# THE EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE ON STALLION REPRODUCTIVE PARAMETERS

by

MARION WHITNEY TRAYLOR

(Under the Direction of Gary Heusner)

#### ABSTRACT

Six stallions were used in this study to determine the effects of endophyte-infected tall fescue on reproductive parameters. One experimental diet consisted of endophyte-infected tall fescue and the other consisted of a non-toxic endophyte tall fescue, Flecha AR-542, known as MaxQ. Stallions were fed one of two diets at 45% of the grain diet, at a rate of 1% of their body weight. Three stallions were blindly assigned each experimental diet and fed each diet for 70 days. After a seventy day rest period treatments were switched and fed for another 70 days. Stallions were supplied with fresh water, Common Bermuda, and Annual Ryegrass ad libitum. Results showed no differences in stallion reproductive parameters measured when feed endophyte-infected or MaxQ. Urinary alkaloid and creatinine tests were performed on urine samples to make sure stallions were receiving toxic levels of ergot alkaloids. Stallions consuming endophyte-infected seed had higher (P<0.0002) alkaloid/creatinine levels.

INDEX WORDS: Tall fescue, Endophyte, Ergot alkaloids, Horses, Stallions, Reproductive parameters, Semen parameters

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## DEDICATION

I dedicate this thesis to my parents, John and Karen Traylor, for their unconditional love and support. I also dedicate this to my husband, Timothy Wiggins, for his unconditional love, support, and timeless hours of help at the barn.

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#### CHAPTER 1

#### INTRODUCTION

Breeding horses is a precarious business. Horse fertility and management practices greatly influence pregnancy and foaling rates for a given stallion and are often difficult to assess or control. Despite great strides in research, overall conception rates are not high. In most cases, fertility issues are blamed on the mare for numerous reasons. Some common reasons that breeders blame mares for reproductive problems have to do with: age, uterine cysts, scar tissue from previous foaling, abnormal peritoneal conformation, and/or a malfunctioning endocrine system. However, some reproductive problems are caused by the stallion. In this study, we examined the reproductive performance of stallions and evaluated if endophyte-infected tall fescue affected stallion reproductive parameters. There has been a significant amount of research conducted on the mare, but no research has been conducted on the stallion concerning effects of ergot alkaloids on reproductive parameters. The most noticeable reproductive issues due to endophyte-infected tall fescue lie within the mare. The stallion has been overlooked because there have been no noticeable problems with fertility when consuming endophyteinfected tall fescue. Determining whether stallions reproductive parameters are affected when consuming endophyte-infected tall fescue is an important issue, since almost 700,000 horses graze on tall fescue, most of it being endophyte-infected. Eradication of endophyte-infected tall fescue is not a practical solution to toxicosis problems because of the extent of endophyteinfected tall fescue establishment and because of its persistence. Substitution of non-toxic endophyte tall fescue is a good solution but is difficult, since it can easily be infected with seed

heads of endophyte-infected tall fescue. Endophyte-infected tall fescue can be in a neighboring farmer's field and wind can disperse infected seed heads into the non-toxic endophyte pastures causing them to become infected. Once endophyte-infected tall fescue is established it will easily take over an entire non-toxic endophyte tall fescue pasture due to endophyte-infected tall fescues persistence. The best chance at eliminating the detrimental effects of endophyte-infected tall fescue is by first understanding the action of the active ergot alkaloid compounds. The next step is to find out how the ergot alkaloids are affecting each livestock animal. The last step is to devise a way to block these devastating toxicosis effects on reproduction and growth.

This research is going to conclude if there are reproductive problems in stallions consuming endophyte-infected tall fescue. We know there are reproductive problems in the mare due to endophyte-infected tall fescue. This research will determine if endophyte-infected tall fescue affects stallion reproductive parameters. The hypothesis is that stallion reproductive parameters will not be affected when consuming endophyte-infected tall fescue.

## **CHAPTER 2**

#### **REVIEW OF LITERATURE**

#### **Tall Fescue**

Tall fescue (Festuca arundinacea) originated in Europe and was introduced into North and South America in the mid-1800's (Thompson et al., 2001). A special variety of tall fescue became popular because it was introduced after the depression, was good at reducing erosion, and supplying forage to livestock. This special variety of tall fescue was known as "Kentucky 31." It was released in the United States in 1942 as a productive tall fescue grass of high nutritive value (Bacon, 1995). Farmers widely accepted this wonder-grass due to its drought tolerance, insect resistance, nematode resistance, ease of establishment, tolerance of poor soil conditions, long grazing season, and tolerance of heavy animal traffic. The name for "Kentucky 31" came about in 1931, when a doctor named E.N. Fergus from the University of Kentucky visited W.M. Suiter farm in Menifee County, Kentucky (Lacefield et al., 1986). He was impressed by a tall fescue ecotype growing on a mountainside pasture. He collected seed samples and planted these samples in many different locations in Kentucky to determine the hardiness of the ecotype. It proved to be hardy and was released to the public in 1942 (Lacefield et al., 1986). Currently, tall fescue is grown on over 35 million acres and is one of the most widely used grazing crops in the mid-west and southern United States (Thompson et al., 2001). Over 8.5 million beef cattle and nearly 700,000 horses are maintained on tall fescue hay and pasture (Thompson et al., 2001).

## Genetics

The genetics of tall fescue go back to two different plants. It is referred to as an Autoallo hexaploid, meaning there are two plant species combined to form one. One progenitor is a diploid and the other is a tetraploid. The diploid progenitor is *Festuca mareii* and the tetraploid progenitor is *Festuca glaucescens*. Each of the two plants has seven chromosomes but tall fescue has two sets of chromosomes from *Festuca glaucescens* (n=21, 2n=42). Tall fescue is also an obligate cross pollinator, meaning there is much genetic variability in the plant. This is the reason that tall fescue can adapt too many different regions in the United States. However, there are some downfalls in being an obligate cross pollinator. They are difficult to fix a genetic form for future generations, there are seed production issues, and they are difficult to breed.

## Growth

Tall fescue is a cool season perennial bunchgrass. It has dense tiller production, lots of leaf area close to the surface, is tolerant to grazing, is a long term perennial, and is weakly rhizomatous (Franzluebbers et al., 1999). It is best adapted to cool moist climates that have fertile well-drained soils. However, it tolerates infertile drought prone soils, as well as, poorly drained flood plains. It also tolerates acidity in soils ranging from a pH of 5.1 to 8.4 and is not susceptible to iron or magnesium deficiency. It's durability and plentiful leaf area close to the surface is the reason livestock producers love it. Some tall fescue fields date as far back as fifty to sixty years. Tall fescue also grows well with companion species such as legumes.

In Georgia, establishment of tall fescue should be made in mid-September to mid-October, sometimes as late as November. It is best to plant 9.1 kg an acre at 0.006 to 0.013 meters deep (Hancock et al., 2003). Grazing should be light the first year, starting only after June 1<sup>st</sup>, when the plant reaches 0.125 meters tall and it should not be grazed below 0.038 meters.

#### **Tall Fescue Disorders**

Soon after the widespread use of "Kentucky 31," farmers started reporting health issues in livestock grazing these lush pastures and fields. Horse breeders noticed reproductive and foaling problems in mares that were grazing tall fescue pastures or being fed tall fescue hay. Cattle producers reported that steers appeared unthrifty and that milk production in lactating dairy and beef cows was reduced. Several common disorders that were noticed in cattle are referred to as fescue foot, bovine fat necrosis, and fescue toxicosis (Thompson et al., 2001). Fescue foot is caused by the vasoconstriction of blood vessels; it usually occurs in extremities and can cause sloughing of hooves, tails, and ear tips. Other symptoms of fescue foot are rough hair coat, weight loss, elevated body temperature, and increased respiration rate. This usually occurs in the winter and may be noticed a few days after the first cold snap of winter. Bovine fat necrosis is characterized by the presence of necrotic fat lesions in the abdominal cavity and usually results in disturbance of digestion, kidney function, and birth. Fat necrosis occurs when blood flow to the body core decreases causing fat to harden and die (Thompson et al., 2001). It is commonly seen in cattle grazing tall fescue pastures with high rates of nitrogen fertilizer. Fescue toxicosis occurs mostly in the summer and is the common name that refers to all of the other problems seen in livestock such as; poor gains, reduced conception rates, decreased feed intake, decreased milk production, reproductive losses, intolerance to heat, increased respiration, increased body temperature, excessive salivation, retention of hair, decrease in prolactin levels, and listlessness. Increased body temperature and respiration are due to heat intolerance which is defined as the inability of an animal to adjust to increased ambient temperatures (Thompson et

al., 2001). Heat intolerance is thought to be due to vasoconstriction of the blood vessels leading to decreased flow to peripheral tissues. When cattle experience toxicosis, cortisol, which is stress induced, is released into the blood and leads to increased intake of ergot alkaloids in endophyte-infected tall fescue causing even more negative toxicosis effects (Thompson et al., 2001). Cortisol is a steroid hormone that regulates carbohydrate metabolism and maintains blood pressure. In cattle, there is a lot of absorption of ergot alkaloids in the rumen, but little to no absorption in the small intestines or abomasum. There are more reproductive issues in the mare than in the cow or ewe (Porter et al., 1992). Poor reproductive performance in mares includes prolonged gestation, spontaneous abortion, premature separation of the chorion (red bag), dystocia, thickened placenta, retained placenta, aglactia, and poor foal maturation (Porter et al., 1992). Foals can be dysmature with overgrown hooves, have large poorly developed muscled skeletal frames, irregular incisors, and a long hair coat (Porter et al., 1992).

#### The Endophyte

In 1977, there was a breakthrough discovery of an endophyte in tall fescue that caused reproductive and production issues in livestock. The man credited with discovery of the endophyte is Charles Bacon, PhD, a plant pathologist at the United States Department of Agriculture at the Richard Russell Research Center in Athens, GA (Roberts et al., 2005). His discovery of the endophyte lead to research on cattle. He was the first to document the detrimental effects of endophyte-infected tall fescue, known as fescue toxicosis (Roberts et al., 2005). Early studies were carried out at Auburn University. Several of his studies on cattle provided clear evidence that fescue did in fact have harmful effects on cattle consuming endophyte-infected tall fescue hay or pasture (Roberts et al., 2005). In the 1980's, research was conducted investigating the negative reproductive effects on mares consuming endophyte-

infected tall fescue. This research documented the detrimental reproductive effects that endophyte-infected tall fescue caused in mares.

It is suggested that over 90% of tall fescue pastures in the U.S. are endophyte-infected (E+); this is based on extensive state surveys (Ball et al., 1993). The endophyte is derived from a fungus and the fungal endophyte was originally called *Epichloe typhina* (Belesky et al., 2009). The endophyte was later reclassified and called Acremonium coenophialum (Morgan-Jones & Gams, 1982). A few years later the endophyte was once again reclassified and is now known as Neotyphodium coenophialum (Glenn et al., 1996). The endophyte is seed disseminated and causes toxicity by producing toxic alkaloid substances called ergot alkaloids. So infected plants yield infected seeds, therefore, plant-to-plant transmission does not occur (Lewis, 2005). The endophyte lives in a mutalistic relationship with the plant obtaining housing and soluble nutrients from the plant (Thompson et al., 2001). It does not penetrate the plant cells but rather grows inbetween cell walls. Therefore, every plant cell, seed, and stem contains endophytes but roots and leaves do not. Endophyte-infected tall fescue grows better than endophyte-free tall fescue. Almost 95% of all "naturalized" populations of tall fescue pastures contain endophytes (Yates et al., 1988). It is estimated that 80% of tall fescue growing in the United States is infected to varying degrees, usually at levels of 70% or more in pastures.

Tall fescue produces two diazaphenanthrene alkaloids independent of the endophyte fungus, called perloline and perlolidone (Thompson et al., 2001). Perloline was ruled out as a causative agent of fescue toxicosis because it was found in endophyte-free (E-) tall fescue (Bush et al., 1976). Researchers think that these diazaphenanthrene alkaloids decrease the number of toxic ergot alkaloids by inhibiting their growth. They have also been thought to decrease

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palatability and intake, caused by bitterness. All of these effects are thought to be mild compared to effects of ergot alkaloids.

Loline alkaloids such as N-formylloline loline and N-acetyl loline are saturated pyrrolizidine alkaloids. They are thought to be the agents in tall fescue that cause insect resistance (Siegel et al., 1989, 1990). It is also speculated that they can cause mild vasoconstriction, possibly suppress prolactin secretions, and are a weak D2-Dopamine receptor agonist. They possibly cause hyperthermia and fescue foot observed in cattle and sheep poisoned by fescue. However, lolines are not thought to be the causative agent in fescue toxicosis.

As mentioned before alkaloids are produced by a fungus and therefore are referred to as ergot alkaloids. There are several different ergot alkaloids that are produced: ergovaline, ergotamine, ergonovine, and lysergic acid amides (Thompson et al., 2001). These have been divided into three main groups of biologically active alkaloids that have been studied in the search of the cause of fescue toxicosis: ergopeptines, clavine alkaloids, and ergoline alkaloids (Hill, 2005). The ergopeptines include ergovaline, ergotamine, ergosine, ergocomine, ergonine, and ergocryptine. The clavine alkaloids include elymoclavine and argoclavine. The ergoline alkaloids include lysergic acid, lysergol, and ergonovine. Ergopeptines are believed to be the primary cause of fescue toxicosis since they account for atleast 50% or more of the total ergot alkaloid concentration in endophyte-infected tall fescue (Lyons et al., 1986). They are only found in endophyte-infected tall fescue and can be tested for by looking for ergovaline, since it accounts for more than 80% of the ergopeptine fraction (Paterson et al., 1995). Ergopeptines can interact with D-2 receptors,  $\alpha$ -2 adrenergic receptors, and serotonin-2 receptors (Fayrer-Hosken et al., 2008). Thus altering normal homeostasis, which results in multiple fescue toxicosis signs

and symptoms such as; vasoconstriction, hyperthermia, vomiting, decreased milk production, abortions, serotonin antagonism, and decreased prolactin levels. Ergopeptines also decrease serum prolactin concentrations through dopaminergic and antiserotonergic activities (Berde et al., 1978). Most symptoms and signs of fescue toxicosis can be mimicked by administration of natural and synergic ergopeptines (Garner, 1989, Ireland et al., 1989). Vasoconstriction is due to ergopeptines antagonizing  $\alpha$ -adrenergic receptors and decreasing prolactin secretion in the pituitary. This is due to ergopeptines interacting with dopamine receptors (Strickland et al., 1993). Clavine alkaloids can also produce some of the same symptoms of fescue toxicosis as ergopeptines, however, they are not thought to be the causative agent in fescue toxicosis. Another alkaloid, ergoline alkaloids, compose the other 50% of the total ergot alkaloid pool in endophyte-infected tall fescue (Hill, 2005). Thus, it is impossible to ignore ergoline alkaloids as a potential suspect in fescue toxicosis, along with ergovaline or in place of it.

The chemical structure of the three classes of ergot alkaloids impacts their ability to transport across gastric tissue, causing toxicity in livestock (Hill, 2005). Therefore, it is important to understand their chemical structure. The ergoline ring structure is common to all ergot alkaloids, it results from one molecule of tryptophan and one molecule of mevalonic acid (Hill, 2005). Tryptophan and mevalonic acid go through decarboxylation, oxidation, and isomerization steps forming the lysergic moiety that is common to all ergot alkaloids. The lysergic moiety can have a tricyclic peptide ring attached through a carbonyl at the 8<sup>th</sup> position in the D-ring forming a number of ergopeptine alkaloids (Figure 1) (Hill, 2005). Ergovaline, the



Figure 1: Basic structure of ergot alkaloids (Hill, 2005).

predominant ergopeptine is chemically different from other ergopeptines because it has a methyl group at  $R_1$  and an isopropyl at  $R_2$ . Ergoline alkaloids have simpler chemical structures than other alkaloids. They can have a carboxylic acid, methyl alcohol, azide, or amide group attached to the No. 8 carbon in the D-ring (Rutschmann et al., 1978). It is important to see the structural similarities between biogenic amines norepinephrine, epinephrine, dopamine, and serotonin with the lysergic moiety of ergot alkaloids and clavine alkaloids (Figure 2) (Paterson et al., 1995). Therefore, clavine, ergoline, and ergopeptine alkaloids all have the chemical structures necessary



Figure 2: Structural similarity between biogenic amines norepinephrine, dopamine, and serotonin with the lyserigic moiety of ergot alkaloids (Hill, 2005).

to elicit fescue toxicosis in livestock. The difference between clavine and ergoline alkaloids, and ergopeptine alkaloids is their polarity and relative solubility (Hill, 2005). Solubility of ergot alkaloids ranges from freely soluble to insoluble. Clavine and ergoline alkaloids are polar, have lower molecular weights, and are more soluble then ergopeptine alkaloids (Hill, 2005). When ergoline and ergopeptine are administered intravenously to mice, rabbits, and rats they are equally toxic, but when ergoline is administered orally it is much more toxic (Griffith et al., 1978). Ergoline alkaloids have a greater capacity for transport regardless of the tissues tested. This leads to the suggestion that ergoline alkaloids are more conducive to metabolism and transport through monogastric digestive systems. It also leads to the question of whether ergopeptine alkaloids such as ergovaline, are solely responsible for fescue toxicosis in livestock (Hill, 2005).

There are two main strategies that have been formed to determine the specific ergot alkaloid that causes a particular sign or symptom seen in fescue toxicosis. The first strategy is to isolate tissues or cells to look at effects in vivo (Larson et al., 1995). These studies indicate that ergoline and ergopeptines are similar because they both result in vasoconstriction and dopamine receptor activity. The second strategy is to administer alkaloids intravenously; Browning et al. (1997) used intravenous administration of ergonovine and ergotamine and found that both caused similar prolactin, growth hormone, and blood pressure responses. However, respiration rates varied between ergonovine and ergotamine (Browning et al., 1997).

