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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

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July 24, 2008
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THESIS

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

By

Kathleen Scarlett Black

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

Lexington, Kentucky

2008

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I dedicate this work in honor of all of those that have supported and encouraged me in all 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 25°C for 8 hours followed by 10 to 15 °C 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).
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 C₃ plant, meaning that it assimilates CO₂ by the Calvin cycle with 3-phosphoglyceric acid as the primary product (Wolf et al., 1979). As a C₃ plant and displaying some characteristics of photorespiration, tall fescue becomes light saturated at only 25 to 50% full sunlight (Wolf et al., 1979), unlike C₄ 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).

![Endophyte](image)

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).
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.,
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 E₂ (PGE₂) and F₂α (PGF₂α) and increase in uterine myometrial oxytocin receptors number. PGE₂ acts to soften the cervix while PGF₂α 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 (T3) 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.
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 (Burfenning, 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* (Burfenning, 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 (Burfenning, 1973). The damage inflicted can result in loss of extremities as seen in “fescue foot.”

The ergopeptines inhibit prolactin secretion via binding to D2-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 D2-dopamine receptors and initiate cAMP production to decrease prolactin.
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).
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 \textit{in vitro} (Abney et al., 1993; Solomons et al., 1989). Ergonovine, another lysergic acid amide, also caused constriction of bovine pedal veins \textit{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 \textit{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 D2 dopamine receptors (Larson et al., 1999). However, NAL did cause vasoconstriction in the equine lateral saphenous vein \textit{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 $4.4 \times 10^{-8}$M for ergotamine and 1.4 to $2.2 \times 10^{-7}$M for ergosine). NAL caused vasoconstriction of the cranial branch of the lateral saphenous vein via $\alpha_2$-adrenergic receptors (where the catecholamines norepinephrine and epinephrine normally bind to cause vasoconstriction) \textit{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).

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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-formynorloline 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 cellulolytic 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).

![Chemical structure of metsulfuron methyl](image)

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

This chemical compound is a member of the sulfonyleurea 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
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, whereas 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).

![Chemical structure of imazapic](image)

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 multiparous 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).

<table>
<thead>
<tr>
<th>North</th>
<th>1A - Control - Methylated Seed Oil @ 473.0mL/0.4hectare</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1B - Control - Activator 90 surfactant @ 0.25% v/v</td>
</tr>
<tr>
<td></td>
<td>2A – Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473.0mL/0.4hectare</td>
</tr>
<tr>
<td></td>
<td>2B – Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v</td>
</tr>
<tr>
<td></td>
<td>3A – Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473.0mL/0.4hectare</td>
</tr>
<tr>
<td></td>
<td>3B – Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v</td>
</tr>
</tbody>
</table>

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/cm² (30 pounds per square inch) using a CO₂ pressurized plot sprayer (Figure 2.1). Cimarron® assigned pastures were sprayed at 28.4g Cimarron® per 0.4 hectare (1 weight ounce per area).
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 CO₂ 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](image)

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, “T₄”). 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 T₄) 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](image-url) 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 multiparous 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.

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).

