoveland *et al.*, 1983; Howard *et al.*, 1992; Peters *et al.*, 1992). Lambs had a linear decrease in feed intake as the amount of E+ fescue seed in the diet was increased (Gadberry *et al.*, 2003). Decreases in the weight gain of lambs were found to be up to 66% lower than normal when lambs grazed E+ fescue (Parish *et al.*, 2003a). Yearling horses also had decreased weight gain when grazing E+ fescue (Aiken *et al.*, 1993). However, there was no effect on weight in mature geldings ingesting E+ fescue hay for 14 days when compared to controls consuming non-infected hay (Redmond *et al.*, 1991). Cattle consuming E+ fescue seed had a decrease in feed intake only when ambient temperatures were in excess of 32°C (Peters *et al.*, 1992). Similar studies conducted in mice also showed a decrease in feed intake when temperatures were in excess of 32˚C (Larson *et al.*, 1994).

Fescue Toxicosis Alterations in Blood Components

In addition to the effects of E+ fescue ingestion on feed intake and weight gain, E+ fescue also affects blood components. Decreased serum concentrations of cholesterol have been reported in both cattle and horses (Stuedemann *et al.*, 1985; Youngblood *et al.*, 2004). Components of the immune system may also be affected by consumption of E+ fescue. For example, decreased total leukocyte count, monocytes phagocytosis, and eosinophil counts have been observed in cattle (Oliver *et al.*, 2000; Saker *et al.*, 1998). However, Waller and coworkers (2002) reported that there were no effects on hemoglobin, hematocrit, platelets, mean platelet volume, white blood cells, polymorphonuclear-leukocytes, lymphocytes, monocytes, or basophils. Missouri workers determined that ingestion of E+ fescue seed can cause down regulation of expression of genes involved in immune function in rat livers (Settivari *et al.*, 2006).

One of the hallmarks of fescue toxicosis is decreased serum prolactin concentrations, regardless of species. Hypo-prolactin has been reported in cattle (heifers, steers, and bulls), lambs, and pregnant mares (Aldrich *et al.*, 1993a; Boosinger *et al.*,

1995b; Gadberry *et al.*, 2003; Lipham *et al.*, 1989; Schuenemann *et al.*, 2005a). However, recent research conducted by Schultz and coworkers (2006) found that mature geldings ingesting E+ fescue did not have decreased serum prolactin concentrations, while bulls grazing E+ fescue did (Schuenemann *et al.*, 2005). Decreases in prolactin concentrations can occur in as little as 3 days in horses (McCann *et al.*, 1992) and can eventually reach the lower detection limit in cattle (Parish *et al.*, 2003b). Prolactin, secreted from lactotrophs in the anterior pituitary gland, is regulated by tonic inhibition via dopamine from the hypothalamus and is stimulation by thyroid releasing hormone (Ben-Jonathan and Hnasko, 2001; Lothrop, Jr. *et al.*, 1987). Prolactin stimulates growth and development of the mammary gland and is necessary for lactogenesis and galactopoiesis (Freeman *et al.*, 2000) in gestating and postpartum livestock. Prolactin concentrations in horses normally increase as day length increases, such that it is higher in the summer months (Thompson, Jr. *et al.*, 1986). Increasing temperature may also be involved in the increase of prolactin from the anovulatory levels (Johnson, 1987). Prolactin may also be involved in signaling the start of the ovulatory season (Ginther, 1992). A decrease in prolactin can contribute to decreases in milk production and milk persistence in dairy cattle and agalactia in pregnant or postpartum mares grazing E+ fescue (Monroe *et al.*, 1988; Seath *et al.*, 1954).

Fescue Toxicosis and Reproduction

Other reproductive effects in cattle caused by the consumption of E+ fescue include delayed onset of puberty, decreased conception rates, and decreased calving rates (Gay *et al.*, 1988; Lechtenberg *et al.*, 1975; Washburn *et al.*, 1989). In the mare, prolonged luteal phases have also been reported (Brendemuehl *et al.*, 1994). Studies involving early embryonic loss are less clear. Brendemuehl *et al.* (1994) reported increased losses with mares in the first 14 to 21 days of gestation, though the increase was not statistically significant. While Youngblood *et al.* (2003) reported no increase in pregnancy loss in mares at 65 to 100 days of gestation ingesting E+ fescue seed for 10 days. Cattle also have been reported to have problems during early gestation. When conditions of fescue toxicosis (elevated rectal temperatures and decreased prolactin concentrations) were simulated by feeding ergotamine tartate (a synthetic alkaloid, Sigma

Chemicals, St Louis, MO, that is similar to ergovaline) to cows, decreased embryo recovery, development, and quality were reported (Schuenemann *et al.*, 2005b).

E+ fescue may also affect male reproduction. For example, bulls grazing E+ fescue had decreased testicular temperature which may have been due to a decrease in blood flow to the testes, although there was no significant effect on sperm progressive motility or morphology (Schuenemann *et al.*, 2005a). In stallions grazing E+ fescue for 14 days, the catecholamine norepinephrine decreased slightly (Olsen *et al.*, 2005). Normally norepinephrine peaks around the time of ejaculation (Terada *et al.*, 2005), and decreased norepinephrine could result in decreased ejaculatory function through decreased sympathetic nervous system activity (McKinnon and Voss, 1993). The decrease in norepinephrine concentrations could be due to decreased release, increased uptake, or increased binding to receptors. *In vitro* studies with E+ fescue have shown that α-2 adrenergic receptors in the cranial branch of the lateral saphenous vein are more sensitive to norepinephrine than are E- fescue treated veins (Oliver *et al.*, 1998). Oliver and coworkers (1998) suggested that the ergot alkaloid derivatives may have a preference for the α-2 adrenergic receptors and may be involved with decreased heat regulation. Alterations to heat regulation in the testes could affect spermatogenesis. In rats, ingestion of E+ fescue seed caused a decrease in daily sperm production and epididymal weight (Zavos *et al.*, 1986). Evans et al (1988), however, did not find an effect of E+ consumption on testosterone concentrations or on epididymal weight in bulls.

The largest and best-documented impact of E+ fescue intake in reproduction involves mares in late gestation. Prolonged gestation is one of the most well documented problems of E+ fescue consumption in pregnant mares (Earle *et al.*, 1989; Monroe *et al.*, 1988; Putnam *et al.*, 1991). Gestation can be prolonged 20 to 27 days in mares (Earle *et al.*, 1989; Monroe *et al.*, 1988; Putnam *et al.*, 1991) accompanied by alterations in hormone concentrations (Figure 1.6 b). Late gestation mares on E+ fescue pastures have decreased serum progesterone and prolactin concentrations and increased estradiol 17β concentrations (Boosinger et al., 1995b; Evans et al., 1991; Monroe et al., 1988; Redmond et al., 1994); (Figure 1.6 a and b). In mares, normally estrogens in the maternal circulation decrease in the last 60 days of gestation and progestagens increase. Approximately 24 hours prior to parturition progestagens decrease, while estrogens

remain constant (Ginther, 1992). The change in the progestagens to estrogens ratio may signal for the normal production of prostaglandins E_2 (PGE_2) and $F_2\alpha$ ($PGF_2\alpha$) and increase in uterine myometrial oxytocin receptors number. PGE_2 acts to soften the cervix while $PGF_2\alpha$ stimulates myometrial contractions. At parturition, a finely orchestrated cascade of events involving PGF2α and oxytocin occurs to initiate, sustain, and complete parturition. Mares suffering fescue toxicosis do not experience the normal decrease in estrogens and increase in progestagens in the third trimester at the expected time (Boosinger *et al.*, 1995b; Brendemuehl *et al.*, 1996; Redmond *et al.*, 1994; Vivrette, 1994). Alterations to these patterns can be a contributing cause of prolonged gestation in the mare.

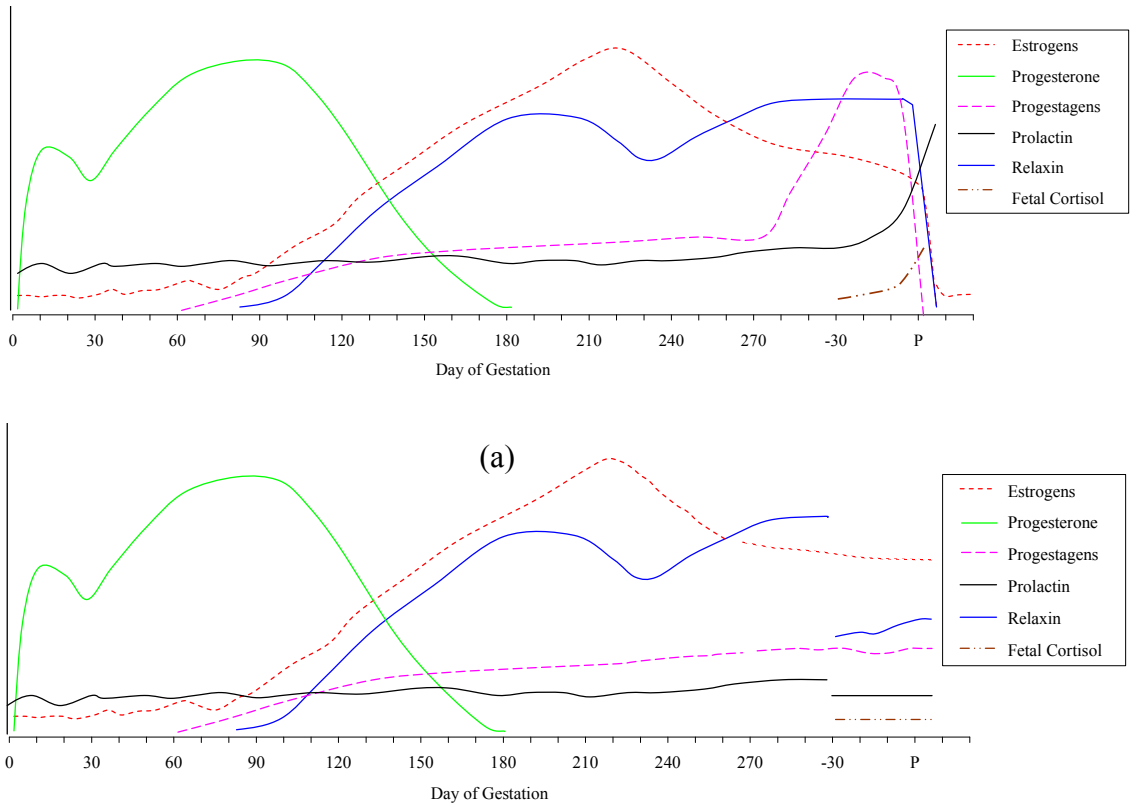


Figure 1.6. The normal endocrinology (a) (adapted from Ginther, 1992; Vivrette, 1994) is altered in mares suffering from fescue toxicosis (b) with decreased progestagens, prolactin, relaxin, and fetal cortisol, and increased estrogens near the time of parturition (adapted from Boosinger *et al.*, 1995b; Brendemuehl *et al.*, 1995; Brendemuehl *et al.*, 1996; Redmond *et al.*, 1994; Ryan *et al.*, 2001b; Vivrette, 1994). Due to the variability in gestation lengths, the x-axis represents the day of ovulation (day 0) through day 270 of gestation. It is then normalized from 30 days pre-partum to parturition (P).

Additionally, mares grazing E+ fescue have fetuses with altered adrenal gland cortisol production. Fetal cortisol normally is not detectable prior to day 310 of gestation, possibly due to a lack of 17-hydroxylase activity which converts progesterone to 17α-hydroprogesterone (Chavatte *et al.*, 1995). A rise in fetal cortisol occurs approximately 2 to 4 days pre-partum (Vivrette, 1994); (Figure 1.6 a). This increase in fetal cortisol may trigger fetal liver, thyroid gland, lung, gut and adrenal gland maturation, which occurs 2 to 3 days pre-partum (Ousey, 2006) and acts as a signal to the

mare of readiness for birth by initiating prostaglandin production by the maternal uterus (Ginther, 1992). However, in mares grazing E+ fescue postpartum studies have determined that cortisol, along with adrenocorticotropic hormone (ACTH) (Brendemuehl *et al.*, 1995) and tri-iodothyronine (T_3) concentrations (Boosinger *et al.*, 1995a) are decreased in the newborn foal. These results suggest that fetal cortisol levels pre-partum are also reduced (Figure 1.6 b). Pashen *et al.* (1984) concluded that the increase in cortisol in foals immediately following normal parturition is necessary for postnatal survival. Fetal cortisol has also been suggested to be involved with the synthesis and metabolism of placental progestagens, since exogenous cortisol has been shown to increase plasma progestagens (Ousey *et al.*, 2000; Ousey, 2004). Thus, alterations in fetal cortisol could be another explanation for prolonged gestation and for decreased progestagens in the maternal circulation.

E+ fescue also affects relaxin levels in pregnant mares. Relaxin is produced by the mare's placenta and functions to prepare the reproductive tract for parturition through myometrial relaxation, pubic separation, and pelvic relaxation (Ginther, 1992; Stewart *et al.*, 1982). Relaxin normally increases around day 75 of gestation, peaks at day 175, and remains elevated until parturition (Stewart et al., 1992; Figure 1.6 a). Mares grazing E+ fescue, however, frequently have decreased relaxin concentrations in the last 30 days of gestation (Ryan *et al.*, 2001a); (Figure 1.6 b). In the absence of increased relaxin concentrations, the mare will not undergo the necessary changes to prepare for parturition and expulsion of the foal.

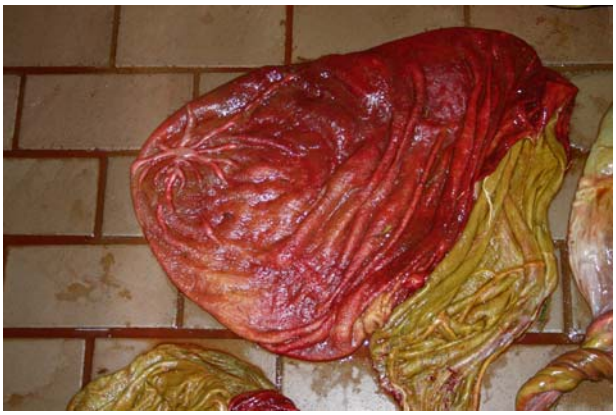
Alterations in hormone concentrations and patterns can result in a lack of preparation of the reproductive tract for parturition, ultimately leading to dystocias, or difficult births. Dystocias can range from bruising, to cervical tears, to soft tissue trauma, or to death. E+ fescue-related prolonged gestation often leads to abnormally large framed foals (Monroe *et al.*, 1988) that are referred to as dysmature. Dysmature foals are born at full term, but show signs of being premature (Rossdale, 2004). These foals often are large-framed, but have poor muscling, long fine coats, overgrown hooves, and irregular incisors (Putnam *et al.*, 1991). Additionally, these large-framed foals can have a 90 to 180 degree rotation from the normal dorsal-sacral presentation for parturition (Monroe *et al.*, 1988; Taylor *et al.*, 1985). Thus, an abnormally oriented, large-framed foal attempts

to exit the mare's body through an unprepared reproductive tract. Putnam and coworkers (1991) reported that 91% of mares grazing pastures of greater than 80% infected tall fescue experienced a dystocia with only one of eleven foals surviving the neonatal period.

Another form of dystocia that often occurs with mares on E+ fescue pastures is placenta previa commonly referred to as "red bagging." Normally the chorioallantois of the fetal placenta invades the uterine endometrium and develops villi in the form of microcotyledons that interdigitates with the maternal microcaruncles. The fetal-placental unit gains access to nutrients from the maternal circulation through these microcotyledons as well as through the uterine glands (Ginther, 1992). Normally, the placenta ruptures at the cervical star and as the foal progresses through the birth canal, the umbilical cord pulls the placenta "inside out," such that the shiny, smooth interior of the allantois is observed at parturition, (Figure 1.7 a). In a "red bag," however, the placenta does not rupture and the red colored velvet appearing microcotyledonary surface is seen at parturition, (Figure 1.7 b and c). Often placentas from E+ fescue consuming mares are heavy, resist breaking, and have increased weights (Loch *et al.*, 1987; Monroe *et al.*, 1988). Brendemuehl and coworkers (1995) reported a premature separation of the chorioallantois from the uterus in mares with fescue toxicosis and only seven of twelve foals survived postpartum for more than 2 hours. Often foals are born still encapsulated in the placenta due to its failure to rupture. These foals require human intervention in order to escape the placenta and avoid asphyxia (severe lack of oxygen possibly leading to brain damage) or suffocation (death from lack of oxygen). Sometimes foals that do survive parturition become "dummies" possibly due to asphyxiation during parturition and immediately postpartum. Dummy foals will act normal for the first 24 hours, but then revert. They will not nurse, may appear uncoordinated, and may die without assistance in feeding via nasogastric tube and medications.

(a)

(b)



(c)

Figure 1.7. Normally the amnion precedes the foal and chorioallantois resulting in the inversion the chorioallantois, such that the fetal surface of the chorioallantois is seen following the foal's birth (a). In a red bag dystocia, the cervical star does not rupture (b) and the endometrial surface of the chorioallantois presents first (c). Photo b is courtesy of Dr. Dale Paccamonti (Beilts, 2004 with permission). Photo c is courtesy of Dr. Neil Williams (University of Kentucky Department of Veterinary Science, with permission).

Mares grazing E+ fescue lack normal mammary gland development. Horse owners frequently watch for mammary gland development to determine which mares to observe more closely, or "night-watch" for impending parturition. Without proper mammary gland development these mares may foal unattended. Frequently in pregnancies complicated by fescue toxicosis neither the mare nor the foal may survive parturition. Putnam *et al.* (1991) reported a 72% mortality for foals (8 of 11) during parturition, and of the three foals that did survive parturition only one lived beyond the

neonatal period. Additionally, of the eleven mares grazing E+ fescue, four died due to complications during delivery.

For mares and foals that survive parturition the next problem to overcome may be agalactia. As stated previously, mares grazing E+ fescue have decreased prolactin levels in the last 30 days of gestation. Without prolactin to stimulate lactogenesis and galactopoiesis (Freeman *et al.*, 2000) and oxytocin to cause milk let down (Ginther, 1992) agalactia results. Normal mammary development 10 to 5 days pre-partum (Worthy *et al.*, 1986) does not occur and the mare becomes agalactic. Newborn foals must receive colostrum within the first 24 hours of life to receive passive immunity, with maximum absorption occurring with the first 6-8 hours of life (Tizard, 2004). Beyond this time, the foal's intestines are unable to absorb the macromolecules. Green and coworkers (1991) hypothesized that even if mares do have colostrum after ingesting E+ fescue, their foals may have decreased immunoglobulin G absorption. If levels are below 4g/L, the transfer is believed to have failed (Rossdale, 2004), leaving foals more susceptible to diseases.

The Alkaloids Contained in Endophyte Infected Tall Fescue

The above-mentioned signs of fescue toxicosis and its impact on the livestock industry have fostered extensive research into its cause and methods of prevention. Alkaloids that are made by the plant and/or endophyte are the target of many studies. Some alkaloids are made by the endophyte and some are made by the plant in response to the endophyte's presence (Siegel and Bush, 1994). The alkaloid group receiving the most attention is the ergopeptines produced by the endophyte (Figure 1.8).

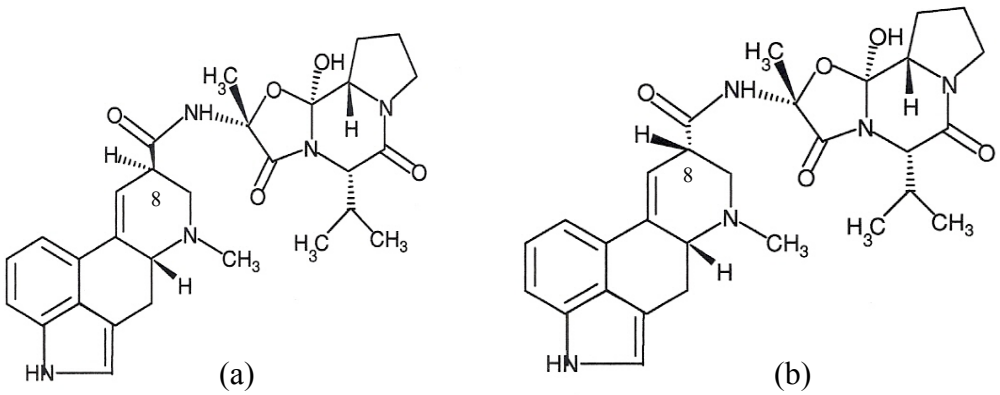


Figure 1.8. The structure of the ergopeptine alkaloids ergovaline (a) and its isomer, ergovalinine (b), differ in their arrangement at carbon 8.

