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Effects of Fescue Toxicosis on Bovine Acrosomal Integrity and Semen Cryopreservation

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EFFECTS OF FESCUE TOXICOSIS ON BOVINE ACROSOMAL INTEGRITY AND
SEMEN CRYOPRESERVATION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Animal and Veterinary Sciences

by
Callie Rebecca Burnett
May 2016

Accepted by:
Dr. Scott L. Pratt, Committee Chair
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ABSTRACT

Tall fescue is the most widely used cool-season perennial bunchgrass in the southeastern United States and serves as forage for approximately 8.5 million cattle. Through a mutualistic relationship with the endophyte *Epichloë coenophiala*, tall fescue is bestowed insect resistance and disease, drought, and grazing tolerance. In spite of these desirable agronomic traits, the endophyte produces ergot alkaloids that are harmful to the physiology of animals consuming tall fescue. Accumulation of ergot alkaloids in animal systems results in a syndrome known as fescue toxicosis. Among other symptoms, reproductive inefficiencies are reported for beef cattle consuming toxic tall fescue. The objectives of this research were to assess the influence of toxic tall fescue consumption on bull sperm by evaluating acrosomal integrity and survival of spermatozoa following cryopreservation. Semen was collected and fixed from bulls that were either fed a ration containing toxic or nontoxic tall fescue seed or grazing toxic or nontoxic tall fescue pasture. Fluorescent-labeled peanut agglutinin was used to evaluate sperm acrosomal integrity. According to our methodologies and data, subtle, if any, differences due to treatment were detected. Semen was also collected, extended, and frozen from bulls grazing toxic or nontoxic tall fescue. Differences due to treatment post-thaw were detected for sperm progressive motility. Significant treatment by day interactions were detected for sperm concentration, motility, total motile sperm per dose, and total progressive motile sperm per dose post-thaw. Our results indicate that acrosomal integrity is not greatly affected by fescue toxicosis, and that grazing toxic tall fescue negatively

impacts spermatozoa physiology as measured by survival of sperm following cryopreservation.

DEDICATION

I dedicate my thesis to my family. Special thanks go to my parents, Susan and Dennis Burnett, for being great role models, prayer warriors, and my biggest fans. I thank you from the bottom of my heart for the overwhelming outpouring of love, support, and encouragement that has been shown to me throughout this journey in life. My sister, Carrie, deserves my wholehearted thanks as well. You have been a constant source of love and support throughout my life and I am thankful for the incredible bond that we share. I also dedicate this thesis to my grandparents, Ruth Burnett and Barbara and Ramsey Taylor, whose love for me knows no bounds. Thank you for helping to instill in me the value of hard work, for believing in me, and for your countless prayers.

Kirk, thank you for being in P&A at the right time and for your unwavering support throughout the writing stages. Thank you for being my sounding board and for believing in me.

Thank you, Lord, for being a promise maker and a promise keeper. All glory goes to You.

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I want to thank my committee members for their help and expertise, for allowing me this opportunity, and for providing me with a unique and challenging graduate school experience.

TABLE OF CONTENTS

	Page
TITLE PAGE	i
ABSTRACT.....	ii
DEDICATION.....	iv
ACKNOWLEDGMENTS	v
LIST OF FIGURES	viii
CHAPTER	
I. LITERATURE REVIEW	1
Tall Fescue	
Introduction.....	1
History.....	2
Adaptability and Suitability	2
Uses.....	4
Fescue Toxicosis	
Overview.....	4
Toxicity Symptoms.....	5
Pioneer Studies.....	9
Endophyte	9
Ergot Alkaloids	12
Effects on Male Reproduction	14
Pasture Management.....	16
Summary.....	18
Acrosomal Integrity	
History of the Sperm Cell.....	19
Basic Sperm Cell Structure and Function.....	19
Acrosome	20
Methods Used to Evaluate Acrosomal Integrity.....	23
Conclusion	27

Table of Contents (Continued)	Page
II. USING FLUORESCENT-LABELED PEANUT AGGLUTININ.....	28
TO ASSESS ACROSOMAL INTEGRITY OF BEEF	
BULLS CONSUMING TOXIC TALL FESCUE	
Abstract.....	28
Introduction.....	29
Materials and Methods.....	30
Results.....	33
Discussion.....	40
Acknowledgements.....	41
III. EFFECTS OF TOXIC TALL FESCUE ON BOVINE.....	42
SEMEN CRYOPRESERVATION	
Abstract.....	42
Introduction.....	43
Materials and Methods.....	44
Results.....	47
Discussion.....	56
Acknowledgements.....	57
IV. CONCLUSION.....	58
APPENDICES	60
A: Copyright Permission from Current Conceptions, Inc.	61
B: Copyright Permission from John Andrae	62
REFERENCES	63

LIST OF FIGURES

Figure		Page
1.1	Tall fescue adaptation in the United States.....	3
1.2	Sloughed hoof as a result of the fescue foot malady	6
1.3	Fat necrosis in a beef cow.....	8
1.4	Basic sperm cell structure	20
1.5	Acrosomal membranes.....	21
1.6	Conceptual visualization of mammalian capacitation	22
2.1	Different acrosomal staining patterns of formalin-fixed and PNA-Alexa Fluor 594 stained bull spermatozoa, as assessed with fluorescence microscopy.....	35
2.2	Mean percentages for acrosomal staining pattern 1 are given on the y-axis and day of treatment is on the x-axis.....	36
2.3	Mean percentages for acrosomal staining pattern 2 are given on the y-axis and day of treatment is on the x-axis.....	37
2.4	Mean percentages for acrosomal staining pattern 3 are given on the y-axis and day of treatment is on the x-axis.....	38
2.5	Mean percentages for acrosomal staining pattern 4 are given on the y-axis and day of treatment is on the x-axis.....	39
3.1	Mean serum prolactin concentrations are given on the y-axis and day of treatment is on the x-axis	49
3.2	Mean urinary alkaloid concentrations are given on the y-axis and day of treatment is on the x-axis	50

List of Figures (Continued)

Figure		Page
3.3	Means for post-thaw progressive motility are given on the y-axis and day of treatment is on the x-axis.....	51
3.4	Means for post-thaw sperm concentration are given on the y-axis and day of treatment is on the x-axis.....	52
3.5	Means for post-thaw sperm motility are given on the y-axis and day of treatment is on the x-axis	53
3.6	Means for post-thaw total motile sperm are given on the y-axis and day of treatment is on the x-axis.....	54
3.7	Means for post-thaw total progressive motile sperm are given on the y-axis and day of treatment is on the x-axis.....	55

CHAPTER ONE

LITERATURE REVIEW

TALL FESCUE

Introduction

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh. = *Schedonorus arundinaceus* (Schreb.) Dumort., formerly *Festuca arundinacea* Schreb. var. *arundinacea* Schreb.] is the most widely used perennial, cool-season bunchgrass in the southeastern United States (Buckner et al., 1979; Pendlum et al., 1980; Bouton, 2000), occupying approximately 14 million hectares (Sleper and Buckner, 1995). Heavily utilized in livestock production systems, the grass serves as a primary source of forage for more than 8.5 million cattle in the United States (Hoveland, 1993). Along with its wide range of adaptation, ease of establishment, tolerance to poor management, and extended grazing season (Stuedemann and Hoveland, 1988), tall fescue is also a plant well-known for possessing desirable agronomic traits bestowed to it via a mutualistic relationship with the fungal endophyte, *Epichloë coenophiala* (Young et al., 2014). The endophyte produces ergot alkaloids (Trethewie et al., 1954; Maag and Tobiska, 1956), which are beneficial to the plant, but harmful to the physiology of the animals that consume it. Consumption of endophyte-infected fescue results in a syndrome known as fescue toxicosis (Bacon et al., 1977), costing the grazing livestock industry an estimated \$1 billion yearly in animal production losses (Allen and Segarra, 2001; Strickland et al., 2011).

History

Although the exact date in which tall fescue was introduced to the U.S. is still unknown, it has been proposed that the plant was brought by accident as a contaminant in meadow fescue [*Lolium pretense* (Huds.) Darbysh. = *Schedonorus pratensis* (Huds.) Beauv., formerly *Festuca pratensis* Huds.] seed from Europe before 1880 (Stuedemann and Hoveland, 1988). The United States National Herbarium recorded the first specimen of tall fescue in 1886 (Cowan, 1956); however, an established cultivar of tall fescue was not discovered to be growing on U.S. soil until 1931 by Dr. E. N. Fergus, an agronomist at the University of Kentucky (Fergus and Buckner, 1972). On a farm belonging to Mr. W. M. Suiter located in Menifee County, Kentucky, Dr. Fergus noticed a lush, green stand of grass growing on a steep hillside in cold weather. It was at that time that Dr. Fergus identified the grass as tall fescue and took seed samples back to the University of Kentucky for testing and further examination. In 1943, after years of analysis, the plant was registered and released as what is now the most commonly used cultivar, “Kentucky-31” (Fergus and Buckner, 1972).

Adaptability and Suitability

Grown from Florida to Canada, tall fescue is highly adapted to a multitude of environments and predominates in the transition zone between the temperate northern and subtropical southern regions of the eastern United States (Aiken and Strickland, 2013). Its persistence as a cool-season grass in a principally humid and drought-prone “fescue belt” (Buckner and Bush, 1979) is unparalleled to other cool-season grasses of

the like, with minimum rainfall requirements ranging from 35 to 85 cm (Buckner and Cowan, 1973). Tolerant of flooding, poor drainage, alkalinity, and salinity (Cowan, 1956; Seay, 1960), tall fescue can also be ideal for pastureland in the Intermountain Region of the United States, ranging from southern California to northern Washington (Bush and Buckner, 1973). While grown on claypan and various other shallow soils, as well as on moist lowlands and sandy loam uplands, tall fescue exhibits its best growth in heavy or medium-textured soils containing large quantities of humus (Bush and Buckner, 1973).

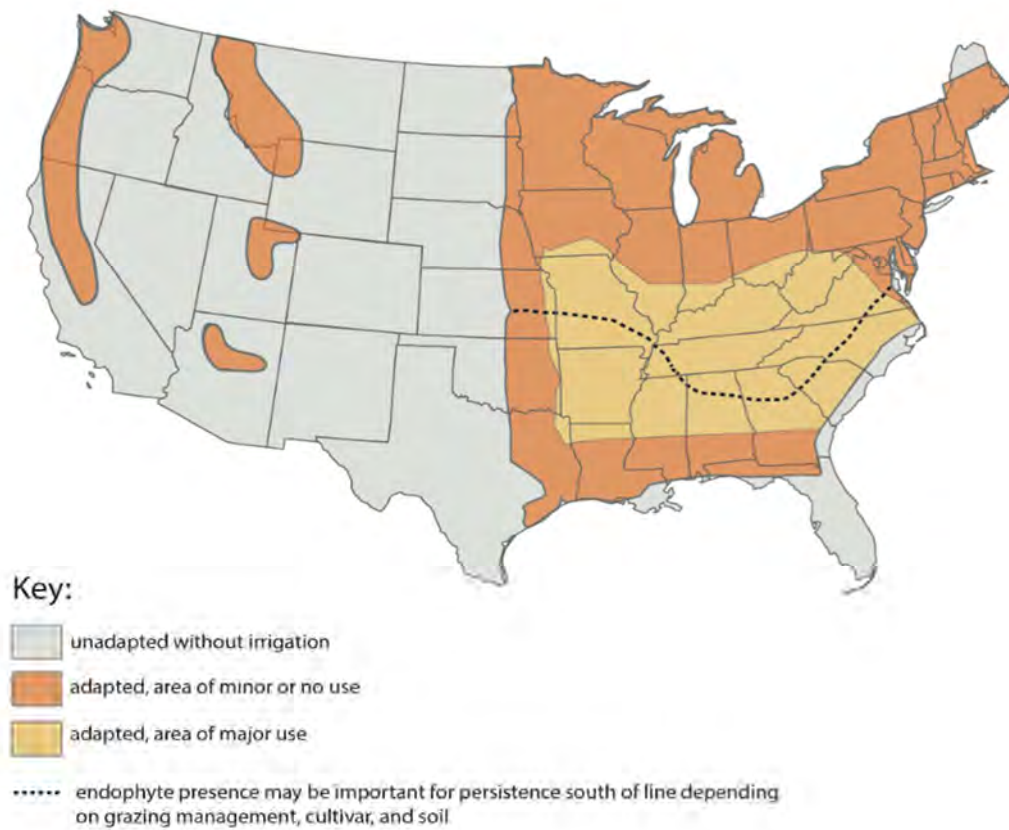


Figure 1.1. Tall fescue adaptation in the United States. Adapted from West, 1998.