<table>
<thead>
<tr>
<th>North</th>
<th>1A – Control – Methylated Oil @ 473mL/0.4 hectare</th>
<th>1B - Control - Activator 90 surfactant @ 0.25% v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2A – Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v</td>
<td>2B – Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473mL/0.4 hectare</td>
</tr>
<tr>
<td></td>
<td>3A – Plateau® @ 295.7mL/0.4 hectare plus methylated seed oil @ 473mL/0.4 hectare</td>
<td>3B – Cimarron® @ 28.4g/0.4 hectare plus Activator 90 @ 0.25% v/v</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect</th>
<th>Pr &gt; F</th>
<th>Variable</th>
<th>Effect</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Urea Nitrogen</td>
<td>Trt</td>
<td>0.0690</td>
<td>Albumin to Globulin Ratio</td>
<td>Trt</td>
<td>0.8390</td>
</tr>
<tr>
<td></td>
<td>Week</td>
<td>&lt;0.0001</td>
<td></td>
<td>Week</td>
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</tr>
<tr>
<td></td>
<td>Trt*week</td>
<td>0.2814</td>
<td></td>
<td>Trt*week</td>
<td>0.9336</td>
</tr>
<tr>
<td>Calcium</td>
<td>Trt</td>
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<td>Aspartate Aminotransferase</td>
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<tr>
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<td>Week</td>
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<tr>
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<tr>
<td>Phosphorus</td>
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<td>Creatine Phosphokinase</td>
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</tr>
<tr>
<td></td>
<td>Week</td>
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<td>γ Glutamyl Transferase</td>
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<tr>
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<td>Sodium</td>
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<tr>
<td></td>
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<td>Week</td>
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<tr>
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<td>Trt*week</td>
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<tr>
<td>Potassium</td>
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<td>Glucose</td>
<td>Trt</td>
<td>0.4848</td>
</tr>
<tr>
<td></td>
<td>Week</td>
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<td></td>
<td>Week</td>
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<tr>
<td></td>
<td>Trt*week</td>
<td>0.4471</td>
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<td>Trt*week</td>
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<tr>
<td>Chloride</td>
<td>Trt</td>
<td>0.8399</td>
<td>Creatinine</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>Total Protein</td>
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<td>Cholesterol</td>
<td>Trt</td>
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</tr>
<tr>
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<td>Week</td>
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<td>Total Bilirubin</td>
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<td></td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>Trt*week</td>
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</tr>
<tr>
<td>Globulin</td>
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<td>Cholesterol</td>
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</tr>
<tr>
<td></td>
<td>Week</td>
<td>&lt;0.0001</td>
<td></td>
<td>Week</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Trt*week</td>
<td>0.2767</td>
<td></td>
<td>Trt*week</td>
<td>0.5599</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect</th>
<th>Pr &gt; F</th>
<th>Variable</th>
<th>Effect</th>
<th>Pr &gt; F</th>
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</thead>
<tbody>
<tr>
<td>Packed Cell Volume</td>
<td>Trt</td>
<td>0.9993</td>
<td>Lymphocyte</td>
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<td>Week</td>
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<tr>
<td></td>
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<td>White Blood Cell</td>
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<td>Monocyte</td>
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<td>Week</td>
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<tr>
<td></td>
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<td>Hemoglobin</td>
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<td>Eosinophil</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>Week</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
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<td>Trt*week</td>
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<td>Red Blood Cell</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trt*week</td>
<td>0.4804</td>
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</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
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</tr>
<tr>
<td></td>
<td>Week</td>
<td>0.0911</td>
</tr>
<tr>
<td></td>
<td>Trt*week</td>
<td>0.4391</td>
</tr>
<tr>
<td>Progesterone</td>
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<td>0.8667</td>
</tr>
<tr>
<td></td>
<td>Week</td>
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</tr>
<tr>
<td></td>
<td>Trt*week</td>
<td>0.4501</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Trt</td>
<td>0.7715</td>
</tr>
<tr>
<td></td>
<td>Week</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Trt*week</td>
<td>0.2848</td>
</tr>
</tbody>
</table>

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).
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.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Combined</th>
<th>Spring 2005</th>
<th>Fall 2005</th>
<th>Spring 2006</th>
<th>Fall 2006</th>
</tr>
</thead>
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<tr>
<td>Pr &gt; F</td>
<td>Pr &gt; F</td>
<td>Pr &gt; F</td>
<td>Pr &gt; F</td>
<td>Pr &gt; F</td>
<td>Pr &gt; F</td>
</tr>
<tr>
<td>Trt</td>
<td>0.2458</td>
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<td>0.0636</td>
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<td>Week</td>
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<td>&lt;0.0001</td>
<td>0.0015</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>Trt*week</td>
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<td>0.9758</td>
<td>0.6528</td>
<td>0.0134</td>
<td>0.3156</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Combined</th>
<th>Spring 2005</th>
<th>Fall 2005</th>
<th>Spring 2006</th>
<th>Fall 2006</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trt</td>
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<td>0.8576</td>
<td>0.2095</td>
<td>0.5541</td>
</tr>
<tr>
<td>Week</td>
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<td>0.0265</td>
<td>0.0007</td>
<td>0.0031</td>
</tr>
<tr>
<td>Trt*week</td>
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<td>0.0387</td>
<td>0.6215</td>
<td>0.5064</td>
<td>0.8543</td>
</tr>
</tbody>
</table>

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).