Normally, what is referred to as an ergot is the sclerotium formed by the fungus. The sclerotium replaces the seed or kernel in other species of plants (Burfening, 1973), for example wheat and oats that are infected by the *Claviceps purpura.* Ergotism, or ergot toxicosis, can present in two forms in animals. The first is nervous ergotism, where neurologic signs such as vertigo, staggers, convulsions, and/or incomplete rigor mortis, are often seen in animals grazing perennial ryegrass (*Lolium perenne*) infected by the endophyte *Neotyphodium lolii* (Burfening, 1973). The compounds responsible for the above mentioned signs are tremorgens, specifically lolitrem B, produced by *N. lolii* (DiMenna *et al.*, 1992; Miles *et al.*, 1992). The other form of ergotism is gangrenous. Animals suffering from gangrenous ergotism have arterial spasms, anoxemia, and capillary endothelial degeneration (Burfening, 1973). The damage inflicted can result in loss of extremities as seen in "fescue foot."

The ergopeptines inhibit prolactin secretion via binding to D_2-dopamine receptors (Larson et al., 1999; Strickland et al., 1992). Dopamine, released by the hypothalamus, acts to tonically inhibit prolactin secretion (Ben-Jonathan and Hnasko, 2001). By acting as dopamine agonists, alkaloids lower prolactin concentrations. Intravenous (IV) infusion of ergopeptine mixtures can also reduce reticulorumenal contractions in sheep (McLeay and Smith, 2006). One specific ergopeptine, ergovaline, represents up to 90% of the total ergopeptines found in E+ fescue (Lyons *et al.*, 1986). It has been shown to bind to D_2-dopamine receptors and initiate cAMP production to decrease prolactin

production (Larson *et al.*, 1995). Mature geldings receiving 15µg ergovaline per kg body weight IV had excessive sweating, prostration, and difficulty urinating (Bony *et al.*, 2001). A synthetic ergot alkaloid, bromocriptine, when administered to mares resulted in the same signs (increased gestation length, decreased prolactin, agalactia, retained placentas, and dystocias) as those seen with mares grazing endophyte-infected fescue, suggesting that it is an ergopeptine alkaloid that causes fescue toxicosis in mares (Ireland *et al.*, 1991). Rats ingesting E+ fescue seed containing 4100µg ergovaline per kg seed had decreased expression of genes involved in energy metabolism (ATP synthase), growth (insulin like growth factor 1), and immune function (interferon beta 1) compared to rats on E- fescue seed (Settivari *et al.*, 2006). Ergovaline also has been found in *in vitro* studies to cause vasoconstriction of the bovine lateral saphenous vein (Klotz *et al.*, 2007a; Klotz *et al.*, 2008).

Ergovaline may not be the only alkaloid responsible for all the signs of fescue toxicosis. E+ fescue seed containing 0.5mg ergovaline and 0.3mg lysergic acid (another alkaloid group) per kg of fescue seed did not have an effect on rectal temperatures, prolactin concentrations, or on many blood chemistry values in geldings (Schultz *et al.*, 2006). Additionally, comparison of lambs fed diets of E- fescue seed, E+ fescue seed, or ergovaline added, indicated that lambs on the E+ fescue seed diet had a greater reduction in prolactin serum concentrations than those on the ergovaline added treatment, leading researchers to conclude that ergovaline is not the sole alkaloid that causes the signs of fescue toxicosis (Gadberry *et al.*, 2003). Vasoconstriction was greatest in bovine lateral saphenous veins exposed to ergovaline, N-acetylloline, and lysergic acid combined *in vitro* (Klotz *et al.*, 2008).

Lysergic acids, another group of alkaloids produced by the endophyte, may be primarily responsible for fescue toxicosis. It has greater transport potential across sheep rumen and omasum than ergopeptine alkaloids *in vitro*, though ergovaline specifically was not tested (Hill et al., 2001; Figure 1.9).

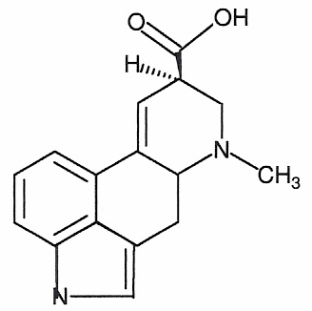
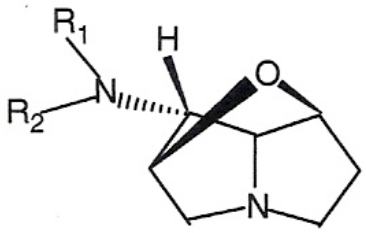


Figure 1.9. Lysergic acid may be the main alkaloid involved in fescue toxicosis.

Ergotamine tartate, a lysergic acid amide, caused vasoconstriction of both the equine dorsal metatarsal artery and lateral saphenous vein and of the bovine dorsal pedal veins *in vitro* (Abney et al., 1993; Solomons et al., 1989). Ergonovine, another lysergic acid amide, also caused constriction of bovine pedal veins *in vitro* (Oliver *et al.*, 1992). Finally, lysergamide, a lysergic acid amide similar to ergonovine, also caused vasoconstriction of the cranial branch of the bovine lateral saphenous vein and the dorsal metatarsal artery *in vitro*, but effects were more pronounced in the vein than in the artery (Oliver *et al.*, 1993).

Another class of alkaloids is the pyrrolizidines, commonly referred to as "lolines" (Figure 1.10). These alkaloids do not appear to be as toxic as the ergopeptines or lysergic acids. Pyrrolizidines, specifically N-formylloline and N-acetylloline (NAL), did not affect cAMP production at D_2 dopamine receptors (Larson *et al.*, 1999). However, NAL did cause vasoconstriction in the equine lateral saphenous vein *in vitro*, but not in the dorsal metatarsal artery (Abney *et al.*, 1993). Solomons *et al.* (1989) concluded that a mixture of loline and its derivatives at concentrations of 10^{-9} to 10^{-5}M did not cause vasoconstriction in the bovine dorsal pedal vein to the same extent as a mixture of ergopeptines (1.2 to 4.4 X 10^{-8}M for ergotamine and 1.4 to 2.2 X 10^{-7}M for ergosine). NAL caused vasoconstriction of the cranial branch of the lateral saphenous vein via α_2-adrenergic receptors (where the catecholamines norepinephrine and epinephrine normally bind to cause vasoconstriction) *in vitro* (Oliver *et al.*, 1990). Larsen *et al.* (1999) explained that loline and its derivatives do not have an effect on prolactin secretion due to a lack of the alkaloids binding to biogenic amine receptors (such as dopamine receptors).

However, increasing loline concentrations have been correlated with decreasing cholesterol concentrations in cattle (Stuedemann *et al.*, 1985).



	R_1	R_2
Norloline	H	H
Loline	H	CH_3
N-methylloline	CH_3	CH_3
N-formylloline	CH_3	HCO
N-formylnorloline	H	HCO
N-acetylloline	CH_3	CH_3CO
N-acetylnorloline	H	CH_3CO

Figure 1.10. The basic structure of pyrrolizidines and reactive groups required to create the structures of specific pyrrolizidines. Of the alkaloids above N-formylloline and N-acetylloline are the most abundant lolines in the E+ fescue plant, but N-methylloline, N-acetylnorloline, and N-formylnorloline are also present (Yates *et al.*, 1990).

The final alkaloid to be discussed is diazaphenanthrene, namely perloline, which is produced by the tall fescue plant not by the endophyte, since it is found in both E+ fescue and E- fescue (Bush et al., 1976). At concentrations of greater than 10^{-4}M perloline can inhibit "*in vitro* ruminal cellulose digestion, production of fatty acids, and growth of steer ruminal cellulytic bacteria" and reduced apparent crude protein and cellulose digestibility in lambs *in vivo* (Boling et al., 1975; Bush et al., 1970). However, the effects of the other alkaloids produced by *N. coenophialum* overshadow perloline's effects.

Fescue Toxicosis Treatment and Management

There are four methods of handling fescue toxicosis in animals. First, do nothing and suffer monetary losses due to decreases in weight gain in growing livestock and mare/foal morbidity/mortality. Second, remove the animal from the infected field. Another choice is to treat affected animals pharmacologically. Finally, renovate the pasture to remove the E+ fescue or decrease its consumption by adding another forage to the pasture. Animals can recover from fescue toxicosis if they are removed from a diet containing E+ fescue and are placed on a diet that does not contain E+ fescue. In cattle,

blood flow to coronary bands on the forelimbs increased 8 days after animals were removed from a high endophyte infected fescue diet (Rhodes *et al.*, 1991). Pregnant mares had fewer delivery complications if removed from E+ fescue pasture prior to day 300 of gestation (Boosinger *et al.*, 1995b; Putnam *et al.*, 1990). In mares beyond their expected date of parturition, depressed prolactin levels returned to normal within 2 to 11 days after removal from E+ fescue, and the mares began to show signs of parturition (Earle *et al.*, 1990; McCann *et al.*, 1992; Youngblood *et al.*, 2004). Additionally, estrogens decreased and progestagens increased after only 7 days of removal from an E+ fescue diet (Redmond *et al.*, 1994). However, if an animal is removed from a field containing E+ fescue, a quarantine period of 3 days should be observed, due to the ability of the endophyte and tall fescue seed to survive the digestive tracts of cattle and horses (Shelby and Schmidt, 1991).

Animals that cannot be removed from the E+ fescue field can be treated by other methods. Vaccination against the lysergic ring of the ergopeptine alkaloids has been attempted (Filipov *et al.*, 1998; Hill *et al.*, 1994). However, currently vaccination shows little promise due to short lived effects of active anti-ergot alkaloid immunization in rabbits (Filipov *et al.*, 1998) or passive anti-ergot alkaloid immunization of cattle (Hill *et al.*, 1994). Estradiol 17-β implants in steers consuming E+ fescue haylage has been shown to be beneficial, with improvements in average daily gain when compared to non-implanted steers on E+ fescue (Beconi *et al.*, 1995). Ammoniation (sprayed with or soaked in 3% anhydrous ammonia) of E+ fescue hay was found to be beneficial by decreasing the ergopeptine and loline alkaloid concentrations in the hay (Roberts *et al.*, 2002; Simeone *et al.*, 1998). Improvements in prolactin serum concentrations and lower rectal temperatures were seen in cattle, and increased feed intake and weight gains were seen in rats ingested treated E+ fescue (Kerr *et al.*, 1990; Simeone *et al.*, 1998).

Another option for fescue toxicosis management is use of a dopamine antagonist. The efficacy of several medications to treat signs of fescue toxicosis have been tested, including phenothiazine, thiabendazole, reserpine, metoclopramide, sulpiride, perphenazine, acepromazine, and domperidone (Aldrich *et al.*, 1993b; Altom *et al.*, 1995; Dooley *et al.*, 1999; Evans *et al.*, 1999; Oliver *et al.*, 1992; Redmond *et al.*, 1994). Many of these were shown to be beneficial, with improvements in mammary gland

development, increased prolactin and progestagen concentrations, improved dry matter intake and body weight gain (Aldrich *et al.*, 1993b; Cross *et al.*, 1995; Jones *et al.*, 2003; Kouba *et al.*, 1995; Lipham *et al.*, 1989; Nihsen *et al.*, 2004; Redmond *et al.*, 1994). While body temperature, gestation length, and estrogens concentrations were decreased with the antagonist treatment (Cross *et al.*, 1999; Dooley *et al.*, 1999; Parish *et al.*, 2003a). However, unlike domperidone, many of these antagonists are able to cross the blood brain barrier and can result in a sedative effect or diarrhea (Bouton *et al.*, 2002; Gunter and Beck, 2004; Parish *et al.*, 2003b), making domperidone the current antagonist of choice.

The final method in the treatment and prevention of fescue toxicosis is pasture renovation. Pasture renovation can be anything that changes the plant population in a field. E- fescue can be used in place of E+ fescue, however, it lacks the hardiness and stress tolerance of E+ fescue (Aiken *et al.*, 1993). Novel endophyte infected tall fescue cultivars (NE+ fescue) breach the gap between E+ and E- fescue. NE+ fescue has decreased alkaloid concentrations while retaining the positive characteristics of E+ fescue. For example, MaxQ (Pennington Seeds, Madison GA), a NE+ fescue, has been cultivated to have low to zero levels of ergovaline. To make NE+ fescue, the endophyte producing high concentrations of ergovaline is killed and removed from the plant and is replaced with a novel endophyte that produces low to zero concentrations of ergovaline. Pastures of NE+ fescue appear to resist volunteer E+ fescue better than E- fescue (McCann *et al.*, 1991). Cattle grazing NE+ fescue had similar average daily weight gain, rectal temperatures, mean respiration rates, hair coat scores, and prolactin, cholesterol, and creatinine serum concentrations to those on E- fescue (Fuller *et al.*, 1971; Nihsen *et al.*, 2004). Lambs benefit from consuming NE+ fescue verses E+ fescue with similar prolactin and average daily weight gains as lambs on E- fescue (Fuller *et al.*, 1971).

Pasture renovations can also include the addition of another forage to dilute the current E+ fescue stand. The addition of a legume, such as white clover, *Trifolium repens*, can result in higher weaning weights and grades in beef cattle (Aiken *et al.*, 1993). In horses, however, Cross (1997) postulated that diluting with a legume will not eliminate the E+ fescue effects on horses, because horses still exhibit signs when only ingesting small quantities of E+ fescue. Supplementing yearling horses with a

concentrated grain ration did improve growth rates while they were consuming E+ fescue (Aiken *et al.*, 1993). However, a diet of 40% E+ fescue had no effect on average daily weight gain in yearling horses (McCann *et al.*, 1991). Nevertheless, the best line of defense with fescue toxicosis is complete removal of E+ fescue from the pasture.

Weeds, or any unwanted plant such as E+ fescue, can be difficult to eliminate. Typical weed control methods are biological (insects), cultural (mowing, grazing practices, or seeding), and use of herbicides (Green *et al.*, 2006). The best way to eliminate weeds from invading pastures is to develop and maintain a dense stand of desirable forages that will out compete weeds (Tu *et al.*, 2001). However, this can be difficult, especially with high stocking rates or periods of droughts. E+ fescue is frequently more resistant to biological and cultural control than other plants, leaving herbicidal use as the best option. There are approximately 945 million acres (approximately 382 million hectares) in the US devoted to agricultural usage, with 1.9 million individual farms (Aspelin, 1997). Of these farms, 1.4 million use chemicals to control insects, disease, and weeds (Aspelin, 1997). To select an appropriate herbicide several elements must be considered. These are the type of forage grown, the waiting period after application before animals can graze the treated forage, the type of weeds to eliminate, the timing of application, and the cost of treatment. With space restrictions, pasture renovations using an application of a broad-spectrum herbicide (such as Roundup®, Monsanto, a 5-enolpyruvylshikimate-3-phosphate synthase competitive inhibitor) to remove all vegetation and reseeding later, is difficult. Pasture renovations can take up to 1 to 2 years, leaving the farmer with limited grazing space for his livestock. Manufacturers such as BASF™ and DuPont™ have developed herbicides that can, under optimal conditions, suppress or kill tall fescue and several other weeds, but leave desired forages, such as Kentucky bluegrass, unharmed.

Herbicide Information

Herbicides can have different modes of action, such as mitosis inhibitors, photosynthesis inhibitors, or amino acid synthesis inhibitors (Tu *et al.*, 2001). All must be applied at a time of plant growth so that the herbicide will be taken up and translocated within the plant. The herbicide Cimarron® is manufactured by DuPont™

and has the active ingredient metsulfuron methyl (methyl 2-[[[[(4-methoxy-6-methyl-1, 3, 5-triazin-2yl) amino] carbonyl] amino] sulfonyl] benzoate); (Figure 1.11).

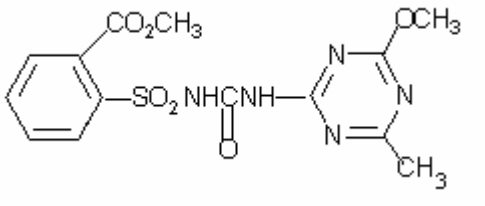


Figure 1.11. The chemical structure of metsulfuron methyl, the active ingredient in Cimarron®.

This chemical compound is a member of the sulfonylurea family, which uses the amino acid synthesis inhibitor mode of action (DuPont, 2005). Metsulfuron methyl specifically inhibits the enzyme acetolactate synthase (ALS), also referred to as acetohydroxyacid synthase. This enzyme is necessary for the formation of the branch chain amino acids leucine, valine, and isoleucine, which are required for normal plant growth (Environmental Protection Agency, 1998). Initially the plant growth is stunted, but the plant will continue to live using branch chain amino acid reserves for physiological function. However, without the proper amino acids to make required proteins the plants will begin to die. Cimarron® is labeled for use in pastures, grass hayfields, fencerows, and ungrazed cropland (Green *et al.*, 2006) and is recommended for pastures containing bluestems, indiangrass, orchard grass, switchgrass, and wheat grasses (DuPont, 2005). It is labeled for control of pre- and post-emergent Canada thistle, chickweed, dandelion, henbit, pigweed, clover, black henbane, honeysuckle, yucca, bull thistle, St. Johnswort, and poison hemlock (DuPont, 2005). Cimarron® is not actually labeled for tall fescue control; however, studies by Witt (2006) demonstrated that when Cimarron® is used at higher concentrations tall fescue will be significantly harmed. Unfortunately, one of the disadvantages of using Cimarron® is that it will severely injure or kill legumes (Green *et al.*, 2006) and stunt timothy growth (DuPont, 2005).

Currently, there are no grazing restrictions on Cimarron® since animals do not have the enzyme acetolactate synthase, thus it is believed that Cimarron® would have little effect on them. DuPont™ has conducted several studies on the safety of this product with laboratory and food animals and these studies were published by the

Environmental Protection Agency (EPA) (Environmental Protection Agency, 1998). Acute ophthalmic exposure caused irritation, blurred vision, or pain, which resolves within 72 hours. Repeated exposure to greater than 125mg of metsulfuron methyl per kg of body weight per day (mg/kg/d) caused skin irritation in rabbits, but there were no other observable effects with doses up to 2000mg/kg/d. A few studies evaluated the effects of a single exposure to high concentrations of Cimarron® or metsulfuron methyl. The single oral dose of metsulfuron methyl required to kill half of the study rats (LD50) was greater than 5000mg, where as the single topical LD50 was greater than 2000mg/kg in laboratory rabbits. There were discrepancies in studies conducted on feed intake and long-term exposure to low doses of metsulfuron methyl. Male dogs had a small depression in feed intake, but no effect on weight after 12 months. There was no effect on female dogs. In rats, feed intake and body weight were decreased over a 2-year period, but there were no effects in mice that ingested metsulfuron methyl over 18 months. Concentrations of greater than 1000 mg metsulfuron methyl per liter of culture media caused chromosome aberrations in hamster ovary cells cultured *in vitro*, but there were no significant effects in *in vivo* studies with mice.

In goats and cattle less than 0.1% of the daily dose of metsulfuron methyl was detected in milk samples (Environmental Protection Agency, 1998). Cattle exposed to metsulfuron methyl 12 hours prior to slaughter had traces of the metsulfuron methyl in their muscle. The amount was very low because approximately 70% of metsulfuron methyl was excreted in the urine and feces within 72 hours of ingestion. Cimarron®'s active ingredient had reproductive effects on laboratory animals. Laboratory rats experienced reduced parental weights and feed intake at doses of 340 to 420mg/kg/d, but fertility, litter size, pup survival, and lactation were not affected at this dose. Rat fetuses were not affected at doses up to 1000mg/kg/day of metsulfuron methyl. However, rabbits had reduced feed intake and increased maternal mortality at doses of metsulfuron methyl greater than 100mg/kg/d. No effects on the fetus were observed at higher doses (<700 mg/kg/d) (Environmental Protection Agency, 1998).

In addition to Cimarron®, another herbicide can also significantly harm fescue. Plateau®, manufactured by BASF™, contains an ammonium salt of imazapic as its

active ingredient (BASF, 2004). Imazapic is a member of the imidazolinone family (Figure 1.12) and also kills by inhibiting ALS (BASF, 2004).

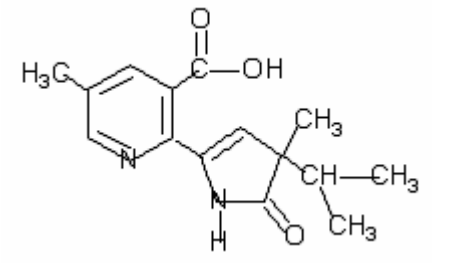


Figure 1.12. The ammonium salt of imazapic, a member of the imidazolinone family, kills by inhibiting ALS. The ammonium salt of imazapic's chemical structure is shown above.