Uses

While tall fescue did not become used extensively for animal feed (pasture, hay, silage) in the United States until the 1940s and 50s, it now occupies upwards of 14 million hectares of land (Sleper and Buckner, 1995). Producing a tough sod able to withstand being trampled by livestock (Cowan, 1956; Seay, 1960), tall fescue is a valuable forage grass to livestock producers. Referred to as a wonder grass, stands of fescue can even be established on hillsides, allowing for better use of land that would otherwise be unproductive (Cowan, 1956). The cultivated grass is also commonly used as a turf grass for lawns and is planted in waterways and ditches, and on hillsides, airfields, athletic fields, and pond banks to prevent soil erosion.

FESCUE TOXICOSIS

Overview

Forage grass toxicities have been observed since the biblical periods (Matthew 13:25 – 40), when fungus infected seed of darnel was considered toxic to animals and humans (Bacon, 1995). Despite tall fescue's popularity as valuable, high quality forage, it can be toxic to grazing livestock. Negative effects observed in cattle grazing tall fescue, which include unthrifty appearance, decreased performance, as well as other arbitrary symptoms, were believed by early researchers to be a result of fescue poisoning (Merriman, 1955). Although fescue toxicosis is the most common problem, conditions such as fescue foot and fat necrosis also occur as a result of toxic tall fescue consumption.

Toxicity Symptoms

Fescue Foot

The term “fescue foot” is used to describe a non-infectious, potentially life-threatening phenomenon that involves necrosis and sloughing of hooves due to toxicosis. Mainly occurring in colder climates or during winter months (Goodman, 1952), symptoms begin with weight loss or reduced weight gain, rough hair coat, soreness of the rear limbs, and arched back (Jacobson et al., 1963). Hyperemia of the coronary band occurs between the dewclaw and hoof, which causes swelling (Hemken et al., 1984) that is often hot to the touch and painful to the animal (Cunningham, 1949; Cowan, 1956). The animal eventually loses its ability to control the rear leg muscles (Cunningham, 1949; Pulsford, 1950; Merriman, 1955; Fowler and Kingrey, 1956).

As the condition progresses, drying and hardening of the skin occurs. Subsequently, a line of demarcation forms, causing distal portions of the limb to become cold, insensitive to pain, and discolored. Dry gangrene occurs, due to a restriction of blood supply to the hind limbs (Goodman, 1952; Jacobson et al., 1963), and peripheral portions of the limb may be sloughed (Cunningham, 1949; Pulsford, 1950; Cowan, 1956). Sloughing of the tips of ears and switches of tails has also been known to occur (Goodman, 1952; Jensen et al., 1956; Ashley, 1958; Jacobson and Miller, 1961).

Initial reports of fescue foot stemmed from New Zealand (Cunningham, 1948), Australia (Pulsford, 1950), and the United States (Goodman, 1952), providing early evidence that tall fescue is poisonous forage for grazing animals. Reports have also indicated that the left rear leg is typically affected first (Cunningham, 1949; Klussendorf,

1955; Cowan, 1956; Watson et al., 1957; Hore, 1961); however, a lack of understanding still exists regarding the mechanisms involved in fescue toxicosis. Nevertheless, the ailment can involve both hind limbs (Cunningham, 1949; Pulsford, 1950; Goodman, 1952; Stearns, 1953; Merriman, 1955; Cowan, 1956; Jensen et al., 1956; Watson et al., 1957; Ashley, 1958). There has only been one documented case in which the front legs were the only limbs affected (Hore, 1961). Differences in susceptibility to the ailment (Cunningham, 1948) and sporadic occurrences of fescue foot in the southeastern U.S. have been observed in cattle, which may help to explain why it is the least common form of fescue toxicity.



Figure 1.2. Sloughed hoof as a result of the fescue foot malady. Reproduced with permission from John Andrae (Roberts and Andrae, 2004).

Fat Necrosis

Fat necrosis is a less visible condition associated with fescue toxicosis that was first diagnosed in a north Georgia cattle herd grazing toxic tall fescue in winter and spring (Williams et al., 1969). The condition is characterized by hard masses of fat within the adipose tissue comprising the abdominal, pelvic, and perirenal regions of the animal (Williams et al., 1969; Stuedemann et al., 1975), and is attributed to excessive nitrogen (Stuedemann et al., 1985) and poultry litter application on pastureland (Williams et al., 1969; Wilkinson et al., 1971; Stuedemann et al., 1975).

Although the mechanism is still unknown, it has been documented that ergot alkaloids produced by the endophyte within the tall fescue plant can be retained in the fat tissue of beef cattle (Realini et al., 2005). Serving as a potential reservoir for such compounds, fat deposits may allow for the steady release of toxins long after grazing livestock have been removed from endophyte-infected tall fescue (Roberts and Andrae, 2004). The retention and gradual release of ergot alkaloids from fat tissue could provide justification as to why symptoms of fescue toxicosis are present in summer months, and why cattle in feedlots exhibit rough hair coats after being removed from toxic pasture (Roberts and Andrae, 2004).

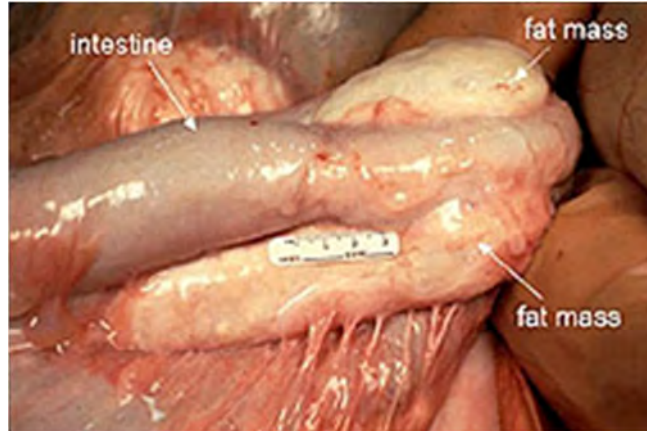


Figure 1.3. Fat necrosis in a beef cow. Reproduced with permission from John Andrae (Roberts and Andrae, 2004).

Fescue Toxicosis

The most common syndrome associated with cattle grazing tall fescue is fescue toxicosis, also referred to as “summer slump” or “summer syndrome” because of an animal’s unthrifty appearance and poor performance during summer months (Bush and Buckner, 1973; Hoveland et al., 1983). Although exacerbated by heat stress (Hemken et al., 1981), the condition is known to transpire year-round. The disorder is characterized by poor gains (Hoveland et al., 1983), nervousness, heat intolerance, excessive salivation, rough hair coat (Merriman, 1955), increased body temperature (Oliver, 2005), reduced conception rates, and decreased milk production (Pratt and Haynes, 1950). Clinical signs, such as vasoconstriction (Solomons et al., 1989; Klotz et al., 2010) and reduced serum prolactin concentrations have been reported, as well. Behavior changes are also seen in animals affected by fescue toxicosis, which often include less time spent grazing and

prolonged congregation in wet and shaded areas (Schmidt and Osborn, 1993; Strickland et al., 1993; Oliver, 2005).

Pioneer Studies

Increased use of tall fescue as forage for grazing animals lead to a rise in resulting livestock health issues (Pratt and Haynes, 1950). Sparking scientists to identify any toxic agent(s) associated with the plant and attempt to ameliorate harmful effects, research began in the 1950s on alkaloids, rumen toxins, and anions (Bush et al., 1979).

Unfortunately, those research efforts (Bush et al., 1979) provided no solutions to fescue toxicosis, with the earliest reports of ergot alkaloids (Trethewie et al., 1954; Maag and Tobiska, 1956) being disregarded. Preliminary observations did, however, lead scientists to study endophyte-infected grasses.

Endophyte

Breakthrough

Research on plant fungi began in the 1970s due to fescue toxicosis symptoms being analogous to those from ergot toxicity (Robbins, 1983). In 1973, a group of scientists at the USDA Russell Research Center isolated three endophytic fungi of the species *Balansia* – *B. epichloe* (Weese), *B. henningsiana* Moell, and *B. Myriogensopora atrementosa* (Berk. & Curt.) Diehl – from pasture in Newton County, Georgia (Bacon et al., 1975). Because the fungi lived within (“endo”) the plant (“phyte”) and were not harmful to the host, they were referred to as “endophytes” and served as the basis for

grass toxicity studies (Bacon et al., 1975). Interestingly, the same group of fungi had first been linked to grass toxicosis in India (Nobindro, 1934).

Examination

Toxicological investigation of *Balansia* fungi provided evidence that the species was poisonous to chick embryos and had the potential to produce ergot alkaloids (Bacon et al., 1975, 1979; Porter et al., 1979). These discoveries lead to the subsequent hypothesis that a fungal endophyte was the culprit of fescue toxicosis, promoting further research (Bacon, 1995). Microscopic analysis of plant tissue from tall fescue pasture in Newton County revealed a 100% infection rate with the fungal endophyte *Epichloe typhina* (Pers., Fr.) Tul. (Bacon et al., 1977), which was subsequently renamed *Acremonium coenophialum* (Morgan-Jones and Gams, 1982). Fourteen years later, the endophyte was renamed *Neotyphodium coenophialum* (Glenn et al., 1996), and is presently known as *Epichloë coenophiala* (Young et al., 2014).

Field Trials

Grazing trials in central Alabama further supported the hypothesis that a fungal endophyte was the underlying cause of fescue toxicity (Hoveland et al., 1983). The first trial was a three-year study in which steers were allotted to tall fescue paddocks with 18% and 80% endophyte infection (Hoveland et al., 1980). Results showed that steers grazing tall fescue with lower endophyte infection resulted in average daily gain (ADG) values 51% higher than those grazing 80% endophyte-infected fescue (Hoveland et al.,

1980). The second experiment was a four-year trial in which steers grazed tall fescue pasture with 5% and 94% endophyte infection, showing similar results (Hoveland et al., 1983). Coincidentally, animals consuming tall fescue pasture with higher endophyte levels displayed signs of toxicosis.