*Neotyphodium 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.
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 reticuloruminal 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 D2-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 4100 μg 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 D2 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 acetylacetate 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 brome grass, 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).

<table>
<thead>
<tr>
<th></th>
<th>Day</th>
<th>Number of pots</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td></td>
<td>7</td>
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</tr>
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<td>14</td>
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<td></td>
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<tr>
<td></td>
<td>28</td>
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</tr>
<tr>
<td>Plateau</td>
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<td>4</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>4</td>
</tr>
<tr>
<td>Cimarron</td>
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</tr>
<tr>
<td></td>
<td>14</td>
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<tr>
<td></td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>4</td>
</tr>
</tbody>
</table>

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).
On day 0 (April 4, 2006), 4 Control pots were sampled by placing a ruler at soil level and everything above 10.2 cm in height was cut using scissors. The clippings from each pot were placed in individually labeled paper bags and stored in a -20°C 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 295.7 mL per 0.4 hectare (174.8 g ammonium salt of imazapic per hectare) and Cimarron® was applied at 28.4 g per 0.4 hectare (42.3 g 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 94.6 L of herbicide per 0.4 hectare, 43.2 cm above the plants, at 2.1 kg/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 -20°C 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).](image)

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 x 10^-4M 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 14.6 cm (5 3/4 inch) pipets containing kimwipes into gas chromatography (GC) vials. One microliter of sample was injected into a 15 m fused silica dimethyl polysiloxane GC column with 0.5 μm film thickness and 530 μm internal diameter. The GC column was set to a flow of 4.0 mL/minute, at a purge rate of 2.2 minutes. Hydrogen was used as the carrier gas at 40 mL/minute. Air flow was set at 300 mL/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 90°C then increased to 155°C at 4°C/minute.
2. Rate of temperature change was changed to 30°C/minute until reaching 280°C, 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).

<table>
<thead>
<tr>
<th>Plot #</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Control</td>
</tr>
<tr>
<td>11</td>
<td>Cimarron®</td>
</tr>
<tr>
<td>10</td>
<td>Plateau®</td>
</tr>
<tr>
<td>9</td>
<td>Plateau®</td>
</tr>
<tr>
<td>8</td>
<td>Cimarron®</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
</tr>
<tr>
<td>6</td>
<td>Cimarron®</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
</tr>
<tr>
<td>4</td>
<td>Plateau®</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>Cimarron®</td>
</tr>
<tr>
<td>1</td>
<td>Plateau®</td>
</tr>
</tbody>
</table>

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 -20°C 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 -20°C 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 CO₂ 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 -20°C for further analysis.

Figure 3.8. Sampling timeline 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).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ergovaline</td>
<td>Trt</td>
<td>0.0492</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.0032</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.2201</td>
</tr>
<tr>
<td>N-acetylloline</td>
<td>Trt</td>
<td>0.0076</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.0857</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.5760</td>
</tr>
<tr>
<td>N-formylloline</td>
<td>Trt</td>
<td>0.0100</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.0031</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.7599</td>
</tr>
<tr>
<td>N-acetylnorloline</td>
<td>Trt</td>
<td>0.0976</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.0078</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.2741</td>
</tr>
<tr>
<td>Total Lysergic Acid</td>
<td>Trt</td>
<td>0.0245</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.0036</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.1787</td>
</tr>
</tbody>
</table>