Plateau® is marketed for use in grasslands, pastures, rangeland, and other noncrop areas and controls crabgrass, foxtail, johnson grass, timothy, ryegrass, tall fescue, and other undesirable plants (BASF, 2004). Unfortunately, Plateau® can suppress the growth of orchardgrass, Kentucky bluegrass, and bromegrass, but they will survive, as will legumes (BASF, 2004). The safety of Plateau® and its active ingredient, an ammonium salt of imazapic, underwent testing with laboratory and food animals prior to EPA approval. These studies were published by Syracuse Environmental Research Associates (2001). Ocular exposure caused irritation, but that resolved within 48 to 72 hours. The EPA registration process also required testing at extremely high oral doses. The LD50 oral dose of the ammonium salt of imazapic in rats was greater than 5000mg/kg. The LD50 for a single topical application in rabbits was greater than 5000mg/kg, but 2000mg/kg had no effect. Repeated topical exposure of 0.43g to guinea pigs was without effect. There were conflicting results with longer-term studies conducted at extremely high doses. Nevertheless, there was no significant effect in rats ingesting the ammonium salt of imazapic for 2 years, nor was there an effect in mice fed the ammonium salt of imazapic for 18 months. However, dogs ingesting an extremely high dose for 1 year had increased incidence of vomiting, decreased body weight, and decreased feed intake. In addition, these dogs had decreased hemoglobin and increased liver damage. A lower dose of the ammonium salt of imazapic only minimal skeletal muscle alternations occurred.

The ammonium salt of imazapic had no effect on fetal development in rats exposed to up to 1000 mg/kg/d for 10 days (Syracuse Environmental Research Associates, 2001). In addition, there were no effects on rat body weight, feed intake, mortality, or reproductive performance at doses up to 1200mg/kg/d for 14 weeks. However, another study reported increased incidence of maternal mortality in rabbits ingesting 700mg/kg/d or higher on days 7 through 19 of gestation, and only 40% of rabbits ingesting 700mg/kg/d survived the trial. Additionally, at a maternal intake of greater than 700mg/kg/d, embryo and fetal toxicity were reported. Finally, acute and short term exposure of 350mg/kg/d ammonium salt of imazapic to pregnant rabbits resulted in fetuses with rudimentary ribs (Environmental Protection Agency, 1999).

In conclusion, fescue toxicosis results from a multifarious relationship between the animal, the plant, the environment, and the fungus. Conditions have to be right, or wrong depending on one's point of view, for the toxicosis to occur. Proactive grazing management and herbicide use may reduce the occurrence of fescue toxicosis. Currently there are no studies published on the reproductive effects of Cimarron® or Plateau®, on livestock, specifically in the pregnant mare, necessitating the research projects reported here. The following experiments were designed to examine the effects of real world application and exposure of these herbicides to pregnant mares and E+ fescue by testing the following hypotheses:

Hypotheses

1. There is an increased incidence of fetal loss or foal mortality/morbidity in broodmares grazing pastures treated with either Cimarron® or Plateau® herbicides when compared to broodmares and their newborn foals grazing control pastures.
2. As Cimarron® or Plateau® herbicide cause the death of tall fescue, the alkaloid concentrations contained within the plant are decreased when compared to controls.

CHAPTER TWO
EFFECTS OF CONSUMING PLATEAU® OR CIMARRON® ON PREGNANT MARES

INTRODUCTION

Ingestion of tall fescue (*Festuca arundinacea* Schreb.) infected with the endophyte *Neotyphodium coenophialum* (Glen *et al.*, 1996) (E+ fescue) causes deleterious effects in livestock. With the horse, the largest impact of E+ fescue occurs in the pregnant mare. Fescue toxicosis in pregnant mares can cause prolonged gestation, altered hormonal concentrations and patterns (decreased prolactin, progestagens, relaxin, and increased estrogens in the maternal circulation), decreased mammary gland development, thickened placenta, placenta previa or "red bagging," dystocia, dysmature foals, and even foal and mare death (Altom *et al.*, 1995; Boosinger *et al.*, 1995b; Brendemuehl *et al.*, 1995; Brendemuehl *et al.*, 1996; Earle *et al.*, 1989; Monroe *et al.*, 1988; Putnam *et al.*, 1991; Redmond *et al.*, 1994; Ryan *et al.*, 2001b; Taylor *et al.*, 1985; Vivrette, 1994).

Management of pregnant mares and E+ fescue can involve removal from E+ fescue pastures. If mares are removed from E+ fescue pastures prior to day 300 of gestation they frequently foal normally (Boosinger *et al.*, 1995b; Putnam *et al.*, 1990). When beyond the expected date of parturition, increased prolactin concentrations, and signs of impending parturition have been reported within 2 to 11 days of removal from E+ fescue fields (Earle *et al.*, 1990; McCann *et al.*, 1992; Youngblood *et al.*, 2004). However, if removal to an uninfected field is not an option, medication with a dopamine antagonist, for example domperidone, can be beneficial to pregnant mares suffering from fescue toxicosis. Pregnant mares receiving domperidone had improved mammary gland development, increased prolactin, and progestagen concentrations, while gestation length, and estrogens concentrations were decreased (Cross *et al.*, 1999; Dooley *et al.*, 1999; Evans *et al.*, 1999; Evans, 2002; Kouba *et al.*, 1995; Redmond *et al.*, 1994).

Thirty-five million acres (approximately 14 million hectares) are estimated to contain tall fescue in the United States, with Kentucky alone containing 5.5 million acres (approximately 2 million hectares) (Buckner *et al.*, 1979; Lacefield *et al.*, 1993). Of this,

it is estimated that 85% of tall fescue plants are infected with the *N. coenophialum* (Lacefield *et al.*, 1993) making removal of E+ fescue from pastures with herbicides a focus for many farm owners. Herbicides fall into one of three classes based on their mechanism of action: 1) photosynthesis inhibitors, 2) mitosis inhibitors, and 3) amino acid synthesis inhibitors (Tu *et al.*, 2001). Two herbicides, Cimarron® (DuPont™) and Plateau® (BASF™), inhibit the enzyme acetolactate synthase, which is the catalyst for the production of the branch chain amino acids leucine, isoleucine, and valine (BASF, 2004; DuPont, 2005). These herbicides stunt plant growth and the plant begins to die as amino acid reserves are depleted. Both of these herbicides have undergone testing for EPA approval, which included laboratory animal testing for oral and topical toxicities, short and long term effects on feed intake, weight loss, morbidity/mortality, and reproductive effects on fetal well-being, and fetal and maternal morbidity/mortality (Environmental Protection Agency, 1998; Syracuse Environmental Research Associates, 2001). However, this testing involved only laboratory animals and high to extremely high doses of the active ingredients of Cimarron® and Plateau®. No research has been found that evaluated livestock under normal herbicide application to pastures and grazing conditions, which lead to the following research experiment. The first experiment was designed to test the hypothesis that there is an increased incidence of fetal loss or foal mortality/morbidity in broodmares grazing pastures treated with either Cimarron® or Plateau® herbicides when compared to broodmares and their newborn foals grazing control pastures.

MATERIALS AND METHODS

Pasture and Tall Fescue Analysis

The experiment was divided into 4 studies conducted over 2 years (Spring 2005, Fall 2005, Spring 2006, and Fall 2006), using 60 pregnant mares in a research protocol approved by the University of Kentucky Institutional Animal Care and Use Committee. Three large pastures, located at the University of Kentucky Veterinary Science Research Station located in Lexington, Kentucky, were divided into 6 smaller pastures (East to West), approximately 0.7 hectares each, using 2.5 cm electrical nylon tape. These 6 pastures contained plant populations of approximately 57% Kentucky bluegrass, 11% tall

fescue, and 32% of various other species of grass and weed (estimated using line transection prior to the start of the Spring 2005 study). The presence of the endophyte in tall fescue tillers in experimental pastures was determined by immunoblot assay as described by Gwinn et. al. (1991) prior to the Spring 2005 study.

Spring 2005 - Broadcast herbicide application

Eighteen multiparious broodmares were leased from a local nurse mare provider and placed onto one of the 6 pasture plots described above. Mares were assigned to pastures based on stage of gestation (early, mid, or late) and mare body type (light verses heavy) such that each plot contained mares at each stage of gestation and of each body type. Pastures were assigned to one of three treatments: Control, Cimarron®, or Plateau® (Table 2.1).

Table 2.1. Pasture assignments to treatments: Control, Cimarron®, or Plateau® (diagram not drawn to scale).

North ⟶

1A - Control - Methylated Seed Oil @ 473.0mL/0.4hectare
1B - Control - Activator 90 surfactant @ 0.25% v/v
2A – Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473.0mL/0.4hectare
2B – Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v
3A – Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473.0mL/0.4hectare
3B – Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v

Pastures assigned to be treated with Plateau® were sprayed at 295.7mL Plateau® per 0.4 hectare (10 fluid ounces per acre) (174.8g ammonium salt of imazapic per hectare). Plateau® was mixed with a methylated seed oil surfactant, resulting in a total of 94.6L (25 gallons) of the mixture applied to each of the assigned pastures at 2.1kg/cm (30 pounds per square inch) using a CO_2 pressurized plot sprayer (Figure 2.1). Cimarron® assigned pastures were sprayed at 28.4g Cimarron® per 0.4 hectare (1 weight ounce per

acre) (42.3g metsulfuron methyl per hectare). Cimarron® was mixed with a non-ionic surfactant, Activator 90, and a total of 94.6L (25 gallons) of the mixture was applied to the assigned pastures at 2.1kg/cm. The Control pastures were sprayed with either Activator 90 or the methylated seed oil mixed with water for a total of 94.6L, applied at 2.1kg/cm.

Figure 2.1. A CO_2 pressurized plot sprayer was used to apply the assigned treatments in the Spring 2005 through Fall 2006 studies.

The assigned pastures were sprayed with their respective treatments on June 9, 2005 (day 0) in the early afternoon by Dr. William W. Witt, the University of Kentucky Department of Plant and Soil Science. The treated pastures were given 20 minutes to dry and then the pregnant mares were placed on their assigned pastures to graze, with *ad libitum* access to water, salt blocks, and trace mineral blocks. After approximately 3 weeks the mares had consumed the majority of the vegetative material in the pastures and a mixed grass hay was provided. Mares and foals remained on their respective pastures until July 19, 2005 (54 days after spraying).

Pregnancies were monitored during the study using transrectal palpation and transrectal real time ultrasonography with a Pie Medical Digital Cineloop Scanner 200, using a 6 to 8 MHz linear probe. Mares were examined on study days -9, -2, 5, 12, 26,

and 40 (Figure 2.2). Stage of pregnancy, fetal movement, fetal heartbeat, and fetal fluid echogenicity were recorded on each examination day.

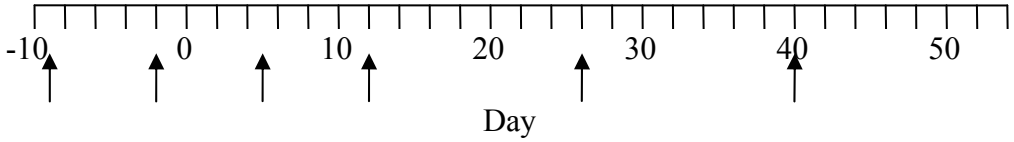


Figure 2.2. Time line of Spring 2005, with day of spraying indicated as day 0 (June 9, 2005). Arrows indicate days that mares and foals were examined and blood samples drawn. Mares and foals were removed 54 days after spraying.

Blood samples were taken from mares and any foals present on the examination days via jugular veno-puncture to evaluate blood clinical chemistry, hematology, and maternal circulating concentrations of estrogens (E), progestagens (P), and thyroid hormone (Thyroxin, "T_4"). Serum was extracted from 10mL of whole blood for blood chemistry panel and hormone concentration analyses. Serum samples were analyzed for blood urea nitrogen, calcium, phosphorus, sodium, potassium, chloride, total protein, albumin, globulin, albumin to globulin ratio, aspartate aminotransferase, creatine kinase, gamma glutamyltransferase, alkaline phosphatase, glucose, creatinine, total bilirubin, and cholesterol. Three milliliters of whole blood were mixed with ethylenediaminetetraacetic acid for hematology analysis including packed cell volume and total white blood cell count, hemoglobin content, red blood cell count, and white blood cell differential counts (segmented neutrophil, lymphocyte, monocytes, eosinophil, and basophil). Blood chemistry and the hematological analyses were performed by the University of Kentucky Livestock Disease Diagnostic Center (LDDC). Hormone concentrations (E, P, and T_4) were determined by radioimmunoassay at a commercial laboratory (Bluegrass Embryo Transplant, Lexington, Kentucky).

Four mares foaled while on study and their placentas were submitted to the LDDC for gross examination, histopathology, and bacteriology. Because the mares were leased and not owned by the University of Kentucky Department of Veterinary Science, further sampling was not possible after they were returned to their owner.

Fall 2005 - Spot spray application

For any unwanted fescue that is remaining in the fall it is often necessary to spot spray. For the second study, 12 broodmares owned by the University of Kentucky Department of Veterinary Science were used. Stages of pregnancy were estimated by transrectal palpation, ultrasonography, and knowledge of exposure to stallions to be in mid-gestation (approximately days 115 to 230 of gestation). The same pastures used in Spring 2005 were spot sprayed on October 20, 2005 (day 0), with the same treatments previously assigned. To achieve uniform application of treatments to all fields, spot spraying was performed by activating the sprayer to apply treatment for 6.1m then deactivating it for 6.1m. Mares were placed on the pasture 20 minutes following treatment application. Mares had *ad libitum* access to water, salt blocks, and trace mineral blocks throughout the study.

Fetal well-being was monitored using trans-rectal palpation and ultrasonography on study day -1, 0, 1, 6, 13, and 32 (Figure 2.3). When mares had consumed the majority of the vegetation in the pastures (approximately 3 weeks post spraying), a mixed grass hay was provided. On examination days, blood samples were collected for blood chemistry panel, hematology panel, and hormone concentrations, as described previously. When mares foaled the following spring, placentas were submitted to the LDDC for gross examination, histopathology, and bacteriology the day after parturition. Additionally, a postpartum blood sample was taken from the mare and from the foal within 1 week following parturition.

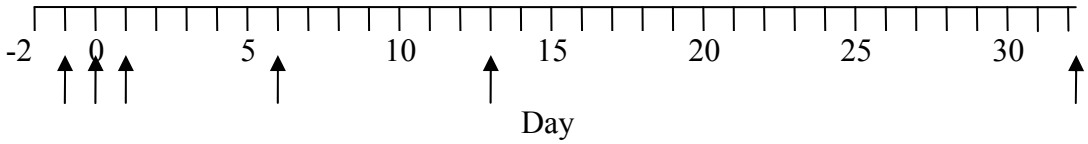


Figure 2.3. The time line for Fall 2005. Days are labeled relative to the day of spraying (October 20, 2005 = day 0); arrows indicate days of examination and blood sample collection. Mares were removed from the pastures on day 32 post spraying.

Spring 2006 - Broadcast herbicide application

The protocol for the Spring 2006 study was a replicate of the Spring 2005 study with some modifications. Eighteen multiparious mares in late gestation were leased from

the nurse mare provider (different mares from the Spring 2005 study). The same paddocks used in Study 1 and 2 were used again. For the Spring 2006 study the entire paddocks were again sprayed with the same treatments as used in Spring 2005 (Table 2.1). These treatments were applied on May 16, 2006 (day 0). Mares were placed on assigned pastures 20 minutes following treatment application. Mares had *ad libitum* access to water, salt blocks, and trace mineral blocks throughout the study.

Fetal well-being was monitored via transrectal palpation and ultrasonography and blood samples were taken on days -8, -5, -1, 2, 7, 14, 21, 28, and 35 of study relative to the spray date, May 16, 2006 (Figure 2.4). Following these examinations of all mares and foals present, the pregnant mares were examined every two weeks (day 49, 63, 77, 91, 106, 120, 134, and 148) and blood samples were taken on days 49, 91, 120, 134, and 148 for blood chemistry panels, hematology panels, and hormone concentration assays. Mares with foals at their sides were examined and blood samples were taken on days 42, 63, 127, and 155.

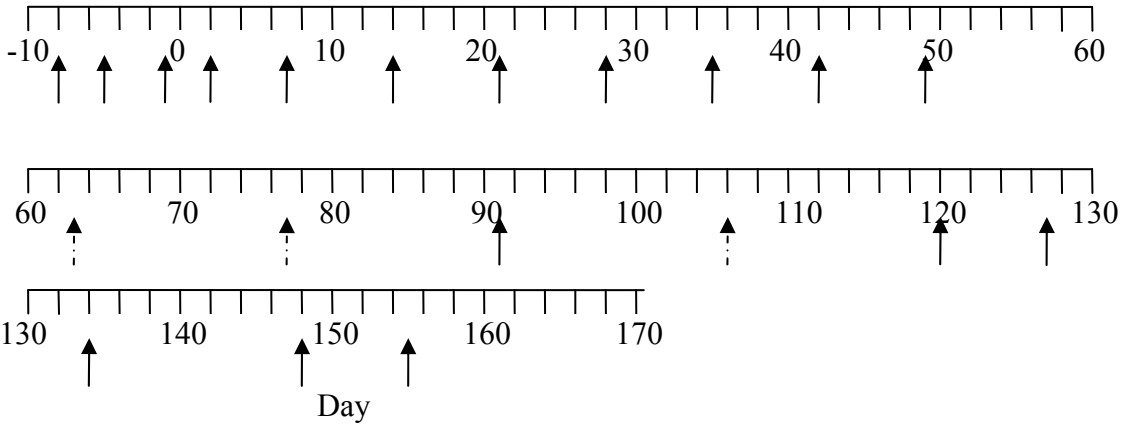


Figure 2.4. The time line for Spring 2006. Days are labeled relative to the spraying date of May 16, 2006 (day 0). Solid arrows indicate examination and blood sample collection days. Dashed arrows indicate days where only examinations were performed. Mares were removed from the pastures on day 28 post spraying.

After nearly 3 weeks of study, the majority of the vegetation in the pastures treated with herbicides had been consumed and a mixed grass hay was then provided to all mares and foals. All mares foaled while on study and placentas were submitted to the LDDC for gross examination, histopathology, and bacteriology.

Fall 2006 - Spot spray application

The Fall 2006 study was a replicate of the Fall 2005 study with some minor modifications. Twelve mares at mid-gestation from the University of Kentucky Department of Veterinary Science broodmare herd were used. After three previous applications of herbicides, the plant population had changed in several of the pastures. To keep vegetation species consistent across study pastures, the treatment map was altered. In the Spring 2005 through Spring 2006 studies, the original large fields were each divided in half in an east to west direction. In the Fall 2006 study the large fields were divided in north to south (Table 2.2) The 6 pastures were spot sprayed as described in Fall 2005 on September 27, 2006. Mares were placed on the pasture 20 minutes following treatment application and had *ad libitum* access to water, salt blocks, and trace mineral blocks throughout the study. Once pregnant mares had consumed the majority of the vegetation in the pastures (approximately 3 weeks post spraying) a mixed grass hay was provided.

Table 2.2. Pasture assignments to treatment, Fall 2006: Control, Cimarron®, or Plateau® (diagram not drawn to scale).

North ⟶

1A – Control – Methylated Oil @ 473mL/0.4 hectare	**1B** - Control - Activator 90 surfactant @ 0.25% v/v
2A – Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v	**2B –** Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473mL/0.4 hectare
3A – Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473mL/0.4 hectare	**3B –** Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v

Mares and fetuses were monitored by transrectal palpation and ultrasonography on days -7, 0, 1, 7, 14, 21, 28, 35, and 42 (Figure 2.5). Blood samples were taken on the examination days. Blood chemistry panels, hematology panels, and hormone concentration assays were performed as described in Spring 2005. Mares were

monitored monthly until parturition and at that time blood samples were taken from the mares and foals. Placentas were submitted to the LDDC for gross examination, histopathology, and bacteriology.

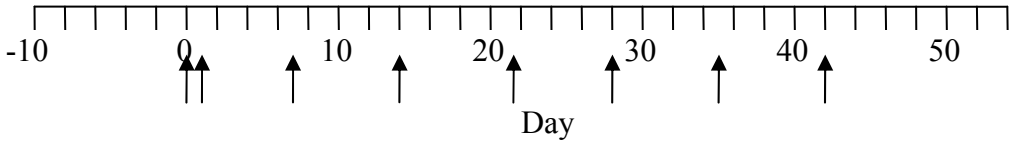


Figure 2.5. The time line for the Fall 2006 study with examination days indicated by arrows. Days are labeled relative to the spraying date of September 27, 2006 (day 0). Mares were removed from the pastures on day 54 of study.