Economic Impacts

Research has gauged the mean endophyte infection rate of tall fescue pastures in the United States to be 58% (Shelby and Dalrymple, 1987). Therefore, it is estimated that cattle grazing tall fescue lose 0.63 kg ADG due to fescue toxicity (Shelby and Dalrymple, 1987). On a broader scale, production losses, reproductive inefficiencies, and mortalities are thought to cost the beef cattle industry over \$600 million as a result of the fescue toxicosis syndrome (Hoveland, 1993). Accounting for inflation, more recent evidence suggests those economic losses to be approximately \$1 billion for the grazing livestock industry (Allen and Segarra, 2001; Fribourg and Waller, 2005; Strickland et al., 2011).

Advantages in the Southeast

Unfortunately, the same endophyte that causes fescue toxicity in grazing livestock also gives tall fescue its desirable agronomic traits via a mutualistic relationship it has with the plant (Siegel et al., 1984; Bacon and Siegel, 1988; Latch, 1997). As a result, tall fescue is known as the forage cattle producers “can’t live with”, but “can’t live without” (Browning, 2003). Research by Bouton et al. (1993) and West et al. (1993) showed that tall fescue infected with the endophyte was more resistant to drought conditions than its

endophyte-free counterparts. A favorable attribute in the southeastern United States, the endophyte helps tall fescue persist by prompting roots to grow deeper into the soil (Richardson et al., 1990). Other means of facilitating drought survival include the endophyte's encouragement of carbohydrate accumulation in leaf sheaths (Richardson et al., 1991) and ability to lower plant photosynthetic rates in order to conserve water and nutrients (Belesky et al., 1987). Also, through the production of ergot alkaloids, the endophyte is able to bestow pest resistance to the tall fescue plant, thereby reducing insect (Pownall et al., 1995) and aphid herbivory (Johnson et al., 1985).

Ergot Alkaloids

Structure

Toxins produced by the endophyte are fungal metabolites and belong to a class of compounds known as ergot alkaloids. Including clavine alkaloids, lysergic acid amides, and ergopeptines (Bacon et al., 1977), these compounds have a wide spectrum of activities due to the fact that they are D-Lysergic acid-derived and possess a tetracyclic ergoline ring structure (Tudzynski et al., 2001). Interestingly, the structures of ergot alkaloids are similar to those of serotonin, dopamine, and noradrenaline, which make ergot alkaloids able to bind receptors for neurotransmitters (Berde, 1980; Weber, 1980), and elicit undesirable biological activity in grazing livestock.

Prevalence

Numerous ergot alkaloids have been found present in tall fescue (Strickland et al., 2011). However, ergovaline, a member of the ergopeptine class of ergot alkaloids, is the most prevalent and researched compound that is believed to be the cause of fescue toxicosis (Yates et al., 1985; Lyons et al., 1986; Belesky et al., 1988). More recent studies have shown that derivatives of lysergic acid may also be involved (Hill et al., 2001).

Ergot alkaloids are present in leaf and stem tissue of wild-type tall fescue, with highest concentrations found in the seed (Rottinghaus et al., 1991). Research has shown that ergot alkaloid concentrations fluctuate during the growing season. Grazing studies (when fescue is in its non-vegetative state) have indicated that ergopeptine alkaloid concentrations peak in late spring, decrease during the summer months, and rise to maximum concentration in the fall (Belesky et al., 1988; Rottinghaus et al., 1991). However, recent research has shown that when tall fescue is maintained in a vegetative state of regrowth, ergovaline concentrations are low in spring, steadily increase throughout the spring and summer months, and greatly increase during fall (Rogers et al., 2011).

Physiological Functions

Little information is known regarding the metabolism of ergot alkaloids in ruminants (Strickland et al., 2011). However, it has been documented that ergot alkaloid absorption occurs across the gastrointestinal epithelia of the rumen and/or in the small intestine (Strickland et al., 2011). Subsequently, the alkaloids are transported by the

lymphatic system into systemic circulation (Eckert et al., 1978). This information is evidenced by the *in vitro* analysis of excreted waste (combined with estimates of animal intake) due to a lack of highly sensitive analytical methods and difficulty in accessing tissues *in vivo* (Strickland et al., 2011). In beef cattle, specifically, studies have shown that approximately 96% of ergopeptine alkaloids ingested by grazing toxic tall fescue are excreted in urine (Stuedemann et al., 1998). Furthermore, very small amounts of those alkaloids consumed were found present in bile (Stuedemann et al., 1998).

Effects on Male Reproduction

To date, few studies have evaluated the effects of toxic fescue on male bovine reproduction. Moreover, results have been inconsistent. For example, field trials have shown that sperm concentration (Pratt et al., 2015), motility (Jones et al., 2004; Looper et al., 2009), and morphology (Pratt et al., 2015) are reduced as a result of grazing toxic tall fescue. Semen cryopreservation experiments yielded similar results for motility post-thaw (Pratt et al., 2015). In contrast to these findings, other studies revealed no detrimental effects of toxic fescue intake on semen production or quality parameters (Evans et al., 1988; Schuenemann et al., 2005a, Stowe et al., 2013).

It has also been reported that bulls consuming toxic fescue exhibit reduced serum prolactin concentrations (Schuenemann et al., 2005a,b; Stowe et al., 2013; Pratt et al., 2015). This is a useful tool for determining whether or not animals are consuming ergot alkaloids, as ergot alkaloids are known to suppress prolactin release. In addition, prolactin has long been speculated to play a role in male reproduction of other species;

however, the effect of suppressed prolactin in circulation on bull reproduction is unknown.

Conflicting reports involving reductions in scrotal circumference measurements (Jones et al., 2004; Schuenemann et al., 2005a,b; Looper et al., 2009; Stowe et al., 2013) due to fescue toxicity have also been described, with either no effect being observed (Schuenemann et al., 2005 a,b; Looper et al., 2009) or a decrease in prolonged exposure to ergot alkaloids (Stowe et al., 2013). Testosterone is required for maintenance of spermatogenesis, and *in vitro* experiments have shown that testosterone may be regulated by prolactin. However, *in vivo* studies have shown that, when serum prolactin concentrations are reduced due to the consumption of ergot alkaloids, testosterone concentrations are seemingly unaffected (Schuenemann et al., 2005a,b; Looper et al., 2009; Pratt et al., 2015).

Research concerning the direct effects of ergot alkaloids on male fertility has likewise been minimal. A study by Wang and colleagues (2009), involving the incubation of motile spermatozoa with ergotamine and dihydroergotamine, showed that both ergot alkaloids decreased sperm motility via alpha adrenergic receptors. In a semen freezing study, Gallagher and Senger (1989) documented a reduction in the number of intact acrosomes prior to freezing, as well as post-thaw, when ergonovine was used in semen extender. Spermatozoa motility was also decreased post-thaw (Gallagher and Senger, 1989).

Reduced embryo cleavage rates have been documented using sperm from bulls that were either administered ergotamine tartrate (Schuenemann et al., 2005a) or grazing

toxic fescue (Schuenemann et al., 2005b). Interestingly, no differences in motility or morphology were noted between treatments. This information may suggest, due to fescue toxicosis, structural and/or physiological changes to the sperm cell. Further, these changes may not be detected using traditional breeding soundness evaluation techniques.

Ultimately, the mechanisms by which fescue toxicosis negatively impact reproduction are not well understood and warrant further research. Because fertility has only been assessed *in vitro*, it is unknown what effects ergot alkaloids have, if any, on sperm structure and function *in vivo*.

Pasture Management

Approaches

Since most tall fescue pastures in the United States are infected with the ergot alkaloid-producing fungal endophyte (Shelby and Dalrymple, 1987), the seemingly simple and obvious solution to fescue toxicosis would be to replace pastures with endophyte-free tall fescue. However, unless all existing endophyte-infected fescue is eliminated, pasture will not remain endophyte-free (Shelby and Dalrymple, 1993) due to the invasive nature of the wild type. Furthermore, when compared to toxic tall fescue, endophyte-free fescue is not as persistent (Hill et al., 1991), is less tolerant of drought conditions (West et al., 1993), and is more vulnerable to plant pathogens, insect herbivory, and overgrazing (Latch, 1993; Malinowski and Belesky, 2000). Because of these issues, alternative approaches aimed at alleviating fescue toxicosis through the management of ergot alkaloids can be implemented (Roberts and Andrae, 2004).

For instance, interseeding toxic tall fescue pastures with other grasses or legumes has been shown to increase steer ADG by as much as 50 – 80% (Hoveland et al., 1981; McMurphy et al., 1990). Although dilution is considered a good practice, it often masks the toxic effects of ergot alkaloids, and serves as only a partial remedy to the problem (Roberts and Andrae, 2004). Also, research has shown that tall fescue seedheads contain five times more ergovaline than leaves or stems (Rottinghaus et al., 1991). Therefore, keeping seedheads clipped can help alleviate fescue toxicosis by reducing the concentration of ergot alkaloids ingested by grazing animals (Roberts and Andrae, 2004). Rotational grazing practices, in which cattle are placed on nontoxic forage during the summer months, may mitigate heat-stress problems associated with fescue toxicity as well. In addition, stockpiling tall fescue for winter grazing when ergovaline concentrations have decreased (Kallenbach et al., 2003) may also help relieve fescue toxicosis.

Novel Endophytes

Another management practice receiving increased attention is the replacement of toxic tall fescue stands with novel endophyte-containing cultivars (commonly referred to as “beneficial endophytes”). These cultivars contain endophytes that are members of the same fungal species as the endophyte found in toxic tall fescue (Roberts and Andrae, 2004). However, they produce little to no ergot alkaloids and are considered to be nontoxic (Bouton et al., 2002; Parish et al., 2003; Nihsen et al., 2004). Studies have shown that novel endophyte cultivars not only increase plant persistence and animal

gains, but are also grazed for longer periods of time and consumed in larger quantities on a per day basis (Parish et al., 2003).

To date, replacement of toxic tall fescue stands with novel endophyte cultivars may be the most cost-effective and time-efficient solution to the fescue toxicosis problem, affording producers the benefits of the endophyte without toxic effects. However, for some producers, replacement may not be worthwhile. Several aspects (including toxicity level, field terrain, class of livestock, and grazing management) should be taken into consideration before replacing established tall fescue stands (Roberts and Andrae, 2004).

Summary

Tall fescue is the predominant forage for millions of cattle and livestock in the southeastern United States. In spite of its desirable agronomic traits, the cool-season grass has been rendered toxic due to an ergot alkaloid-producing endophyte. Among other symptoms, reproductive inefficiencies are reported for beef cattle consuming toxic tall fescue. Moreover, little consideration has been given to researching the potential effects of fescue toxicosis on male reproductive physiology. Therefore, our lab is interested in studying spermatozoa from bulls consuming toxic tall fescue. Specifically, we are interested in evaluating the integrity of the acrosome – an enzyme-containing structure that is required for fertilization purposes.

ACROSOMAL INTEGRITY

History of the Sperm Cell

Since Leeuwenhoek's microscopic discovery of spermatozoa in the late 1600s and Spallanzani's ensuing observation that sperm are necessary for fertilization, human knowledge of sperm cell structure and function has been greatly improved. Though it wasn't realized until 1875 by Hertwig that nuclei of the sperm and egg fused during the process of fertilization, his discovery laid the foundation for the concept of genetic inheritance. Years later, in the early 1950s, the acrosome reaction of spermatozoa was discovered by J.C. Dan using marine invertebrates (Dan, 1954). While still somewhat of an enigma in most species, it has been recognized that the acrosome is essential for fertilization in many animals, including mammals.