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 (P=0.0076), 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>
<thead>
<tr>
<th>Table Section</th>
<th>Treatment</th>
<th>% Dead</th>
<th>Table Section</th>
<th>Treatment</th>
<th>% Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Con 0</td>
<td>0</td>
<td>C</td>
<td>Con 0</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Con 7</td>
<td>0</td>
<td>C</td>
<td>Con 7</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Con 14</td>
<td>0</td>
<td>C</td>
<td>Con 14</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Con 21</td>
<td>0</td>
<td>C</td>
<td>Con 21</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Con 28</td>
<td>0</td>
<td>C</td>
<td>Con 28</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Plat 7</td>
<td>50</td>
<td>C</td>
<td>Plat 7</td>
<td>75</td>
</tr>
<tr>
<td>A</td>
<td>Plat 14</td>
<td>45</td>
<td>C</td>
<td>Plat 14</td>
<td>95</td>
</tr>
<tr>
<td>A</td>
<td>Plat 21</td>
<td>70</td>
<td>C</td>
<td>Plat 21</td>
<td>99</td>
</tr>
<tr>
<td>A</td>
<td>Plat 28</td>
<td>90</td>
<td>C</td>
<td>Plat 28</td>
<td>90</td>
</tr>
<tr>
<td>A</td>
<td>Cim 7</td>
<td>20</td>
<td>C</td>
<td>Cim 7</td>
<td>5</td>
</tr>
<tr>
<td>A</td>
<td>Cim 14</td>
<td>60</td>
<td>C</td>
<td>Cim 14</td>
<td>60</td>
</tr>
<tr>
<td>A</td>
<td>Cim 21</td>
<td>95</td>
<td>C</td>
<td>Cim 21</td>
<td>20</td>
</tr>
<tr>
<td>A</td>
<td>Cim d28</td>
<td>40</td>
<td>C</td>
<td>Cim d28</td>
<td>40</td>
</tr>
<tr>
<td>B</td>
<td>Con 0</td>
<td>0</td>
<td>D</td>
<td>Con 0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Con 7</td>
<td>0</td>
<td>D</td>
<td>Con 7</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Con 14</td>
<td>0</td>
<td>D</td>
<td>Con 14</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Con 21</td>
<td>0</td>
<td>D</td>
<td>Con 21</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Con 28</td>
<td>0</td>
<td>D</td>
<td>Con 28</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Plat 7</td>
<td>60</td>
<td>D</td>
<td>Plat 7</td>
<td>75</td>
</tr>
<tr>
<td>B</td>
<td>Plat 14</td>
<td>80</td>
<td>D</td>
<td>Plat 14</td>
<td>45</td>
</tr>
<tr>
<td>B</td>
<td>Plat 21</td>
<td>30</td>
<td>D</td>
<td>Plat 21</td>
<td>75</td>
</tr>
<tr>
<td>B</td>
<td>Plat 28</td>
<td>75</td>
<td>D</td>
<td>Plat 28</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>Cim 7</td>
<td>45</td>
<td>D</td>
<td>Cim 7</td>
<td>40</td>
</tr>
<tr>
<td>B</td>
<td>Cim 14</td>
<td>5</td>
<td>D</td>
<td>Cim 14</td>
<td>25</td>
</tr>
<tr>
<td>B</td>
<td>Cim 21</td>
<td>10</td>
<td>D</td>
<td>Cim 21</td>
<td>65</td>
</tr>
<tr>
<td>B</td>
<td>Cim 28</td>
<td>50</td>
<td>D</td>
<td>Cim 28</td>
<td>60</td>
</tr>
</tbody>
</table>
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).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ergovaline</td>
<td></td>
</tr>
<tr>
<td>Trt</td>
<td>0.7315</td>
</tr>
<tr>
<td>study day</td>
<td>0.1849</td>
</tr>
<tr>
<td>Trt * day</td>
<td>0.6581</td>
</tr>
<tr>
<td>N-acetylloline</td>
<td></td>
</tr>
<tr>
<td>Trt</td>
<td>0.1260</td>
</tr>
<tr>
<td>study day</td>
<td>0.5845</td>
</tr>
<tr>
<td>Trt * day</td>
<td>0.1670</td>
</tr>
<tr>
<td>N-formylloline</td>
<td></td>
</tr>
<tr>
<td>Trt</td>
<td>0.5628</td>
</tr>
<tr>
<td>study day</td>
<td>0.1725</td>
</tr>
<tr>
<td>Trt * day</td>
<td>0.1556</td>
</tr>
<tr>
<td>N-acetylnorloline</td>
<td></td>
</tr>
<tr>
<td>Trt</td>
<td>0.6119</td>
</tr>
<tr>
<td>study day</td>
<td>0.9109</td>
</tr>
<tr>
<td>Trt * day</td>
<td>0.3274</td>
</tr>
<tr>
<td>Total Lysergic Acid</td>
<td></td>
</tr>
<tr>
<td>Trt</td>
<td>0.4858</td>
</tr>
<tr>
<td>study day</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Trt * day</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Plot number</th>
<th>Treatment</th>
<th>Percent infected tillers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Plateau</td>
<td>20%</td>
</tr>
<tr>
<td>2</td>
<td>Cimarron</td>
<td>42%</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>33%</td>
</tr>
<tr>
<td>4</td>
<td>Plateau</td>
<td>25%</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>22%</td>
</tr>
<tr>
<td>6</td>
<td>Cimarron</td>
<td>23%</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>45%</td>
</tr>
<tr>
<td>8</td>
<td>Cimarron</td>
<td>50%</td>
</tr>
<tr>
<td>9</td>
<td>Plateau</td>
<td>20%</td>
</tr>
<tr>
<td>10</td>
<td>Plateau</td>
<td>30%</td>
</tr>
<tr>
<td>11</td>
<td>Cimarron</td>
<td>30%</td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>14%</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th></th>
<th>Effect</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ergovaline</td>
<td>Trt</td>
<td>0.0460</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.1761</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.8714</td>
</tr>
<tr>
<td>N-acetylloline</td>
<td>Trt</td>
<td>0.2775</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.6642</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.3255</td>
</tr>
<tr>
<td>N-formylloline</td>
<td>Trt</td>
<td>0.0324</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.1780</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.1612</td>
</tr>
<tr>
<td>N-acetyl-norloline</td>
<td>Trt</td>
<td>0.9214</td>
</tr>
<tr>
<td></td>
<td>study day</td>
<td>0.9277</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Total Lysergic Acid</td>
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<td>0.0093</td>
</tr>
<tr>
<td></td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Trt * day</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