#### Solutions

The endophyte in tall fescue gives it cold and drought tolerance, along with insect resistance (Bacon et al., 1997; West et al., 1993). However, the negative effects seen in livestock are far too devastating and solutions to fescue toxicosis are needed. Samples of pasture grass may be taken or sent to laboratories where ELISA or HPLC tests can be run to see if tall fescue is endophyte-infected (Wright et al., 2001). If it is infected, one solution is to try and kill the endophyte. A potential way to kill the endophyte is to treat the endophyte-infected tall fescue pasture with chemicals such as fungicides and herbicides (Hancock et al., 2003). Another solution is to put tall fescue seed under harsh storage conditions before reseeding or replanting a

pasture. This will kill the endophyte and help prevent your new seed planted from being infected. Harsh storage conditions refer to alternating between hot and humid conditions at temperatures between 25° and 35° C, because these temperatures are thought to reduce the endophyte. Endophyte-infected tall fescue pastures can also be mowed to keep them in a vegetative state rather letting them reach the reproductive state. Having diverse forages along with a tall fescue pasture is another way to reduce toxicosis in livestock. This is achieved by mixing in white clover, red clover, or annual lespedeza with endophyte-infected tall fescue. Endophyte-infected tall fescue pastures can be replanted if one has the time and patience to do this. Currently, there are several cultivars of tall fescue available that contain a non-toxic endophyte. These new cultivars are referred to as novel endophyte. Jesup/MaxQ is a novel endophyte that was developed by The University of Georgia and Ag Research in New Zealand, for the upper portion of the Eastern U.S (Hancock et al., 2003). There is also another variety of novel endophyte, MaxQ/Georgia-5, that was developed primarily for the lower portion of the tall fescue belt (Hancock et al., 2003). Both mixtures have the benefits of endophyte-infected tall fescue but contain non-toxic endophytes; therefore, no negative effects are seen in livestock.

Mares experiencing reproduction problems resulting from consuming endophyteinfected tall fescue can be treated with Domperidone, a D-2 dopamine receptor antagonist that combats the endophyte (Thompson et al., 2001). Mares treated with this orally, once daily, for ten to fifteen days prior to the their expected foaling date have shown to have shorter gestation lengths, decreased dystocia, decreased retained placentas, produce milk, and have higher serum prolactin and progesterone concentrations (Blanchard et al., 2003). Mares can also be removed from endophyte-infected pastures forty-five to sixty days before foaling (Blanchard et al., 2003). Currently, Domperidone is not approved for other livestock but there are other dopamine antagonists that they can be treated with (Ireland et al., 1989, Lipham et al., 1989, Rhodes et al., 1989).

#### **Photoperiod Affect on Hormones**

The effects that are seen in livestock are due to ergot alkaloid endocrine disruptors. Changes in environment, photoperiod, and feed can alter normal homeostasis in animals (Rhoades et al., 2003). Homeostasis keeps the body functioning normally, as well as, keeps the animal alive and well. A major regulator of homeostasis is the hypothalamus (Rhoades et al., 2003). It receives input from the internal environment of the body through signals in the blood. The hypothalamus is the major regulator of endocrine function because of its connection with the master gland of the endocrine system, the pituitary gland (Rhoades et al., 2003). Hormones are commonly affected by ergot alkaloids, and changes in hormone levels can have a wide range of effects on an animals' body functioning properly. For both the seasonal and non-seasonal breeder, normal hormonal synchronization with changing season is critical for growth, maturation, and reproductive efficiency (Porter et al., 1992). Therefore, it is very important to know where hormones are produced and released. The pineal gland regulates the release of melatonin, which is affected by season, and acts on the hypothalamus by increasing or decreasing production of hormones (Blanchard et al., 2003). In this way, the hypothalamus is thought to play a major role in regulating the biological clock (Rhoades et al., 2003). The hypothalamus stimulates the pituitary gland, which is composed of the anterior pituitary and posterior pituitary. The hypothalamus is responsible for the production and release of gonadotropin releasing hormone (GnRH) and thyroid releasing hormone (TRH). It is also involved in the production of vasopressin (AVP) and oxytocin, which are stored in the posterior pituitary. Neurons in the preoptic area of the hypothalamus secrete GnRH. These neurons

contain receptors for gonadal steroid hormones, testosterone and/or estradiol, which regulate GnRH secretion in the male or female (Rhoades et al., 2003). The anterior pituitary/adenohypophysis is responsible for the secretion of prolactin, adrenocorticotripic hormone (ACTH), growth hormone (GH), thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), and luteinizing hormone (LH). Each of these hormones synthesis and secretion is controlled by a releasing hormone. Corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), and growth hormone-releasing hormone (GHRH) stimulate the synthesis and secretion of ACTH, TSH, and GH respectively (Rhoades et al., 2003). Luteinizing hormone-releasing hormone (LHRH), also known as gonadotropin-releasing hormone (GnRH) is responsible for stimulating the synthesis and release of FSH and LH. Somatotropin release inhibiting factor (SRIF) inhibits the secretion of GH. All of these releasing hormones are peptides with the exception of dopamine (Rhoades et al., 2003).

Dopamine is a neurohormone released by the hypothalamus. Its main function as a hormone is to regulate the release of prolactin from the anterior lobe of the pituitary. Dopamine is a catecholamine that inhibits the synthesis and secretion of PRL. The posterior pituitary/neurohypophysis stores oxytocin and vasopressin. When stimulated, the posterior pituitary transmits one of these hormones to nearby capillary circulation, which then carries the hormone to systemic circulation (Rhoades et al., 2003). The pituitary gland is a complex endocrine organ that secretes peptide hormones that have important actions on almost every aspect of the body (Rhoades et al., 2003). These actions include but are not limited to; the animals' ability to grow, reproduce, and respond to stress and trauma. Stimuli that can affect the secretion of the pituitary hormone can originate in or out of the body (Rhoades et al., 2003). The hypothalamus and pituitary work together. For example, the release of target gland hormones begins with production of a releasing hormone by the hypothalamus. This releasing hormone stimulates the production of a tropic hormone by the anterior pituitary. This in turn stimulates the production of the target gland hormone by the target gland. The target gland sends positive or negative feedback back to inhibit or activate the releasing hormone from the hypothalamus, which then acts on a tropic hormone in the anterior pituitary (Rhoades et al., 2003). Two common tropic hormones that stimulate the ovaries and testes are FSH and LH (Figure 3).



Figure 3: Reproductive hormone model for feedback control in a stallion (From Blanchard TL, Varner DD: Evaluating breeding soundness in stallions. IV. Hormonal assay and testicular biopsy, *Vet Med* April: 358, 1996).

The olfactory system is directly related to photoperiod because it has a direct connection with the limbic system and facilitates coordination of behavioral, endocrine, and autonomic responses involved in mating (Rhoades et al., 2003). Olfactory cues are affected by the biological clock, which are affected by photoperiod, and are important in initiating mating especially in the seasonal breeder. Olfactory cues are driven by the hypothalamus's endogenous seasonal clock, and initiate hormonal control on the gonads (Rhoades et al., 2003). In horses, this hormonal release leads to the secretion of odorants, called pheromones, by the mare signaling to the stallion that she is receptive. Pheromone cues are powerful and initiate mating behavior in stallions, even at very low concentrations (Rhoades et al., 2003).

## Photoperiod

Photoperiod is one of the most important cues for livestock, allowing them to determine the season (Blanchard et al., 2003). Photoperiod is the duration of light and dark and is changed by the amount of light and dark there is during a day, month, or season (Blanchard et al., 2003). For instance during the summer months it stays light longer and during the winter is gets dark earlier. The reproductive system of the horse responds positively to increasing amounts of daylight and negatively to decreased amounts of daylight. In this way photoperiod has a profound effect on the reproductive performance of a seasonal breeder (Blanchard et al., 2003). In seasonal breeders such as the horse, reproduction is primarily regulated by photoperiod, while nutrition and climate are modifiers. The retina is very important in helping the horse perceive photoperiod (Rhoades et al., 2003). The retina is able to perceive light and produce electrical activity because light stimulates the retina. This electrical activity is then passed from the retina through the superachiasmatic nucleus (SCN), a center in the hypothalamus that serves as the brain's biological clock (Rhoades et al., 2003). An important pathway that influences the SCN is the afferent retinohypothalamic tract of the optic nerve. It originates in the retina and enters the brain through the optic chiasm and terminates in the SCN. This pathway is the principal means by which light signals from the outside world transmit the day/night rhythm to the brain's internal clock (Rhoades et al., 2003). This entire process is referred to as the retinohypothesis process (RHP), because it allows interpretation and adjustments of incoming photic signals before sending them to the pineal gland, thus regulating production of melatonin (Figure 4) (Rhoades et al., 2003). RHP even allows animals that are visually impaired or blind



**Figure 4:** Hormonal regulation by photoperiod in a mare and stallion (Blanchard et al., 2003). to perceive and respond to the photoperiod (Blanchard et al., 2003). The length of the photoperiod regulates reproduction in the seasonal breeder through regulation of GnRH secretions (Blanchard et al., 2003). GnRH secretions are dependent on the release of melatonin from the pituitary gland.

## Melatonin

Melatonin is a neurotransmitter hormone whose secretion is dependent on photoperiod (Blanchard et al., 2003). The pineal gland is responsible for secretion of melatonin. Synthesis of melatonin and secretion from the pineal gland is dependent on periods of light and dark (Reiter, 1981; Ebadi, 1984). In contrast to prolactin, melatonin secretions are increased during the night. Melatonin is secreted from the pineal gland during periods of darkness. The pineal gland is able to measure day light and adjust secretion of melatonin accordingly. The effect of melatonin on the reproductive system is said to be anti-gonadotrophic. Much of this inhibitory effect is due to inhibition of GnRH from the hypothalamus, which is necessary for the secretion of anterior pituitary hormones. Melatonin indirectly inhibits secretion of gonadotrophic hormones; prolactin, LH and FSH from the anterior pituitary. FSH in the stallion is responsible for

stimulating Leydig cells which produce testosterone. Testosterone in turn affects the reproductive tract and stimulates Sertoli cells. Impaired or altered secretion of melatonin and prolactin will affect a horses' reproductive performance, seasonal cycle of feed intake, and coat/hair growth (Porter et al., 1992). Without the pineal gland the horse would have a hard time knowing what season it was, which would ultimately affect reproduction. The reverse happens when there is increased day light, melatonin secretions are reduced and the inhibitory influence melatonin has on GnRH synthesis and secretion is removed. This leads to GnRH secretion and stimulation that acts on LH and FSH in the anterior pituitary, thus initiating the breeding season in seasonal breeders. In this way the pineal gland signals the hypothalamus through secretion of melatonin (Blanchard et al., 2003). The pineal gland is also dependent on activation converts serotonin to N-acetylserontonin, the precursor of melatonin. The pineal gland may function as a transducer or interface between seasonal changes and multiple adjustment associations with acclimation and reproduction, such as temperature and photoperiod (Heldmaier et al., 1986).

### Prolactin

Prolactin is produced and secreted from the anterior pituitary, upon proper stimulation from the hypothalamus by lactotrophs (Rhoades et al., 2003). Prolactin levels usually increase during the day and decrease during the night (Tucker et al., 1982; Tucker et al., 1984; Critser et al., 1988). Prolactin secretions from the anterior pituitary are dependent on light acting on the hypothalamus. Darkness inhibits production and secretion of prolactin from the anterior pituitary (Rhoades et al., 2003). Prolactin production and secretion can also be inhibited by dopamine (Rhoades et al., 2003). Prolactin is an important hormone that helps the horse recognize what season it is, is involved in milk production in the mare, and has some supportive action on androgenic hormones in the reproductive tract of a stallion (Rhoades et al., 2003). Therefore, stimulation of prolactin due to photoperiod is very important in the seasonal breeder.

Since the horse is a seasonal breeder, photoperiod is very important in regulating their breeding season. The physiologic breeding season can be manipulated to fit into the operational breeding season by artificially increasing the photoperiod (Blanchard et al., 2003). Artificial light helps the horse prepare for the breeding season by decreasing melatonin production and increasing the production of prolactin. Prolactin is an important hormone that is involved in reproduction, coat growth, feed intake, and respiration rate. There are several different ways to apply artificial light. One way is to put horses on lights eight to ten weeks before the breeding season. There must be a minimum of 3.048 meter candles at eye level; this is achieved when one can read a black and white newspaper in the darkest spot of the stall (Sharp et al., 1993). A horse needs light for fourteen to sixteen hours per day for eight to ten weeks, and it is best to add light at the end of the day or split it between the morning and evening (Sharp et al., 1993). Another way to supply artificial light is to give one-hour pulses of light eighteen and a half hours after the onset of daylight. Not only does light increase the production and secretion of prolactin, it helps to increase GnRH secretion by decreasing melatonin production. This allows GnRH to stimulate secretion of LH and FSH in the anterior pituitary (Rhoades et al., 2003). Therefore, impaired or altered secretion of prolactin may compromise the animals' physiological adaptation to seasonal changes and adversely affect reproduction.

#### Testosterone

Testosterone is the major steroid produced by the testis. Production of testosterone is the primary function of the testes (Blanchard et al., 2003). It is the sex hormone in the male and is responsible for primary and secondary sex characteristics (Rhoades et al., 2003). Primary characteristics include structures promoting sperm development, preservation, and delivery. The secondary characteristics include masculine behavior, large muscles, and big bone mass. Testosterone diffuses into the blood immediately after being synthesized (Rhoades et al., 2003). Once testosterone is released into circulation it functions as a pro-hormone, and it is converted to dihydrotestosterone (DHT) by the action of enzyme 5alpha-reductase (Rhoades et al., 2003). DHT is the most biologically potent natural androgen. Testosterone is necessary for normal sexual behavior and testicular function.

Spermatogenesis requires high intra-testicular levels of testosterone, secreted from LH stimulated Leydig cells. Testosterone diffuses across the blood testes barrier, complexes with antigen-binding protein, and then binds to Sertoli cell receptors (Rhoades et al., 2003). This is one reason concentrations of testosterone are higher in the testes rather than in systemic concentrations. Sertoli cells are responsible for maintaining the necessary testicular concentrations so that spermatozoa production occurs (Blanchard et al., 2003). Testosterone concentrations from the testes control the release of GnRH and gonadotropins through a negative feedback loop. If the concentration of testosterone is high in the testes inhibin is released by Sertoli cells. Inhibin acts on the hypothalamus by inhibiting release of GnRH which inhibits the release of FSH from the anterior pituitary, thereby, stopping the production and release of testosterone in the testes, activin is released that acts on the hypothalamus and anterior pituitary. This causes FSH to be released, which leads to production and release of testosterone.

Testosterone in stallions has been proven to have diurnal variation in concentration (Sharma, 1976). In a study, thirty-two stallions were used to test for diurnal variation of testosterone. Plasma testosterone was measured in peripheral blood of normal stallions by

radioimmunoassay (RIA). Twenty-four of the stallions had a single blood sample taken between 0930 and 1030 hours, the mean testosterone concentration was 3.06 +/- 1.27 ng/ml (range 1.08 to 5.14 ng/ml). In the other eight stallions blood was collected every four hours and there was evidence of diurnal rhythm. Evidence of diurnal variation in testosterone concentration results from the fact that the highest mean testosterone concentration was observed at 0800 hours and the lowest at 2100 hours P<0.01 (Sharma, 1976). All stallions showed low levels of testosterone between 0400 and 1200 hours. Levels then increased to a peak average of 3.49 +/- 0.89 ng/ml (range 2.74 to 5.2 ng/ml) at 0800 hours. The mean plasma testosterone (3.054 +/- 1.27 ng/ml) observed in this study was similar to another study conducted by Berndtson et al. in 1974, on stallions during the breeding season. In Berndtson et al. study, synthesis and secretion of testosterone was shown to be influenced by season (Berndtson et al., 1974). In another study, three stallions were bled every hour for twenty-five hours at twenty-eight intervals throughout one year. The results showed that testosterone pulse frequency and pulse amplitude were higher in the summer months P<0.01 (Byers et al., 1983). Mean testosterone levels were also highest in the summer but showed a secondary smaller increase in the autumn. Semen characteristics were also analyzed from two hundred and twenty-two stallions with the greatest volume in the summer P<0.01, but concentration P<0.01 and the total number of spermatozoa per ejaculate P<0.05 were highest in the autumn (Byers et al., 1983). These results show that the highest testosterone concentrations in peripheral plasma are not necessarily associated temporally with optimum semen quality (Byers et al., 1983).

#### **Animal Effects**

The greatest economic impact of endophyte-infected tall fescue on livestock is decreased productivity and reduced reproductive efficiency (Hoveland, 1992). Whether endophyte-infected tall fescue greatly reduces reproductive performance or gains it has devastating effects on livestock (Hoveland, 1992). It is well known how it affects cattle (bulls and cows), sheep (ewes and rams), and horses (mares) but little or no research has been conducted on stallions. Fescue toxicosis is more noticeable during the summer months when the temperature exceeds 31° C (Porter et al., 1992). Therefore, grazing of tall fescue pastures should be avoided during late June, July, and August since this is when the endophyte is most prevalent. However, the extent to which livestock are affected by endophyte-infected tall fescue depends on the type of livestock, environmental temperature, and the amount and chemical species of alkaloids consumed.