Basic Sperm Cell Structure and Function

The mature mammalian sperm cell is a small, compact, and specialized male germ cell comprised of two principal and morphologically distinct regions referred to as the "head" and "tail" (Figure 1.4). Surrounded by a continuous plasma membrane, the cell's sole purpose is to deliver the male's genetic information to the female gamete quickly and efficiently. Although size and shape of spermatozoa tend to be species-specific (Fawcett, 1975), basic structure is similar for all mammals. The "head", or "anterior portion" of the cell consists mainly of the nucleus and acrosome, which function to facilitate fertilization. The "midpiece", though less morphologically distinct, contains mitochondria that provide the energy needed for flagellum propulsion. The "tail", or

“flagellum”, is an appendage that serves to propel the sperm up the female reproductive tract toward a non-motile egg, or ovum.

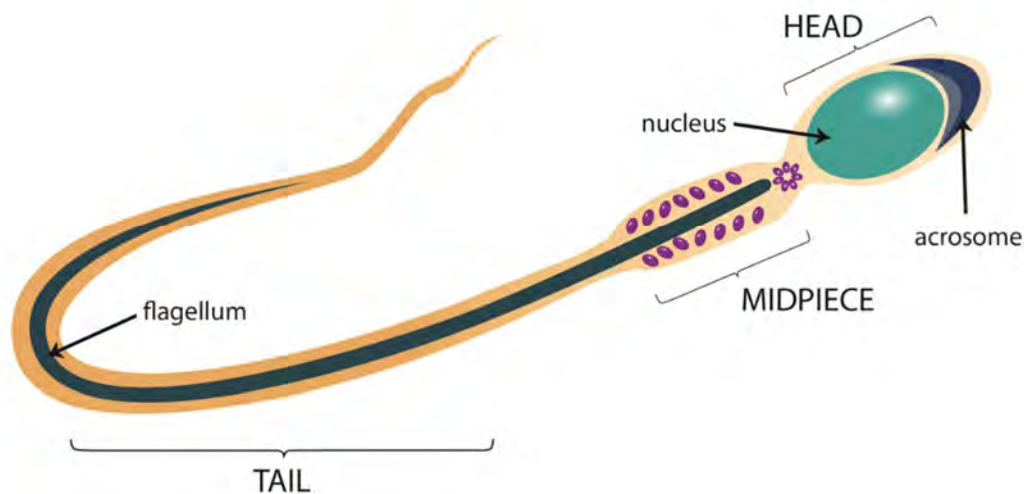


Figure 1.4. Basic sperm cell structure. Adapted from Proceptin Healthcare, Inc.

Acrosome

The acrosome is a cap-like structure derived from the Golgi apparatus that lies over the anterior portion of the nucleus of a sperm cell. Structurally, the acrosome consists of an inner acrosomal membrane (closest to the nucleus), outer acrosomal membrane, and acrosomal matrix (located between the inner and outer acrosomal membranes) (Zaneveld and De Jonge, 1991), as shown in Figure 1.5. Once believed by researchers to be a modified lysosome (Hartree, 1975) due to its acidic nature, the acrosome contains enzymes analogous to those found in lysosomes (Allison and Hartree, 1970; Zaneveld and De Jonge, 1991; Yanagimachi, 1994). More specifically, the

acrosome contains digestive enzymes that aid in sperm penetration through the outer membrane of the ovum (Yanagimachi, 1994), referred to as the zona pellucida.

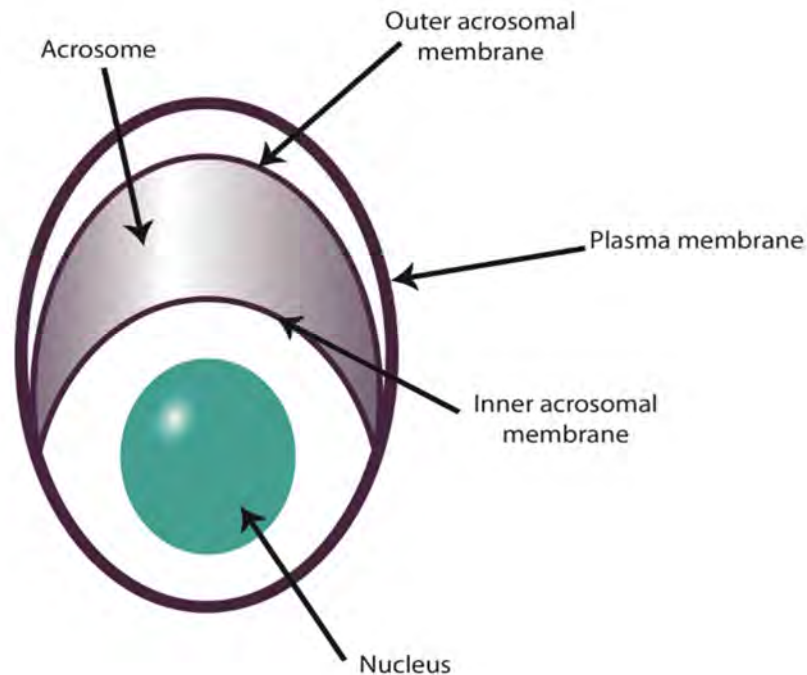


Figure 1.5. Acrosomal membranes. Adapted from Anifandis et al., 2014.

Capacitation

Spermatozoa begin to acquire the ability to fertilize an egg as they pass through the epididymis and undergo a process known as epididymal maturation (Courot, 1981; Orgebin-Crist, 1981; Olson and Orgebin-Crist, 1982). However, the changes that occur during maturation do not make sperm cells entirely fertile. Following maturation and ejaculation, sperm must reside in the female reproductive tract for a certain period of time

(Austin, 1951; Chang, 1951) where they undergo physiological changes in order to render them fertile. These changes are collectively known as capacitation (Austin, 1952). While capacitation is not completely understood, it has been recognized that the event involves alterations to the surface of sperm cells (Reviewed by Yanagimachi, 1994). More specifically, change in or removal of a “protective coat” (Figure 1.6) from the surface of the sperm plasma membrane occurs, permitting sperm to interact with an egg and subsequently undergo the acrosome reaction (Piko, 1967).

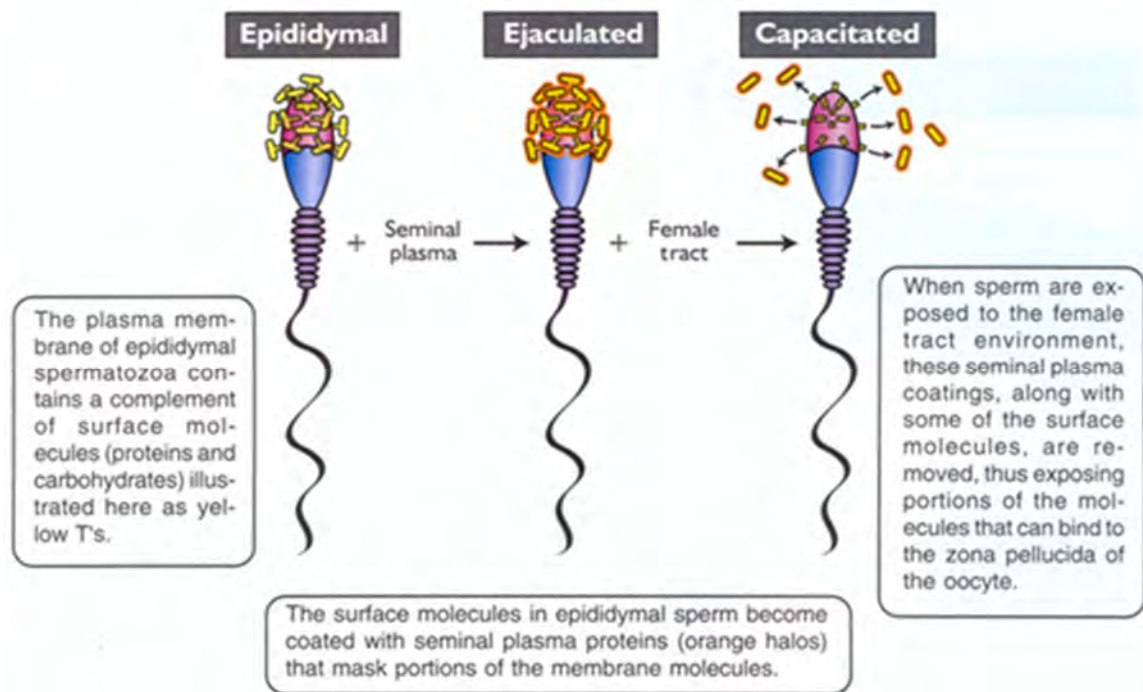


Figure 1.6. Conceptual visualization of mammalian capacitation. Reproduced with permission from Current Conceptions, Inc. (Senger, 2003).

Acrosome Reaction

The acrosome reaction is an exocytotic event that is initiated when sperm bind to the zona pellucida of an ovum. Once sperm binding occurs, the outer acrosomal membrane fuses with the overlying plasma membrane (Barros et al., 1967). Fusion of these membranes triggers vesiculation, a process by which many small vesicles are created that allows for the dispersal of acrosomal enzymes (Saacke and Almquist, 1964; Franklin et al., 1970; Meizel, 1984). The release of acrosomal enzymes enables the sperm cell to “digest” its way through the zona pellucida and begin the process of fertilization (Senger, 2003).

Importance of an Intact Acrosome

The ability of a sperm cell to undergo capacitation, the acrosome reaction, and a fertilization event requires an intact acrosome at the time of ejaculation. Disruption of or damage to the acrosome is permanent and results in premature loss of acrosomal contents, which ultimately prevents fertilization (Senger, 2003). Moreover, damaged acrosomes do not undergo vesiculation properly, but rupture spontaneously (Senger, 2003).

Methods Used to Evaluate Acrosomal Integrity

Overview

The study of acrosomal integrity in mammalian species is receiving increased attention as a valuable tool in evaluating male subfertility and infertility. With

advancements in microscopic visualization and cell staining technology, methods for determining acrosomal integrity have been developed. However, many of these methods are time-consuming, involve expensive equipment and reagents, and are not optimal for evaluating bovine acrosomal status.

Phase-Contrast and DIC Microscopy

Phase-contrast and differential interference contrast (DIC) microscopy are techniques that enhance the contrast of transparent and colorless specimens for improved visualization. Using these methods, few spermatozoa are required, and cell viability and acrosomal integrity can be evaluated simultaneously (Cross and Meizel, 1989). Another advantage to using these techniques is that partial and/or complete acrosome reactions can be easily detected in viable sperm. However, these methods are optimal for examining sperm cells with large acrosomes (such as guinea pigs and hamsters) (Cross and Meizel, 1989). Also, in most species it is essential to reduce sperm velocity by examining spermatozoa at room temperature or in single frames of videos (Cross and Meizel, 1989).

While phase-contrast and DIC microscopy can be used to assess the acrosomal status of live bull sperm, it is difficult due to the small size of the acrosome (Cross and Meizel, 1989). Using DIC microscopy, studies have shown that the presence or absence of the apical ridge (characteristic of an intact acrosome) can be determined in motile bovine sperm (Saacke and Marshall, 1968; Aalseth and Saacke, 1986). Both methods can also be utilized in detecting intermediate stages of the acrosome reaction and

degenerative acrosomal loss, to some extent, in viable spermatozoa (Hancock, 1952; Saacke and Marshall, 1968).