STATISTICAL ANALYSIS

Experiment 1: All Combined

Data collected over the four studies (Spring 2005, Fall 2005, Spring 2006, and Fall 2006) in Experiment 1 were combined for statistical analyses. To make comparisons across all four studies, only samples collected the week prior to herbicide application and samples collected six weeks following herbicide application were used, such that the data analyzed was from weeks 0, 1, 2, 3, 4, 5, and 6. Because of the high degree of variability among mares in the pre-study samples data were normalized to the pre-study sample, by subtracting the pre-study sample from all subsequent samples. Such that an increase from the pre-study sample would be a positive number, while a decrease would be negative. The normalized data were analyzed via the Mixed Procedure of SAS (2006) for the effects of treatment (Cimarron®, Control, and Plateau®) and time (weeks 0, 1, 2, 3, 4, 5, and 6) and the interaction of treatment by time. Least square means were calculated for all parameters and their differences were examined. If differences in individual parameters were detected using the combined data, the studies were then analyzed separately to determine if differences observed with the combined data were consistent across studies.

RESULTS

Foaling

Of the 60 mares in Experiment 1, the foaling status was known for 46 mares. The remaining mares were returned to their owner prior to foaling. Of the 46 mares, 40 produced normal singleton foals, and all mares foaled unattended. In the Fall 2005 study, two mares aborted their foals on January 11, 2006 and May 5, 2006, respectively. One mare had villous atrophy of the placenta (Plateau® treated pasture); while the second had chronic-active placentitis (Cimarron® treated pasture). One mare, from the Fall 2005 study, died from apparent dystocia on April 21, 2006 (Control – Activator 90 surfactant treated pasture). In the Fall 2006 study, one mare experienced an idiopathic abortion on January 17, 2007 (Plateau® treated pasture). Finally, two mares from the Fall 2006 study died, the first on April 28, 2007 from trauma resulting in a fractured pelvis and left femur, uterine laceration and tearing, and hemoperitoneum (Cimarron® treated pasture). The second mare died on May 22, 2007 with uterine and rectal prolapse and hemorrhage (Plateau® treated pasture).

Blood Analysis

Statistical analyses for blood chemistry panel, hematology blood panel, and hormone concentrations are reported in Tables 2.3, 2.4, and 2.5 respectively.

Table 2.3. Chemistry blood panel statistical analysis using the combined data for weeks 0, 1, 2, 3, 4, 5, and 6 examining the effects of treatment (trt), week, and the interaction of treatment and week.

Variable	Effect	Pr > F	Variable	Effect	Pr > F
Blood Urea Nitrogen	Trt	0.0690	Albumin to Globulin Ratio	Trt	0.8390
	Week	<0.0001		Week	<0.0001
	Trt*week	0.2814		Trt*week	0.9336
Calcium	Trt	0.8157	Aspartate Aminotransferase	Trt	0.4600
	Week	<0.0001		Week	0.0061
	Trt*week	0.2191		Trt*week	0.4195
Phosphorus	Trt	0.9055	Creatine Phosphokinase	Trt	0.9578
	Week	0.0009		Week	0.1480
	Trt*week	0.9093		Trt*week	0.3415
Sodium	Trt	0.7581	γ Glutamyl Transferase	Trt	0.2652
	Week	0.0269		Week	<0.0001
	Trt*week	0.4593		Trt*week	0.8787
Potassium	Trt	0.2597	Alkaline Phosphatase	Trt	0.4848
	Week	0.0016		Week	<0.0001
	Trt*week	0.4471		Trt*week	0.6260
Chloride	Trt	0.8399	Glucose	Trt	0.9974
	Week	0.0044		Week	<0.0001
	Trt*week	0.3195		Trt*week	0.9106
Total Protein	Trt	0.2667	Creatinine	Trt	0.2458
	Week	<0.0001		Week	<0.0001
	Trt*week	0.7945		Trt*week	0.0003
Albumin	Trt	0.3433	Total Bilirubin	Trt	0.0835
	Week	<0.0001		Week	<0.0001
	Trt*week	0.9657		Trt*week	0.0737
Globulin	Trt	0.1781	Cholesterol	Trt	0.2426
	Week	<0.0001		Week	<0.0001
	Trt*week	0.2767		Trt*week	0.5599

Table 2.4. Hematology blood panel statistical analysis using the combined data for weeks 0, 1, 2, 3, 4, 5, and 6 examining the effects of treatment (trt), week, and the interaction of treatment and week.

Variable	Effect	Pr > F	Variable	Effect	Pr > F
Packed Cell Volume	Trt	0.9993	Lymphocyte	Trt	0.8135
	Week	<0.0001		Week	<0.0001
	Trt*week	0.3512		Trt*week	0.3250
White Blood Cell	Trt	0.9578	Monocyte	Trt	0.7474
	Week	<0.0001		Week	0.0040
	Trt*week	0.3844		Trt*week	0.9334
Hemoglobin	Trt	0.9547	Eosinophil	Trt	0.2019
	Week	<0.0001		Week	<0.0001
	Trt*week	0.2284		Trt*week	0.7896
Red Blood Cell	Trt	0.9819	Basophil	Trt	0.5722
	Week	<0.0001		Week	0.1166
	Trt*week	0.2310		Trt*week	0.9426
Neutrophils	Trt	0.6111			
	Week	0.0872			
	Trt*week	0.4804			

Table 2.5. Hormone concentration assay statistical analysis using the combined data for weeks 0, 1, 2, 3, 4, 5, and 6 examining the effects of treatment (trt), week, and the interaction of treatment and week.

Variable	Effect	Pr > F
Estradiol	Trt	0.8475
	Week	0.0911
	Trt*week	0.4391
Progesterone	Trt	0.8667
	Week	0.2793
	Trt*week	0.4501
Thyroid	Trt	0.7715
	Week	<0.0001
	Trt*week	0.2848

There were differences by week in most of the parameters assayed. However, of all the analyzed blood components only creatinine differed among treatment by week interactions (P=0.0003). Mares grazing Cimarron® treated pastures had elevated creatinine levels compared to mares grazing Plateau® treated (P=0.0030) or Control (P<0.0001) pastures. Time was also found to be different overall (P<0.0001). When the

four studies were analyzed separately, only in Spring 2006 was there a treatment by week interaction for creatinine (P = 0.0134; Table 2.6 and Figures 2.6). When least square means differences were examined, the observed treatment time interaction could be attributed largely to time's influence where samples from weeks 1, 2, 3, 4, 5, and 6 were different overall (P <0.0001). Total bilirubin approached a treatment by time interaction significance at P=0.0737, but when the individual studies were examined this difference was detected only in Spring 2005 (P=0.387; Table 2.7 and Figure 2.7).

Table 2.6. Creatinine analyses for combined and individual studies. Spring 2006 was the only study with a significant treatment by week interaction.

	Combined	**Spring 2005**	**Fall 2005**	**Spring 2006**	**Fall 2006**
Effect	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F
Trt	0.2458	0.5099	0.8358	0.1332	0.0636
Week	<0.0001	<0.0001	0.0015	<0.0001	<0.0001
Trt*week	0.0003	0.9758	0.6528	0.0134	0.3156

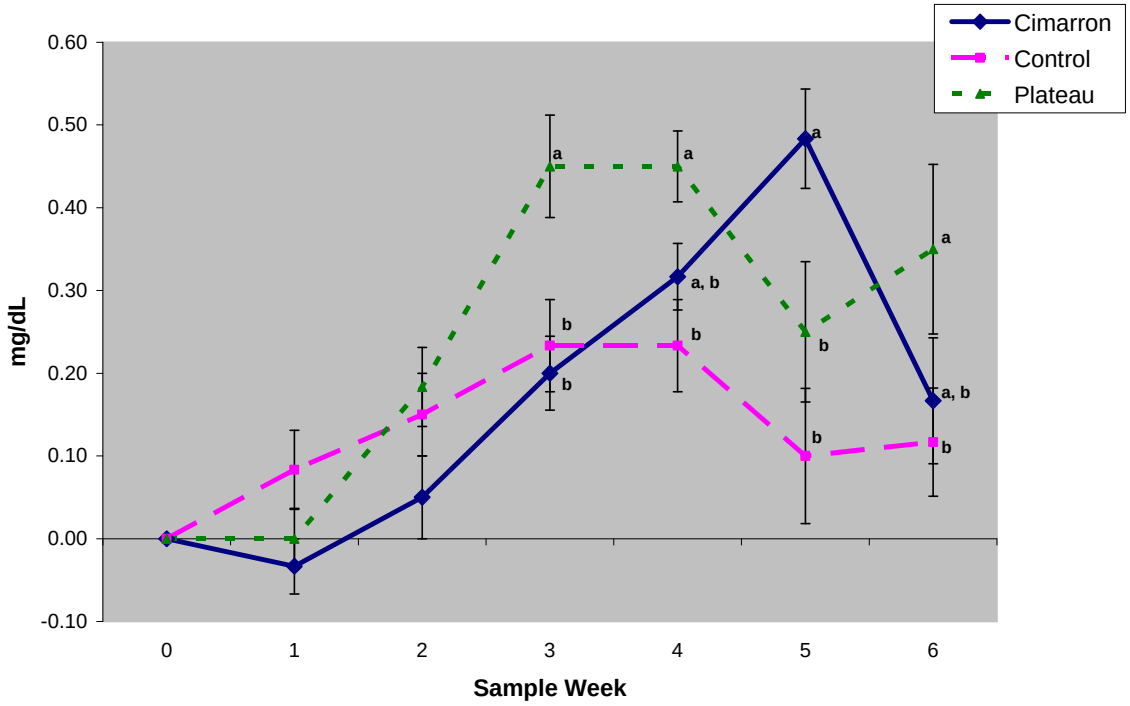


Figure 2.6. Creatinine for Spring 2006 normalized data (means and SEM). There was a difference in week (P < 0.0001) and in the treatment week interaction (P = 0.0134). Treatment differences within each time point are indicated by different letters (P < 0.05).

Table 2.7. Total bilirubin analyses for combined and individual studies. Spring 2005 was the only study with a significant treatment by week interaction.

	Combined	Spring 2005	Fall 2005	Spring 2006	Fall 2006
Effect	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F
Trt	0.0835	0.1797	0.8576	0.2095	0.5541
Week	<0.0001	0.0001	0.0265	0.0007	0.0031
Trt*week	0.0737	0.0387	0.6215	0.5064	0.8543

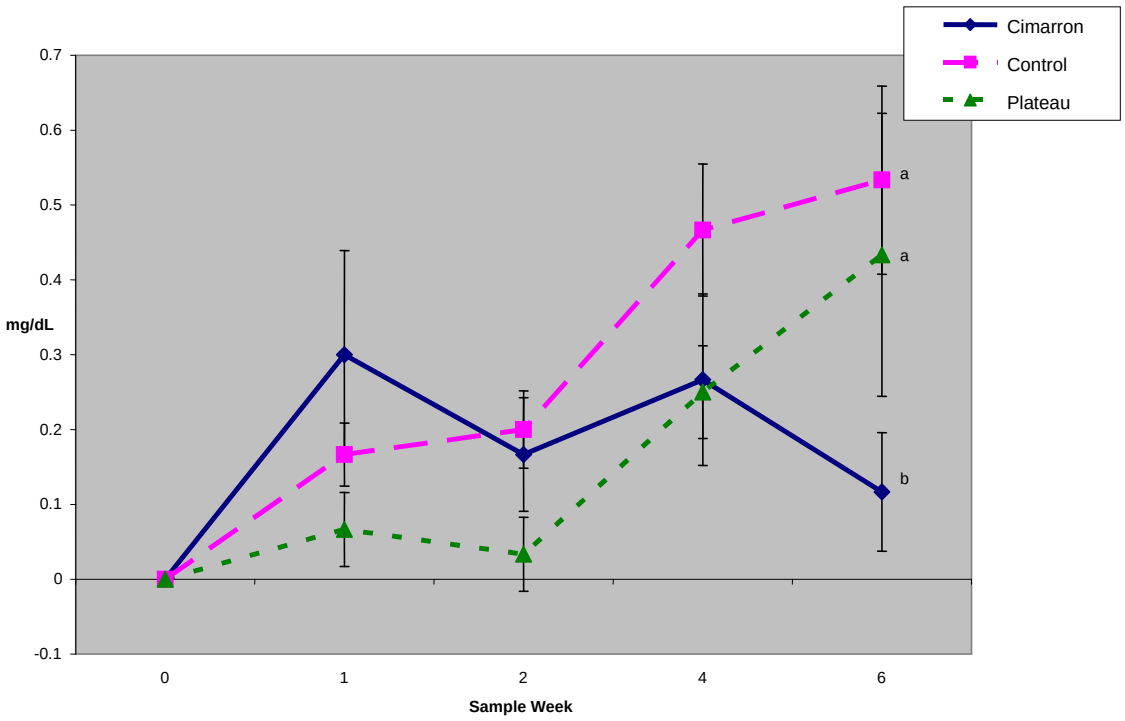


Figure 2.7. Total bilirubin for Spring 2005 (means and SEM). There was a difference in week (P = 0.0001) and in the treatment week interaction (P = 0.0387). Treatment differences within each time point are indicated by different letters (P < 0.05).

DISCUSSION

The very small amount of tall fescue in the pastures allowed the assumption that differences among treatments were a result of the herbicide treatments and not from fescue toxicosis. Regarding abortions and mare deaths, since these occurred in all treatment groups approximately 83 to 237 days post herbicide application it can be concluded that the herbicides were not the cause. Sampling time was found to be different for 25 of 30 blood components (Tables 2.3, 2.4, and 2.5). This significance of

time in the combined normalized data analysis was not a surprise. Concentrations of blood components varied from mare to mare and from day to day. Mare's blood components can be affected by numerous factors including diet, stress or exercise, pregnancy, stage of estrous cycle, medication (Ginther, 1992; Lees *et al.*, 1983; Marlin *et al.*, 2002; Passantino *et al.*, 2005; Zeyner *et al.*, 2006), and numerous other factors. The only blood component with a significant treatment by time interaction was creatinine, though total bilirubin approached significance. Creatinine is a by product of muscle metabolism, where creatine or phosphorylcreatine are non-enzymatically converted to creatinine within the muscle (Wyss and Kaddurah-Daouk, 2000). The observed treatment by time interaction could be attributed largely to the influence of time. Creatinine and blood urea nitrogen (BUN) are both measures of kidney function and alterations to their blood concentrations can indicate kidney dysfunction (Meyer and Harvey, 2004). The kidneys are also involved in the homeostasis of sodium, calcium, and potassium (Meyer and Harvey, 2004). However, in this experiment BUN, sodium, potassium, and calcium were not affected by treatment. Thus, the interaction of time and treatment in creatinine concentrations can be attributed to time, since the kidneys were functioning normally.

Trends of the plotted normalized data for creatinine for all treatments behaved in a similar manner until sample week 5 where horses on Cimarron® treated pastures had an increase in creatinine, while those on Control and Plateau® treated pastures were decreased. The increase was traced back to samples taken from mares on Cimarron® treated pastures in the Spring 2006 study. However, by sample week 6 in Spring 2006 there was no difference between Cimarron® and Control mares (Figure 2.6). Regardless of the treatment time interaction in the combined and Spring 2006 normalized data, creatinine raw concentrations remained within the normal physiological range (0.5 - 2.0mg/mL; Duncan and Prasse, 1986).

Bilirubin is the product of natural hemoglobin breakdown that is removed from the blood by the liver. An increase in bilirubin could indicate liver dysfunction, however no treatment effect was observed (P=0.0835) in Experiment 1 (Thompson, 2007). Other indicators of liver function include alkaline phosphatase and gamma glutamyl transferase, neither of which were effected by the herbicide treatments (P=0.4848 and P=0.2652

respectively) and as such the near significant interaction of time and treatment for total bilirubin (P=0.0737) can largely be attributed to time (P<0.0001). When the studies were analyzed individually the only treatment by time interaction occurred in Spring 2005. Differences in Spring 2005 occurred only in week 6 where Cimarron differed from Control and Plateau (P=0.0013 and P=0.0134 respectively). Regardless of the treatment by time interaction in the Spring 2005 study, total bilirubin raw concentrations remained within the normal physiological range (0.2 - 5.0mg/mL; Duncan and Prasse, 1986).

The general lack of effect of the herbicide on mare health can be attributed to the lack of enzyme acetolactate synthase (ALS) in mammals. This enzyme is only found in plants, which is why ALS inhibition by the herbicides did not affect mare or foal health or pregnancy maintenance. Because the mares consumed all palatable plant material in treated pastures with 3 to 4 weeks post spraying, we can conclude that they consumed all applied herbicides. Environmental Protection Agency testing of these herbicides or their active ingredients was conducted at very high to extremely high concentrations. The lowest concentration used was 100mg metsulfuron methyl per kg of body weight per day for Cimarron® and 350mg ammonium salt of imazapic per kg of body weight per day for Plateau® (Environmental Protection Agency, 1998; Environmental Protection Agency, 1999). However, in real world application the exposure concentrations are very low. Mares on this experiment were exposed to only 174.8g of ammonium salt of imazapic (Plateau®'s active ingredient) per hectare or 42.3g of metsulfuron methyl (Cimarron®'s active ingredient) per hectare. Mares consumed approximately 5.1mg per kg of body weight per day (mg/kg/d) of ammonium salt of imazapic or 1.25mg/kg/d of metsulfuron methyl in the Fall studies and 3.4mg/kg/d of ammonium salt of imazapic or 0.83mg/kg/d of metsulfuron methyl® in the Spring studies, which was vastly different from prior testing in small laboratory animals (see Appendix for calculations).

In conclusion, Cimarron® and Plateau® did not alter broodmare blood concentrations out of physiological range and did not affect fetal loss or foal mortality/morbidity in broodmares grazing pastures treated with these herbicides when compared to those broodmares grazing control pastures. However, it is well known that alkaloids in E+ fescue cause reproductive problems in broodmares, but the effects of these herbicides on the alkaloid levels in E+ fescue is not known.

CHAPTER THREE
EFFECTS OF CIMARRON® AND PLATEAU® ON ALKALOID CONTENT OF ENDOPHYTE INFECTED TALL FESCUE

INTRODUCTION

Tall fescue (*Festuca arundinacea* Schreb.) is a seed propagated, perennial, cool season bunch grass. It was imported into the United States in the 1800's (Buckner *et al.*, 1979) and in 1931 a specific ecotype, later named Kentucky 31 (KY31), was discovered in Menifee County, Kentucky. KY31 was released by the University of Kentucky in 1943 and quickly spread due to its dependability, adaptability, grazability, and palatability to livestock (Buckner *et al.*, 1979). In 1940 it was estimated that tall fescue inhabited 40,000 acres (16,187 hectares); however, by 1973 it inhabited approximately 35 million acres (approximately 14 million hectares) in the United States (Buckner *et al.*, 1979). Shortly after its release the benefits of tall fescue began to be overshadowed by the detrimental effects it caused in livestock. Lameness, decreased feed intake, and decreased average daily gain were reported in sheep and cattle (Gallagher *et al.*, 1966; Hoveland *et al.*, 1983; Howard *et al.*, 1992; Jensen *et al.*, 1956; Peters *et al.*, 1992; Sampson, 1933). In 1977, Charles Bacon and coworkers first associated these deleterious effects with tall fescue infected with the endophyte *Epichloe typhina* (Sampson, 1933). The endophyte was later renamed *Neotyphodium coenophialum* (Glen *et al.*, 1996).

This endophyte was found to enhance tall fescue's ability to thrive in numerous soil and climate types, to endure water logging, and to grow at lower temperatures than other cool season grasses (Buckner *et al.*, 1979; Burns and Chamblee, 1979). However, it has been estimated that endophyte infected tall fescue (E+ fescue) consumption results in an estimated annual loss of $600 million dollars to the cattle industry due to decreased weight gains (Jones *et al.*, 2003). Additionally, E+ fescue consumption by pregnant mares can lead to increased gestation length, dystocia, increased foal and mare mortality, altered hormone concentrations, and agalactia (Cross *et al.*, 1995).

N. coenophialum is located intercellularly enabling it to take nutrients from the plant (Bacon *et al.*, 1977; Christensen and Voisey, 2007). The endophyte does not sexually reproduce; instead, it passes to the next plant generation asexually via the plant

seed. The endophyte in the seed invades seedling within 2 days of germination. Once mature, the E+ fescue plant can out compete non-infected tall fescue (E- fescue) and other grass species by "enhanced drought tolerance, increased tillering and growth, and increased resistance to herbivory from mammals and insects" (Bacon, 1994; Bacon and Siegel, 1988; Marks *et al.*, 1991).

Alkaloids (amines made by the plant and/or the endophyte) in E+ fescue cause deleterious signs in animals associated with fescue toxicosis. Four types of alkaloids are found in E+ fescue: ergopeptines, lysergic acid and its derivatives, pyrrolizidines, and diazaphenanthrenes. Of these, ergopeptines have received the most attention for the negative animal responses observed. They have been shown *in vitro* to cause decreased prolactin serum concentrations in rats (Strickland et al., 1992). Infusions of ergopeptine mixtures caused reduced reticulorumenal contractions in sheep (McLeay and Smith, 2006). Ergovaline represents 90% of the total ergopeptines in E+ fescue (Lyons *et al.*, 1986), and it binds to D_2-dopamine receptors and initiates cAMP production (Larson *et al.*, 1995). Intravenous injection of ergovaline into mature geldings caused excessive sweating, prostration, and difficulty in urination (Bony *et al.*, 2001). Rats ingesting E+ fescue seeds containing 4100µg ergovaline per kg of dry matter had decreased expression of genes involving energy metabolism (ATP synthase), growth (insulin like growth factor 1), and immune function (interferon beta 1) compared to rats on E- fescue seed (Settivari *et al.*, 2006).