#### **Beef Cows**

As stated before, the effects of ergot alkaloids in cattle especially cows are more noticeable during the summer months. Cows seem to have the most devastating effects. It is estimated that annually, there is \$800 million impact on the beef industry due to losses induced by fescue toxicosis on reproductive performance and production (Paterson et al., 1995). Some common negative effects seen in cows consuming ergot alkaloids include: fescue foot, decreased average daily gain, fat necrosis, reduced feed intake, decreased milk production, heat intolerance, and reduced/poor reproductive performance. Heat intolerance can be caused by hair retention, which can cause imbalances in melatonin. Melatonin, in turn affects GnRH secretions from the hypothalamus (Stuedemann et al, 1988). These imbalances contribute to heat intolerance by elevating internal temperatures, ultimately causing reproductive issues. In a study, cows and heifers were fed endophyte-infected tall fescue based on a diet of ten to twenty percent. The results showed a decrease in average daily gain and a decrease in plasma prolactin. (Mizinga et al., 1990).

Ergot alkaloids are associated with alterations in reproductive hormones (Browning et al., 1998; Burke et al., 2001), estrous cycle parameters (Jones et al., 2003), follicle diameter prior to ovulation (Burke et al., 2002), and luteal dysfunction. Reproductive function in cows is governed by GnRH secretions from the hypothalamus. GnRH secretion affects gonadotrophic hormones, FSH and LH (Rhoades et al., 2003). These two hormones along with prostaglandin  $(PGF_{2\alpha})$  are the main hormones that affect reproduction. LH is known to possess oxytocic activity by stimulating uterine smooth muscle (Browning et al., 1998). A reduction in LH could be detrimental to the reproductive performance of cows by stimulating uterine contractility, which might induce release of the luteolytic agent  $PGF_{2\alpha}$  from the uterus (Browning et al., 1998). However, the only evidence found between ergot alkaloids and prostaglandin production was found in canine blood vessels (Browning et al., 1998). In one study, ovariectomized heifers consumed endophyte-infected tall fescue or endophyte-free tall fescue for sixty days. The study showed there was a decrease in circulating prolactin and growth hormone secretory profiles (Christopher et al., 1990). In another study, cows were fed a diet of ten to twenty percent endophyte-infected tall fescue seed. This resulted in reduced ADG and a decrease in plasma prolactin (Mizinga et al., 1990). The corpus luteum (CL) from cows grazing endophyte-infected tall fescue had fewer nuclei and greater number of large luteal cells with increased diameter. An ultrasound revealed an increase in cellularity with an increase in the number of mitochondria, lipid droplets, and secretory granules (Porter et al., 1992). So it may be the CL that limits the

animals' ability to maintain pregnancy. This all suggests that modified endocrine function may be one reason that consumption of endophyte-infected tall fescue hinders reproduction.

#### **Bulls and Steers**

In a study, bulls and steers were fed endophyte-infected tall fescue, this resulted in decreased prolactin and melatonin secretions (Porter et al, 1992). The lowered plasma prolactin concentration increased respiration rates. There was a decrease in dopamine (DA) antagonists in the pituitary stalk. There was an increase in dihydroxyphenylacetic acid (DOPAC), which is a metabolite of dopamine, and 5-hydroxyindoleacetic acid, which is a metabolite of serotonin. Therefore, when body temperature increases it may cause an increase in the turnover of pituitary dopamine and serotonin, which results in an increase in DOPAC and 5-hydroxyindoleatic acid. This increased turnover without synthesis overtime would lead to reduced dopamine, serotonin, and metabolites (Porter et al., 1992). Therefore, DOPAC alters neurotransmitter metabolism in the hypothalamus, the pituitary, and pineal gland. Thus impairing metabolism of neurotransmitters responsible for regulation of prolactin secretion, which may be secondary to the actual mechanisms of reduced reproduction efficiency (Porter et al., 1992).

Imbalances in prolactin and melatonin, along with restricted blood flow to internal organs, may be the principal cause of aberrant reproduction, growth and maturation (Porter et al, 1992). The other major issue with steers feeding on endophyte-infected tall fescue is increased basal body temperature and restricted blood flow to internal organs. The reduced blood flow to peripheral and core body tissues can cause a vasoconstrictive effect of N-acetylloline in the bovine lateral saphenous vein (Porter et al., 1992). Several studies show possible effects between N-acetylloline and ergovaline. These effects could cause reduced blood flow, which could alternatively compromise reproduction through hypoxia (Porter et al., 1992). Ergovaline

concentrations are high in tall fescue in the spring and early autumn, which coincide with the growing season for tall fescue and breeding season for livestock.

In a small study, Holstein bull's ages two to thirteen months were fed endophyteinfected tall fescue. No negative effects were seen on testicular weight, testicular dimensions, epididymal weight and length, or seminal vesicle weight. However, in three month old beef bulls it reduced GnRH secretion, which affected FSH and LH. FSH and LH influence testosterone secretion and spermatozoa production (Alamer et al., 1990). The endophyteinfected diet caused a reduction in the amount of Sertoli cells in eight to twelve month olds. In this way it may permanently impair testicular function (Alamer et al., 1990).

In a case study, Angus bulls were fed either a control diet or a toxic fescue diet (contained 1005 ppb of ergovaline) for sixty days. Semen was collected biweekly and was evaluated by looking at motility, morphology, and concentration. Scrotal temperatures and rectal temperatures were also taken biweekly along with scrotal circumference measurements. Significant differences were observed for scrotal temperatures P<0.001, scrotal circumference P<0.01, and spermatozoa concentration P<0.05 (Jones et al., 2004). Statistical evidence showed that scrotal temperatures were greater, scrotal circumferences were smaller, and spermatozoal concentrations were greater in the collections from the bulls fed endophyte-infected tall fescue diets (Jones et al., 2004). Neither sperm motility nor morphology was affected by dietary treatment. These results indicate that bulls consuming endophyte-infected tall fescue may experience reduced fertility due increased testicular temperatures and decreased testicular size (Jones et al., 2004).

In a similar study, thirty yearling beef bulls were used per year, in a two-year study. The purpose of the study was to determine the effects of grazing endophyte-infected tall fescue

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pastures on endocrine profiles, semen quality, and fertilization potential. Bulls were grouped according to scrotal circumference, bodyweight, breed composites, and age. They were allowed to graze fescue pastures from mid-November to the end of July, within each year. Blood samples, bodyweight, scrotal circumference, and rectal temperature were collected every fourteen days. Semen was collected every sixty days and motility and morphology were evaluated (Schuenemann et al., 2005). Bulls grazing endophyte-infected tall fescue had decreased bodyweight P<0.01, increased rectal temperature P<0.01, and decreased prolactin P<0.01, compared to bulls not grazing endophyte-infected tall fescue (Schuenemann et al., 2005). Sperm motility and morphology did not differ between the between the groups (Schuenemann et al., 2005).

Urinary analysis is a good way to test experimental livestock to make sure they are consuming toxic levels of endophyte-infected tall fescue (Hill et al., 2000). Urinary ergot alkaloid concentrations are usually measured during endophyte-infected tall fescue studies. These measurements are a means of assessing ergot alkaloid load, using ELISA employing monoclonal antibodies that are specific to the lysergic moiety of all ergot alkaloids (Hill et al., 1994). In beef steers, ergot alkaloid concentrations can rise rapidly within 12-48 hours of exposure to endophyte-infected tall fescue pastures. Ergot alkaloid concentration in the urine rapidly decreases within 24-48 hours after removal from endophyte-infected tall fescue pastures (Stuedemann et al., 1998; Hill et al., 2000).

# **Rams, Wethers, and Ewes**

In rams fed endophyte-infected tall fescue there were decreased levels of prolactin and delayed testicular growth (Barenton et al., 1980). Prolactin is associated with molting and regrowth of wool, as well as, reproduction in rams (Lincoln et al., 1985). Therefore, the relationship between melatonin and reproduction is prevalent in photoperiodic breeders like sheep (Tucker et al., 1984). In wethers, castrated rams, there was reduced blood flow to peripheral and core body tissues. Flow restriction can be improved by administration of a dopamine antagonist metoclopramide. Ewes consuming endophyte-infected tall fescue have decreased prolactin, decreased milk production, and lengthened intervals from introduction of the ram until conception (Bond et al., 1988). There were no observed differences in plasma or pituitary LH, FSH, GH, and TSH in the ewe.

### Mink, Mice, and Rats

In a study, male rats were fed a fifty percent endophyte-infected tall fescue diet. The study found decreased daily sperm output (DSO), decreased testicular parenchyma, and decreased epididymal size (Zavos et al., 1986). There was also a decrease in prolactin, which can affect the growth of male accessory reproductive glands. In the female rat fed endophyte-infected tall fescue there was prolonged estrus, decreased litter weights, decreased number of pups, and decreased prolactin levels. Prolactin is vital because it regulates CL function and pituitary gonadotropin release (Varney et al., 1988; Smith, 1980). In the mouse, the female was more sensitive to the negative effects of ergot alkaloids rather than the male (Porter et al., 1992). However, lowered fertility was much greater when the mice were fed in pairs. In mink the major effects of consuming endophyte-infected tall fescue was decreased prolactin production, which affected fertility, coat growth and shedding (Porter et al., 1992).

# Mares

Mares grazing endophyte-infected tall fescue have more detrimental effects on reproductive efficiency than any other livestock (Porter et al., 1992). Mares are sensitive to ergopeptine alkaloids at levels as low as 50-100 ppb, while cattle do not show signs of toxicosis until ergot alkaloid levels reach 1000-2000 ppb (Wright et al., 2001). It has been reported that 40% of all mares grazing endophyte-infected tall fescue have decreased reproduction efficiency (Porter et al., 1992). Some major reproductive issues seen after a mare has been bred are: decreased prolactin and progestagens, prolonged gestation from eleven to twelve months, spontaneous abortion, dystocia, thickened placenta, retained placenta, aglactia (suppression of lactation or no milk production), premature separation of the chorion, and abnormal foal maturation (Cross et al., 1995; Cross, 1997). Gestation can be increased to thirteen months, which can cause difficulties during parturition since fetal size is most likely increased (Putnam et al., 1991). The placenta is typically thickened due to an increase in connective tissue, which can cause stillbirth with the foal still encased in the fetal sac (Blanchard et al., 2003). Other placental abnormalities include edema, fibrosis, and mucoid degeneration, which result from hypoxia and decreased blood flow to this organ (Poppenga et al., 1984). Ergot alkaloids affect dopamine, which in turn blocks fetal cortisol decreasing estrogen and pregnancy rates (Putnam et al., 1990). However, if a mare is removed from an endophyte-infected tall fescue pasture forty-five to sixty days before foaling or at day 300 of gestation the deleterious effects usually resolve (Putnam et al., 1990).

Other physiological markers that have to do with reproduction that are reduced when consuming endophyte-infected tall fescue are prolactin and progesterone (McCann et al., 1992). These reductions occur because the pregnant mare produces a biochemical known as dopamine. Dopamine occurs naturally throughout the body and affects the function of glands, organs, muscles, and nerves. The endophyte produces toxic substances called ergot alkaloids that imprison dopamine receptors causing them to not function normally in the pregnant mare. This results in decreased production of both prolactin and progesterone. Prolactin, as stated above, is secreted from the pituitary gland and is responsible for stimulating milk production.

Progesterone is a key hormone responsible in maintaining normal pregnancy. Therefore, when these two hormones are reduced the mare can fail to produce milk and fail to carry the pregnancy to full term. Mares can be treated orally, once daily, with a dopamine-D2 receptor antagonist called Domperidone, ten to fifteen days before foaling (Blanchard et al., 2003). The negative effects of ergot alkaloids are usually alleviated after administration of this drug. Urinary ergot alkaloids and creatinine levels are elevated in mares consuming endophyte-infected tall fescue (Hill et al., 2000, Youngblood et al., 2004).

In a study conducted by Youngblood et al., (2004), twelve mares were used to evaluate the detrimental effects of consuming endophyte-infected tall fescue during early gestation. Mares were paired up according to their stage of gestation, day 65 to 100, and were assigned one of two diets and fed that diet for ten days. After removal from endophyte-infected tall fescue the mares were monitored from day eleven until day twenty-one. In the morning and evening rectal temperatures were recorded. Blood samples were collected on days 0, 5, 10, and 21 to measure progesterone and prolactin (Youngblood et al., 2004). Other blood samples were collected on alternate days to measure 3,4-dihydroxyphenyl acetic acid. Daily urine samples were collected for urinary alkaloid analysis. Rectal temperatures, serum prolactin concentrations, and progesterone concentrations did not differ between treatments P=0.96 (Youngblood et al., 2004). Plasma 3,4-dihyroxyphenyl acetic acid concentrations decreased P<0.05 but no fetal losses were observed (Youngblood et al., 2004). Urinary alkaloid concentrations were greater P<0.01 in mares consuming endophyte-infected tall fescue compared with mares consuming endophytefree tall fescue (532.12 +/- 52.51 and 13.36 +/- 2.67 ng/mg of creatinine). Elevated concentrations of urinary alkaloids were consistent with depressed endogenous catecholamine

activity. This suggests an endocrine disruptive effect of hypothalamic origin (Youngblood et al., 2004). Ergot alkaloids are absorbed rapidly from the equine digestive tract and translate into catecholamine metabolite 3,4-dihyroxyphenyl acetic acid concentrations, even after short exposure to endophyte-infected tall fescue (Youngblood et al., 2004). Elevated concentrations of urinary alkaloids significantly decreases endogenous catecholamine activity (Youngblood et al., 2004).

In 1986, a study was conducted at Auburn University. Twenty-two pregnant mares were fed either endophyte-infected tall fescue or endophyte-free tall fescue. Mares were on experimental diets during gestation, foaling, and after foaling. All mares except one being fed endophyte-infected tall fescue had foaling problems. Only three of eleven foals were born alive, and only one foal survived past the first week (Putnam et al., 1990). Only seven of the mares that were fed endophyte-infected tall fescue survived, while all of the mares on endophyte-free tall fescue survived and had normal pregnancies and foals (Putnam et al., 1990).

## Foals

Some major issues seen in foals are due to mares not being removed from endophyteinfected tall fescue pastures forty-five to sixty days before they foal (Blanchard et al., 2003). If mares are not removed from endophyte-infected tall fescue pastures before they foal the percentage for their foal to be born normal is low. Gestation length is usually increased causing dystocia because of increased foal and placental weights. Foals can be born dysmature with overgrown hooves, have poor and irregular incisor eruption, have a long hair coat, be extremely weak, have reduced immunity, and have large poorly developed muscled skeletal frames (Porter et al., 1992).

#### **Ruminants vs. Non-Ruminants**

Livestock animals have one of two digestive systems; they are either ruminants or nonruminants. Equines/horses and porcines/pigs are referred to as non-ruminants or monogastrics meaning they have one stomach. Bovines/cattle, ovines/sheep and caprines/goats are referred to as ruminants or gastrics, meaning they have a four chambered stomach. Ruminant animals are also referred to as foregut fermenters and non-ruminants are also referred to as hindgut fermenters. It is thought that ruminant metabolism of ergot alkaloids differs drastically from that of the non-ruminant system (Hill, 2005). By examining the two different digestive systems it is easy speculate that ergot alkaloids are absorbed differently. In ruminant animals ruminant microorganisms may alter chemical structures of alkaloids through metabolism (Hill, 2005). This could be one reason why cattle have more production issues when consuming endophyteinfected tall fescue or it could be the reason why horses experience more devastating reproductive issue than any other livestock. Toxicosis in livestock is directly related to exposure and ingestion of toxic forage (Hill, 2005).

### **Bovine Digestive Tract**

Cattle have no upper teeth so they use their tongues to rip up forages. Once the forage is swallowed it enters the reticulum. The reticulum resembles a honeycomb structure because it has a grooved design with a large surface area. Here undigested plant matter is compressed and regurgitated back up with saliva. It is then re-chewed and re-swallowed, this is called chewing cud. The purpose of this is to produce smaller food particles so that it can be better fermented by bacteria, protozoa, and fungi. After forage is swallowed for the second time it is deposited in the rumen. The rumen is the largest compartment of the stomach, where the most nutrients are absorbed. Digesta will remain here for fifteen to forty-eight hours. Microbial fermentation of

cellulose occurs here. The rumen has a large surface area, that is covered by stratified squamous epithelium and papillae that aid in nutrient absorption. The rumen is very sensitive to its environment because it has multiple layers that contain hairs. These hairs can poke into the layers causing ulcers. The rumen is thought to be the primary site of absorption of ergot alkaloids (Hill, 2005). Absorption of ergot alkaloids is an active system and favors ergoline, in particular lysergic acid over ergopeptine alkaloids. Ergovaline, an ergopeptine alkaloid, is less soluble in ruminal fluid than lysergic acid or ergonovine, and does not transport across the ruminal, reticular, or omasal tissues (Hill, 2005).

After digesta is fermented in the rumen it gathers at the top of the rumen where it enters the omasum. The entrance to the omasum is about the size of a dime and opens periodically to aspirate forage in. The reticular, ruminal, and omasal tissues are all capable of ergot alkaloid transport but the vast majority of alkaloids must transport across the ruminal/reticular tissues before exiting into the omasum (Hill et al., 2001). The omasum has an abundance of folds; therefore, it has a very large surface area that aids in water re-absorption. Water absorption here helps produce saliva, which is phosphate buffer. After leaving the omasum digesta enters the glandular stomach called the abomasum. This is where mucosal tissues secrete pepsin and acids aid in protein degradation. At the end of the abomasum is the pyloric sphincter, which leads to the small intestines. The small intestines contain microvilli, and proteins are absorbed at the polar tips of the microvilli through active transmission. Lipids are absorbed in-between the microvilli through passive transmission.