Bright-Field Microscopy

Bright-field microscopy is another method used in the assessment of acrosomal integrity and is the simplest microscopic technique to date, in which light is either passed through or reflected off of a specimen. This technique is used to view fixed or live cells, utilizes cost-effective equipment and stains, and allows for the creation of permanent slides (Cross and Meizel, 1989). Furthermore, bright-field microscopy often employs double staining procedures. One dye is used to detect the presence or absence of the acrosome, whereas another dye of a different color is used to evaluate plasma membrane integrity. Unfortunately, there are a limited number of stains available that are specific for the acrosomal region of sperm cells, and assays best suited for bright-field microscopy are often time-consuming (Cross and Meizel, 1989). However, there are dual stain dyes, such as trypan blue with giemsa (Didion et al., 1989), as well as naphthol yellow with erythrosin B (Cross and Watson, 1994), that have been verified for use in the bovine species.

Fluorescence Microscopy

Fluorescence microscopy involves labeling cells with fluorescent probes, some of which are specific for intracellular content. After labeling, a fluorescence microscope is used to irradiate the cells with specific wavelengths of light, and then separate the emitted

fluorescence from the excitation light. The emission light reaches the eye or detector and the resulting fluorescent cells are superimposed with great contrast against a black background.

One method of assessing bovine acrosomal status is through the use of fluorescent-labeled lectins. Lectins are proteins, mainly of plant origin, that bind specific carbohydrate moieties. Two lectins that are specific for intracellular acrosomal contents have been verified for assessing acrosomal integrity in bull spermatozoa (Cross and Watson, 1994). These lectins are peanut agglutinin (PNA) and pisum sativum agglutinin (PSA), which may be conjugated to several fluorochromes. Peanut agglutinin is derived from the peanut plant and binds exclusively to terminal β -galactose, which is localized on the outer acrosomal membrane of sperm cells (Jaiswal and Eisenbach, 2002). Pisum sativum agglutinin comes from the edible pea plant and is specific for α -mannose moieties within the acrosomal matrix.

Fluorescence microscopy is advantageous in that it allows for vivid visualization of specimens and is suitable for a multitude of different species, including bovine. However, staining procedures are often time-consuming and take hours to complete, which is not ideal for large numbers of samples. Fluorescence microscopy also involves expensive equipment, and only allows for the evaluation of small numbers of sperm.

Flow Cytometry

Flow cytometry is a laser-based technology that measures and analyzes characteristics of individual cells as they flow in a fluid stream through a beam of light.

This technique is used for fluorescent cell sorting and counting, and can also be utilized in evaluating bovine acrosomal status (Nagy et al., 2003). This method also offers a quick and objective assessment of specimens, analyzing thousands of cells per second (Graham, 2001). Furthermore, flow cytometers afford users the ability to separate cell debris from actual cells through a process called “gating”, which ensures more accurate data.

Conclusion

Being able to visualize and assess the acrosomal status of spermatozoa can be beneficial in evaluating semen quality. It is possible that male subfertility or infertility could be caused by a lack of spermatozoa with intact acrosomes at the time of ejaculation. The effects, if any, of toxic tall fescue consumption on male bovine acrosomal integrity are unknown. Evaluating the acrosomal status of bulls on toxic tall fescue could help elucidate the reproductive inefficiencies observed due to the fescue toxicosis syndrome.

CHAPTER TWO

USING FLUORESCENT-LABELED PEANUT AGGLUTININ TO ASSESS ACROSOMAL INTEGRITY OF BEEF BULLS CONSUMING TOXIC TALL FESCUE

ABSTRACT

Little research exists concerning the effects of fescue toxicosis on bovine male reproduction. The objective of this study was to assess the influence of toxic tall fescue consumption on acrosomal integrity of beef bulls. Semen was collected and fixed from bulls that were either fed a ration containing toxic (E+) or nontoxic (E-) tall fescue seed or grazing toxic (E+) or nontoxic (E-) tall fescue pasture. Beef bulls in 2011 (n = 14), 2012 (n = 21), 2013 (n = 12), and 2014 (n = 25), having passed a breeding soundness exam (BSE), were blocked and allotted to one of two treatments. Blood samples were collected to determine serum prolactin (PRL) concentrations. Semen samples were collected via electro-ejaculation and evaluated using a computerized sperm quality analyzer specific for bull semen. Semen was fixed in neutral buffered formalin and stored at room temperature (RT) until further analysis. Fluorescent-labeled peanut agglutinin (PNA) was used to evaluate sperm acrosomal integrity. For acrosome assessment, fixed spermatozoa were incubated with PNA-Alexa Fluor 594, counterstained with DAPI, air-dried onto microscope slides, photographed under fluorescence microscopy, and counted manually. Four staining patterns were observed following the exposure of spermatozoa to PNA-Alexa Fluor 594. Analysis revealed significant TRT effects for staining patterns 2

and 3 ($P < 0.05$). No TRT or TRT x d interactions were detected for staining patterns 1 and 4 ($P > 0.05$).

Key words: acrosomal integrity, fescue toxicosis, peanut agglutinin

INTRODUCTION

Tall fescue is the most widely used cool-season bunchgrass in the southeastern United States (Bouton, 2000), well known for its wide range of adaptation, ease of establishment, tolerance to poor management, and extended grazing season (Stuedemann and Hoveland, 1988). Through a mutualistic relationship with the ergot alkaloid-producing endophyte, *Epichloë coenophiala* (Young et al., 2014), tall fescue is able to withstand drought and disease conditions, insect herbivory, and grazing pressure (Hoveland, 1993). Despite these desirable agronomic traits, ingestion of ergot alkaloids by grazing livestock causes fescue toxicosis (Thompson and Porter, 1990), a condition that, due to production losses and reproductive inefficiencies, is estimated to cost \$1 billion yearly in economic losses (Allen and Segarra, 2001; Fribourg and Waller, 2005; Strickland et al., 2011).

Little research exists concerning the effects of fescue toxicosis on bovine male reproduction. Moreover, few studies have detected differences in semen quality due to treatment. For example, field trials have shown that sperm concentration (Pratt et al., 2015), motility (Jones et al., 2004; Looper et al., 2009), and morphology (Pratt et al., 2015) are reduced as a result of grazing toxic fescue. In addition, studies by

Schuenemann et al. documented a reduction in fertility as assessed by IVF when bulls were administered ergotamine tartrate (2005a) or grazing toxic fescue (2005b). The objective of this study was to assess the influence of toxic tall fescue consumption on acrosomal integrity of beef bulls consuming toxic Kentucky 31 (KY31) compared to a novel endophyte cultivar, Texoma Max Q II (NE).

MATERIALS AND METHODS

Experimental Design

All animal research was approved by the Clemson University Institutional Animal Care and Use Committee (IACUC protocol #ARC2010-45 and #ARC2010-68).

Treatment

All reagents were purchased from Sigma Scientific (St. Louis, MO), unless stated otherwise. In 2011, yearling beef bulls (n = 14) were fed a ration containing tall fescue seed that either lacked ergot alkaloids (E-) or possessed ergot alkaloids (E+) at a fixed concentration (0.8 µg of ergovaline & ergovalanine/g diet DM) formulated to give a 1.0 kg per day gain (Stowe et al., 2013). For years 2012 (n = 21), 2013 (n = 12), and 2014 (n = 25), Angus bulls were allotted to treatment and subjected to grazing ergot alkaloid-producing tall fescue (E+) or a novel endophyte cultivar that does not produce ergot alkaloids (E-). An ELISA was used to test for ergot alkaloids (Agrinostics, Ltd., Co, Watkinsville, GA) on 50 tillers per pasture, and the E+ pasture exhibited a 98% infection rate. Two weeks before the start of each study, bulls were adjusted to a concentrate or

forage diet. The dietary treatment period for 2011 was 126 d (April to August). Grazing periods for 2012, 2013, and 2014 were 155 (April to August), 60 (February to April), and 168 (February to August) d, respectively. In 2011, bulls were subjected to electro-ejaculation every 21 d, and in all other years at 28 or 30 d intervals. For all years, prior to treatment allotment, bulls were weighed and subjected to a body condition score (BCS) and breeding soundness exam (BSE). Only bulls passing the BSE were allotted to treatment, and allotment was conducted by blocking for body weight (BW) and BCS. To assess the effectiveness of treatment, blood samples were collected via caudal venipuncture and assayed for serum prolactin (PRL) in 2011, 2012, and 2014. Blood was allowed to clot and placed at 4° overnight, and serum was harvested by centrifugation at 2000 x g for 15 min. at 4° C. Serum was placed in vials and stored at -20° C until used for RIA. Prolactin assays were performed by the F. Neal Schrick laboratory as previously described (Bernard et al., 1993) with mean inter- and intra-assay CV of 9.7% and 6.0%, respectively.

Semen Collection, Evaluation, and Fixation

Bulls were restrained in standard animal handling chutes and subjected to electro-ejaculation using the Pulsator IV (Agtech, Manhattan, KS) on the preprogrammed collection mode (Stowe et al., 2013). Ejaculate volume was recorded and semen quality parameters were estimated on-site using a computerized sperm quality analyzer (SQA-Vb; A-Tech, Los Angeles, CA) specific for bull semen. Semen quality parameters evaluated were as described by Stowe et al. (2013). Bulls failed the BSE if semen

samples exhibited < 30% motility or < 70% morphology, or if scrotal circumference (SC) measurements were < 30 cm. Following collection and analysis, 500 µl of each semen sample was fixed in 9.5 mL of 10% neutral buffered formalin and stored at room temperature (RT) until further analysis.

Staining Procedure

For each sample, two 500 µl aliquots of fixed semen were washed separately by centrifugation at 5000 x g for 5 min. in 1X phosphate-buffered saline (PBS). Supernatants were discarded, and one sperm pellet was re-suspended in 200 µl of PNA-Alexa Fluor 594 (1 mg/ml) (Molecular Probes, Eugene, OR) diluted 1:1000 in 1X PBS containing 0.5% bovine serum albumin (BSA). The second sperm pellet served as a negative control and was re-suspended in 200 µl of 1X PBS containing 0.5% BSA. Sperm suspensions were subsequently incubated in the dark at RT for 1 h, and washed twice under the same conditions. Supernatants were discarded and all sperm pellets were counterstained in 200 µl of the DNA dye, DAPI (1 mg/ml) (Molecular Probes, Eugene, OR), diluted 1:2000 in 1X PBS containing 0.5% BSA. Sperm suspensions were incubated in the dark at RT for 15 – 20 min., and washed twice. Supernatants were discarded and final sperm pellets were re-suspended in 150 µl of 1X PBS.

Fluorescence Microscopy, Photography, and Cell Counting

Stained spermatozoa were smeared onto glass microscope slides in 15 µl aliquots using another glass slide. Slides were air-dried and coverslips were placed on slides using

1X PBS. Sperm cells were examined using the 40x objective and appropriate filters for Alexa Fluor 594 and DAPI on an Axio Imager 2 fluorescence microscope (Zeiss, Germany). Five fields per slide were selected at random and photographed using an Axio Cam MRm (Zeiss, Germany). Files were saved as tagged image files (.tif) for later counting. Photographs were uploaded into Infinity Analyze software (Lumenera Corporation, Canada) and all sperm cells in focus were counted manually.

Statistical Analysis

The response variables of interest were the cell counts of each staining pattern expressed as a percentage of the total cells counted. For each response variable, a statistical model was developed consistent with the experimental setup and consisted of terms for TRT, year, month, d, and interactions of those terms. An analysis of variance (ANOVA) was performed to determine which model terms were statistically significant. When TRT or TRT x d terms were significant, follow-up t-tests were performed to determine the nature of the differences. All calculations were conducted using JMP (SAS Inst. Inc., Cary, NC). Statistical significance was defined as $P < 0.05$.