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).

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®.

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).
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 (Rasmussen 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 (0.5-2.0 mg/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 (0.2-5.0 mg/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 = 0.454 grams  
weight oz = 28.35 grams

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

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

Cimarron was applied at 1 weight ounce per acre
Cimarron contained 60% metsulfuron methyl (active ingredient)
0.6 lb metsulfuron methyl/1 lb Cimarron * 1 lb/16 oz = 0.0377 lb/oz
0.0377 lb/A * 16 oz/lb * 28.35 g/1 oz * 1 A/0.4047 Ha = 42.3 g/Ha
Cimarron pastures had 42.3 g metsulfuron methyl applied per 0.4 hectare
42.3 g/hectare * 0.7 hectare/pasture = 29.6 g/pasture = 29,610 mg/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 -- 122,400 mg/pasture / 3 mares = 40,800 mg/mare/pasture
Metsulfuron methyl -- 29,610 mg/pasture / 3 mares = 9,870 mg/mare/pasture

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

Spring ingestion per kg of mare body weight – over approximately 3 weeks
Ammonium salt of imazapic -- 40,800 mg/mare / 567.5 kg BW = 71.9 mg/kg BW
Metsulfuron methyl -- 9,870 mg/mare / 567.5 kg BW = 17.4 mg/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/kg BW/day
Metsulfuron methyl – 17.4mg/kg BW / 21 days = 0.8mg/kg BW/day

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


Zavos, P. M., B. Salin, J. A. Jackson, Jr., D. R. Varney, M. R. Siegel, and R. W. Hemken. 1986. Effects of feeding tall fescue seed infected by endophytic fungus

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