Ergot alkaloids are thought to affect assimilation of nutrients by altering motility and absorption. It has been demonstrated that ergovaline increases ruminal fluid dilution rate and outflow (Thompson et al., 1993), thereby possibly altering digestibility in cattle. However, evidence suggests that ergovaline may not be the only ergot alkaloid responsible for toxicosis or may not be the cause at all (Hill, 2005). Ergoline is suggested as the prime suspect in fescue toxicosis because it composes 50% of the ergot alkaloid fraction. It is more soluble in aqueous solutions that are typical to the gastric system of ruminants than ergovaline (Hill, 2005). Ergovaline is less soluble in the rumen because ruminal microorganisms have trouble metabolizing it (Hill, 2005), and the final form of the metabolite is unknown. Further studies have proved that ergoline is more toxic when administered. Urine analysis of ruminant's urine shows that the renal tubules are the primary site of excretion of ergot alkaloids. The primary alkaloid found in the urine was lysergic acid, which is an ergoline alkaloid (Hill, 2005).

# **Equine Digestive Tract**

Horses are non-ruminant hindgut fermenters. This means that most digestion is occurring in the hindgut, mostly in the large intestines and cecum. Horses have very small stomachs that do very little digesting. The reason that the stomach does very little digesting is because horses are grazers and are flight animals, meaning they run from predators or when they are frightened. This flight response results in direct activation of the sympathetic system, which leads to secretion of epinephrine from the adrenal medulla (Rhoades et al., 2003). The flight response causes a rise in blood pressure due to increased cardiac output. Increased blood pressure results in redistribution of blood flow that allows the muscles and heart to receive more blood, while the stomach and skin receive less (Rhoades et al., 2003). Horses use the flight response a lot and blood flow is redistributed to the heart and muscles resulting in the stomach receiving less blood, which results in decreased digestion. Therefore, it benefits the horse to have small amounts of food in their stomachs. Horses use their lips for prehension and chew their food in a circular motion. The more chewing that occurs the more saliva that is produced, which makes it easier for the horse to digest its food. Saliva is used for lubricating the esophagus and for buffering the proximal stomach, which is very acidic. Saliva contains sodium bicarbonate and posses little to no enzyme activity. A horse can produce as much as 9.08 to 12.11 liters of saliva a day (Lewis, 2005). Once food is swallowed it travels down the very long, four to five foot, esophagus which is located on the left side of the trachea. Since the esophagus is very long muscular contraction called peristalsis aid in transporting the food to the stomach, food enters the stomach through the cardiac sphincter (Lewis, 2005). The cardiac sphincter closes tightly after food enters the stomach. It does this because the stomach is very acidic and if acid entered the esophagus it could easily damage it. The cardiac sphincter also prevents the horse from vomiting or burping (Lewis, 2005).

The stomach composes eight to ten percent of the gastrointestinal tract and digesta remains here for a short time. Forages remain in the stomach for approximately thirty minutes and grains remain in the stomach for approximately fifteen to twenty minutes (Lewis, 2005). Since there is not a lot of digestion and absorption going on in the stomach, horses are extremely sensitive to a change in their diet. The stomach is composed of two different areas, each being covered by a different mucosal surface. There are also two different types of digestion that occur in the stomach. The first area of the stomach is called the squamous area and is responsible for fermentative digestion. The squamous area is composed of the esophageal and fundic regions, which contain microbes and lactic acid bacteria that break down food. The squamous area is found at the top of the stomach, is not protected from acid because of little mucus production, and is really more of a storage area rather than a digestion area. Fermentation stops as soon as

food falls into the glandular area. The glandular area composes the middle and bottom of the stomach, and it is responsible for bacterial digestion (Lewis, 2005). This area is known for secreting lots of mucus, acid, and enzymes. It is also divided into two different regions. The first region is the fundic region, which is composed of parietal cells that secrete HCL and chief cells that secrete pepsin. The next region is the pyloric region, it secretes gastrin that aids in breaking down food. There is continuous acid release in the glandular area; therefore, it benefits horses to have small amounts of food in their stomachs at all times. If horses did not have small amounts of food in their stomachs at all times ulcers might occur due to acid being continuously produced (Lewis, 2005). At the end of the stomach is the pyloric sphincter, which has a loose opening where digesta can freely flow in and out of the small intestines.

The small intestine makes up thirty percent of the digestive tract and is approximately seventy feet long. There is a rapid rate of passage in the small intestines due to neural and hormonal control. There are three different sections in the small intestines; the duodenum, the jejunum, and the ileum. The small intestines are the primary site of digestion and absorption of fat, soluble carbohydrates, and protein (Lewis, 2005). It is composed of epithelial cells that contain microvilli which project from the cell membrane (Anderberg et al., 1994). Located on the exterior of the microvilli is a protective barrier known as the glycocalyx barrier. This barrier protects the microvilli from damage as digesta passes through the small intestines. Subtending this protective layer thus surrounding the microvilli is the lipid layer. All absorbed substances must pass through this lipid layer. Once through this layer substances can diffuse through the cell membrane through pores or leaky gaps in adjacent cells (Figure 5). Digesta remains here



**Figure 5:** Microvilli surfaces of mucosal cells in the intestines taken by a transmission electron micrograph (Hill, 2005). approximately ninety minutes. Several enzymatic secretions are released from the small intestines; pancreatic juice, bile, and disaccharidases. Pancreatic juice has enzymes, fluid, electrolytes, and bicarbonate. Bicarbonate is released to help buffer acid from the stomach as it enters the small intestines. Bile comes from the liver and aids in digesting fat (Lewis, 2005). Bile does not come from the gall bladder because horses do not have a gall bladder. The reason they do not have a gall bladder is because their diets are low in fat and they only have small amounts of food in their stomach at a time. Disaccharidases aid in digesting disaccharides, and the amount of disaccharides digested changes with age.

The large intestine makes up approximately sixty-five percent of the digestive tract. It is composed of the cecum, large colon, and the small colon. Digesta remains in the large intestine approximately thirty-two to sixty hours due to fermentation (Lewis, 2005). Fermentation of digesta is beneficial in breaking down and absorbing structural/insoluble carbohydrates such as; gas, water, electrolytes, B vitamins, vitamin K, microbial crude protein, and volatile fatty acids. Horses need to be fed quality protein feed in order to get the proper nutrients since they cannot create their own protein like cattle can (Lewis, 2005). In the large intestines, digesta enters the cecum then the large and small colon. The cecum is around four to five feet long and is referred to as a blind sack because digesta first travels up the cecum and then travels down the cecum (Lewis, 2005). Digesta is broken down by microbes and protozoa

here, and this is where most water re-absorption occurs. Digesta then travels to the colons where it is further broken down by fermentative digestion.

A horse's diet should consist mostly of forages, since this is necessary to help maintain a healthy gut. Horses need atleast one percent of their body weight in long stem dry matter forage a day (Lewis, 2005). However, care must be taken when feeding endophyte-infected tall fescue since different amounts of endophyte-infected tall fescue toxins can be present in hay or pasture. Toxicosis is related to continuous exposure to the toxin, resident time in digesta, and continuous ingestion of toxic fresh forage (Hill, 2005). Mares are sensitive to ergot alkaloids at low levels. Most horses can eat around three percent of their body weight a day. Lysine is the first limiting amino acid, and this is why protein quality is more important than quantity. Grain should be used as a supplement to pasture and hay, when these are not providing enough nutrition. Grain should not be fed more than 0.75% of the horse's body weight per meal (Lewis, 2005).

#### **Stallion Anatomy**

It is important to understand the unique anatomy and physiology of the stallion. Only then can we understand what affects stallion reproduction and fertility. Some important things to understand that will be discussed below are; how testicular size can influence sperm production, why stallion libido and sperm production are seasonally oriented, and how anabolic steroids can have an effect on reproduction and fertility. It is also important to understand why older stallions can produce and store more spermatozoa than younger stallions, how injury and body temperature can affect fertility, why artificial light affects the breeding season, and the effects frequent breedings can have on sperm quality and production.

# Testes

The testes are first formed within the colt's abdominal cavity and descend right before or after birth. The testes have two main functions, spermatogenesis and steroidogenesis. Both of these functions are regulated by pituitary gonadotropins, LH and FSH (Rhoades et al., 2003). The testes are normally oval shaped, have a smooth outline, and are slightly turgid with a resilient texture. A stallion with soft/mushy or excessively hard testes is potentially a poor producer of spermatozoa and generally has abnormal seminal characteristics (Lewis, 2005). The average stallion's testicle should measure 0.085 to 0.11 m in length, 0.045 to 0.06 m in width, 0.05 to 0.065 m in height, and weight around 0.225 kg in a stallion four years of age or older (Blanchard et al., 2003). The total scrotal width which is the largest measurement taken across both testes and scrotal skin is approximately 0.095 to 0.115 m. Testicular size is highly heritable in stallions (Lewis, 2005). The testes are covered and protected by the scrotum which is composed of two sacs that are separated by a septum. The scrotum should be thin and elastic with a distinct neck, and the testes should be able to move freely in their scrotal pouches (Blanchard et al., 2003). The scrotum is composed of four layers. The first layer is external and is composed of many sweat glands; this layer is called the skin. This layer is involved in the cooling process during hot weather. The next layer forms the outermost component of each scrotal sac and is composed of smooth muscle. This layer is called the tunica dartos. The smooth muscle in this layer allows for movement of the testes, which is important for temperature control. The third layer is composed of loose connective tissue and stands true to its name being called the connective tissue layer. Connective tissue allows for vertical and horizontal movement up to 180° (Blanchard et al., 2003). The last layer is a membranous sac and is considered part of the testes and not as much a layer. This layer is called the parietal

vaginal tunic, which extends all the way from the abdominal cavity to the bottom of the scrotum and forms a covering for the spermatic cord, testis, and epididymis. Between the testes and parietal vaginal tunic is a space called the vaginal cavity, it contains a watery serous fluid that serves as a lubricant and facilitates movement of the testes within the sac. The primary function of the scrotum is to regulate temperature. It is able to do this with the help of the pampiniform plexus and cremaster muscle (Rhoades et al., 2003). The pampiniform plexus is where there is a plexus of veins and the testicular artery is coiled within the plexus of veins. This serves as a countercurrent heat exchanger between warm arterial blood reaching the testes and cooler venous blood leaving the testes (Rhoades et al., 2003). The pampiniform plexus is very important for temperature regulation. The cremaster muscle also responds to temperature change by moving the testes closer or farther away from the body. A stallion's testes need to stay between 37-38° C, if they get too hot or cold negative effects can be seen as early as three to four days (Rhoades et al., 2003). Elevated temperatures can lead to temporary or permanent sterility (Rhoades et al., 2003).

After the so called, "parietal vaginal tunic layer" you will find the tunica albuginea which is fused to the parietal vaginal tunic. Next there is a non-capsular part called the parenchyma, which consists of seminiferous tubules. These tubules are found inside the testes and are involved in production and conveying spermatozoa. Each testis contains hundreds of tightly packed seminiferous tubules that are arranged in lobules. These lobules are separated by extensions of the tunica albuginea and open up on both ends into the rete testes (Rhoades et al., 2003). Each seminiferous tubule is composed of two somatic cell types, myoid cells or Sertoli cells, and germ cells. The seminiferous tubules are surrounded by a basement membrane called the basal lamina with myoid cells on the perimeter (Rhoades et al., 2003). Located in the interstitial tissue between seminiferous tubules are Leydig cells that are involved in the production of testosterone which cause the stallion to become sexually aggressive (Rhoades et al., 2003). Leydig cells are large polyhedral cells that are often found in clusters near blood vessels in the interstitium between seminiferous tubules. Leydig cells primary role is the production and secretion of the steroid hormone, testosterone. They are well equipped to produce steroids because they have numerous mitochondria, a prominent smooth ER, and conspicuous lipid droplets (Rhoades et al., 2003). Leydig cells contain receptors for prolactin, which may synergize with LH to stimulate testosterone production by increasing the number of LH receptors (Rhoades et al., 2003). Testosterone can diffuse from Leydig cells crossing the basement membrane and enter Sertoli cells and bind to ABP. This results in high levels of testosterone within the seminiferous tubules. Different amounts of testosterone are secreted depending on the levels in the peripheral blood. For instance, if there is an increase in testosterone due to increased production by the testes or after an injection of a hormone, feedback is relayed to the hypothalamus and anterior pituitary to suppress discharge of GnRH and LH (Blanchard et al., 2003). Therefore, Leydig cells produce less testosterone and the concentration of testosterone around the seminiferous tubules drops. Testosterone is mandatory for spermatogenesis and proper functioning of Sertoli cells (Rhoades et al., 2003). Within the seminiferous tubules, on the inside of the basement membranes are Sertoli cells. They are involved in the production of spermatozoa. Sertoli cells are attached to one another at tight junctions, and these tight junctions divide each tubule into a basal and adluminal compartment. Mitosis of spermatogonia occurs in the basal compartment of the seminiferous tubule. The early meiotic cells move across the junctional complexes into the adluminal compartment where they mature into spermatozoa after meiosis (Rhoades et al., 2003). Spermatozoa that develop in the

adluminal compartment are not recognized as "self" by the immune system. Since they are not recognized at "self" by the immune system sometimes antibodies are developed against them destroying these spermatozoa. The function of Sertoli cells is influenced by FSH and testosterone (Blanchard et al., 2003); however, around the cells testosterone levels will be much higher than detected in the blood. FSH and testosterone must be at adequate levels so that Sertoli cells produce an environment appropriate for spermatogenesis. Androgen-binding protein (ABP) serves as a carrier of testosterone in Sertoli cells, as a storage protein for androgens in the seminiferous tubules, and as a carrier of testosterone from the testis to the epididymis (Rhoades et al., 2003). FSH acts on Sertoli cells which secrete two protein hormones: inhibin, which acts on the adenohypophysis to suppress the amount of FSH secreted in response to GnRH, and activin, which stimulates FSH secretion (Blanchard et al., 2003). The number of Sertoli cells affects how many spermatozoa are produced, therefore, the greater the number of Sertoli cells the greater the number of spermatozoa produced (Rhoades et al., 2003). Some functions of these cells are: to provide structural support, provide nutrition to germinal cells, help with movement of developing germinal cells, discharge mature spermatozoa, phagocytosis of degenerating germinal cells, and production of estradiol. Sertoli cells are also involved in the secretion of fluids and proteins that bathe cells and convey spermatozoa through the tubules (Rhoades et al., 2003). Sertoli cells also synthesis an iron-transport protein that is important for sperm development.

Located between the non-proliferating Sertoli cells are germ cells that are at various stages of division and development. Spermatogenesis is the process of transformation of germ cells into spermatozoa (Rhoades et al., 2003). Cells at different stages of development are spaced along each tubule in a "spermatogenic wave." Since the sperm cells in each tubule are rapidly dividing and undergoing meiosis they are extremely sensitive to external agents that could alter cell division (Rhoades et al., 2003). The rapid dividing and "spermatogenic wave" helps to ensure continuous production of fresh spermatozoa. Germ cell development is critically dependant on Sertoli cells. Sertoli cells also function as the blood testes barrier. They function as this because they form tight junctions with each other which limits the transport of fluid and macromolecules from the interstitial space into the tubular lumen (Rhoades et al., 2003). Anything that interferes with Sertoli cells interferes with spermatogenesis. It is postulated that abnormally high levels of plasma FSH concentration accompanied with high plasma LH concentration indicates testicular degeneration in older stallions (Blanchard et al., 2003). Conversely, it is postulated that low plasma LH concentrations are associated with poor fertility, low libido, and impotence in stallions even in the presence of normal testosterone. It is important to remember that hormones are released in a pulsatile manor when drawing blood. It is recommended to collect hourly blood samples between 0900 and 1300 hours, since this is when gonadotropin and testosterone usually peak in a stallion (Blanchard et al., 2003).

Stallion testes weigh between five and ten grams at birth and remain this weight and size during the first ten months. There is slight growth between eleven and sixteen months of age, and rapid development at eighteen months. Sometimes testes do not reach final maturation until the stallion is twelve or thirteen years old (Sellnow, 2001). Spermatozoa can first appear in a stallion's ejaculate at 11 to 15 months of age. A stallion may be used in breeding as young as two to three years of age, however, spermatozoa production is generally lower in the younger stallion (Berndtson et al., 1989).

# **Production of Semen**

There is a specific order in which spermatozoa are produced. Production starts in the convoluted seminiferous tubules, and then travels to the straight tubules which converge in a group of interconnecting rete tubules. The rete tubules penetrate the tunica albuginea and fuse with the efferent ducts, which merge with the epididymal duct (Rhoades et al., 2003). There are three phases that occur in the seminiferous tubules. First there is spermatocytogensis which takes around 19.4 days, then there is meiosis which leads to spermatids which takes around 19.4 days, and last there is spermiogenesis which takes 18.6 days. The total Spermatogenic cycle takes fifty-six to fifty-seven days (Blanchard et al., 2003).

After development occurs in the testes the spermatozoa enter the epididymis, in particular the ductus epididymis. There are several functions of the epididymis; storage, protection, transport, and maturation of sperm cells (Rhoades et al., 2003). The epididymis is composed of three sections. The first section is the Caput and this is where the efferent ducts combine into one duct and re-absorption of fluids and secretions occurs. Next is the Corpus, this is where sperm maturation occurs which takes three to four days. Here the sperm acquire surface glycoprotein's that act as stabilizing factors but also prevent sperm-egg interactions until capacitation and the acrosome reaction occurs (Rhoades et al., 2003). This area of the epididymis is not affected by ejaculation. Lastly, there is the Cauda epididymis, this is where spermatozoa mature and are stored prior to ejaculation. This area of the epididymis is affected by ejaculation (Blanchard et al., 2003). For example, if a stallion is not collected for a long period of time old spermatozoa are pushed out in the urine. Another important thing to remember is that if a stallion is not collected over the winter months his first ejaculation could have abnormal sperm and low motility (Blanchard et al., 2003). The total

maturation time spent in the epididymis is usually around seven to ten days. After the mature sperm leave the epididymis through the epididymal duct they travel into the deferent duct. This extends through the spermatic cord to the pelvic urethra. The urethra is a long mucus secreting tube that extends from the bladder to the free end of the penis. It has a thick muscle overlay that allows for powerful contractions that help aid in ejaculation. The urethra contains the vas deferens and ampulla.