RESULTS

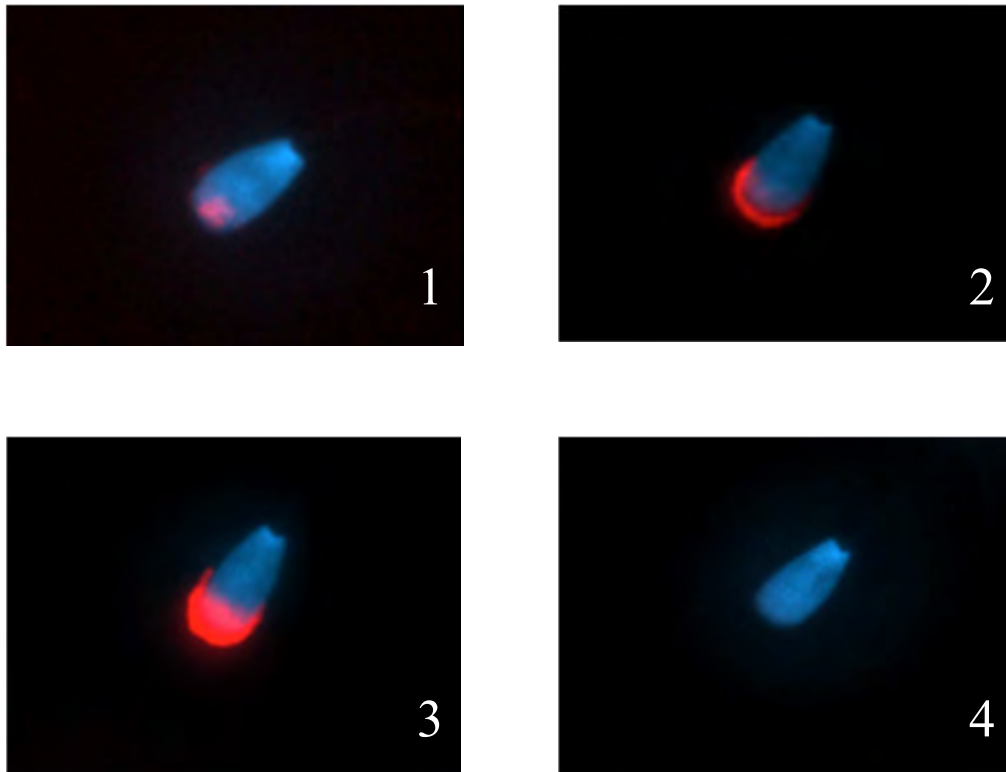
Induction of Fescue Toxicosis

Blood samples collected in 2011, 2012, and 2014 were used to determine serum PRL concentrations. Serum PRL concentrations were lower in bulls on the E+ treatment

compared to E- bulls in 2011 and 2012, as described by Stowe et al. (2013) and Pratt et al. (2015), respectively. Responses in 2014 were as expected (data not shown).

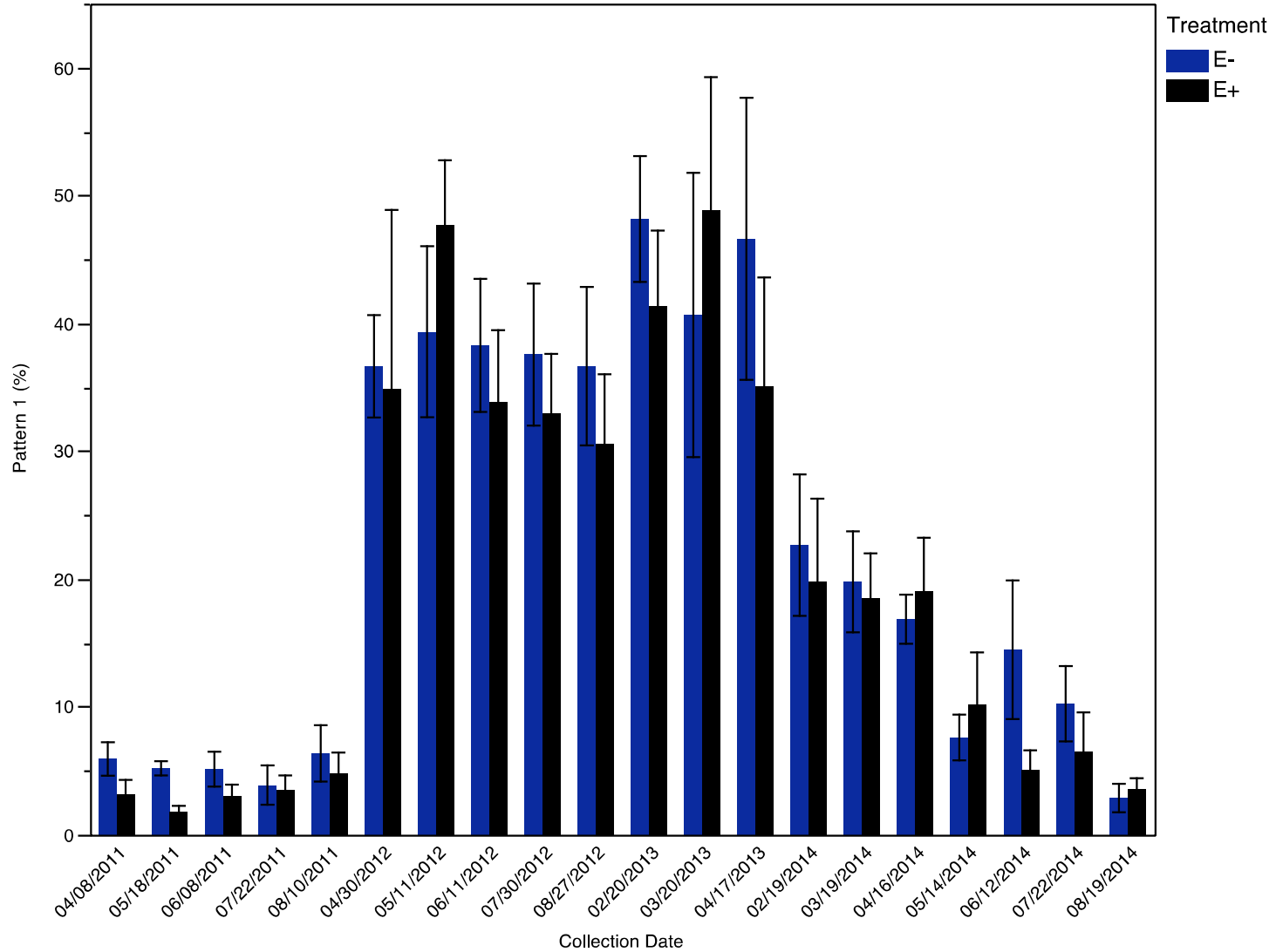
Staining Results

Four staining patterns were observed following the exposure of spermatozoa to PNA-Alexa Fluor 594, as shown in Figure 2.1. Patterns were characterized by labeling of the probe to the acrosomal region and were as follows: pattern 1, irregular staining of the anterior head, producing patchy fluorescence; pattern 2, uniform staining of the anterior head; pattern 3, staining of the entire acrosomal region; pattern 4, no staining in the acrosomal region. Analysis revealed significant TRT effects for staining patterns 2 and 3 ($P < 0.05$). No TRT or TRT x d interactions were detected for staining patterns 1 and 4 ($P > 0.05$).



Different acrosomal staining patterns of formalin-fixed and PNA-Alexa Fluor 594 stained bull spermatozoa, as assessed with fluorescence microscopy. The acrosome region displayed (1) patchy fluorescence, (2) uniform staining of the anterior head, (3) staining of the entire acrosomal region, (4) no staining in the acrosomal region. Sperm cells exhibited blue fluorescence due to counterstaining with the DNA dye DAPI.

Staining Pattern 1



Mean percentages for acrosomal staining pattern 1 are given on the y-axis and day of treatment is on the x-axis. No TRT or TRT x d interactions were detected for pattern 1 ($P > 0.05$).

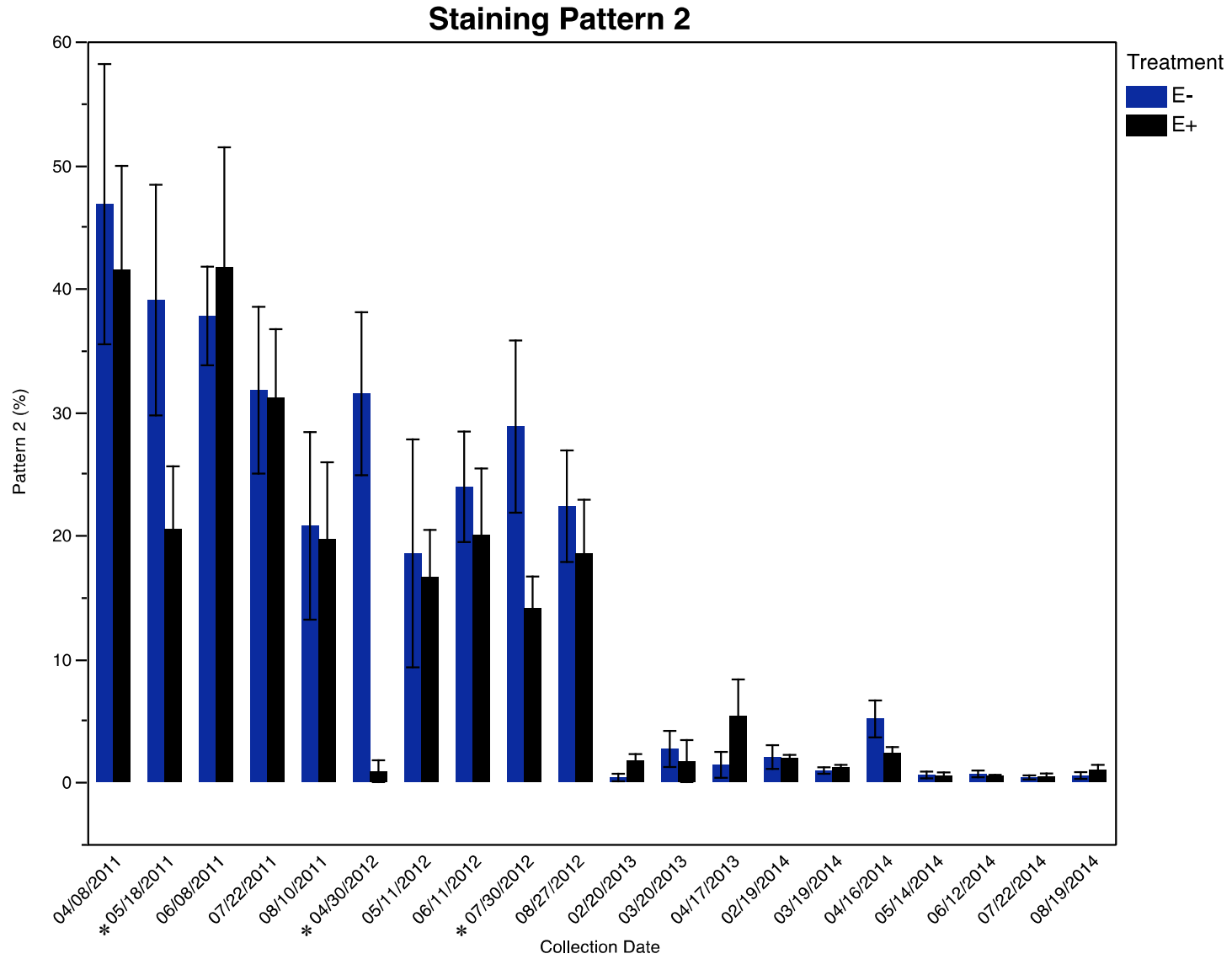


Figure 2.3. Mean percentages for acrosomal staining pattern 2 are given on the y-axis and day of treatment is on the x-axis. An overall TRT effect was detected for pattern 2 ($P < 0.05$). Specific days with a TRT effect ($P < 0.05$) are indicated by an asterisk (*).