Other alkaloids, in addition to the ergopeptines, may be responsible for the signs of fescue toxicosis. Lysergic acids were shown to have a greater transport potential across sheep rumen and omasum *in vitro* than the ergopeptine alkaloids (Hill *et al.*, 2001). In *in vitro* studies, lysergic acid amides caused vasoconstriction of equine dorsal metatarsal arteries and lateral saphenous veins, and bovine pedal veins, lateral saphenous veins, and dorsal metatarsal arteries (Abney et al., 1993; Klotz et al., 2007a; Klotz et al., 2008; Oliver et al., 1992; Oliver et al., 1993; Solomons et al., 1989).

Pyrrolizidines, commonly referred to as the lolines, are another class of alkaloids, but they are not as toxic as the ergopeptines. Although N-formylloline (NFL) and N-acetylloline (NAL) (specific lolines) do not affect cAMP production at D_2 dopamine receptors (Larson *et al.*, 1999), they did cause vasoconstriction of the equine lateral

saphenous vein *in vitro* (Abney *et al.*, 1993). However, when NAL was tested *in vitro* it did not elicit vasoconstriction in equine dorsal metatarsal arteries (Abney *et al.*, 1993). Nor did *in vitro* studies with NAL or NFL result in vasoconstriction of bovine dorsal pedal veins (Solomons et al., 1989). However, rats had the greatest decrease in weight gain when ingesting diets containing high amounts of NAL and ergot alkaloid (Jackson *et al.*, 1996). Cattle ingesting tall fescue seed containing high concentrations of lolines had decreased feed intake, weight loss, and increased rectal temperatures when compared to controls ingesting endophyte free tall fescue seed (Jackson, Jr. *et al.*, 1984).

The final class of alkaloids is diazaphenanthrene, namely perloline, which is produced by the tall fescue plant not the endophyte. Perloline is found in both non-infected and infected tall fescue and can inhibit "*in vitro* ruminal cellulose digestion, production of fatty acids, and growth of steer ruminal cellulytic bacteria" at concentrations greater than 10^{-4}M (Bush et al., 1970), and reduced apparent crude protein and cellulose digestibility in lambs *in vivo* (Boling *et al.*, 1975).

Consumption of E+ fescue causes fescue toxicosis, regardless of which alkaloids are involved. Management, treatment, and prevention of these deleterious effects are paramount for livestock owners. Treatment options include removal of the animal from E+ fescue containing fields or treatment with dopamine antagonists. However, these are only short term options. Pasture renovations with either dilution of the tall fescue stand or complete removal of tall fescue are the most plausible options for long term management. Dilution with a legume, such as white clover (*Trifolium repens*), can result in higher weaning weights and grades in beef cattle (Aiken *et al.*, 1993). In horses, however, Cross (1997) postulated that diluting with a legume will not eliminate the E+ fescue effects on horses, because horses still exhibit signs when only ingesting small quantities of E+ fescue. The best line of defense with fescue toxicosis, therefore, is complete removal of E+ fescue from the pasture.

Weeds, or any unwanted plant, can be difficult to eliminate. Typical weed control methods are biological (insects and livestock), cultural (mowing, grazing practices, or seeding), and use of herbicides (Green *et al.*, 2006). The best way to eliminate weeds from invading pastures is to develop and maintain a dense stand of desirable forages that will out compete weeds (Tu *et al.*, 2001). However, this can be difficult, especially with

high stocking rates or periods of drought. E+ fescue is frequently more resistant to biological and cultural control than other plants, leaving herbicidal use as the best option. To select an appropriate herbicide, several elements must be considered: the type of forage grown, the waiting period after application before animals can graze the treated forage, the type of weeds to eliminate, the timing of application, and cost of treatment. When under space restriction, pasture renovations using an application of broad-spectrum herbicide (such as Roundup®, Monsanto, a 5-enolpyruvylshikimate-3-phosphate synthase competitive inhibitor) to remove all vegetation and reseeding later, is difficult. Pasture renovations can take 1 to 2 years, leaving the farmer with limited grazing space. BASF™ and DuPont™ have developed herbicides that can, under optimal conditions, suppress or kill tall fescue and several other weeds, but leave desired forages, such as Kentucky bluegrass, unharmed.

The herbicide Cimarron® is manufactured by DuPont™ and has the active ingredient metsulfuron methyl (methyl 2-[[[[(4-methoxy-6-methyl-1, 3, 5-triazin-2-YL) amino] carbonyl] amino] sulfonyl-benzoate). This chemical compound is a member of the sulfonylurea family, which causes plant death by inhibiting branch chain amino acid production (DuPont, 2005). Metsulfuron methyl specifically inhibits the enzyme acetolactate synthase (ALS), also referred to as acetohydroxyacid synthase, which is necessary for the formation of leucine, isoleucine, and valine, which are required for plant growth (Environmental Protection Agency, 1998). Initially after Cimarron® application, the plant growth is stunted. However, the plant will continue to live using branch chain amino acid reserves for energy supplies. Once these reserves are depleted, the plant begins to die.

Cimarron® is labeled for use in pastures, grass hayfields, fencerows, and ungrazed cropland, and is recommended for pastures containing bluestems, indiangrass, orchard grass, switchgrass, and wheat grasses (DuPont, 2005; Green *et al.*, 2006). It is labeled for control of pre- and post-emergent Canada thistle, chickweed, dandelion, henbit, pigweed, clover, black henbane, honeysuckle, yucca, bull thistle, St. Johnswort, and poison hemlock (DuPont, 2005). Cimarron® is not labeled for tall fescue control; however, studies by Witt (2006) found that at higher concentrations tall fescue will be significantly harmed. Unfortunately, a disadvantage of using Cimarron® is that it will

severely injure or kill legumes and stunt timothy growth (DuPont, 2005; Green *et al.*, 2006).

The other herbicide used in these experiments was Plateau®, manufactured by BASF™. Plateau® contains an ammonium salt of imazapic as its active ingredient, is a member of the imidazolinone family, and kills by inhibiting ALS (BASF, 2004), as does Cimarron®. Plateau® is marketed for use in grasslands, pastures, rangeland and other noncrop areas and controls crabgrass, foxtail, johnson grass, timothy, ryegrass, tall fescue, and other undesirable plants (BASF, 2004). Unfortunately, Plateau® can suppress the growth of orchardgrass, Kentucky bluegrass, and bromegrass, but they will survive, as will legumes (BASF, 2004).

The concentrations of the alkaloids contained within E+ fescue are not static. The activity of the endophyte increases as the activity of the plant increases. For example, spring time growth of the plant is followed by an increase in ergovaline concentrations (Rottinghaus *et al.*, 1991). Loline alkaloids tend to increase when soil water is decreased, for example in the dry hot summer (Belesky *et al.*, 1989). Fertilization of fields also influences alkaloid concentrations. Increasing nitrogen fertilization can increase ergovaline concentrations (Rottinghaus *et al.*, 1991). However, excessive phosphorus fertilization can decrease ergovaline concentrations (Malinowski *et al.*, 1998). Stress such as a drought can affect ergovaline concentrations. When E+ fescue grown in a greenhouse was subjected to water stress, ergovaline increased (Arachevaleta *et al.*, 1992). Also, mowing at lower heights and more frequently can reduce ergovaline concentrations (Salminen *et al.*, 2003; Salminen and Grewal, 2002).

Alkaloid concentrations are dynamic and can be influenced by many factors, but the effect of Cimarron® and Plateau® on the alkaloids in E+ fescue is currently unknown. Based on the results from Experiment 1 farm owners may be willing to utilize these herbicides to control tall fescue. However, if the herbicides are not a threat to pregnant mares, could the alkaloids in the E+ fescue plant be as it is dying? This question led to Experiments 2 and 3. Experiment 2 was designed to examine the concentrations of ergovaline/ergovalinine (total ergovaline), NAL, NFL, N-acetylnorloline (NANL), and lysergic acids in E+ fescue following the application of either Cimarron® or Plateau® under greenhouse conditions. Experiment 3 was designed

to examine the effects of Cimarron® and Plateau® on the same alkaloids under field conditions. Both experiments were designed to test the hypothesis: as Cimarron® or Plateau® herbicide cause the death of tall fescue, the alkaloid concentrations contained within the plant are decreased when compared to controls.

MATERIALS AND METHODS

Experiment 2: Herbicide Greenhouse Spring 2006

To determine the effect of the herbicides, Plateau® and Cimarron®, on the alkaloid content of the plant post spraying the following experiment was performed. Fifty-two, 20.3cm diameter plastic pots of tall fescue infected with the endophyte *Neotyphodium coenophialum* were used in an environmentally controlled greenhouse (Greenhouse 2 Zone 5) located on the southern end of the University of Kentucky's campus (Lexington, Kentucky). The greenhouse temperature was held at 18.3 to 21.1ºC at night and 23.9 to 26.7ºC during the day. Pots were numbered 1 through 52 and were filled with Pro-mix potting soil. On February 7, 2006, six tillers of E+ fescue were placed in each pot. Glen Weinberger, University of Kentucky Department of Plant and Soil Sciences, watered all pots daily, fertilized with Peters 20-10-20 (commercial fertilizer containing 20% nitrogen, 10% phosphorus, and 20% potassium) 3 times a week, and applied a general systemic insecticide (Marathon) after the initial potting. To promote continuous growth of the plant during the study, emerging inflorescences (seed heads) were removed, as were any other plant species that were growing in the pots. After 7 weeks of growth, the plants were cut to 20.3cm in height from the soil surface (on March 27, 2006) to simulate farm mowing conditions. Clippings from each pot were placed in individually labeled paper bags for alkaloid analysis. E+ fescue pots were randomly assigned to table location (Section A, B, C, or D), treatment (Cimarron®, Control, or Plateau®), and sampling day (0, 7, 14, 21, or 28) (Figure 3.1, Table 3.1). This resulted in 1 pot from each treatment, within each section, being sampled every 7 days (Table 3.2). Pots were arranged in rows of 4 to 5 pots in 3 columns within each table section (Table 3.2).

Figure 3.1. Experiment E+ fescue pots were randomly assigned to one of four sections on the table (A, B, C, or D) in the greenhouse.

Table 3.1. Each pot was randomly assigned to 1 of 3 treatments (Cimarron®, Control, or Plateau®) within each section. Within each section and treatment 1 pot was sampled every 7 days (7, 14, 21, 28), such that each pot was sampled only once. This resulted in 16 pots sprayed with Plateau®, 16 pots sprayed with Cimarron® and 20 pots of Control (not sprayed).

	Day	Number of pots
Control	0	4
	7	4
	14	4
	21	4
	28	4
Plateau	7	4
	14	4
	21	4
	28	4
Cimarron	7	4
	14	4
	21	4
	28	4

Table 3.2. Pots of E+ fescue were arranged in rows of 4 to 5 pots and 3 columns within each table section. Pots were randomly assigned to 1 of 3 treatments: Cimarron® (Cim), Control (Con), or Plateau® (Plat). Within each of these treatments 1 pot was sampled every 7 days (0, 7, 14, 21, or 28 relative to spraying day), such that each pot was sampled only once. (Not drawn to scale).

Section A			**Section B**			**Section C**			**Section D**			
Con 7		Plat 28	Cim 7		Cim 28	Con 21		Con 0	Plat 28		Con 21	North
Cim 14	Plat 14	Con 28	Con 14	Con 0	Con 28	Cim 14	Plat 7	Con 14	Plat 21	Cim 21	Con 7	↓
Con 0	Cim 7	Plat 7	Con 7	Con 21	Plat 28	Con 28	Con 7	Cim 28	Con 14	Plat 7	Cim 14	
Plat 21	Cim 21	Con 21	Cim 21	Plat 21	Plat 7	Plat 14	Cim 7	Cim 21	Cim 7	Con 28	Cim 28	
Cim 28	Con 14		Plat 14	Cim 14		Plat 21	Plat 28		Con 0	Plat 14		

On day 0 (April 4, 2006), 4 Control pots were sampled by placing a ruler at soil level and everything above 10.2cm in height was cut using scissors. The clippings from each pot were placed in individually labeled paper bags and stored in a -20ºC freezer until later analysis. All pots assigned to the Plateau® and Cimarron® treatments were then sprayed with their respective treatments. Plateau® was applied at the equivalent of 295.7mL per 0.4 hectare (174.8g ammonium salt of imazapic per hectare) and Cimarron® was applied at 28.4g per 0.4 hectare (42.3g metsulfuron methyl per hectare) using a research track sprayer (Allen Machine Works, Midland, MI). The research track sprayer was programmed to deliver the equivalent of 94.6L of herbicide per 0.4 hectare, 43.2cm above the plants, at 2.1kg/cm (Figure 3.2). These were the same treatments described in Experiment 1 (same concentrations, pressure, and height) except in Experiment 2 Control pots were not sprayed.

Figure 3.2. Dr. William W. Witt operating the research track sprayer. This machine applied the Cimarron® and Plateau® treatment to the E+ fescue pots at a rate and concentration equivalent to those used in Experiment 1.

Samples were then taken from assigned pots (Table 3.2) on days 7, 14, 21, and 28 of study (Figure 3.3). These samples were collected, processed, and stored similar to the

Control samples taken on study day 0. All samples taken were stored at -20ºC for further analysis.

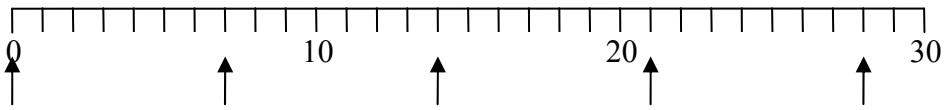


Figure 3.3. Time line for experiment 2 is shown above with sampling days indicated. Days are labeled relative to the spraying date of April 4, 2006 (day 0).

Plant material was lyophilized (Botanique Freeze Drier Model 18DX40 Automatic), ground through a 1mm screen (Thomas Scientific Wiley Mill Model 174931), and analyzed for alkaloid content (total ergovaline, NAL, NFL, NANL, and total lysergic acid).

Briefly, for ergovaline/ergovalinine analyses, tall fescue (0.5g freeze-dried, powdered) was incubated in 10mL of 80% methanol for 2 hours with shaking (Eberbach Corporation Shaker, Ann Arbor, Michigan). Samples were filtered through cotton plugs in 22.9cm (9 inch) pipets then through PrepSep columns (SPE, C18 disposable columns 100mg/mL; Fisher Science.) prior to analysis by High Performance Liquid Chromatography (HPLC). Quality control standards of EJ, an internal laboratory standard seed with high concentrations of ergovaline and ergovalinine, were also processed in a similar manner. Twenty microliters of the filtered solution was injected into a reverse phase C18 column (3µ particle size, 150mm length, 4.6mm external diameter; Alltech Altima). The HPLC program for the ergovaline/ergovalinine analysis was as follows:

1. Initial flow rate was set at 1.25mL/minute with an elution gradient of 95% solvent A (0.075M ammonium acetate in HPLC grade water: acetonitrile (75:25, v/v)) and 5% solvent B (100% Acetonitrile) for 1.0 minute.
2. The solvent ratios were changed to 60% solvent A and 40% solvent B for 17.0 minutes.
3. A column wash was performed by running 100% solvent B at a flow rate of 1.2mL/minute for 6.5 minutes.

4. The solvent ratios were changed to 95% solvent A and 5% solvent B for at least 7.0 minutes or until the next sample was injected.

Total run time for each tall fescue sample or quality control was 31 minutes with the wash included. Detection was performed with a fluorescence detector (Perkin Elmer Series 200) with excitation at 310nm and measurement at above 370nm. Ergovaline and ergovalinine were identified with approximate retention times of 12 to 14 minutes and 18 to 20 minutes respectively (Figure 3.4). Once the concentrations of the separate ergovaline and ergovalinine alkaloids were calculated, they were added together (due to being epimers) for the total ergovaline concentration.

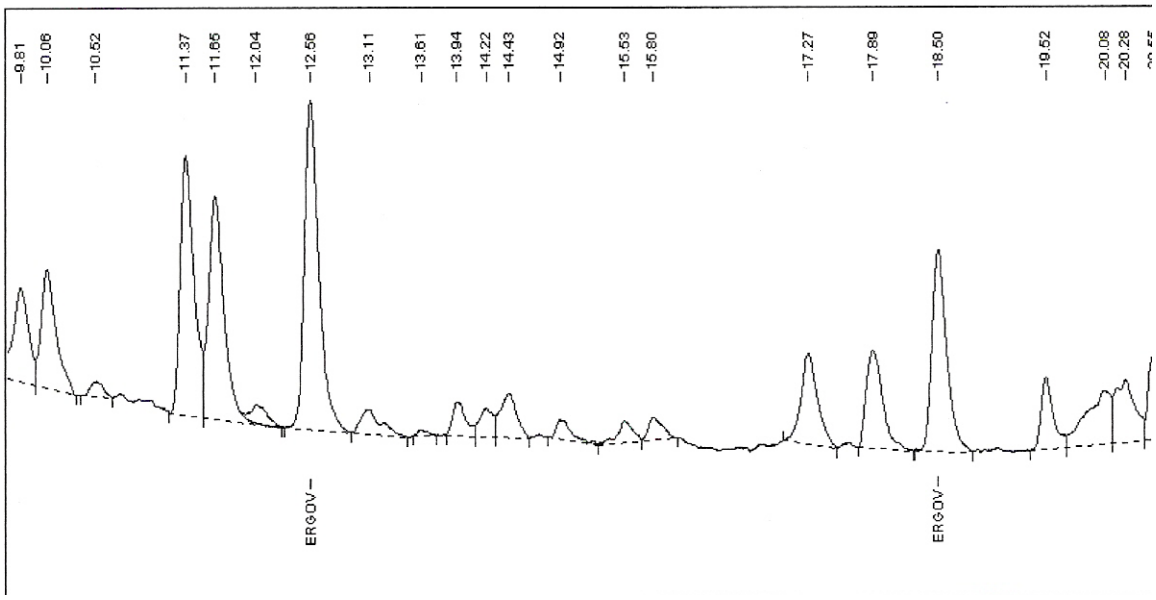


Figure 3.4. Typical HPLC output for the separation and quantification of ergovaline (at 12.56 minutes) and ergovalinine (18.50).

Preparation of the tall fescue or quality control sample for lysergic acid analysis was similar to the ergovaline/ergovalinine preparation, but the HPLC detection differed from the ergovaline/ergovalinine detection. First, only 10µL of the filtered solution (either tall fescue or quality control) was injected into the HPLC, and solvent A was a ratio of 95%, 0.091M ammonium acetate in HPLC grade water to 5% acetonitrile. For the lysergic acid separation and detection, the HPLC program analysis was performed with a constant flow rate of 1.2ml/minute with changes occurring to the gradient elution. Two components – LA1 (lysergic acid) and LA2 (isolysergic) – were detected, and they

both were quantified with a lysergic acid standard. The only difference between these two is the epimerization at carbon 8. The carboxyl group is forward or back from the plane of the ring moiety. The concentrations of these two were added together in the results. The HPLC program for the lysergic acids analysis was as follows:

1. Gradient elution of 100% solvent A and 5% solvent B (100% Acetonitrile) held for 0.5 minutes.
2. Gradient elution changed to 98% solvent A and 2% solvent B for 13.0 minutes.
3. Gradient elution changed to 95% solvent A and 5% solvent B for 7 minutes.
4. A column wash was then performed by 100% solvent B for 7 minutes and then 100% solvent A for at least 7 minutes or until the next sample was injected.

Total run time, including the wash, was 34.5 minutes with detection of the lysergic acid isomers at the approximate retention times of 13 to 14 minutes and 21 to 22 minutes (Figure 3.5).