There are three accessory sex glands in the stallion that help make up the seminal plasma (Blanchard et al., 2003). The first accessory sex gland is the prostate gland. The prostate gland is a single firm nodular gland that contains two lobes. This gland secretes a then watery fluid that helps to clean the urethra, boost the volume of the seminal plasma, and contains proteins that help to increase fertility. The next accessory sex gland is the Bulbourethral gland otherwise known as the Cowper's gland. It is positioned on either side of the pelvic urethra and is responsible for secretions that contribute to the seminal plasma. The final gland is the vesicular gland otherwise known as the seminal vesicles. The vesicular glands are elongated hollow pouches that are 0.15 m long by 0.05 m in diameter. These glands secrete a gelatinous material. This does not affect semen quality but should be filtered off after the stallion is collected. If not filtered off it will result in low motility and a low sperm count reading (Rhoades et al., 2003). The amount of gel fraction produced is highly related to the individual stallion. Typically older stallions have more gel fraction.

Semen is sensitive to physical trauma, light, cold, shock, or extensive heat. It needs to be kept at 37-38° C, which is pre-warmed body temperature (Sellnow, 2001). There are two different ways to determine how much semen a stallion produces. One way to determine a stallions output is to calculate daily sperm production (DSP), this is the amount of spermatozoa produced within a twenty-four hour period (Blanchard et al., 2003). This varies among stallions and is influenced by testicular size. The other way to determine a stallions sperm output is to measure daily sperm output (DSO), this is the number of spermatozoa that can be collected in a twenty-four hour period and is determined by collecting once in a twenty-four hour period for ten days (Blanchard et al., 2003). The average is then taken for days eight, nine, and ten. When evaluating semen one must consider that the number of sperm can be affected by numerous changes such as; it can change from season to season, with age, with testicular size, and with the size of extra-gonadal sperm reserves (Blanchard et al., 2003). Spermatozoa numbers are usually higher in older stallions that are on an infrequent breeding schedule compared to younger stallions on a more frequent breeding schedule.

# Penis

The penis is composed of three regions. The first region is called the root which is the site of attachment to the skeletal system, which is stabilized by two suspensory ligaments. Next is the main portion of the penis, the body/shaft called the corpus cavernosum (Rhoades et al., 2003). The body is composed of spongy erectile tissue and veins. This part of the penis is very sensitive and can easily be irritated when the stallion is being collected. The last part of the penis is the Glans penis, otherwise known as the corpus spongiosum (Rhoades et al., 2003). It is the enlarged free end of the penis which is composed of spongy erectile tissue that surrounds the urethra.

The penis is responsible for ejaculation of seminal plasma. Ejaculation involves three different processes. First, there must be an erection which is caused by blood flowing to the penis either from visual or sensory stimulus of a mare in heat. Next there is emission which involves the movement of; spermatozoa, fluids from the ductus deferents and cauda epididymis,

and fluids from accessory sex glands into the pelvic urethra (Rhoades et al., 2003). Lastly, there is ejaculation which is the expulsion of semen through the urethra. Ejaculation involves not only sperm excretion but secretions from the accessory glands to help promote sperm survival and fertility (Rhoades et al., 2003). There are three accessory glands that add to the seminal fraction in a certain order. First, there is a watery fluid ejaculated as a pre-spermatozoal fraction that comes from the prostate gland. Next, there are three to six discharges of the spermatozoal fraction that contain prostatic and Bulbourethral gland fluids. Lastly, there is a gel fraction released from the vesicular glands. The amount of ejaculation can depend on the time spent stimulating/teasing the stallion with the mare (Blanchard et al., 2003). Sometimes stallions do not have a complete ejaculation and the only way to tell this is by collecting the stallion an hour later. The second ejaculation should be half of the first ejaculation, if it is then the first ejaculation was a complete ejaculation.

# **Stallion Collection**

Before collecting a stallion an artificial vagina (AV) must be chosen and properly prepared. There are several different types of AV's available to use. There is the Colorado, the Missouri, the Japanese, the Roanoke, and disposable AV's. AV selection is based on specific requirements and personal preference. The model used at the University of Georgia is the Missouri model AV. It is one of the most commonly used AV's in the United States because it is inexpensive, easy to assemble and clean, and has a lightweight design. The Missouri is composed of a double-walled heavy duty rubber liner containing a permanently sealed water chamber, which is placed in a leather carrying case (Blanchard et al., 2003). The heavy duty rubber and sealed water chamber help prevent water leakage. The temperature in this AV can be adjusted to a higher level without causing damage to the sperm, because the glans penis should be beyond the water jacket during ejaculation (Blanchard et al., 2003). Therefore, it can be advantageous for stallions that prefer a higher temperature. The AV is also pressurized so that it can be equipped with water and air to help reduce weight (Blanchard et al., 2003). The main disadvantage to this model is that it does not regulate temperature but this is usually not a problem for stallions that are collected regularly.

The proper way to prepare the Missouri AV is to first attach a sterile semen collection bottle or bag to the end of the AV. Then a sterile glove must be used to apply a sterile lubricant to one third of the inside of the AV, leaving the sterile glove in the AV until use so that dirt and other outside debris will not contaminate the AV. Tap water should be heated to 45-50° C to provide an internal AV temperature of 44-48° C and the AV should be filled up to a determined pressure level and caped off (Blanchard et al., 2003). Luminal pressure is very important and the AV should be adjusted so that pressure is uniform around the penis. Some stallions may prefer a higher temperature but one must be careful of this since anything above 45° C could cause damage to the spermatozoa. This is usually not a problem when using the Missouri model since the glans penis protrudes outside the water jacket when ejaculation occurs. After filling and capping off the valve, it is important to wipe off the outside of the AV since water kills spermatozoa (Blanchard et al., 2003). The AV should now be placed in its protected leather jacket and a thermometer should be used to check for correct temperature.

To prepare the stallion for collection a teaser mare is needed to excite the stallion. Once the stallion is fully erect he can then be walked to the phantom which he will mount. Breeding phantoms are used because they allow for more consistent collections and reduce the likelihood of injury to the stallion, mare, and collector (Blanchard et al., 2003). After the stallion mounts it is the collector's job to deflect the penis into the AV. Allowing the stallion to thrust into the AV, making sure that the AV is kept at a proper angle and that the proper pressure is applied. This helps promote consistent stallion performance and maximal spermatozoa harvest (Blanchard et al., 2003). There are several signs to know when a stallion has ejaculated. The stallion may flag his tail, pulsation of the urethra at the base of the penis can be felt or seen, and the collector will see semen in the collection bag (Blanchard et al., 2003). At this point it is important to let the stallion pull out of the AV, and then the collector can head to the lab to process the semen as quickly as possible. There are special techniques designated by every breeding barn across the United States designed to work best with the stallions at that barn.

In the lab it is important to remember that semen is sensitive to light, physical trauma, cold, or excessive heat. It needs to be kept at 37-38° C, which is pre-warmed body temperature (Blanchard et al., 2003). It is best to have an incubator set to this temperature so that the semen can be stored in here while measurements are taken. It should be handled quickly and properly as to not interfere with any measurement. One of the first things that should be done in the lab after a collection is to filter the semen with a nontoxic filter in a graduated cylinder to remove the gel fraction and any debris. Filtration allows for the maximum number of spermatozoa in each semen collection (Blanchard et al., 2003). The volume and color of the gel-free semen is recorded. Volume by itself is seldom an important determinant of stallion fertility, it is mostly important to measure because it is used to calculate the total number of sperm in an ejaculate (Blanchard et al., 2003). Volume can be increased by increasing the time spent teasing the stallion, however, this will not change the sperm number per ejaculate. Ejaculate volume is affected by season, smaller volumes are usually produced in the winter and larger volumes are produced in the summer (Blanchard et al., 2003). A small sample of semen is taken with a sterile dropper and placed on a pre-warmed slide and observed under a microscope where

motility is accessed and recorded. Motility is achieved by looking at the percentage of progressive spermatozoa moving in a rapid, linear manner (Blanchard et al., 2003). This should observed in several different areas of the slide. Spermatozoal motility in general reflects the viability of the sperm population. Although not absolute, there have been some positive correlations between spermatozoal motility and fertilizing capacity (Blanchard et al., 2003). Motility is not affected by season (Lewis, 2005). In a majority of studies conducted, there have been poor correlations seen between motility and morphology. A Densimeter (Animal Reproduction Systems) can be used to calculate the spermatozoal concentration of the raw semen (Figure 6). The Densimeter should first be standardized for 100% transmittance through a dilute



Figure 6: A densimeter (Animal Reproduction Systems) can be used for estimation of spermatozoal concentration, ratio, and dose in a stallion (Blanchard et al., 2003).

cuvette, 180  $\mu$ L of mixed gel-free raw semen is pippetted into the cuvette (Blanchard et al., 2003). The cuvette is then capped off and is gently inverted several times to evenly mix the semen with the diluent. It is then placed in the Densimeter and the door is closed. The Densimeter will then read concentration and display it on the screen. This concentration can be used to determine the volume/ratio required to inseminate one mare, after motility and volume are entered into the machine. The total sperm number/concentration calculated in the Densimeter is one of the most important measurements used to estimate a stallion's fertility

(Blanchard et al., 2003). This is subject to seasonal variation but can also be affected by numerous factors such as rate of ejaculation, age, testicular size, and disease. The total number of sperm obtained from a mature stallion's ejaculate can range from 4 to 12 billion, but can exceed 15 to 20 billion in a sexually rested stallion (Blanchard et al., 2003).

Morphology and bacteria slides can be made to determine how much bacteria is in the semen and to see if there are any abnormalities in sperm. Morphology slides are usually background stains that are stained with an eosin-nigrosin stain. Sperm morphology is important to look at and can give insight in predicting stallion fertility. Morphology slides of spermatozoa are usually examined with a light microscope at 1000x magnification using the oil immersion lens. Atleast 100 spermatozoa should be evaluated for evidence of defects (Blanchard et al., 2003). The type and number of abnormalities should be recorded, along with the amount of normal sperm. Abnormalities in sperm morphology have traditionally been broken down into primary, secondary, and tertiary effects. Primary defects are anything associated with a defect in spermatogenesis, therefore, of testicular origin. Secondary defects occur in the excurrent duct system. Tertiary defects develop in vitro as a result of improper semen collection or handling procedures (Blanchard et al., 2003). However, the most current trend is to record the number of specific morphologic defects, such as detached heads, proximal or distal droplets, bent or irregular midpieces, and bent or coiled tails (Figure 7). This type of recording reveals more



Figure 7: Morphology of normal and abnormal sperm (Blanchard et al., 2003).

specific information about a population of sperm avoiding assumptions about origin of defects and the possibility of classifying an abnormality wrong (Blanchard et al., 2003). However, the value of sperm morphology studies are met with a degree of skepticism since some stallions have sperm morphological abnormalities and still achieve good pregnancy rates (Blanchard et al., 2003). Or vice versa, stallions with little or no abnormalities have decreased fertility. A recent study on sperm morphological defects on fertility was performed on sixty-four stallions. The study found that the percentage of abnormal heads, midpieces, and proximal droplets significantly affected fertility in a negative way (Sellnow, 2001). However, the good news is that abnormal sperm do not interfere with normal sperm, so it could be argued that the number of normal sperm in an ejaculate is what really matters in predicting fertility of a stallion (Blanchard et al., 2003).

# **Sperm Function**

In relation to other livestock such as the bull, boar, and ram, a stallion's sperm are relatively small. However, the goal of all livestock's sperm is to fertilize an oocyte. In a stallion, fertilization cannot happen until maturation has been accomplished in the epididymis. After leaving the epididymis the sperm are mixed with seminal fluids during ejaculation. At this point the sperm become motile, receiving energy from glucose. However, sperm have to have the right pH to become motile. The pH of normal semen is slightly basic and ranges from 7.2 to 7.7 (Blanchard et al., 2003). Measurements are most accurate when taken with a calibrated pH meter within one hour of collection; however, pH paper may be used. Several factors can influence pH such as season of the year, rate of occurrence of ejaculation, and spermatozoal concentration (Blanchard et al., 2003). An abnormally high pH could be due to contamination of ejaculate with urine, soap, or due to an inflammatory lesion of the internal genital tract. This is

one reason that extenders are used when artificial breeding is preformed. They help to maintain the proper pH that is perfect for breeding. The most common extenders are milk based and have a pH range of 6.6 to 7.2, which helps optimize spermatozoal motility and avoid premature capacitation during the cooling process (Blanchard et al., 2003). Also, the addition of antibiotics to extenders helps in eliminating bacteria.

After entering the mare's reproductive tract two events must occur in the oviduct for the sperm to achieve final maturation (Blanchard et al., 2003). The sperm must undergo capacitation and the acrosome reaction in order to bind and penetrate the zona pellucida. Capacitation is an irreversible process that involves increased motility, removal of surface proteins, loss of lipids, and the merging of the acrosomal and plasma membranes of the sperm head (Rhoades et al., 2003). Capacitation occurs when there are changes in pH and in the plasma membrane of the sperm due to the physiologic concentration of free Ca<sup>++</sup>. This induces vesiculation of the plasma membrane. These changes allow the cell to undergo the acrosome reaction, which is the shredding of the spermatozoal plasma membrane and the outer acrossmal membrane (Blanchard et al., 2003). This allows acrosomal contents to be released which aid in the penetration of cumulus cells and the zona pellucida and then fusion with the plasma membrane. This is also accompanied by an altered beating pattern of the flagellum resulting in hyper activated motility that aids in penetration. Capacitation has a minimum duration of six hours in a stallion, which needs to be taken into account before artificial insemination occurs (Blanchard et al., 2003).

There are several very important structures in a stallion's sperm that need to be understood in order to better understand how fertilization occurs. First, there is the head which contains a nucleus composed of DNA and is surrounded by a double layered membrane called the nuclear envelope. The two main components of the head are condensed chromatin and the acrosome. The acrosome is a lysosome-like structure that buds from the golgi apparatus, flattens, and covers most of the nucleus (Rhoades et al., 2003). The acrosome contains proteolytic enzymes, such as hyaluronidase, acrosin, neuraminidase, phospholipase A, and esterases (Rhoades et al., 2003). These are inactive until the acrosome reaction occurs. The head is covered by a spermatozoal plasma membrane, outer acrosomal membrane, and postacrosomal lamina. Next there is the neck, which connects the head and middle piece of the sperm together. The neck is a very complex structure which is very fragile (Blanchard et al., 2003). Abnormalities can be seen here in the form of cytoplasmic droplets. After the neck there is the middle piece that is composed of nine dense fibers and numerous mitochondria to supply energy for the tail (Rhoades et al., 2003). Lastly, there is the tail which is composed of the principal piece and end piece. The principal piece contains fibers that are a continuation from the middle piece. These fibers slide back and forward past each other producing tail movement which causes sperm movement (Rhoades et al., 2003). The end piece contains the tail fibers end, as well as, the end of the plasma membrane. The entire body of the sperm is coved by a plasma membrane, which has different functions at different locations on the sperm. It is composed of lipids, phospholipids and cholesterol. Damage to the plasma membrane will result in sperm death (Blanchard et al., 2003).

To be able to fertilize an oocyte sperm must be fully developed and several processes must be undergone. The sperm must maintain progressive motility, metabolism, enzymes, proteins, and proper distribution of lipids (Blanchard et al., 2003). Progressive motility is important in order for the sperm to find the oocyte. Enzymes that are located in the acrosome are important for penetration through structures surrounding the oocyte. The proper distribution of lipids in the plasma and acrosomal membrane are responsible for stabilizing structures until fertilization. Lipid are important for viability and fertilization potential of sperm. Sperm lipids are high in polyunsaturated fatty acids, in particular DHA and DPA (Rhoades et al., 2003). These fatty acids are obtained from dietary precursors. In other words the body converts what we feed them. The production of energy through metabolism is vital for fertilization to occur. Proteins such as glycoproteins obtained from the epididymis and seminal proteins obtained from the accessory sex glands during ejaculation are found in the plasma membrane of the sperm (Blanchard et al., 2003). These proteins are very important for survival in the mare's reproductive tract.

# **Factors Determining Sperm Output**

There are numerous factors in the stallion that can affect sperm output. Testicular size can affect output because the number of sperm produced is highly correlated to testicular size, which is a highly heritable trait. Testicular size is affected by the time of the year (Blanchard et al., 2003). Total scrotal width, seminal volume, and the number of sperm per ejaculate can increase from 15-20% in mid-winter to 40-50% in late spring and summer (Lewis, 2005). Since sperm output is highly correlated to testicular size it is easy to predict daily sperm output (DSO) by measuring the testes. DSO determines how many billions of sperm are produced a day. Calipers can be used to measure the length, width, and height of each testicle in centimeters, which is then multiplied by .5233 to determine the total volume in milliliters for each testicle (Blanchard et al., 2003). The total volume of each testicle is then added together and multiplied by .024, which is then subtracted 1.26 to come up with the daily sperm output. If the predicted DSO is significantly less than the actual DSO then low spermatogenic effects are usually present

due to testicular dysfunction (Blanchard et al., 2003). However, an ultrasound examination is the best way to get accurate measurements of the testes to predict output.

Age as well can affect sperm output. Sperm reserves of sexually rested stallions' increases with age, however, age significantly affects testes size (Blanchard et al., 2003). As stated before some stallion's testes do not reach final maturation until the stallion is twelve or thirteen years old.