Staining Pattern 3

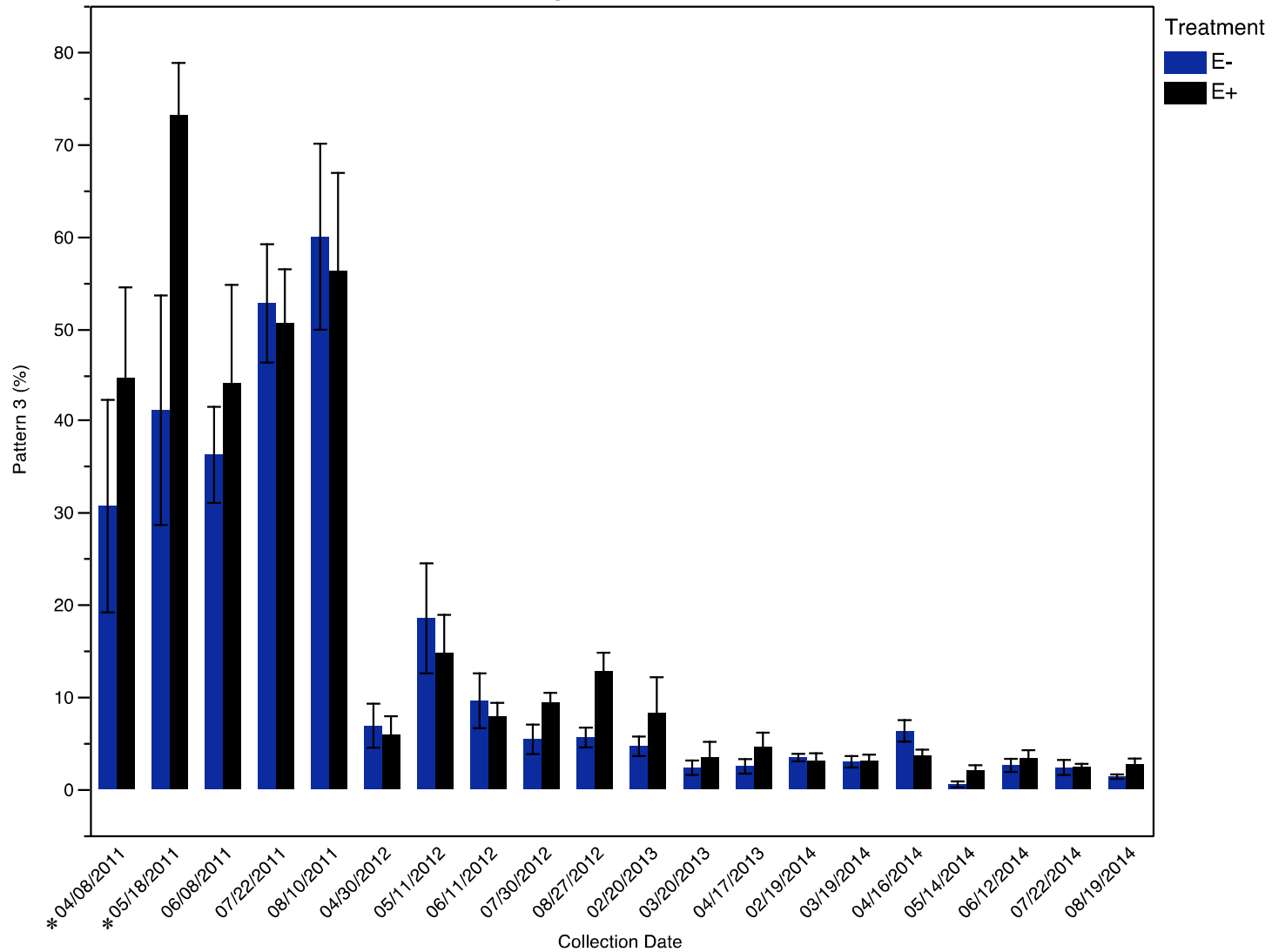
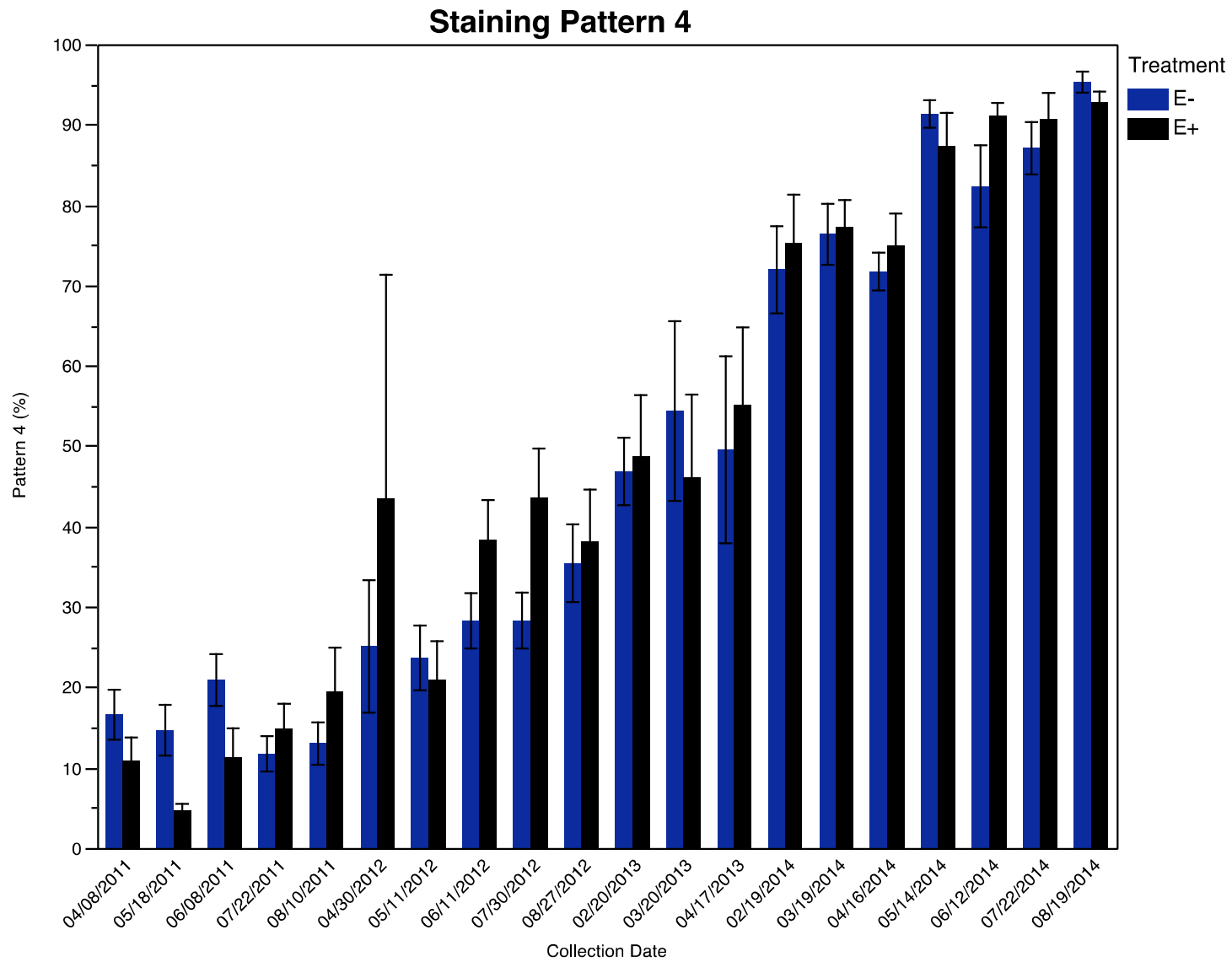


Figure 2.4. Mean percentages for acrosomal staining pattern 3 are given on the y-axis and day of treatment is given on the x-axis. An overall TRT effect was detected for pattern 3 ($P < 0.05$). Specific days with a TRT effect ($P < 0.05$) are indicated by an asterisk (*).



Mean percentages for acrosomal staining pattern 4 are given on the y-axis and day of treatment is on the x-axis. No TRT or TRT x d interactions were detected for pattern 4 ($P > 0.05$).

DISCUSSION

To our knowledge, this is the first study evaluating the acrosomal status of spermatozoa from bulls consuming toxic tall fescue. In 1989, a semen freezing study revealed a decrease in the number of intact acrosomes prior to freezing and post-thaw when ergonovine was used in semen extender, as determined by phase contrast and differential interference contrast microscopy (Gallagher and Senger, 1989). Gallagher and Senger's (1989) findings suggest that ergot alkaloids have a negative impact on acrosomal integrity. However, this information has been the extent of our knowledge concerning the effects of ergot alkaloids on acrosomal status thus far.

In the present study, we used a lectin PNA, Alexa Fluor 594 dye conjugate to assess acrosomal integrity in formalin-fixed spermatozoa from bulls consuming toxic tall fescue. Peanut agglutinin is a plant lectin that has been previously validated as specific for intracellular acrosomal contents in bull sperm (Cross and Watson, 1994). Alexa Fluor 594 is a bright, red-fluorescent dye with high sensitivity and photostability, ideal for use in cell imaging. We used DAPI, a blue-fluorescent DNA dye commonly used to stain fixed cells, as a counterstain and marker for counting sperm. The staining patterns we observed in formalin-fixed spermatozoa were similar to those described by Cross and Watson (1994) using ethanol-fixed spermatozoa. Spermatozoa that exhibited uninterrupted and intense labeling in the acrosomal region were considered to be acrosome-intact, whereas sperm cells displaying little or no labeling in the acrosomal region were regarded as having lost their acrosomal contents (Cross and Watson, 1994).

Using these methodologies and the data presented here, it appears that there are few, if any, differences in acrosomal integrity due to toxic or nontoxic fescue consumption. These data are consistent with previous reports regarding subtle differences observed in semen quality due to treatment (Stowe et al., 2013). More research is required to determine the negative effects of toxic tall fescue consumption on bovine male fertility.

ACKNOWLEDGEMENTS

The author would like to thank Nancy Korn for her assistance throughout this experiment.