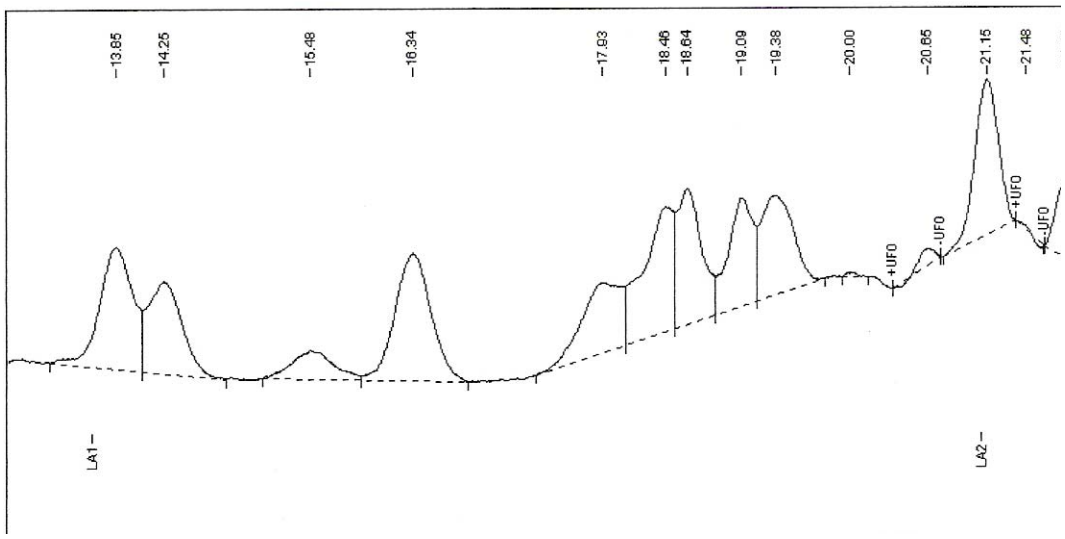


Figure 3.5. Typical HPLC output for the separation and quantification of lysergic acid and isolysergic acid at 13.85 and 21.15 respectively.

In the analysis of pyrrolizidine alkaloids in the tall fescue sample, 0.25g of freeze dried powdered tall fescue was mixed with 5mL of internal standard (1.35×10^{-4}M quinoline in methylene chloride: ethanol (95:5, v/v)) and 250µL of concentrated sodium bicarbonate. The vial was then shaken for 1 hour on an Eberbach Corporation Shaker.

Samples were filtered through 14.6cm (5 3/4 inch) pipets containing kimwipes into gas chromatography (GC) vials. One microliter of sample was injected into a 15m fused silica dimethyl polysiloxane GC column with 0.5µm film thickness and 530µm internal diameter. The GC column was set to a flow of 4.0mL/minute, at a purge rate of 2.2 minutes. Hydrogen was used as the carrier gas at 40mL/minute. Air flow was set at 300mL/minute, and the GC was run in splitless mode with pressure at 0.13 kg/cm (1.8 pounds per square inch) in the column. GC programming for the loline analysis was as follows:

1. Initial temperature was set at 90ºC then increased to 155ºC at 4ºC/minute.
2. Rate of temperature change was changed to 30ºC/minute until reaching 280ºC, where the temperature was held for 30 minutes.

The total run time for the pyrrolizidine separation and detection was 20.42 minutes. Approximate retention times of quinoline (the internal standard) at 7.27 minutes, NANL at 12.28 minutes, NFL at 12.84 minutes, and NAL at 14.24 minutes were used to identify alkaloids of the tall fescue sample (Figure 3.6).

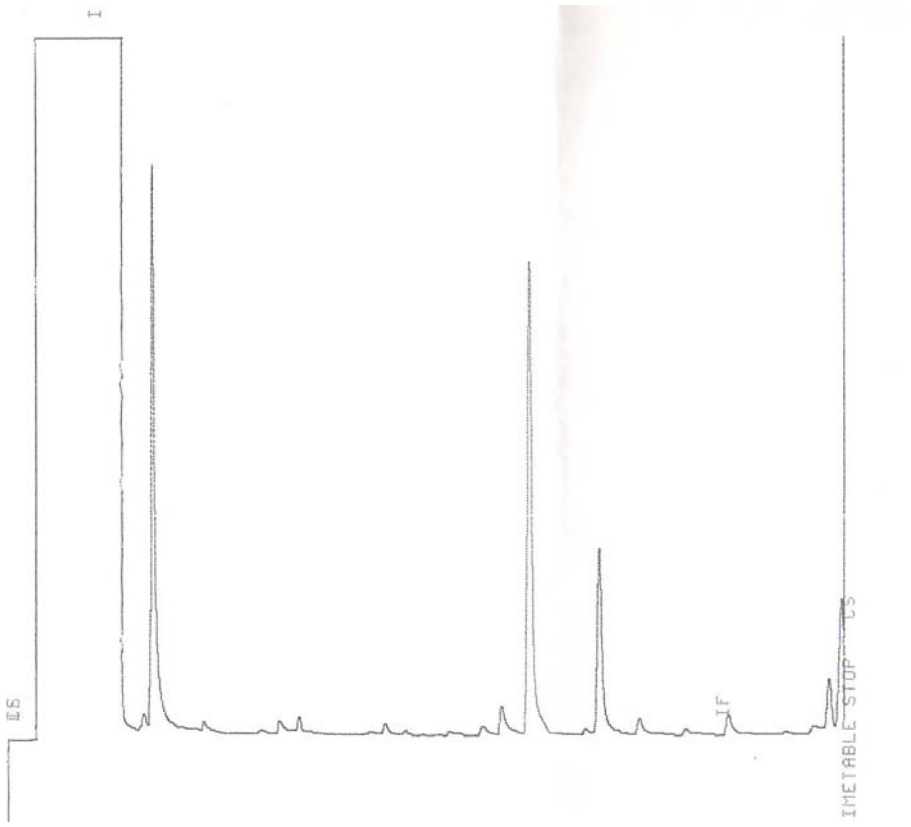


Figure 3.6. Typical GC output for the separation and quantification of the NANL (at 11.810), NFL (at 12.462), and NAL (at 14.156).

Experiment 3: Herbicide Field Summer 2006

Experiment 3 was designed to examine the effect of Cimarron® and Plateau® on the alkaloid content of E+ fescue under field conditions. Twelve, 3 by 6.1 meter plots of land were marked off using a measuring tape and stakes with flags (Figure 3.7). These plots were then randomly assigned to treatments of Cimarron®, Control (not sprayed), or Plateau® (Table 3.3).

Figure 3.7. Twelve plots of land were marked off for Experiment 3. These plots were randomly assigned to one of three treatments (Cimarron®, Control, or Plateau®).

Table 3.3. The twelve plots were randomly assigned to one of three treatments, such that four plots were sprayed with Cimarron®, four plots were sprayed with Plateau®, and four plots were not sprayed (Control). (Not drawn to scale).

Plot #	Treatment
12	Control
11	Cimarron®
10	Plateau®
9	Plateau®
8	Cimarron®
7	Control
6	Cimarron®
5	Control
4	Plateau®
3	Control
2	Cimarron®
1	Plateau®

North ⟶

The plots were composed of mixed forage species, including fescue grass, orchard grass, and some legumes. Plots were mowed to 20.3cm in height on June 5, 2006, to achieve an

even plant stand height. Immediately prior to mowing, approximately 15 tall fescue tillers were taken from random locations within each plot. These tillers were cut just below the ground level, and all tillers from each plot were placed in individually labeled paper bags for each plot. These samples were placed in a -20ºC freezer until testing for endophyte via immunoblotting as described in Experiment 1. On June 8, 2006 (day 0) 15 tillers of tall fescue were selected randomly within each plot. These tillers were cut at the ground level, placed in paper bags labeled for the individual plots, and stored in a -20ºC freezer for later alkaloid content analysis (details described in Experiment 2). After the day 0 samples were taken, the assigned treatments were applied at the equivalent of 94.6L per 0.4 hectare of the herbicide surfactant water mixture at 2.1 kg/cm using a CO_2 pressurized plot sprayer. Herbicides were mixed with their respective surfactants, and Plateau® was applied at the equivalent of 295.7mL per 0.4 hectare (174.8g ammonium salt of imazapic per hectare), while Cimarron® was applied at the equivalent of 28.4g per 0.4 hectare (42.3g metsulfuron methyl per hectare). These were the same treatments described in Experiment 1 and 2 (same concentrations, pressure, and height) except, as in Experiment 2, in Experiment 3 Control pots were not sprayed. Following the application of the herbicides, samples were taken every two weeks (Figure 3.8) by selecting 15 random tillers of tall fescue per plot, cutting them at the ground level, placing them in paper bags labeled for each plot, and storing them at -20ºC for further analysis.

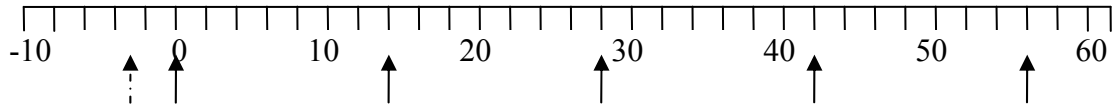


Figure 3.8. Sampling time line for experiment 3 is shown above. Days are labeled relative to the treatment application date of June 8, 2006 (day 0). The dashed arrow indicates tall fescue tiller samples taken for immunoblotting to test for the presence of the endophyte *Neotyphodium coenophialum*. Solid arrows indicate samples taken for alkaloid analysis.

At the conclusion of the study, all samples were lyophilized, ground, and analyzed for alkaloid content described in Experiment 2

STATISTICAL ANALYSIS

Experiment 2: Herbicide Greenhouse Spring 2006

To make comparisons across the three treatments only data collected on days 7, 14, 21, and 28 were used in the statistical analysis. There were an insufficient number of day 0 pots sampled to include them in the analysis. However, day 0 is included in all graphs for reference. The raw data collected on days 7, 14, 21, and 28. The effects examined were treatment (Cimarron®, Control, and Plateau®), sample day, and the interaction of treatment and sample day. These effects were analyzed using the MIXED procedure of SAS (2006) with the Satterthwaite degrees of freedom method used to test the alkaloid concentrations (total ergovaline, NAL, NANL, NFL, and total lysergic acid). Least square means were calculated for all parameters and their differences examined.

Experiment 3: Herbicide Field Summer 2006

Sample days 0, 14, 28, 42, 56 were used in the analysis of Experiment 3. Plot 10 was excluded from the statistical analysis due to accidental lack of treatment application at the start of the experiment. All other plots were included and analyzed using sample day as the repeated term. Raw data were analyzed using the MIXED procedure of SAS and the Satterthwaite degrees of freedom methods to test the alkaloid concentrations (total ergovaline, NAL, NANL, NFL, and total lysergic acid). Due to low concentrations of alkaloids in sampled plant material a second statistical analysis was performed using normalized data. Raw data were normalized using samples from day 0 as the baseline value from which the concentrations changed. Data on days 14, 28, 42, and 56 were subtracted from the initial concentration on day 0, resulting in all plots starting at a concentration of 0 and changing from there. If an individual alkaloid concentration was less on day 14 than on day 0, the resulting value would be negative. If it was higher on day 14 the value, would be positive. The same statistical analysis described for the raw data was used for the normalized data.

RESULTS

Experiment 2: Herbicide Greenhouse Spring 2006

Results of the statistical analyses for all of the alkaloids in the Greenhouse experiment are reported in Table 3.4.

Table 3.4. Experiment 2 alkaloid concentrations. Statistical analysis using the Greenhouse raw data from days 7, 14, 21, and 28 examining the effects of treatment (trt), day (study day), and the interaction of treatment and day (trt * day).

Variable	Effect	Pr > F
Total Ergovaline	Trt	0.0492
	study day	0.0032
	Trt * day	0.2201
N-acetylloline	Trt	0.0076
	study day	0.0857
	Trt * day	0.5760
N-formylloline	Trt	0.0100
	study day	0.0031
	Trt * day	0.7599
N-acetylnorloline	Trt	0.0976
	study day	0.0078
	Trt * day	0.2741
Total Lysergic Acid	Trt	0.0245
	study day	0.0036
	Trt * day	0.1787

There were differences by study day in the total ergovaline (P=0.0032), NFL (P=0.0031), NANL (P=0.0078), and in total lysergic acid (P=0.0036). There were also treatment differences in total ergovaline (P=0.0492), NAL (P0.0076), NFL (P=0.0100), and total lysergic acid (P=0.0245). Differences in treatment in total ergovaline occurred on days 7 and 28, where Control was different from Plateau® on day 7 (P=0.0231) and on day 28 Control was different from Plateau® (P=0.0255) and Cimarron® (P=0.0465, Figure 3.9). Differences in treatments in NAL occurred only on day 28, where Plateau® was different from Control (P<0.0087, Figure 3.10). For NFL, differences in treatment occurred on day 7 where Cimarron® was different from Control (P=0.0494). On day 28 Plateau® was different from Control (P=0.0405, Figure 3.11). Finally, there were differences between Control and Plateau® treatments in total lysergic acid (P=0.0040,

Figure 3.12). However, despite the numerous treatment and day effects seen on specific days there were no treatment study day interactions (P>0.05).

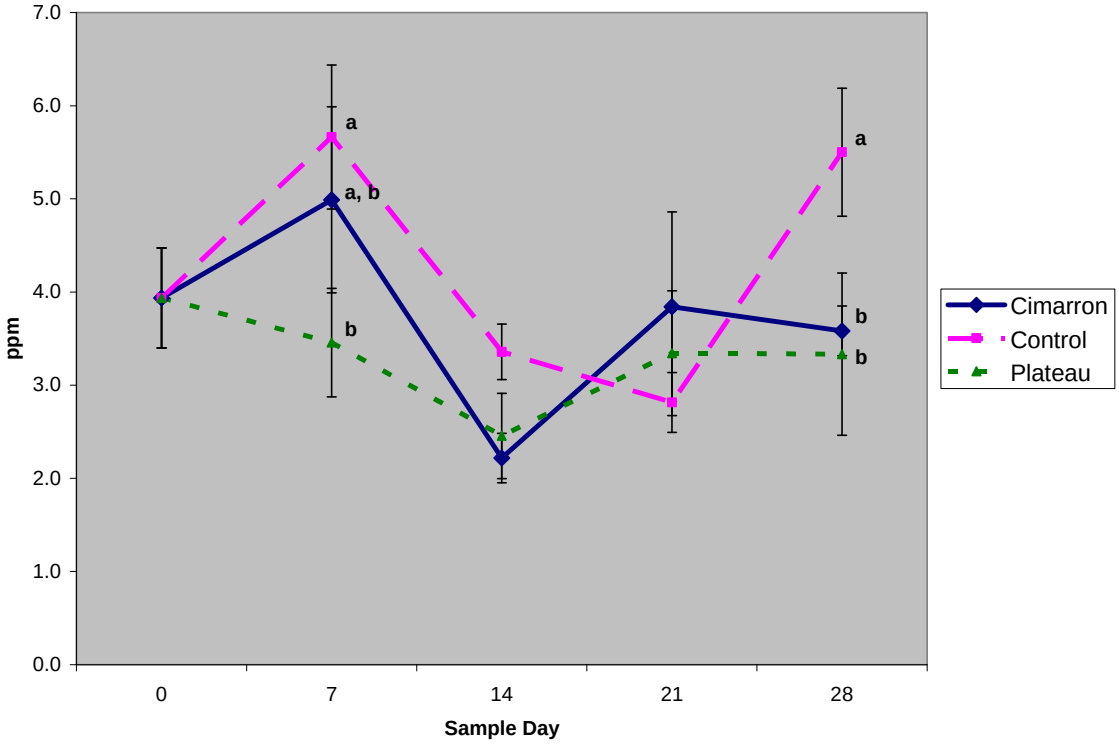


Figure 3.9. Total ergovaline (the sum of ergovaline and ergovalinine) for Greenhouse raw data (means and SEM). Overall there was a treatment (P = 0.0492) and day (P=0.0032) effect. Differences were detected within day 7 and 28 and differences within each day are indicated by different letters (P<0.05). Day 0 is included for a reference point only (from the Control day 0 sampling) and was not included in the statistical analysis.

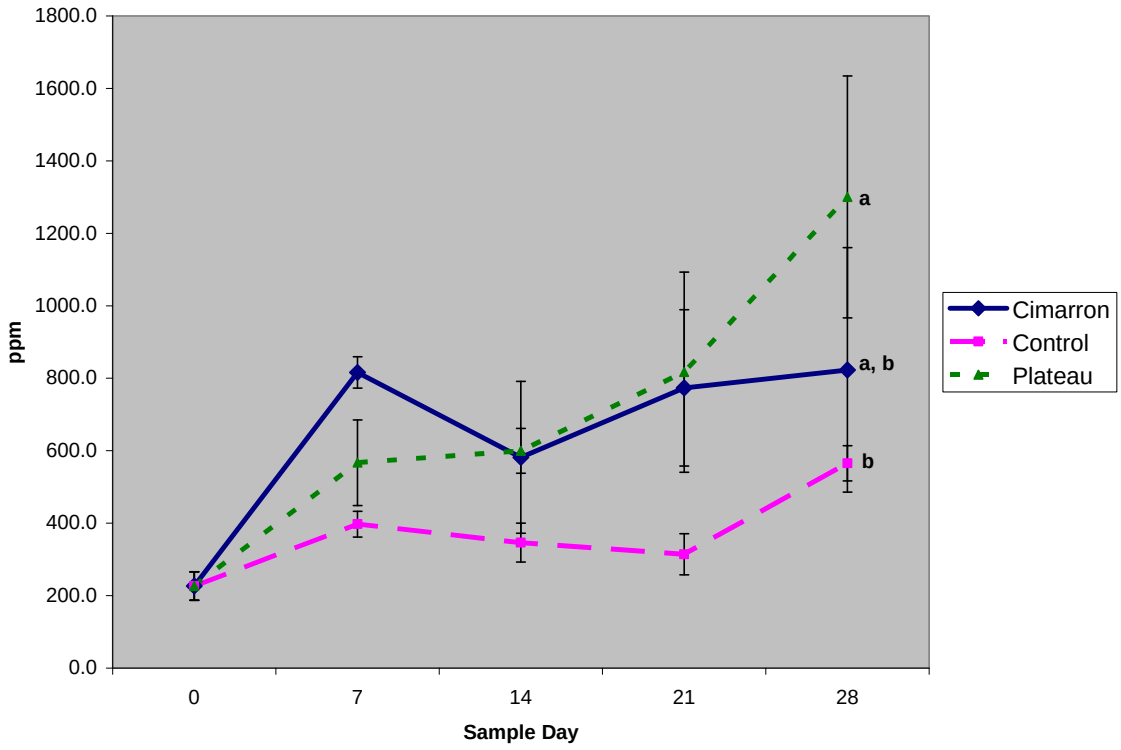


Figure 3.10. NAL for Greenhouse raw data (means and SEM). Differences were detected in treatment (P=0.0076) only. Treatment affects occurred only in sample day 28 and are indicated by different letters (P<0.05). Day 0 is included for a reference point only (from the Control day 0 sampling) and was not included in the statistical analysis.

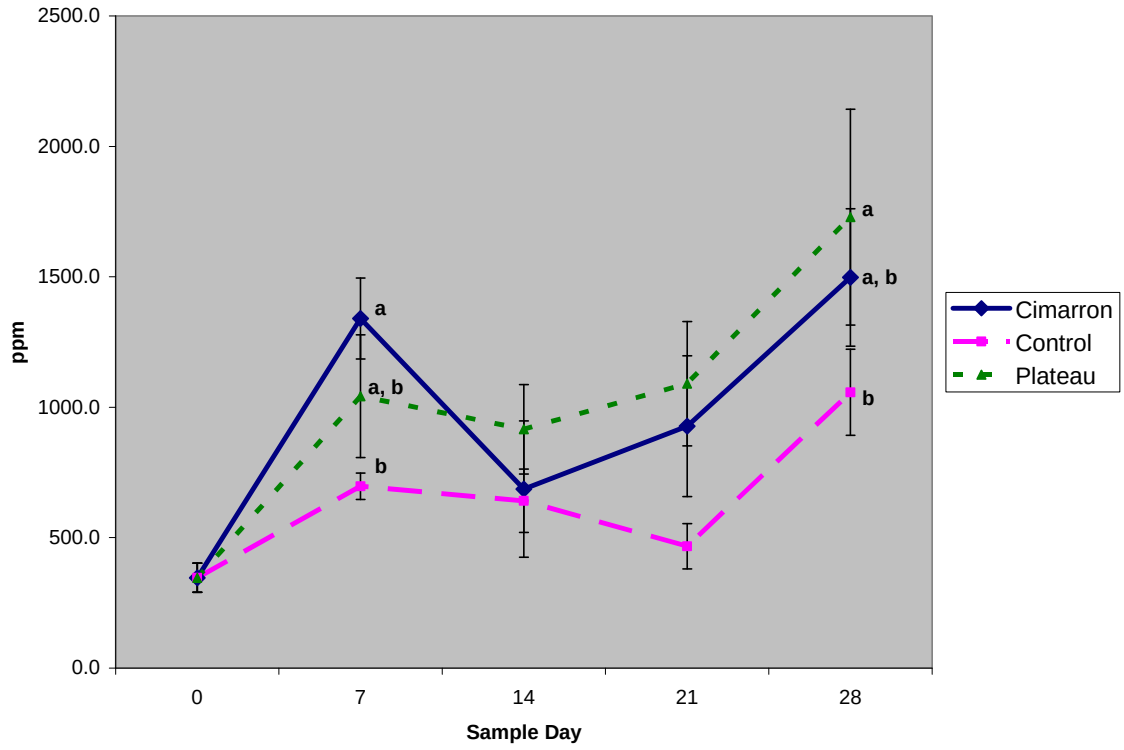


Figure 3.11. NFL for Greenhouse raw data (means and SEM). Differences were detected in treatment (P=0.0100) and day (P=0.0031). Treatment affects occurred only in sample day 7 and 28. Differences within each day are indicated by different letters (P<0.05). Day 0 is included for a reference point only (from the Control day 0 sampling) and was not included in the statistical analysis.