The frequency of ejaculation can also affect sperm output depending on how frequently a stallion is collected. If the frequency of ejaculation is increased from one to two times a day it does not result in increased sperm output neither does collecting from three to six times a week (Blanchard et al., 2003). However, if the stallion is only collected once or twice a week a difference will be seen sperm output. Ejaculation has little to no effect on fertility of individual sperm and increased collections will not cause immature sperm to be ejaculated (Lewis, 2005).

Libido is another important factor that can affect sperm output. Excellent semen quality in a prospective breeding stallion is inconsequential unless the stallion has the desire and ability to deliver the semen (Blanchard et al., 2003). Libido is usually the limiting factor in mature stallions and determines how many mares can be bred naturally in one day. Typically a stallion with good libido will show immediate and intense desire for a mare in estrus when they are first introduced (Blanchard et al., 2003). Most stallions approach a receptive mare with a flexed, extended, trotting-type gait while making throaty sounds. Once introduced to the mare the stallion will develop an erection, become restless, start pawing, vocalizing, sniffing, licking, and nipping at the mare (Lewis, 2005). When smelling the mare's genitalia or urine, stallions will curl their upper lip and extend their head backwards, this is called the Flehmen response (Blanchard et al., 2003). The intensity and duration of contact with the mare is affected by a

stallions' genetic makeup. It can be altered by season, disease, or a learned behavior from a positive or negative experience. Length of teasing and mounts tends to increase during the winter compared to the summer (Blanchard et al., 2003). Teasing stallions to help increase sexual drive will increase the seminal volume but will not increase the total sperm per ml (Lewis, 2005). Libido in the stallion is highest in the spring and decreases in the winter due to decreased daylight and fewer mares being bred during this time. The physiological mechanisms of stallion sexual behavior are not well understood but it involves an intricate relationship between the neural and endocrine systems (Blanchard et al., 2003).

As stated before season has a lot to do with sperm output (Lewis, 2005). Since the horse is a long day breeder the highest output is usually seen in June. Temperature is a small modifier compared to light exposure. Exposures to artificial light two to three months prior to the breeding season will result in increased LH, prolactin, and testosterone, and a decrease in melatonin (Blanchard et al., 2003). This will increase stallion fertility by increasing libido and spermatozoa per ejaculate, however, effects are less pronounced in stallion than they are in mares (Lewis, 2005). Artificial light is a way to prepare a stallion for the breeding season but is not full proof. It is critical to allow stallions to go through periods of darkness, usually in the fall because if they are under lights for excessive periods of time they will respond to lights poorly in the future (Blanchard et al., 2003).

Last but not least, hormones whether natural or drug induced have an effect on sperm output. Levels of testosterone are highly correlated to the time of year. High levels of exogenous testosterone can lead to a negative feedback loop that acts on the hypothalamus and GnRH which decreases testosterone production and release. A human chorionic gonadotropin (hCG) test can be preformed to check testosterone levels. hCG increases LH levels which increases production of testosterone. Other hormones that can be correlated to testicular dysfunction are FSH, inhibin, and estrogen (Rhoades et al., 2003). Blood altering drugs can interfere with Sertoli cells which can alter sperm production. Anabolic steroids can affect Leydig cells ultimately affecting testosterone production, which inadvertently affects sperm production.

Very little is known about the effects of endophyte-infected tall fescue on stallion fertility, and the exact mechanism of ergot alkaloid toxicity is unclear in stallions. No research has been reported in literature other than one review by Fayrer-Hosken et al., (2008), on the effects of endophyte-infected tall fescue on stallion fertility. In this review, it talks about stallions that were treated with bromocriptine, an ergot alkaloid agonist, which caused a decrease in semen volume (Fayrer-Hosken et al., 2008). A preliminary study was conducted with six stallions during the breeding seasons of 1995-1996 in Smithsonia, Georgia. Stallions were fed one of two diets, one consisted of endophyte-infected tall fescue and the other consisted of endophyte-free tall fescue. Ejaculates were collected from each of these stallions. The results showed no difference in any parameter in the spermiogram (Fayrer-Hosken et al., 2008). Some research has been conducted on geldings consuming endophyte-infected tall fescue seed. Ten stallions of mixed breeding were fed one of two diets at 0.7% of body weight a day for twentyone days. One diet consisted of endophyte-free tall fescue seed and the other diet consisted of endophyte-infected tall fescue seed, which contained 0.5 mg ergovaline and 0.3 mg lysergic acid/kg diet/day (Schultz et al., 2006). Once assigned to experimental diet treatments, geldings remained on the same treatments throughout the study. The concentration of alkaloids fed in this study was based on the results of a 4-year survey taken on a horse farm in Kentucky. Here the survey reported an average forage concentration of ergovaline of 0.5 mg/kg dry matter during

summer and autumn, which is when clinical signs of fescue toxicosis typically occur (Long et al., 2004). A concentration of 0.5 mg/kg diet was reported as the threshold level for horses in which clinical signs of fescue toxicosis become evident (Hovermale et al., 2001). Results showed no significant differences between the diets; rectal temperature did not increase, weight loss did not occur, and concentrations of prolactin did not change. Concentrations of prolactin in serum were not affected by treatment; in addition, neither initial nor extended exposure to endophyteinfected tall fescue seed affected prolactin concentrations. However, a day effect was observed (P<0.001) in which concentrations of prolactin in serum decreased in both treatment groups over the course of the experiment (Schultz et al., 2006). These results agree with those found by McCann et al. (1992a), where yearling geldings consumed endophyte-infected or endophyte-free tall fescue hay for a 5-month period. Concentrations of prolactin decreased in both groups over the course of the experiment but did not differ between the two groups. These results lead to contradictory findings observed in mares and geldings, which suggests that concentrations of prolactin in serum may be a better indicator of fescue toxicosis in mares than in geldings (Schultz et al., 2006).

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# CHAPTER 3

# THE EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE ON STALLION REPRODUCTIVE PARAMETERS

#### Abstract

The purpose of this study was to determine whether stallion reproductive parameters were affected by endophyte-infected tall fescue. A total of six stallions were used in this experiment and each stallion was used as their own control. The stallions were randomly assigned to one of two groups, and randomly assigned one of two diets. One diet consisted of endophyte-infected tall fescue seed and the other consisted of non-toxic endophyte tall fescue seed, MaxQ. Stallions were fed one percent of their body weight in experimental grain diets consisting of 45% fescue seed and were also fed Common Bermuda hay and Annual Ryegrass ad libitum. Stallions received the experimental diets for 70 days, October 13, 2008 through December 21, 2008, and were then taken off the experimental diets for 70 days. Then experimental diets were switched between the groups and stallions received the experimental diet for another 70 days, March 9, 2009 through May 17, 2009. Rectal temperatures, testicular temperatures, and prolactin samples were taken every other day for the first two weeks and then once a week for the duration of both trials. Urine samples were collected weekly for the duration of both trials. Stallions were collected once a week, one group was collected on Mondays and the other group was collected on Tuesdays for the duration of both trials. Several semen parameters were measured; gel-free volume, motility, concentration, number of doses, and morphology. Ultrasound measurements of the testes were performed every twenty-third day

during both trials. Human chorionic gonadotropin was administered every twenty-fourth day to measure testosterone levels and responsiveness during both trials.

Stallions consuming endophyte-infected tall fescue had significantly lower (P<0.0004) gel-free volumes than stallions consuming non-toxic endophyte tall fescue. However, gel-free volume does not affect concentration of spermatozoa and motility of spermatozoa, therefore, it does not alter stallion reproductive parameters. Stallions consuming endophyte-infected fescue also had higher (P<0.0002) urinary alkaloid and creatinine levels at 145.126 ng alkaloid/mg creatinine than stallions consuming the non-toxic endophyte fescue (MaxQ) fescue seed at 12.175 ng alkaloid/mg creatinine. Several results had a significant interaction with time and trial; however, these interactions with time and trial were not affected by the treatment they were on. These interactions with time and trial were expected since the stallion is a seasonal breeder and the two trials were conducted in different photoperiods and seasons. Therefore, no matter what season or photoperiod it is stallion reproductive parameters measured in this study, were not affected by endophyte-infected tall fescue.

#### Introduction

Consumption of tall fescue infected with a fungal endophyte *Neotyphodium coenophialum* causes fescue toxicosis in cattle (Glenn et al., 1996). In cattle several signs of fescue toxicosis include: increased body temperature, retained hair coat, reduced ADG, reduced feed intake, decreased serum or plasma prolactin levels, and heat intolerance (Burke et al., 2001). Over the years research has been performed to find solutions to fescue toxicosis. Most of the research investigating fescue toxicosis has been conducted in cattle and has shown that cattle are affected more seriously, than other livestock such as sheep and horses. Studies have also been conducted to determine the main ergot alkaloid affecting livestock and how it affects them. The primary causative agent of fescue toxicosis is believed to be ergovaline (Paterson et al., 1995). Therefore, The University of Georgia and Ag Research in New Zealand developed a non-toxic endophyte that does not produce the ergot alkaloid ergovaline. This non-toxic endophyte is called MaxQ (distributed by Pennington Seed, Inc., Madison, GA) and has shown promising results in grazing studies (Bouton et al., 2002). However, more research suggests that ergoline may be the causative agent of fescue toxicosis or act in conjunction with ergovaline (Hill, 2005).

In recent years, research has been conducted researching the negative effects endophyteinfected tall fescue has on mares. Mares feeding on endophyte-infected tall fescue have shown to have more devastating reproduction anomalies than any other livestock such as; dystocia, retained placenta, aglactia, spontaneous abortion, red bag, and prolonged gestation. Most research has been conducted in mares since this is where the most prominent issues have been observed. Little attention has been paid to stallion to see if he has any negative effects when consuming endophyte-infected tall fescue. No research has been reported in literature other than one review by Fayrer-Hosken et al. (2008). In this review, preliminary tests were conducted on six stallions during the breeding seasons of 1995-1996. Results collected from each stallions ejaculate showed no difference in any parameter in the spermiogram (Fayrer-Hosken et al., 2008). However, some research has been conducted on geldings consuming endophyte-infected tall fescue seed. Results showed that rectal temperature did not increase, weight loss did not occur, and concentrations of prolactin did not change (Fayrer-Hosken et al., 2008). Therefore, more research is needed to conclude if endophyte-infected tall fescue affects stallion reproductive parameters. The objective of this study was to determine if and how endophyteinfected tall fescue affects stallion's reproductive parameters.

### **Materials and Methods**

### **Experimental Design**

Six mature breeding stallions, ranging from 7-12 years old, were used in this experiment to study the effects endophyte-infected tall fescue had on stallion reproductive parameters. Stallions were randomly assigned to one of two groups and an experimental diet was randomly assigned to each group. One diet consisted of endophyte-infected tall fescue. The endophyte-infected tall fescue seed was obtained from Top Notch Farms in Carthage, Missouri. The other diet consisted of a non-toxic endophyte tall fescue, MaxQ. The MaxQ seed was donated by Pennington Seed Company located in Madison, Georgia. Three stallions were fed endophyte-infected tall fescue and the other three were fed MaxQ. Diets were fed to the stallions for 70 days. After the initial 70 days of feeding, the stallions were taken off of the experimental diets for 70 days and were fed Common Bermuda, Annual Ryegrass, and a balanced concentrate mix. Then the experimental diets were switched between the two groups and the stallions were again fed the experimental diets for 70 days. The purpose of the two 70 day experimental trials was to permit the stallions to adjust to normal conditions before receiving the second dietary treatment. The length of the trial was determined by the length of the spermatogenic cycle in the stallion. Spermatozoa production in the testes takes an average of 56 to 58 days, and spermatozoa maturation takes an average of 7 to 10 days. Therefore, the average length of a spermatogenic cycle in a mature stallion is 70 days. This experiment was designed to run the entire length of a spermatogenic cycle in order to accurately assess the effects of endophyteinfected tall fescue on the spermatogenic cycle and reproductive parameters in the breeding stallion. On the first two days of each trial stallions were weighed in the morning to determine an average weight. Concentrate feed intake was set at 1% of body weight. The total experimental diet fed was split between morning feedings, which occurred at 0800 hours, and evening feedings, which occurred at 1600 hours. They were allowed access to Common Bermuda and Annual Ryegrass pastures during the day and fed ad libitum Bermuda grass hay while being stalled. Prior to being placed on experimental diets, the stallion's testes were ultrasounded for baseline measurements: vasculature in the pampiniform plexus, cross section of each testicle, and length of each testicle. Each stallion was housed in a 3.0 m x 7.3 m stall. Stallions were housed in stalls all day for the first two days of each trial while urine samples were collected. After the first two days of each trial stallions were housed in stalls during the night and placed in paddocks during the day, after their morning feeding. One exception to the stallions being stalled was when urine was collected once a week with a collection harness. During this time they were housed in stalls during the day and placed in paddocks at night, after their evening feeding. All care and procedures were approved by the University of Georgia Institutional Animal Care and Use Committee, AUP #A2009-10011-0 and by the Department of Animal Welfare Assurance #A3437-01.

# **Experimental Diet**

Two different experimental diets were set up consisting of tall fescue seed. The first diet consisted of 45% endophyte-infected tall fescue seed and was 90+% infected. It was mixed with cracked corn, whole oats, fat, molasses, rolled wheat, salt, dicalcium phosphate, calcium carbonate, vitamin ADE premix, vitamin E premix, and a trace mineral premix. The second diet consisted of 45% non-toxic endophyte tall fescue seed and was 90+% infected with non-toxic endophyte tall fescue (MaxQ - Flecha Endophyte AR-542). It was also mixed with cracked corn, whole oats, fat, molasses, rolled wheat, salt, dicalcium phosphate, calcium carbonate, vitamin ADE premix, vitamin E premix, and a trace mineral premix (Table 1). Both experimental diets were mixed at the University of Georgia feed mill. Each stallion was fed an experimental diet at 1% of their body weight per day throughout each trial. Fed was provided in equal portions in the morning and evening of each day. Stallions were reweighed every 23 days and diet amounts were adjusted if weight loss or gain occurred. In addition to the experimental grain mix all stallions had access to fresh water, Common Bermuda grass and hay, and Annual Ryegrass ad libitum. Uneaten experimental diets were weighed back each morning and recorded.

Ingredients	per 1000	MaxQ (%)	Endophyte-infected
Cracked corn	250.00	25%	25%
Whole Oats	82.20	8.22%	8.22%
Fat	40.00	4%	4%
Molasses	70.00	7%	7%
Rolled Wheat	80.00	8%	8%
Fescue Seed (endophyte-infected or Max Q)	450.00	45%	45%
Salt	5.00	0.5%	0.5%
Dicalcium phosphate	9.50	0.95%	0.95%
Calcium carbonate	8.00	0.80%	0.80%
Vitamin ADE premix <sup>b</sup>	1.50	0.15%	0.15%
Vitamin E premix <sup>c</sup>	2.50	0.25%	0.25%
Trace mineral premix <sup>d</sup>	1.00	0.10%	0.10%
Total	1000.00	100%	100%

**Table 1:** Composition of Experimental Diets<sup>a</sup>. One diet consisting of 45% endophyte-infected tall fescue/E+ seed and the other diet consisting of 45% non-toxic endophyte tall fescue/MaxQ seed.

<sup>a</sup>Percentage on an as-fed basis of 1% body weight

<sup>b</sup>Provided per kg of mix: vitamin A, 2 million IUs/lb, vitamin D, 1 million IUs/lb, vitamin E, 2,000 IUs/lb <sup>c</sup>Contains 20,000 IUs/lb vitamin E

<sup>d</sup>Provided per kg of mix: Ca, 10.35%, P, 12.40%, Cu, 5%, Fe, 5%, Mn, 12%, Zn, 12%, Se, 600ppm

## **Sample Collection**

Before the start of the each trial all six stallions were taken to the University of Georgia, College of Veterinary Medicine Teaching Hospital where testicular ultrasounds were performed. These ultrasounds were performed to obtain baseline measurements of each testes length, width, height, and vasculature in the pampiniform plexus. After the start of each trial stallions were ultrasounded on day 23, day 46, and day 71 of each trial to determine if changes in testicular size occurred or if blood flow to the testes was altered during the experiment.

Urine was collected from each stallion, in order to do this each stallion had a collection harness placed on him in the morning. Urine was collected at hours one, three, seven, twelve, and twenty-four after the initial morning feeding on day one of each trial. The numerous urine collections on day one of each trial were taken to access how fast creatinine and urinary alkaloid levels increased once stallions consumed endophyte-infected tall fescue. After day one, urine samples were collected weekly on Wednesdays during the third week of each trial and thereafter each week for the remainder of each trial. Collection harnesses were put on the stallions at 0900 hours following jugular venipuncture, rectal temperature, and testicular thermal scanning. Urine samples were taken to ensure that stallions were consuming high enough levels of ergot alkaloids to potentially interfere with reproductive parameters, and to make sure stallions consuming non-toxic endophyte tall fescue were not consuming toxic levels of ergot alkaloids. Once urine was collected in the collection harness, a sterile pipette was used to collect a small sample. The sample was then placed in a cryogenic vial which was labeled and stored in the freezer at -20°C until both trials were completed and the samples could be analyzed. Urine was analyzed for concentrations of creatinine and urinary alkaloids (Hill et al., 2000). Creatinine is a by-product of muscle metabolism, a derivative of muscle creatine phosphate, and is produced continuously in the body and is excreted in the urine (Rhoades et al., 2003). Creatinine is not only filtered by the kidneys but is also secreted by the kidneys. Another purpose of weekly urine collections was to determine how much urinary alkaloid levels changed during the duration of each trial.