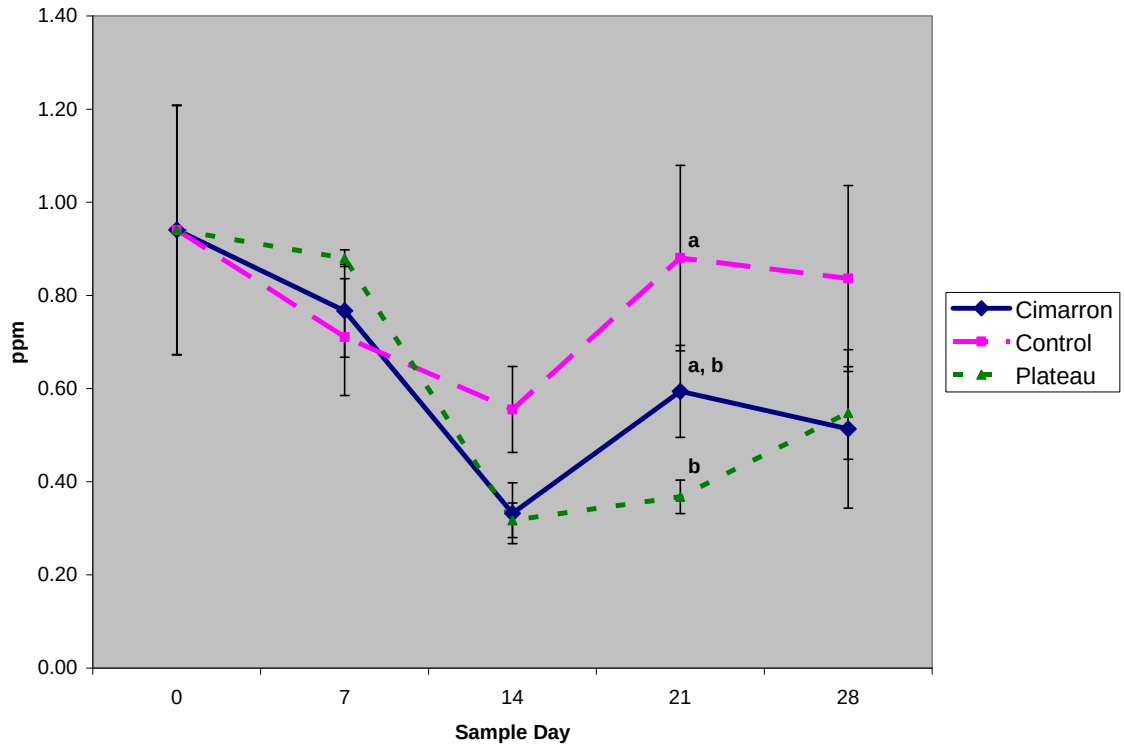


Figure 3.12. Total Lysergic acid (lysergic acid and isolysergic acid combined) for Greenhouse raw data (means and SEM). Differences were detected in treatment (P=0.0245) and day (P=0.0036). Treatment affects occurred only in sample day 21 and are indicated by different letters (P<0.05). Day 0 is included for a reference point only (from the Control day 0 sampling) and was not included in the statistical analysis.

Treatment kill rates were variable (Table 3.5). Plateau® killed anywhere from 20% to 99% of the contents of treated pots. Cimarron® had a wider kill rate of 5% to 95% of the fescue in the pots. Controls remained green and growing throughout the experiment. Kill rates were estimated on 43 days post spraying data. The lack of effective kill in the herbicide treated pots necessitated the Field experiment.

Table 3.5. Estimated kill rates for individual pots treated with Control, Cimarron®, or Plateau® in the Greenhouse experiment.

Table Section	Treatment	% Dead		Table Section	Treatment	% Dead
A	Con 0	0		C	Con 0	0
A	Con 7	0		C	Con 7	0
A	Con 14	0		C	Con 14	0
A	Con 21	0		C	Con 21	0
A	Con 28	0		C	Con 28	0
A	Plat 7	50		C	Plat 7	75
A	Plat 14	45		C	Plat 14	95
A	Plat 21	70		C	Plat 21	99
A	Plat 28	90		C	Plat 28	90
A	Cim 7	20		C	Cim 7	5
A	Cim 14	60		C	Cim 14	60
A	Cim 21	95		C	Cim 21	20
A	Cim d28	40		C	Cim d28	40
B	Con 0	0		D	Con 0	0
B	Con 7	0		D	Con 7	0
B	Con 14	0		D	Con 14	0
B	Con 21	0		D	Con 21	0
B	Con 28	0		D	Con 28	0
B	Plat 7	60		D	Plat 7	75
B	Plat 14	80		D	Plat 14	45
B	Plat 21	30		D	Plat 21	75
B	Plat 28	75		D	Plat 28	20
B	Cim 7	45		D	Cim 7	40
B	Cim 14	5		D	Cim 14	25
B	Cim 21	10		D	Cim 21	65
B	Cim 28	50		D	Cim 28	60

Experiment 3: Herbicide Field Summer 2006

Results of the statistical analyses for all of the alkaloids in the Field experiment using the raw data are reported in Table 3.6.

Table 3.6. Experiment 3 alkaloid concentrations statistical analysis using the Field raw data from days 0, 14, 28, 42, and 56 examining the effects of treatment (trt), day (study day), and the interaction of treatment and day (trt * day).

	Effect	Pr > F
Total Ergovaline	Trt	0.7315
	study day	0.1849
	Trt * day	0.6581
N-acetylloline	Trt	0.1260
	study day	0.5845
	Trt * day	0.1670
N-formylloline	Trt	0.5628
	study day	0.1725
	Trt * day	0.1556
N-acetylnorloline	Trt	0.6119
	study day	0.9109
	Trt * day	0.3274
Total Lysergic Acid	Trt	0.4858
	study day	<0.0001
	Trt * day	0.0005

The only differences seen in the Field experiment were an effect on day and a treatment day interaction in total lysergic acid ($P<0.0001$ and $P=0.0005$ respectively, Figure 3.13).

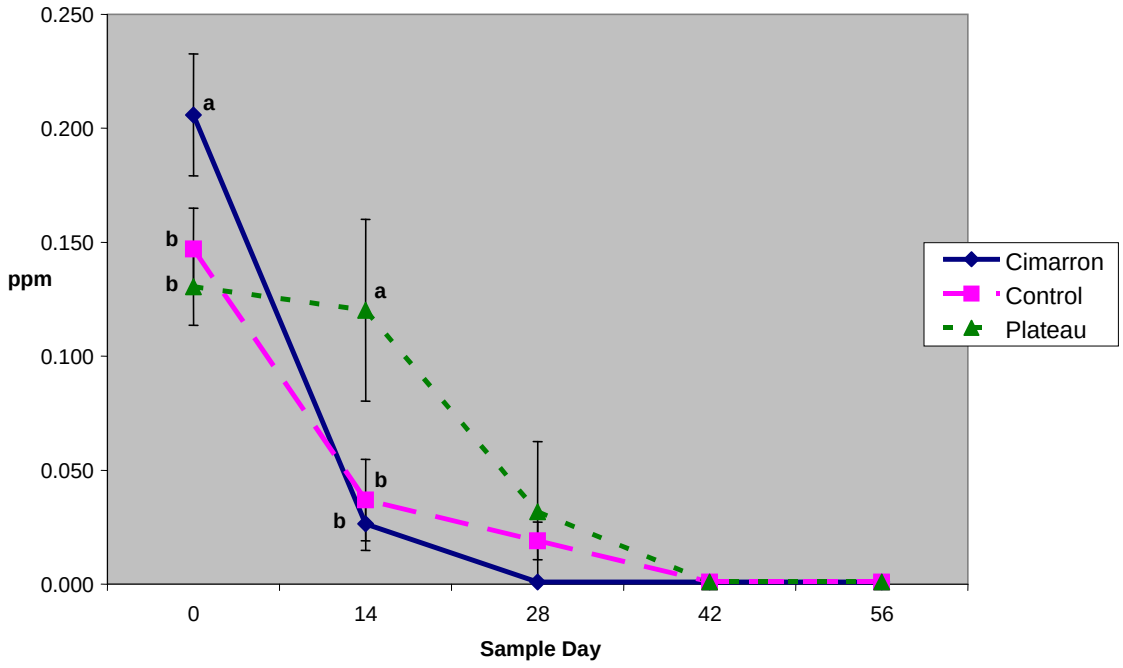


Figure 3.13. Total lysergic acid (lysergic acid and isolysergic acid combined) for Field raw data (means and SEM). Differences were detected in day ($P<0.0001$) and in the interaction between treatment and day ($P=0.0005$). Treatment differences occurred on days 0 and 14 and are indicated by different letters ($P<0.05$).

Many of the samples tested contained alkaloids at concentrations below detection limits. Results from immunoblotting were determined following the start of the Field experiment and are reported in Table 3.7.

Table 3.7. Immunoblotting results for tillers collected from Field plots.

Plot number	Treatment	Percent infected tillers
1	Plateau	20%
2	Cimarron	42%
3	Control	33%
4	Plateau	25%
5	Control	22%
6	Cimarron	23%
7	Control	45%
8	Cimarron	50%
9	Plateau	20%
10	Plateau	30%
11	Cimarron	30%
12	Control	14%

Due to the low infection levels and low alkaloid concentrations in samples, the decision to look at changes from a baseline was made. By analyzing this normalized data set, differences were measured in treatment, study day, and an interaction between the two. Results are reported in Table 3.8.

Table 3.8. Experiment 3. Alkaloid concentrations statistical analysis using the Field normalized data from days 0, 14, 28, 42, and 56 examining the effects of treatment (trt), day (study day), and the interaction of treatment and day (trt * day).

	Effect	Pr > F
Total Ergovaline	Trt	0.0460
	study day	0.1761
	Trt * day	0.8714
N-acetylloline	Trt	0.2775
	study day	0.6642
	Trt * day	0.3255
N-formylloline	Trt	0.0324
	study day	0.1780
	Trt * day	0.1612
N-acetylnorloline	Trt	0.9214
	study day	0.9277
	Trt * day	0.4411
Total Lysergic Acid	Trt	0.0093
	study day	<0.0001
	Trt * day	0.0002

There were differences among treatments in total ergovaline, NFL, and total lysergic acid (Table 3.8). There was a difference between Control and Plateau® on day 28 for total ergovaline (P=0.0437, Figure 3.14). NFL was different on day 42 and 56 between Control and Plateau® (P=0.0030 and P=0.0017 respectively, Figure 3.15). Differences in treatment for total lysergic acid occurred on all days tested (Figure 3.16).

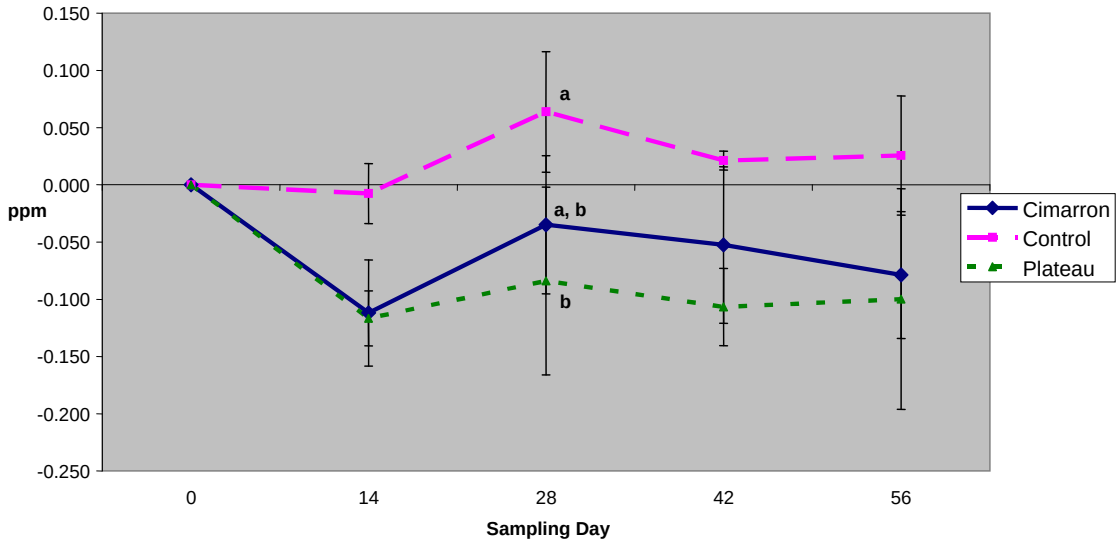


Figure 3.14. Total ergovaline (ergovaline and ergovalinine combined) for Field normalized data (means and SEM). Treatment differences occurred on day 28 and are indicated by different letters (P<0.05). Day 0 is included for a reference point only and was not included in the statistical analyses.

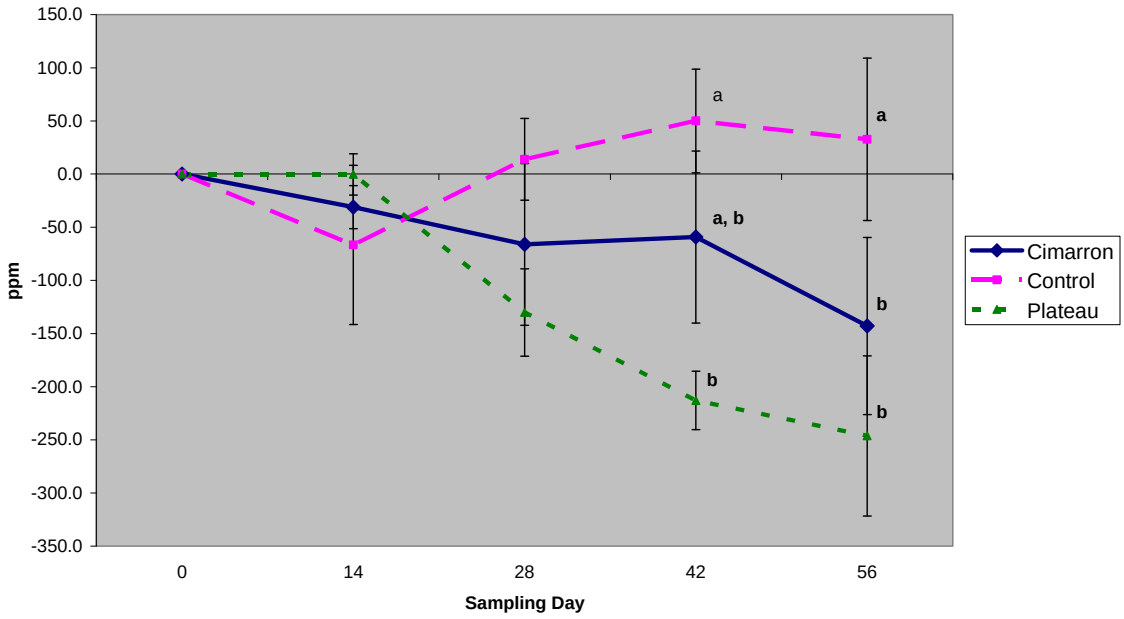


Figure 3.15. NFL for Field normalized data (means and SEM). Treatment differences occurred on days 42 and 56 and are indicated by different letters (P<0.05). Day 0 is included for a reference point only and was not included in the statistical analyses.

There was a day effect in total lysergic acid (P<0.0001). Also, there was an interaction between treatment and day in total lysergic acid, which occurred on all days sampled (Figure 3.16). On day 14, Control was different from both Cimarron® and Plateau® (P=0.0231 and P=0.0035 respectively), and Cimarron® was different from Plateau® (P<0.0001). On day 28, Cimarron® was different from Control and Plateau® (P=0.0126 and P=0.0021 respectively). Cimarron® was again different from Plateau® on day 42 (P=0.0223), but not from Control (P>0.05). Finally, on day 56, Cimarron® was different from Plateau® (P=0.0223).

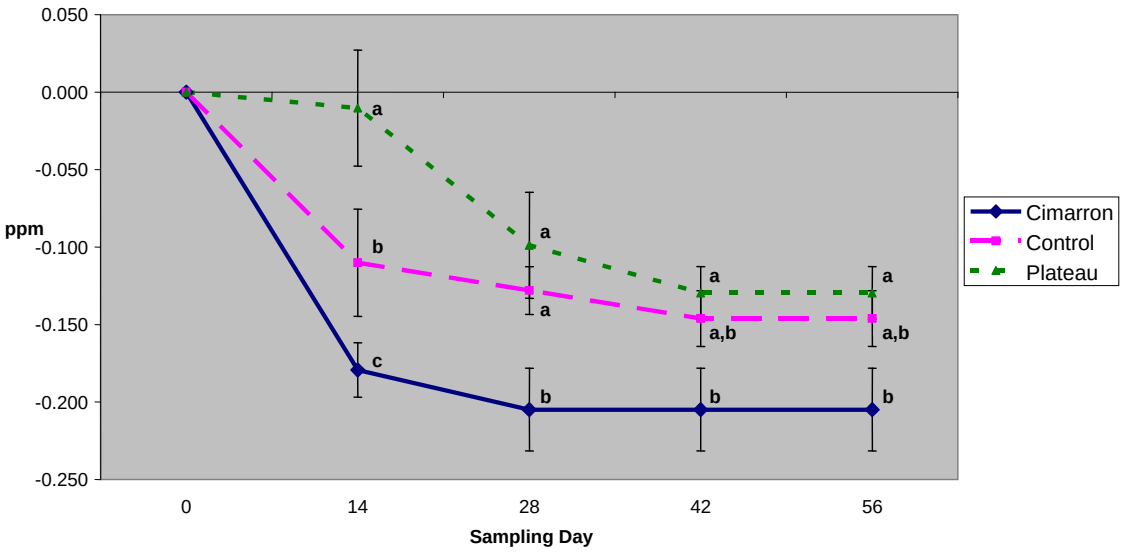


Figure 3.16. Total lysergic acid (lysergic acid and isolysergic combined) for Field normalized data (means and SEM). Treatment differences occurred on days 14, 28, 42, and 56 and are indicated by different letters (P<0.05). Day 0 is included for a reference point only and was not included in the statistical analyses.

DISCUSSION

Alkaloid concentrations are affected by many factors including season, temperature, water availability, fertilization, and mowing practices (Arachevaleta *et al.*, 1992; Belesky *et al.*, 1989; Malinowski *et al.*, 1998; Rottinghaus *et al.*, 1991; Salminen *et al.*, 2003; Salminen and Grewal, 2002). In these experiments, many of these factors were equalized between treatments. For example, pots and plots were only clipped prior to the

start of the experiments, so any growth seen would be equal. In the Greenhouse experiment, all pots were watered and fertilized in the same manner and housed in the same greenhouse. However, not all pots behaved the same. Despite herbicide treatments causing stunted growth, many pots did not completely die while others did (Figure 3.17).