Prolactin levels were measured throughout the course of the each trial. In order to do this blood was collected via jugular venipuncture every other day for the first two weeks of each trial (starting day one and ending day fourteen) and then once a week for the remainder of each trial. In order to accurately access if there were any changes due to experimental diets baseline prolactin samples were taken before each trial. Blood was drawn weekly on Wednesdays at 0900 hours. Blood was allowed to clot in labeled Vacutainer (BD Vacutainer –Serum Collection Tubes; Becton, Dickinson & Company, Franklin Lakes NJ, USA) tubes for several hours and centrifuged at 1,500g for 15 minutes. Serum was pippetted off of the top of the sample and put into appropriately labeled cryogenic vials and stored in the freezer at -20°C. Once both trials were completed serum samples were sent to Clemson University where they were analyzed.

A human chorionic gonadotropin (hCG) challenge was performed on each stallion and blood was drawn to determine if there were changes in testosterone levels between experimental diets. Blood was drawn for this prior to each trial and on day 24, day 48, and day 72. Jugular venipuncture was performed the day after ultrasounding, this was to ensure there were no affects from sedation drugs given during ultrasounding. Before testosterone samples were taken 5cc of hCG was administered to each stallion intravenously in the jugular vein at 0900 hours. In order to ensure appropriate testosterone levels in response to the hCG challenge blood was not drawn until two hours later at 1100 hours. Blood was then allowed to clot for several hours in appropriately labeled vacutainer (BD Vacutainer –Serum Collection Tubes; Becton, Dickinson & Company, Franklin Lakes NJ, USA) tubes. It was centrifuged at 1,500g for 15 minutes, then serum was pippetted from the surface of the tubes. Two milliliters of serum was then placed into appropriately labeled cryogenic vials and stored at -20° C. Samples were sent to BET laboratories in Lexington, Kentucky for analysis.

Stallions' rectal temperatures were taken every other day for the first two weeks of each trial and then once a week on Wednesday mornings at 0900 hours for the duration of both trials. Rectal temperature was taken with a digital thermometer. Barn temperature was taken with an outdoor temperature gauge. The purpose of taking rectal temperature was to determine if stallions were able to regulate their body temperature while consuming an experimental diet consisting of endophyte-infected tall fescue seed or non-toxic endophyte tall fescue seed.

Along with rectal temperature a thermal imaging temperature scan was taken of each stallions testicles every other day for the first two weeks of each trial and then once a week on Wednesday mornings at 0900 hours for the duration of each trial. Each testicle was scanned from top to bottom and side-to-side. The mean highest temperature and mean lowest temperature was recorded for each stallion. The thermal imaging temperature scan was taken to determine whether stallions were able to regulate testes temperature properly or not while being fed the experimental diets.

Stallions were collected once a week for the duration of each trial in order to assess semen parameters. Each trial lasted ten weeks. Three stallions were collected every Monday and the remaining three stallions were collected every Tuesday for the duration of each trial. Each stallion's ejaculate was evaluated for; gel-free volume, concentration, motility, doses, pH, morphology, and bacteria. Gel-free volume was measured in a graduated cylinder after proper filtration of the gel fraction and any debris. A pH measurement was taken with pH paper immediately after filtration. The pH paper was dipped into a small amount of semen that was poured into a sterile container and pH was read immediately. Motility was evaluated using a light microscope to determine the average number of progressive linear motile sperm, which was then recorded. Sperm cell concentration was measured by a Densimeter (Animal Reproduction Systems). Two morphology slides were made for each stallion using an eosin-nigrosin stain. The morphology examination was performed with a light microscope at 1000 x (oil emersion lens). Two cytological slides were made using a Diff Quik® stain to detect any bacteria in the semen. A total of four slides were made for each stallion, two morphology slides and two bacterial slides. One hundred sperm were randomly observed under a light microscope to determine the percentage of; normal sperm, abnormal heads, abnormal midpieces, and abnormal tails. Marks were denoted under the proper category that each individual sperm fell in. A total of fifteen different frames were observed on each bacteria slide; bacteria are shaped like rods and are ranked 0+, 1+, 2+, 3+, and 4+. A ranking was given to the slide after observing all fifteen different frames. A ranking of a 1+ means there are only one or two rods per high power field, a

ranking of 2+ means two to ten rods per high power field, a ranking of 3+ means there are more than ten to thirty rods per high power field, and a ranking of 4+ means there are rods everywhere in the frame. Neutrophils, yeast, lymphocytes, and red blood cells were also noted if seen in the bacteria slides.

#### **Chemical Analysis**

Urinary alkaloids and creatinine were analyzed using commercial test kits (Agrinostics Limited Company, Watkinsville, GA) for both trials. Tests were run over a period of three days. Testosterone blood samples from both trials were sent to BET Labs. Here commercial RIA laboratory kits (BET Labs, Lexington, Kentucky) were used to analyze the testosterone samples. Prolactin samples from both trials were sent to the Endocrine Physiology Laboratory Department of Animal and Veterinary Medicine (Clemson University, Anderson, South Carolina). Here another commercial laboratory kit (Endocrine Physiology Laboratory, Anderson, South Carolina) was used to analyze prolactin samples.

#### **Statistical Analysis**

Statistical analysis of data was performed using the mixed procedure of SAS (SAS Institute Inc, Cary, NC). The model included independent variables: horse, order, trial (1 or 2), treatment (A or B), and time. Time represented the date of sampling through each trial. The design of the statistical analysis in SAS was a cross over design with repeated measures. SAS was used to determine the least squares means and the treatment differences (significance at P<0.05). Results were presented as least squares means for testosterone, collection measurements, morphology, ultrasound measurements, temperature readings, and creatinine and urinary alkaloids.

### **Results and Discussion**

Each trial consisted of a 10-week period. All samples were taken at the same intervals during each trial, however, some were taken every week during the 10-week period and others were taken every couple of weeks. Therefore time, which is really the date that the sample was taken, will differ in some of the results. Samples were taken at the same intervals during each trial so that results could be combined over both trials. Having two trials ensured that all six stallions were fed endophyte-infected tall fescue and non-toxic endophyte tall fescue (MaxQ) for a total of 10 weeks. In order to account for seasonal changes and photoperiod only three stallions were on each treatment, either endophyte-infected tall fescue/HE (high endophyte) or non-toxic endophyte tall fescue/MQ (MaxQ), at a time during each trial. Therefore, when results were combined over trials, time and trial had a significant interaction with some results due to seasonal changes and photoperiod. These interactions with time and trial were expected when the study was designed. However, one result had a treatment by time interaction. There were several independent variables used for statistical analysis. The independent variables used were treatment (HE - high endophyte or MQ - MaxQ), horse (1-6 stallions), trial (1 or 2), order (1 or 2), treatment by time, and time. There was one covariate used for statistical analysis in the temperature data, this was barn temperature.

### **Urinary Alkaloids and Creatinine Data**

The urinary alkaloid and creatinine data results are shown in Table 2. There was a total of 108 observations read and 106 observations used for the urinary alkaloid and creatinine data. Samples were taken over a 10 week period, however, only nine intervals were used for statistical analysis for both experimental diets, therefore, urinary alkaloid and creatinine data time is 9. There were no differences between time, treatment by time, order, and trial for urinary alkaloids

	Treatment	LS Mean	Standard Error	Significance
Alkaloids and Creatinine	HE	145.03	6.26	< 0.0001
(ng alkaloid/mg creatinine)	MQ	12.17	6.15	

**Table 2:** Urinary alkaloid and creatinine (ng alkaloid/mg creatinine) data. Least square means and standard error for all six stallions over both trials consuming endophyte-infected tall fescue/high endophyte (HE) diet and non-toxic endophyte tall fescue/novel endophyte (MQ) diet.

<sup>a</sup> Data are means for all six stallions per treatment throughout both trials

and creatinine. There was a significant difference between treatment for urinary alkaloids and creatinine (P<0.0001) shown in figure 8. This significance was expected between the treatments. These results prove that the experimental diet was toxic (contained ergot alkaloids) when the stallions were fed endophyte-infected tall fescue and that they were non-toxic when fed MaxQ. This was the basis that the experiment was set upon, since we had to make sure stallions were fed toxic enough levels of ergot alkaloids to possibly affect reproductive parameters.



**Figure 8:** Urinary analysis measured in ng alkaloid/mg creatinine for all six stallions over both trials consuming the high endophyte diet (HE) and the MaxQ diet (MQ). A significant difference (P<0.0001) was seen between stallions consuming the HE diet and the MQ diet.

Urine samples were collected on day one of each trial after the initial morning feeding at hours; one, three, seven, twelve, and twenty-four. The purpose of this was access how fast ergot alkaloids were processed in the stallion and at what hour toxic levels were reached in the urine. Negative effects are seen in cattle consuming endophyte-infected tall fescue at urinary levels as low as 20 ng alkaloid/mg creatinine. During trial one (figure 9) two stallions reached toxic levels (ranging from 32.59 -91.97 ng alkaloid/mg creatinine) within seven hours of exposure to endophyte-infected tall fescue. The third stallion reached toxic levels at twelve hours (41.31 ng alkaloid/mg creatinine). This stallion may have not ingested his experimental diet as fast as the



Figure 9: Least square means for urinary alkaloids and creatinine in trial one. Three stallions consumed endophyte-infected tall fescue/high endophyte (HE) and three stallions consumed non-toxic endophyte tall fescue/MaxQ (MQ).

other stallions did. In trial two (figure 10) stallions reached toxic levels (ranging from 38.75 – 45.11 ng alkaloid/mg creatinine) within six hours of consuming the endophyte-infected tall fescue diet. Once again a third stallion did not reach toxic levels until twelve hours (50.14 ng alkaloid/mg creatinine). This could once again be due to how fast this stallion ingested the experimental diet. Therefore, over the duration of both trials ergot alkaloids reached toxic levels on day one of each trial, within 6-12 hours, after consuming endophyte-infected tall fescue.



**Figure 10:** Least square means for urinary alkaloids and creatinine in trial two. Three stallions consumed endophyte-infected tall fescue/high endophyte (HE) diet and three stallions consumed non-toxic endophyte tall fescue/MaxQ (MQ) diet. Some other urine samples were collected four days prior to the start of trial two on March 5, 2009. Raw data suggests that stallions were at non-toxic levels (9.66 ng alkaloid/mg creatinine +/- 6) before the start of trial two on March 9, 2009. Urine samples were also collected on May 19, 2009, two days after the end of trial two on May 17, 2009. The purpose of this was to determine if toxic levels of ergot alkaloids would decrease to non-toxic levels two days post trial. Raw data for the three stallions consuming endophyte-infected tall fescue during trial two showed a decrease in ng alkaloid/mg creatinine post trial, however, levels were not below suggested non-toxic levels of 20 ng alkaloid/mg creatinine.

# **Collection Data**

Seminal collections measured were: gel-free volume, motility, concentration, and number of doses (Table 3). There was a total of 120 observations read and used for the collection data. Samples were taken over a 10-week period for both experimental diets, therefore, collection data

alet.				
	Treatment	LS Mean	Standard Error	Significance
Gel-free volume (mL)	HE <sup>b</sup>	47.45	3.68	.0004
	$MQ^{c}$	62.77	3.68	
Concentration $(1 \times 10^6)$	HE	257.93	52.74	$NS^d$
	MQ	219.40	52.74	
Motility (%)	HE	69.75	6.07	NS
	MQ	66.58	6.07	
Doses $(500 \times 10^6)$	HE	12.57	3.18	NS
	MQ	13.13	3.18	

**Table 3:** Seminal collection data<sup>a</sup>. Least square means and standard error for all six stallions over both trials consuming endophyte-infected tall fescue/high endophyte (HE) diet and non-toxic endophyte tall fescue/novel endophyte (MQ) diet.

<sup>a</sup> Data are means for all six stallions per treatment throughout both trials

<sup>b</sup> HE= high endophyte diet

<sup>c</sup> MQ= endophyte free diet

<sup>d</sup> NS= not significant, P>.05

time is 10 weeks. The gel-free volume throughout both trials showed no difference among time, treatment by time, order, and trial. Gel-free volume was significantly affected by treatment (P<0.0004). When stallions consumed the endophyte-infected tall fescue diet they had significantly lower gel-free volumes, compared to the stallions that consumed the non-toxic endophyte tall fescue diet. However, gel-free volume does not affect concentration; therefore, it does not affect stallion reproductive parameters. Concentration showed no significant difference among time, order, treatment, and trial. However, concentration did approach significance (P<0.09) between treatments. Concentration had a significant treatment by time interaction (P<0.01), shown in Figure 11. When statistically evaluating each treatment at each time, the concentration data only had one time (time five) that approached significance (P>0.1).



**Figure 11:** Concentration least square means for all six stallions over both trials consuming the high endophyte diet (HE) and the MaxQ diet (MQ). Concentration has a significant interaction (P<0.01) between treatment and time. Only time five approaches significance between treatment and time, therefore, the treatment by time interaction is not strong and could be due to error.

Since there is only one treatment by time interaction, this indicates that the interaction between treatment and time is not significant and could be due to random error. However, this interaction could also be due to one stallion that had very high concentrations regardless of the treatment. This particular stallion's sperm cell concentration was always higher than the other stallion's sperm cell concentrations. Trial one was conducted out of the stallions' normal breeding season from October 13, 2008 to December 21, 2008. As stated earlier, output is affected by photoperiod (Thompson et al., 1997). Therefore, it would be normal to see a decrease in concentration in all the stallions from the beginning of trial one to the end of trial one, since the highest output is usually seen in June. This gradual decrease in concentration was expected and was seen in the stallions being fed the HE diet. However, this was not the case with stallions being fed the MaxQ diet (Figure 12). During trial one, the stallion with high concentrations was



Figure 12: Trial one least square means for concentration. Three stallions were fed a HE (high endophyte fescue) seed diet and three were fed MQ (MaxQ fescue) seed diet. Stallions have a general trend for decreased concentration over time. Stallions consuming the MQ diet have higher concentrations than stallions consuming the HE diet.

fed the MaxQ diet. This interaction was also seen in the second trial when this same stallion was fed the HE diet (Figure 13). In trial two, the concentration should gradually increase over time and peak one to two months after the experiment is finished. Stallions fed the MaxQ diet had concentrations that gradually increased or decreased across trial two. Stallions fed the HE diet



**Figure 13:** Trial two least square means for concentration. Three stallions were fed a HE (high endophyte fescue) seed diet and three were fed MQ (MaxQ fescue) seed diet. Stallions have a general trend for decreased concentration over time. Stallions consuming the HE diet have higher concentrations than stallions consuming the MQ diet, which is the opposite of trial one.

had concentrations that drastically changed from one week to the next. Again, this was thought

to be due to one particular stallion that had very high concentration values, since he was

switched to the HE diet during trial two. Since this stallion was suspected as one of the causes of the treatment by time interaction seen in concentration, statistics were re-run on the concentration data leaving his analysis out. The results showed that the treatment by time interaction no longer existed (Figure 14). However, without this stallion's concentration data there is an interaction with trial (P<0.04) seen in the concentration data. This means that there was a significant difference between the concentrations in trial one and trial two. The least square mean for trial one was 210.93 x  $10^6$  spermatozoa/ml and for trial two it was  $174.55 \times 10^6$  spermatozoa/ml.



Figure 14: Concentration least square means for five stallions. Stallions consumed HE (high endophyte fescue diet) and MQ (MaxQ fescue diet), the treatment by time interaction no longer exists without one stallions concentration data.

Motility throughout both trials showed no difference among treatment by time, order, treatment, and trial. Motility did decrease (P<0.004) over time regardless of treatment (Figure 15). In trial one, motility decreased in the HE (high endophyte diet) from 82% to 57% and in the MQ (MaxQ diet) decreased from 87% to 72%. Likewise in trial two, motility decreased in the HE diet from 85% to 62% and in the MQ diet decreased from 73% to 67%.



Figure 15: Percent motile spermatozoa least square means for all six stallions over both trials consuming the high endophyte diet (HE) and the MaxQ diet (MQ). There was a significant decrease (P<0.004) in percent motile spermatozoa over time regardless of the treatment.

The number of doses throughout both trials had no differences between treatment by time, order, and treatment. The number of doses did decrease (P<0.005) over time regardless of treatment (Figure 16). In trial one, the number of doses ( $500x10^6$ ) decreased in the HE diet from 25 doses to 8 doses and in the MQ diet decreased from 29 doses to 12 doses. Likewise in trial two, the number of doses ( $500x10^6$  total motile spermatozoa) decreased in the HE diet from 11 doses to 7 doses and in the MQ diet decreased from 13 doses to 12 doses. The number of doses was also different (P>0.005) between trial one and trial two. In trial one, the least square mean for the number of doses was 14, and the least square mean for trial two was 11 doses.



**Figure 16:** Least square means for number of doses for all six stallions over both trials consuming the high endophyte diet (HE) and the MaxQ diet (MQ). The number of doses significantly decreases (P<0.005) over time regardless of the treatment.

### **Morphology Data**

There were no differences in percent normal sperm, percent abnormal heads, percent abnormal midpieces, and percent abnormal tails between treatments (Table 4). There was a total of 120 observations read and used for the morphology data. Samples were taken over a 10 week period for both experimental diets, therefore, morphology data time is 10. There were no differences between treatment by time, order, and treatment for percent normal sperm,

**Table 4:** Morphology least square means and standard error data<sup>a</sup>. Data is for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ).