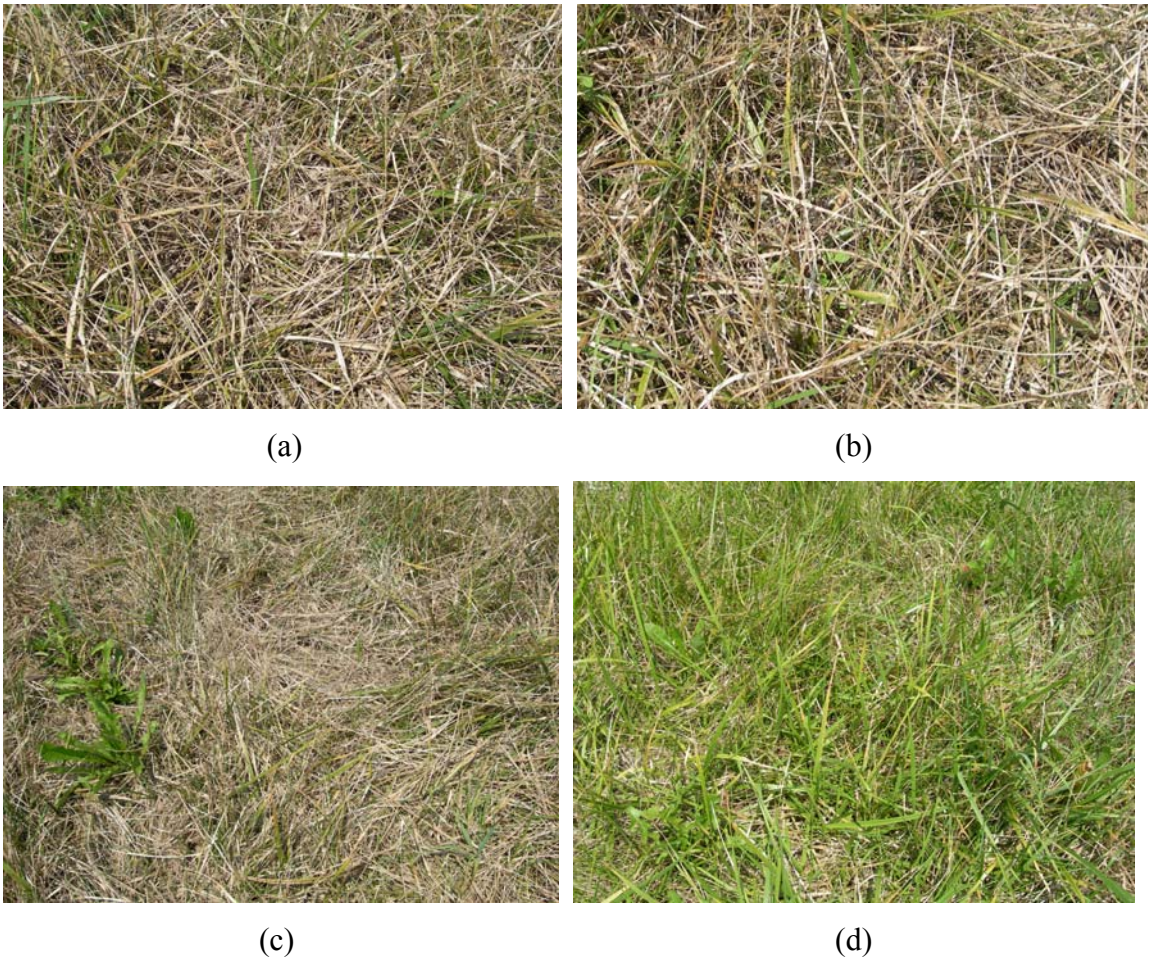
(a)

(b)

Figure 3.17. Greenhouse pots treated with Cimarron® (a) and Plateau® (b) showed varied responses, where some had a greater than 95% kill rate while others had less than 50 % kill rate 43 days post spraying.

To get the most effective kill, plants treated with the herbicides may need to be exposed to ultraviolet light. The greenhouse glass minimized plant exposure to ultraviolet light. This necessitated the Field experiment in which plant response more

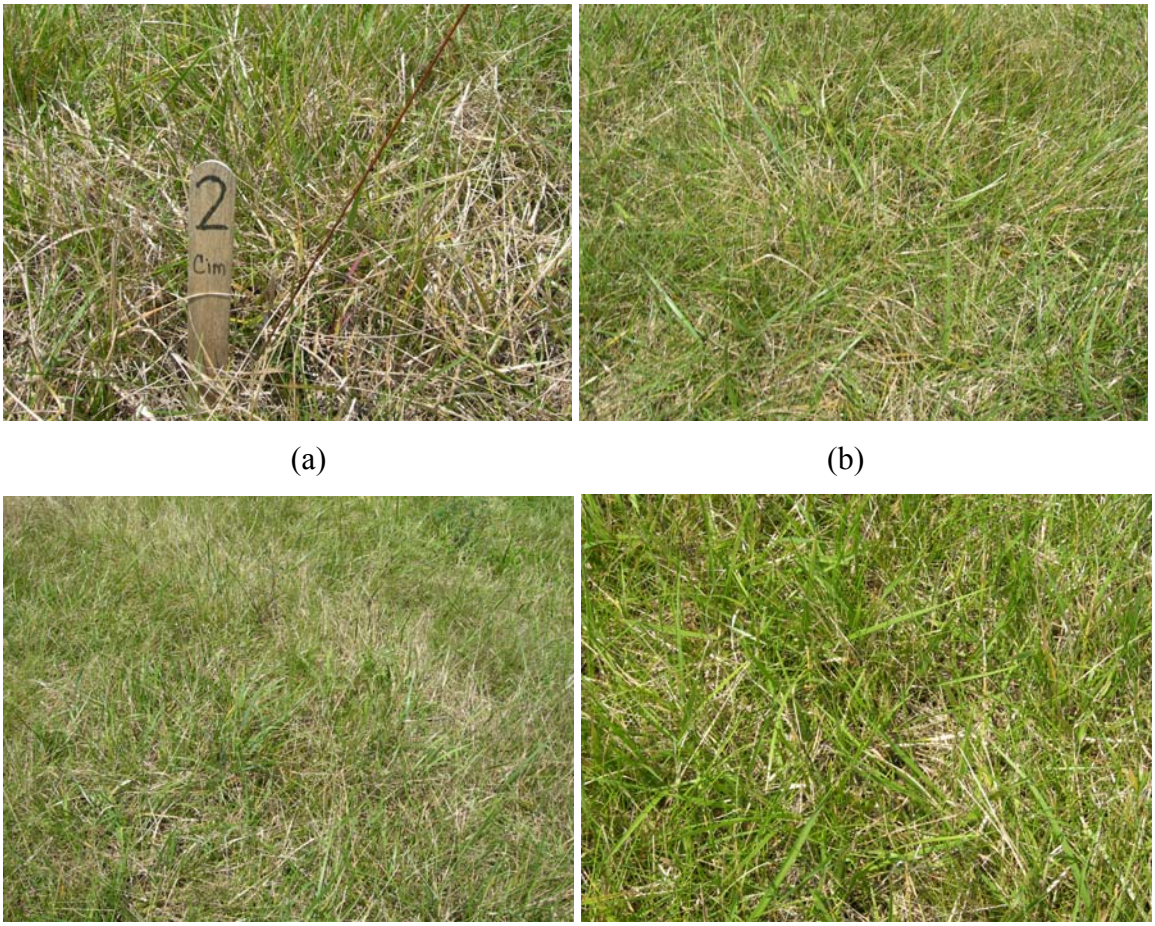
closely mimicked a pasture environment. In the Field experiment, plants treated with the herbicides had stunted growth and most treated plants died, while Controls remained green (Figure 3.18 and 3.19). Green plants within the plots may be recovered orchard grass, new tall fescue growth from seedlings that were not treated, or new weeds. From the Field experiment, it could be seen that Plateau® appeared to be more effective than Cimarron®.



(a) (b)

(c) (d)

Figure 3.18. All Field plots (plot 1 (a), plot 4 (b), and plot 9 (c)) treated with the herbicides Plateau® were stunted and turned brown as the forages died. Plot 10 was excluded from statistical analyses because it was not treated and remained green throughout the experiment (d). All pictures were taken on July 20, 2006 (day 42 post spraying).



(a) (b)

(c) (d)

Figure 3.19. All Field plots (plot 2 (a), plot 6 (b), plot 8 (c) and plot 11 (d)) treated with the herbicides Cimarron® were stunted and turned brown as the forages died. All pictures were taken on July 20, 2006 (day 42 post spraying).

Analyses of the alkaloid concentrations indicated that concentrations of the alkaloids were often lower in Control pots than those of plants treated with the herbicides; for example total ergovaline in both the Greenhouse and Field experiments (Figure 3.9 and 3.14). This maybe explained by dilution of alkaloids within the increased dry matter content of Controls over time, because increasing the availability of nutrients has been shown to reduce alkaloid concentrations (Rassmussen *et al.*, 2007). Additionally, ergovaline and ergovalinine can be metabolized to lysergic acid by microbial action (Duringer *et al.*, 2007; Schultz *et al.*, 2006), which can further explain

why Control pots and plots had higher concentrations of total ergovaline. Thus total ergovaline was in agreement with the original hypothesis.

In the Greenhouse experiment, NAL, and NFL concentrations were higher in pots treated with the herbicides than in Controls by day 7 (Figure 3.10 and 3.11). In the Field experiment when NFL concentrations were evaluated, it was again higher in plots treated with the herbicides than Controls by day 28 (Figure 3.15). Control plants had uniform increased NAL and NFL concentrations, while plants treated with Cimarron® or Plateau® had decreased concentrations. Belesky and coworkers (1989) found that lolines concentrations increased in E+ fescue under water stress. A similar response occurs with these herbicides, only the limiting component is branch chain amino acids. Therefore, the plant should contain a higher concentration of lolines.

As expected, total lysergic acid concentrations in herbicide treated plants, in the Greenhouse experiment, were less than Control plants by day 14 (Figure 3.12). This coincides with a large decrease in total ergovaline concentrations (Figure 3.9). In the Field experiment, Control plants had higher total lysergic acid concentrations than plants treated with Plateau® (Figure 3.13). However, in all treatments total lysergic acid decreased to below detection limits by day 42 post spraying (July 20, 2006). These decreases, since they were seen in all treatments, may be due to growth environment and not the treatments themselves.

The validity of the results from the Field experiment may be questioned, because of the low infection rates (Table 3.7). Samples that had high alkaloid concentrations may have had more infected tillers sampled, while samples that had low alkaloid concentrations may have been mostly uninfected tillers. Also, sampling periods were only 28 and 56 days and the alkaloids concentrations may decrease if later post spraying samples were taken. Further studies using plots of 100% infection rates and a longer sampling time frame are necessary for a definitive decision. However, based on the current overall results from Experiments 2 and 3 the hypothesis that the alkaloid concentrations decrease as the plant dies must be rejected, because total ergovaline and lysergic acids concentrations did decrease as the plant dies, but the lolines tended to increase.

CHAPTER FOUR
OVERALL DISCUSSION

Fescue toxicosis affects cattle and sheep resulting in decreased weight gains and feed intake, lameness, unthriftiness, decreased prolactin, and sloughing of hooves (Gadberry *et al.*, 2003; Gallagher *et al.*, 1966; Hoveland *et al.*, 1983; Howard *et al.*, 1992; Jensen *et al.*, 1956; Peters *et al.*, 1992; Sampson, 1933; Schuenemann *et al.*, 2005b; Tor-Agbidye *et al.*, 2001). However, in horses the most pronounced effect is in the pregnant mare. Fescue toxicosis manifests in the pregnant mare with prolonged gestation, decreased prolactin, agalactia, a larger than normal foal, dystocia, retained placenta, and possibly death mare and/or foal (Cross *et al.*, 1995). Thus, it is to the benefit of livestock owners to eliminate endophyte infected tall fescue (E+ fescue) from their pastures. Plateau® and Cimarron® are able to kill E+ fescue via inhibition of acetolactate synthase (ALS) (BASF, 2004; DuPont, 2005). The manufacturers of these herbicides performed safety studies and the Environmental Protection Agency published these results. However, all of the testing was at extremely high dosages and primarily in laboratory animals. The study reported here examined the effects of Cimarron® and Plateau® on pregnant mares and their foals at the suggested herbicidal concentrations to suppress tall fescue (42.3g metsulfuron methyl (Cimarron®'s active ingredient) per hectare and 174.8g ammonium salt of imazapic (Plateau®'s active ingredient) per hectare respectively).

Analysis of combined data from the four studies of Experiment 1 (Spring 2005 through Fall 2006) determined that sample week was different ($P<0.0001$), and there was a treatment by time interaction ($P=0.0003$) in creatinine concentrations. Sample week differences are to be expected since blood components can be altered by stress/exercise, diet, medication, stage of estrous, pregnancy, or numerous other factors (Ginther, 1992; Lees *et al.*, 1983; Marlin *et al.*, 2002; Passantino *et al.*, 2005; Zeyner *et al.*, 2006). The treatment by time interaction was traced to the Spring 2006 study only ($P=0.0134$). In this study, mares on Plateau® pastures had creatinine concentrations higher than mares on Control pastures on sample week 3, 4, and 6 ($P<0.05$), and a treatment sample week interaction was seen ($P=0.0134$). However, overall treatment differences were not detected ($P>0.05$). Thus, the treatment sample week interaction can largely be attributed

to sample week, which alone was significant (P<0.0001). Creatinine concentrations for mares on Cimarron® treated pastures were not different from mares on Control pastures, except on sample week 5 in Spring 2006. Regardless of treatments, creatinine concentrations remained below upper physiological limits (0.5 -2.0mg/mL; Duncan and Prasse, 1986). Total bilirubin neared a treatment by time interaction (P=0.0737) and this difference was traced back to the Spring 2005 study only (P=0.0387). In the individual studies sample week was significant (P<0.05), but treatment alone was not significant (P>0.05). In the Spring 2005 the only difference among treatments was in sample week 6 where Cimarron® was different from Control and Plateau®; P=0.0013 and P=0.0134 respectively. Thus the treatment by time interaction can again contributed largely to sample week. Regardless of the treatments total bilirubin remained within physiological limits (0.2 - 5.0mg/mL; Duncan and Prasse, 1986). The general lack of effect of these herbicides was expected since mammals do not have the enzyme ALS.

The conclusion that Cimarron® and Plateau® did not alter blood components outside of physiological norms in mares grazing nearly tall fescue free pastures raised the question of what happens to the alkaloids within E+ fescue as it dies. This question was addressed in Experiments 2 and 3. E+ fescue alkaloids are not static and can be affected by time of year, environmental temperature, water availability, fertilization, and mowing/clipping practices (Arachevaleta *et al.*, 1992; Belesky *et al.*, 1989; Malinowski *et al.*, 1998; Rottinghaus *et al.*, 1991; Salminen *et al.*, 2003; Salminen and Grewal, 2002). The Greenhouse experiment allowed these factors to be equalized between treatments. Despite this equalization, pots within treatments did not behave the same. Some herbicide treated pots had greater plant death than others within the same treatment group. Thus, it was concluded that the most effective kill and uniform actions within treatment groups may require ultraviolet light. In Experiment 3, plots of forage treated with either herbicide did show signs of suppression and death within all replicates. Visual assessments of plots determined that Plateau® was a more effective herbicide than Cimarron®.

Alkaloids within E+ fescue are implicated in fescue toxicosis. Ergopeptines cause decreased prolactin concentrations (Strickland et al., 1992), reduced ruminal contractions (McLeay and Smith, 2006), and excessive sweating (Bony *et al.*, 2001). The specific

ergopeptine, ergovaline, binds to D_2 dopamine receptors which can decrease prolactin concentrations (Larson *et al.*, 1995). Pyrrolizidines, commonly called the "lolines," cause vasoconstriction, but not to the extent of the ergopeptines (Solomons et al., 1989). They do not affect prolactin secretion (Larson *et al.*, 1999), but are correlated with decreased cholesterol (Stuedemann *et al.*, 1985). Lolines can also cause decreased feed intake, weight loss, and increased rectal temperatures (Jackson, Jr. *et al.*, 1984). Lysergic acids have greater transport potential than other alkaloids (Hill *et al.*, 2001) and cause vasoconstriction (Abney *et al.*, 1993; Oliver *et al.*, 1992; Oliver *et al.*, 1993). Ergovaline causes more vasoconstriction than lysergic acid or N-acetylloline (NAL) in cattle (Klotz *et al.*, 2007b). Additionally, researchers suggested that lysergic acid may inhibit some of the vasoconstrictive action of ergovaline (Klotz *et al.*, 2007b). Taken together these alkaloids cause a greater reduction of prolactin than individual alkaloids alone (Gadberry *et al.*, 2003). Also, greater decreases in weight gain are seen with the combination of NAL and ergot alkaloid than individual alkaloids (Jackson *et al.*, 1996).

Results of the Field experiment were complicated by low infection rates within treatment plots. Nevertheless, ergovaline concentrations were decreased during the experiment, possibly due to conversion to lysergic acid (Duringer *et al.*, 2007; Schultz *et al.*, 2006), but there was no treatment effect observed ($P>0.05$). Total lysergic acid also decreased over the course of the experiment and a treatment study day interaction was seen ($P=0.0005$), but treatment alone was not significant ($P>0.05$). Control total lysergic acid also decreased over time. So whether the decrease was due to the herbicides or due to weather remains to be seen. Because overall alkaloid concentrations were low, samples were normalized to day 0. Treatment differences were then detected in total ergovaline ($P=0.0460$), N-formylloline (NFL) ($P=0.0324$), and total lysergic acid ($P=0.0093$). Total ergovaline concentrations and NFL were lower in Cimarron® and Plateau® plots than in Control plots, but total lysergic acid were only lower in Cimarron® treated plots. Total lysergic acid still had a treatment study day interaction ($P=0.0002$), but this can be largely attributed to time ($P<0.0001$) since all treatments experienced a large decrease over time, with Cimarron® treated plots reaching lower detection limits.

There are two possible reasons for a decrease in alkaloids concentrations. Alkaloid concentrations in Control plots often decreased during the experiment. This decrease can be attributed to dilution of the alkaloids within the increasing plant material. The plants treated with the herbicides were stunted and did not grow following spraying, therefore, decreases in alkaloid concentration can be attributed to the plants death. However, statistical analysis did not differentiate between the two types of decrease.

In conclusion, the herbicides Cimarron® and Plateau® did not affect fetal/foal mortality/morbidity in broodmares grazing pastures treated with either herbicide and did not alter blood components outside the physiological norm. However, alkaloids in dying E+ fescue may still be a threat to the pregnant mare and her fetus/foal. Monitoring the mare for signs of parturition and the alkaloid concentrations within the pastures may still be necessary to prevent fescue toxicosis. Overall, it may be safest to limit pregnant mare exposure to E+ fescue throughout pregnancy, especially in the last 30 to 60 days of gestation. Additionally, if a livestock owner wants to treat his pastures with either herbicide it would be best to spray in the fall during early to mid gestation when the threat of E+ fescue to pregnancy maintenance is lower.

APPENDIX

Calculations to determine the ingested concentrations of Plateau® or Cimarron®

Conversions
1 acre = 0.4047 hectare 1 pound = 0.454grams
weight oz = 28.35 grams

Mares were 1000 to 1500lbs → an average of 1250lbs (567.5kg)

Pastures were approximately 1.75 acres each
Plateau was applied at 10 fluid ounces per acre
Plateau contained 2lbs ammonium salt of imazapic (active ingredient) per gallon of Plateau
10oz/A = 0.156lb ammonium salt of imazapic/A
0.156lb/A * 16oz/1lb * 28.35g/oz * 1A/0.4047Ha =174.8g/Ha
Plateau pastures had 174.8g ammonium salt of imazapic applied per 0.4 hectare
174.8g/hectare * 0.7hectare/pasture = 122.4g/pasture = 122,400mg/pasture

Cimarron was applied at 1 weight ounce per acre
Cimarron contained 60% metsulfuron methyl (active ingredient)
0.6lb metsulfuron methyl/1lbCimarron * 1lb/16oz = 0.0377lb/oz
0.0377lb/A * 16oz/1lb * 28.35g/1oz * 1A/0.4047Ha = 42.3g/Ha
Cimarron pastures had 42.3g metsulfuron methyl applied per 0.4 hectare
42.3g/hectare * 0.7hectare/pasture = 29.6g/pasture = 29,610mg/pasture

Mares per pasture

Spring 2005 3 mares
Fall 2005 2 mares
Spring 2006 3 mares
Fall 2006 2 mares

Spring ingestion per mare – over approximately 3 weeks
Ammonium salt of imazapic -- 122,400mg/pasture / 3 mares = 40,800mg/mare/pasture
Metsulfuron methyl -- 29,610mg/pasture / 3 mares = 9,870mg/mare/pasture

Fall ingestion per mare – over approximately 3 weeks
Ammonium salt of imazapic – 122,400mg/pasture / 2 mares = 61,200mg/mare/pasture
Metsulfuron methyl – 29,610mg/pasture / 2 mares = 14,805mg/mare/pasture

Spring ingestion per kg of mare body weight– over approximately 3 weeks
Ammonium salt of imazapic – 40,800mg/mare / 567.5kg BW = 71.9mg/kg BW
Metsulfuron methyl – 9,870mg/mare / 567.5 kg BW = 17.4mg/kg BW

Fall ingestion per kg of mare body weight – over approximately 3 weeks
Ammonium salt of imazapic – 61,200mg/mare / 567.5kg BW = 107.8 mg/kg BW
Metsulfuron methyl – 14,805mg/mare / 567.5kg BW = 26.1mg/kg BW

Spring ingestion per kg of mare body weight per day
Ammonium salt of imazapic – 71.9mg/kg BW / 21 days = **3.4mg/kgBW/day**
Metsulfuron methyl – 17.4mg/kg BW / 21 days = **0.8mg/kgBW/day**

Fall ingestion per kg of mare body weight per day
Ammonium salt of imazapic – 107.8mg/kg BW / 21 days = **5.1mg/kgBW/day**
Metsulfuron methyl – 26.1mg/kg BW / 21 days = **1.2mg/kgBW/day**

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