	Treatment	LS Mean	Standard Error	Significance
Percent Normal Sperm	HE	66.1	0.056	NS
	MQ	67.4	0.056	
Percent Abnormal Heads	HE	8.1	0.008	NS
	MQ	8.8	0.008	
Percent Abnormal Midpieces	HE	20.0	0.053	NS
-	MQ	19.1	0.053	
Percent Abnormal Tails	HE	5.8	0.007	NS
	MQ	4.7	0.007	

<sup>a</sup> Data are means for all six stallions per treatment throughout both trials

percent abnormal heads, percent abnormal midpieces, and percent abnormal tails. Trial two had a higher (P<0.0002) percent normal sperm with 69% versus trial one with 64%. Trial two also had a higher (P<0.004) percent abnormal heads with 9.4% versus trial one with 7.5%. Trial one had a higher (P<0.002) percent abnormal midpieces with 21% versus trial two with 18%. Trial

one also had a higher (P<0.0001) percent abnormal tails with 7% versus trial two with 3.6%. There was also a significant decrease (P<0.04) over time in percent abnormal tails for both treatments (Figure 17). In both trials, the percent abnormal tails decreased in the HE diet from



Figure 17: Least square means for percent abnormal tails for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). The number of percent abnormal tails decreases (P<0.04) over time.

11.8% to 3.8% and decreased from 6.8% to 2.4% in the MaxQ diet. The trend for a decrease in percent abnormal tails over time is a good result, because a decrease in abnormalities in spermatozoa is desired.

### **Temperature Data**

The temperature data results that were taken were: rectal temperature, thermal low temperature scan of both testes, and thermal high temperature scan of both testes, shown in Table 5. There was a total of 120 observations read and a total of 114 used for the temperature data. Samples were taken over a 10 week period for both experimental diets, therefore, temperature data time is 10. Barn temperature was added as a covariate in the temperature data. There were no differences between treatment by time, order, and treatment for rectal temperature, thermal low temperature scan of both testes, and thermal high temperature scan of both testes.

	Treatment	LS Mean	Standard Error	Significance
Rectal Temperature (°C)	HE	37.53	0.12	NS
	MQ	37.46	0.12	
Thermal scan low (°C)	HE	30.63	0.44	NS
	MQ	30.60	0.44	
Thermal scan high (°C)	HE	32.68	0.53	NS
	MQ	32.66	0.53	

**Table 5:** Temperature least square means and standard error data<sup>a</sup>. Data is for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ).

<sup>a</sup> Data are means for all six stallions per treatment throughout both trials

Rectal temperature showed a gradual increase over time (P<0.05) shown in figure 18. Mean barn temperatures are listed in the place of time, in the figures shown below. The purpose of this was to show that increased rectal temperature over time was due to increased barn temperatures. In trial one, rectal temperatures showed a general trend of increased temperatures. In the HE diet rectal temperatures increased from 37.56°C to 37.72°C and in the MQ diet rectal temperatures increased from 37.28°C to 37.56°C. Another variable that could have increased rectal temperature in trial one was how fast each stallion grew their winter coat and how thick each stallions coat was. In week nine of trial one, all the stallions rectal temperatures spiked. This was due to a spike in the outdoor temperature that occurred in December. Therefore, a spike in barn temperature with a full winter coat will cause an increase in rectal temperature. In trial two, increased rectal temperatures can be attributed to the breeding season. Some stallions are over active in their paddocks and stalls during the breeding season, which can cause an increase in overall body temperature. This over activeness can be attributed to them seeing and smelling mares in heat. Each stallion reacts differently to the breeding season which leads to the varied rectal temperatures in trial two. Rectal temperature was also significantly different (P<0.0003) between the trials. In trial one, the least square mean for rectal temperature was 37.35°C and in trial two it was 37.64°C.



Figure 18: Rectal temperature least square means for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). Rectal temperature shows a general trend to decrease (P<0.05) over time.

The thermal high temperature scan of the testes showed an interaction with time (P<.0001). The thermal high temperature scan decreased over time (Figure 19). In trial one, stallions fed the HE diet had a decrease in their thermal high temperature scan from 33.52°C to 31.85°C and stallions fed the MQ diet decreased from 33.15°C to 32.41°C. In trial two this same trend was followed, stallion fed the HE diet had decreased thermal high temperature scans from 33.89°C to 32.22°C and stallions fed the MaxQ diet had decreased thermal high temperature scans from 33.33°C to 33.15°C. Testes temperature changes depending on the stallion's body temperature and barn/outdoor temperature.



**Figure 19:** Least square means for the thermal high temperature scan of both testes for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). The thermal high temperature scan of both testes decreases (P<0.0001) over time. The thermal low temperature scan of both testes showed a decrease in temperature over time (P<0.001). It also showed a trend to decrease in correlation to barn temperature (P<0.0001) shown in figure 20. The interaction with barn temperature showed a general trend for the thermal low temperature scan to decrease when barn temperatures were lower and to slightly increase when barn temperatures were higher. The testes regulate their temperature depending on body temperature and barn/outdoor temperature. Therefore, barn temperature directly affects the temperature of the testes, which affects the thermal low temperature scan and how cold the testes get.



**Figure 20:** Least square means for the thermal low temperature scan of both testes for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). The thermal low temperature scan of both testes decreases (P<0.001) over time. The thermal low temperature scan of both testes also decreases or increases (P<0.0001) as barn temperature increases or decreases.

# **Ultrasound Data**

The ultrasound measurements that were taken were: cross section of the left testicle, cross section of the right testicle, total volume of both testicles, vasculature average of the left testicle, and vasculature average of the right testicle, shown in Table 6. There was a total of 48 observations read and used for the ultrasound data. Samples were taken over a 10 week period at four intervals for both experimental diets, therefore, temperature data time is 4. There were no differences in treatment by time, order, and treatment for the cross section of the left testicle, cross section of the right testicle, total volume of both testicles, vasculature average of the left testicle, and vasculature average of the right testicle.

	Treatment	LS Mean	Standard Error	Significance
Cross Section Left (m)	HE	0.283	1.82	NS
	MQ	0.286	1.82	
Cross Section Right (m)	HE	0.292	1.68	NS
	MQ	0.29	1.68	
Total Volume (ml)	HE	276.295	20.44	NS
	MQ	280.326	20.44	
Vasculature Avg. Left (m)	HE	0.00265	0.02	NS
	MQ	0.00278	0.02	
Vasculature Avg. Right (m)	HE	0.00255	0.02	NS
	MQ	0.00265	0.02	

**Table 6:** Ultrasound least square means and standard error data<sup>a</sup>. Data is for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ).

<sup>a</sup> Data are means for all six stallions per treatment throughout both trials

The cross section of the left testicle decreased (P<0.05) over time, shown in figure 21. This same trend was followed during each trial. In trial one, the cross section of the left testicle decreased in the HE diet from 0.2534 m to 0.2399 m and decreased in the MQ diet from 0.3582 m to 0.271 m. Likewise in trial two, the cross section of the left testicle decreased in the HE diet from 0.3434 m to 0.2717 m and decreased in the MQ diet from 0.2536 m to 0.2305 m. Typically the left testicle is larger than the right testicle. There was a significant difference (P<0.006)



**Figure 21:** Least square means for the cross section of the left testicle for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). The cross section of the left testicle shows a general trend of decreasing (P<0.05) over time. seen between the trials in the cross section of the right testicle. In trial one, the least square mean of the cross section of the right testicle was 0.27 m and in trial two it was 0.31 m. The total

volume of both testicles (figure 22) decreased over time (P<0.05). This trend was seen in both trials. In trial one, the total volume of both testes decreased in the HE diet from 237.85 ml to 221.48 ml and decreased in the MQ diet from 314.16 ml to 278.90 ml. In trial two, the total volume decreased in the HE diet from 350.68 ml to 268.69 ml and decreased in the MQ diet from 288.49 ml to 236.24 ml. The trend for decreased total volume is not expected during trial two since this is during a stallion's normal breeding season. The decrease in total volume could explain why there was a decrease in concentration and the number of doses over time, since testicular size is correlated to spermatozoal output. In trial two total testicular volume was greater (P<0.004) at 297.57ml versus trial one at 259.05 ml. There was also a general trend for



Figure 22: Least square means for the total volume of both testicles for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). Total volume of both testes shows a general trend to decrease (P<0.05) over time.

the vasculature of the right testicle to decrease over time (P<0.05), shown in figure 23. In the HE diet, the vasculature of the right testicle decreased from 0.0031 m to 0.0022 m. However, in the MaxQ diet the vasculature of the right testicle remained consistent over time from 0.0027 m to 0.0027 m with a decrease in vasculature 0.0025 m at week three. There was also a significant



Figure 23: Least square means for the vasculature average of right testicle for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). The vasculature of the right testicle decreases (P<0.05) over time.

difference (P<0.001) between the trials in the vasculature of the right testicle. In trial one, the

vasculature of the right testicle was 0.0028 m and in trial two it was 0.0024 m.

# **Testosterone Data**

The testosterone results are shown in Table 7. There was a total of 48 observations read and used for the testosterone data. Samples were taken at four intervals over a ten week period for both experimental diets, therefore, testosterone data time is 4. Results showed that there were no differences in time, treatment by time, order, and treatment for testosterone.

**Table 7:** Testosterone least square means and standard error data<sup>a</sup>. Data is for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ).

Treatment	LS Mean	Standard Error	Significance
HE	2059.45	303.54	NS
MQ	2033.14	303.54	
	Treatment HE MQ	Treatment      LS Mean        HE      2059.45        MQ      2033.14	Treatment      LS Mean      Standard Error        HE      2059.45      303.54        MQ      2033.14      303.54

<sup>a</sup> Data are means for all six stallions per treatment throughout both trials

There was a significant difference (P<0.04) in testosterone levels seen between the trials. In trial one, the least square mean testosterone was 2335.06 pg/ml and in trial two the least square mean was 1757.53 pg/ml.

### **Data not Analyzed**

Some of the data in this experiment was not analyzed. The data that was not analyzed was prolactin, weight, number of mounts, pH, and bacteria slides. Prolactin was not statistically analyzed due to multiple non-detectable prolactin results. Weight was not statistically analyzed due to multiple variables: weight loss during the breeding season, weight loss or gain due to the season, and weight loss or gain due to eating habits. No stallions showed extreme weight gain or loss during the course of this experiment that was due to the diet they were being fed. The number of mounts was not analyzed because of multiple outside variables that we could not control. Those variables included the season in which the stallion was collected, the number of mares in heat, likability of the mares in heat, and stallions learned ability to mount a phantom. pH showed very little increase or decrease during each trial regardless of treatment. It was not statistically analyzed due to this and the fact that we did not have a calibrated pH meter to accurately read the pH of semen. Bacteria found in the bacteria slides were not statistically analyzed due too very little increase or decrease seen in bacteria counts regardless of treatment.

### Implications

Results from this study showed that stallions consuming endophyte-infected tall fescue had no negative effects on their reproductive parameters. Several results had interactions with time and trial, however, these were not affected by treatment. Therefore, results from this study show that endophyte-infected tall fescue does not alter reproductive parameters, measured in this study, in a mature breeding stallion. The first trial was run from October 13, 2008 to December 21, 2008, which was not during the regular breeding season for horses. However, the stallions showed good libido, gave good collection values and percentages, and maintained normal temperatures throughout trial one. The second trial was run from March 9, 2009 to May 17, 2009, which was during their breeding season. Once again the stallions maintained good libido, collection values and percentages, and normal temperatures throughout the trail. Therefore, it is speculated that prolonged use of endophyte-infected tall fescue pastures and hay will not affect stallion reproductive parameters. However, one value was significant (P>0.0004) between the treatments, this was gel-free volume (Figure 24). Results show that stallions consuming endophyte-infected tall fescue/HE diet had significantly lower gel-free volumes than stallions consuming non-toxic endophyte tall fescue/MaxQ diet. However, this significant decrease in



**Figure 24:** Least square means for gel-free volume for all six stallions consuming the high endophyte diet (HE) and the MaxQ diet (MQ). There is a significant difference (P<0.0004) between the HE diet and the MQ diet. Stallions consuming the HE diet tend to have lower gel-free volumes than stallions consuming the MQ diet.

gel-free volume will not affect stallion reproductive parameters. However, consuming endophyte-infected tall fescue will affect the accessory sex organs because they supply volume to the ejaculate, which directly affects gel-free volume. Accessory sex organs do not affect stallion reproductive parameters because they do not affect concentration and motility.

One reason that stallion's reproductive parameters measured in this study were not affected by endophyte-infected tall fescue could be due to their digestive system. Horses are hindgut fermenters and only keep small amounts of fed in their stomach at a time, this alters where and how ergot alkaloids are digested. Urinary analysis of samples from hours one, three, seven, twelve, and twenty-four on day one of each trial suggests that stallions can reach toxic levels within six to twelve hours of exposure to endophyte-infected tall fescue. Forages and concentrates are rapidly processed in the stomach in approximately fifteen to thirty minutes and are rapidly processed in the small intestines in approximately ninety minutes. This puts digesta in the large intestines within two hours of feeding, and digesta remains in the large intestines for approximately thirty-two to sixty hours. Therefore, digesta is in the large intestines in six to twelve hours after being consumed, which is when stallion's excreted toxic levels of ergot alkaloid in their urine. This suggests that ergot alkaloids are being absorbed in the large intestines, particularly in the cecum.

Urinary alkaloid and creatinine tests proved that stallions were consuming toxic levels of ergot alkaloids from endophyte-infected tall fescue. These results also suggest that MaxQ had no effect on stallion reproductive parameters. Urinary alkaloid and creatinine tests also proved that non-toxic endophyte tall fescue, MaxQ, was free of toxic ergot alkaloids. It is believed that stallions rapidly process and get rid of ergot alkaloids once exposure is gone. Since ergot alkaloids are excreted in a stallion's urine, this theory could easily tested. Multiple urine tests were taken during each trail, therefore, we analyzed urine samples that were taken two days post, May 19, 2009, experimental treatment in trial two. There was a large decrease in toxic levels of ergot alkaloids just two days after removal from endophyte-infected tall fescue. If we take a closer look at the three stallions being fed endophyte-infected tall fescue during trial two, we can see that the stallion's urine concentrations greatly decreased two days post trial. One stallion's urine concentration decreased from 140.64 to 24.80 ng alkaloid/mg creatinine. The third stallion's urine concentrations did not decrease as drastically but did decrease from 164.68
to 105.93 ng alkaloid/mg creatinine. This leads to the belief that urine levels will reach nontoxic levels 72 hours post exposure.

In this study, sexually mature stallions' reproductive parameters were not affected when fed endophyte-infected tall fescue. However, this study does not conclude whether sexually immature stallions are affected by endophyte-infected tall fescue. Approximately 700,000 horses graze endophyte-infected tall fescue pastures (Thompson et al., 2001), which includes mares, fillies, stallions, geldings, and colts (sexually immature stallions). Most colts are exposed to endophyte-infected tall fescue at some point during the first year of their life. If endophyteinfected tall fescue has any negative effects on endocrine gonadotrophic production and secretion in colts it could delay sexual maturity, affect testicular size, affect testosterone production, and affect spermatozoa production. Being exposed to endophyte-infected tall fescue prior to maturity could impair or alter normal sexual maturation, which could lead to fertility issues when these colts become sexually mature. This is an important issue to look at since this could be the cause of some fertility issues currently seen in sexually mature stallions.

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## **CHAPTER 4**

## Conclusion

This study was conducted to see if consuming endophyte-infected tall fescue had an effect on stallion reproductive parameters. The results from this study indicate that mature breeding stallions consuming endophyte-infected tall fescue had no negative effects on reproductive parameters, measured in this study. However, the findings in this study do not conclude whether stallion's spermatozoa are affected at the cellular level when consuming endophyte-infected tall fescue. These results are only conclusive for mature breeding stallions. Endophyte-infected tall fescue is still unsafe for broodmares. Special care must be taken to not let mares feed on endophyte-infected tall fescue 45-60 days before they foal. Even if stallions are the only horses grazing on endophyte-infected tall fescue pastures, it is very important to remember that endophyte-infected tall fescue seed can spread to other non-toxic endophyte pastures and infect them.

Through the course of this experiment there were no significant affects on stallion reproductive parameters. Urinary alkaloid and creatinine tests were run on each stallion's urine samples that were taken throughout the course of the experiment. The results were conclusive that each stallion received toxic levels of ergot alkaloids when they consumed endophyteinfected tall fescue seed and they received no ergot alkaloids when they consumed MaxQ seed. Other results showed that testicular size, testicular blood flow, testicular temperature, rectal temperature, testosterone levels, semen concentration, number of doses, motility, and sperm morphology were not significantly affected by order and treatment. This means that stallion reproductive parameters were not affected by experimental diet or the order in which they received the experimental diet. However, several results showed an interaction with time; motility, number of doses, rectal temperature, thermal high temperature scan of testes, thermal low temperature scan of testes, percent abnormal tails, cross section of the left testicle, total volume of both testicles, and vasculature of the right testicle. Several of these results also showed a significant interaction with trial; number of doses, cross section of the right testicle, total volume of both testicles, vasculature of the right testicle, percent normal sperm, percent abnormal heads, percent abnormal midpieces, percent abnormal tails, testosterone levels, rectal temperature, and thermal low temperature scan of testes. These interactions seen between time and trial were expected due to the different seasons throughout the experiment, the different photoperiods the trials were conducted in, and the individual variation of each stallion. However, none of these interactions with time or trial had anything to do with the treatment they were on, therefore, they were not responsible for a change in reproductive parameters. There was one measurement, concentration, that had a treatment by time interaction (P < 0.01). However, this interaction no longer existed when one stallion was taken out of the concentration data, since his concentration levels were consistently higher than the other stallions. Concentration also approached significance between the treatments (P<0.09). Decreased total volume of both testes could be the reason that the number of doses decreased over time. The thermal low temperature scan of the testes had a significant interaction (P<0.0001) with barn temperature. This was also expected and was a good indication that the testes were regulating temperature correctly, since testes temperature is affected by internal and external temperatures. All seasonal changes were expected and were taken into consideration when this experiment was designed.

Further research may need to be conducted on sexually immature stallions to see if endophyte-infected tall fescue pasture or hay can affect their development into sexually mature stallions.