CHAPTER THREE

EFFECTS OF TOXIC TALL FESCUE ON BOVINE SEMEN CRYOPRESERVATION

ABSTRACT

In spite of the positive agronomic traits that make tall fescue a desirable forage, reduced fertility rates are reported for beef cattle grazing pasture containing the ergot alkaloid-producing endophyte, *Epichloë coenophiala*. The objective of this study was to assess the influence of toxic tall fescue consumption on spermatozoa physiology as measured by survival of spermatozoa following cryopreservation. Semen was collected, extended, and frozen from bulls grazing either toxic Kentucky 31 (KY31) or the novel endophyte-containing cultivar, Texoma Max Q II (NE; AR584 Ag Research). Yearling Angus bulls (n = 25), having passed a breeding soundness exam (BSE), were blocked based on body weight (BW) and body condition score (BCS). Bulls were allotted to one of two treatments, grazing KY31 or NE for 112 d. On d 112, all bulls were placed on NE pasture to the end of test (d 168) to evaluate recovery from grazing KY31. Blood, urine, and semen samples were collected every 28 d. There were significant TRT x d interactions for serum PRL concentrations, verifying the effectiveness of treatment ($P < 0.05$). Serum prolactin (PRL) concentrations were decreased in the KY31 TRT vs. NE TRT on d 28, 84, and 112. Urinary alkaloid concentrations were affected by TRT x d interactions, confirming ergot alkaloids were present in animal systems ($P < 0.05$). Bulls on the NE TRT exhibited lower urinary alkaloid concentrations than KY31 on d 28, 84,

and 112. Post-thaw semen analysis revealed that progressive motility was decreased in KY31 vs. NE ($P < 0.05$). There were significant TRT x d interactions for concentration, percent motility, total motile sperm per dose, and total progressive motile sperm per dose post-thaw ($P < 0.05$). The KY31 TRT was significantly lower than NE for concentration on d 84; for percent motility on d 28, 84, and 168; for total motile sperm per dose on d 28, 84, and 168; and for total progressive motile sperm per dose on d 28 and 84. Motility was impacted post-thaw for at least 56 d following removal from toxic pasture.

Key Words: tall fescue, endophyte, semen freezing

INTRODUCTION

Heavily utilized in livestock production systems, tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh. = *Schedonorus arundinaceus* (Schreb.) Dumort.] is the primary source of forage for an estimated 8.5 million cattle in the southeastern and mid-Atlantic sectors of the United States (Hoveland, 1993). Well-known for having a wide range of adaptation, being easy to establish, and tolerating poor management (Stuedemann and Hoveland, 1988), tall fescue is also renowned for a number of desirable agronomic traits that are bestowed to the plant via its mutualistic relationship with a fungal endophyte. The endophyte, *Epichloë coenophiala* (Young et al., 2014), confers disease and insect resistance, as well as drought and grazing tolerance to the plant (Hoveland, 1993). Unfortunately, the endophyte produces toxic compounds, including ergot alkaloids (Maag and Tobiska, 1956; Trethewie et al., 1954), which are harmful to

the physiology of grazing livestock. Exposure to ergot alkaloids results in a syndrome known as fescue toxicosis, a condition that costs the entire grazing livestock industry approximately \$1 billion yearly in economic losses (Strickland et al., 2011).

Research efforts concerning the effects of toxic tall fescue consumption on bovine male reproduction have been minimal (Jones et al., 2004; Schuenemann et al., 2005a,b; Looper et al., 2009; Stowe et al., 2013; Pratt et al., 2015). Further, the effects, if any, of fescue toxicosis on semen quality and physiology are not well understood (Pratt and Andrae, 2015). The objective of this study was to assess the influence of toxic tall fescue consumption on spermatozoa survival post-thaw using standard semen cryopreservation procedures, comparing bulls grazing toxic Kentucky 31 (KY31) to those grazing a novel endophyte cultivar, Texoma Max Q II (NE).

MATERIALS AND METHODS

All animal research was approved by the Clemson University Institutional Animal Care and Use Committee (IACUC; IACUC protocol number 2014-60).

Experimental Design

All reagents were purchased from MOFA Global (Verona, WI) unless stated otherwise. Yearling Angus bulls (n = 25), having passed a breeding soundness exam (BSE), were blocked based on body weight (BW) and body condition score (BCS). Bulls were allotted to one of two treatments, grazing toxic Kentucky 31 (KY31) or nontoxic Texoma Max Q II (NE). Bulls were evaluated at 28 d intervals for semen quality

parameters. Parameters included concentration, percent motility, progressive motility, total motile sperm per dose, and total progressive motile sperm per dose. Semen quality data collected at d 56 was excluded due to a breach in experimental protocol.

Treatment

Grazing treatments consisted of ergot alkaloid-containing KY31 or a novel endophyte cultivar lacking ergot alkaloids (NE). Prior to the start of the study, bulls were adjusted to an all-forage diet and grazed NE pasture for two weeks. At the start of the test, bulls were weighed and subjected to a BCS and BSE. All bulls were subsequently allotted to KY31 or NE treatments and remained on treatment for 112 d. On d 112, all bulls were placed on NE pasture to the end of test (d 168) to evaluate recovery from grazing KY31. To assess the effectiveness of treatment, blood and urine samples were collected. Blood was collected via caudal venipuncture and assayed for serum prolactin (PRL). Blood was allowed to clot and placed at 4° overnight, and serum was harvested by centrifugation at 2000 x g for 15 min. at 4° C. Serum was placed in vials and stored at -20° C until needed for RIA. Prolactin assays were performed by the F. Neal Schrick laboratory as previously described (Bernard et al., 1993) with mean inter- and intra-assay CV of 9.7% and 6.0%, respectively. Urine samples were collected in conical tubes, stored at -20° C, and later analyzed using an ELISA (Agrinostics, Ltd. Co., Watkinsville, GA).

Semen Evaluation

Bulls were restrained in standard animal handling chutes and subjected to electro-ejaculation using the Pulsator IV (Agtech, Manhattan, KS) on the preprogrammed collection mode (Stowe et al., 2013). Ejaculate volume was recorded and semen quality parameters were estimated on-site using a computerized sperm quality analyzer (SQA-Vb; A-Tech, Los Angeles, CA) specific for bull semen. Bull ejaculates were extended and frozen if they met the minimum requirements of 30% motility and 70% morphology.

Semen Extension and Cryopreservation

In addition to having enough volume and concentration to extend and freeze 10-20 0.5 mL doses, semen with similar and acceptable quality was extended to 30×10^6 motile sperm/mL per manufacturer instructions using Andromed CSS 1 Step extender at 32° C. Following extension, semen was allowed to cool to room temperature (RT) and immediately packaged in 0.5 mL French straws. Straws were refrigerated overnight at 4° C and frozen horizontally in liquid N₂ vapor 16-20 h later for 10 min. Subsequently, straws were plunged directly into liquid N₂, transferred into goblets, and stored in liquid N₂ until further analysis. Forty-eight h post-freezing, three straws per bull were thawed at 37° C for 1 min. and subjected to computerized sperm quality analysis.

Statistical Analysis

Urinary alkaloid excretion, serum PRL, and post-thaw data were analyzed using ANOVA to test the effects of TRT, d, and TRT x d interactions, followed by the

appropriate t-tests using a significance level of 0.05. Assumptions for ANOVA and t-tests were checked. When any possible violations of the assumptions were detected, a nonparametric rank transformed ANOVA was used to confirm the original ANOVA results. All calculations were conducted using JMP (SAS Inst. Inc., Cary, NC).

RESULTS

Serum Prolactin and Urinary Alkaloid Concentrations

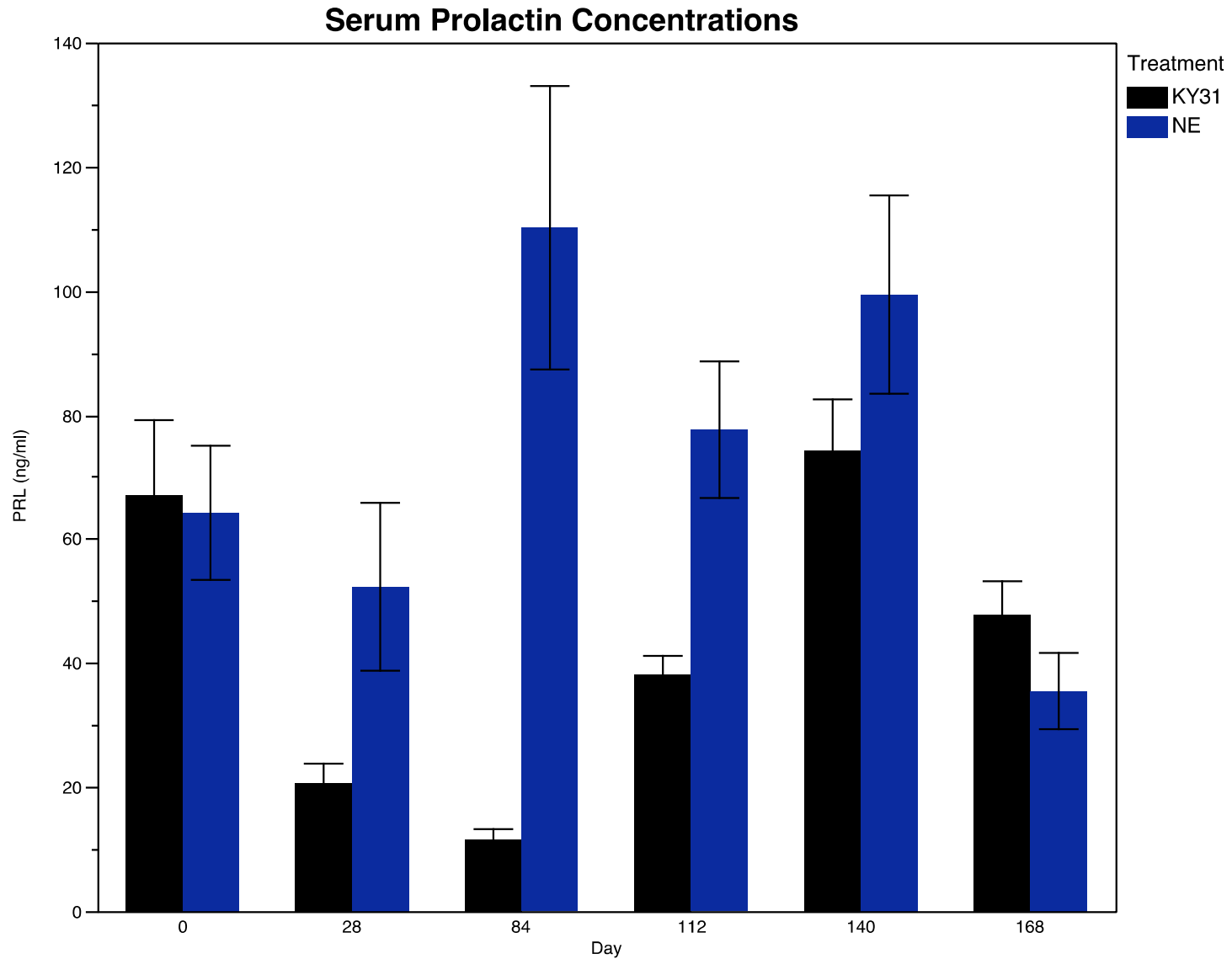
Blood samples were collected and serum was assayed for PRL concentrations to verify the effectiveness of treatment. Serum PRL concentrations were affected by TRT x d interactions ($P < 0.05$). Serum PRL concentrations were decreased in the KY31 TRT vs. NE TRT on d 28, 84, and 112 ($P < 0.05$). No significant differences were detected at the start of test (d 0) or after removal from toxic pasture (d 140 and 168) ($P > 0.05$).

Urine samples were collected and used as diagnostic tools to determine if ergot alkaloids were present in animal systems. Urinary alkaloid excretions were affected by TRT x d interactions ($P < 0.05$). Urinary alkaloid excretions were lower in the NE TRT vs. KY31 TRT on d 28, 84, and 112 ($P < 0.05$). No significant differences were detected at the start of test (d 0) or after removal from toxic pasture (d 140 and 168) ($P > 0.05$).

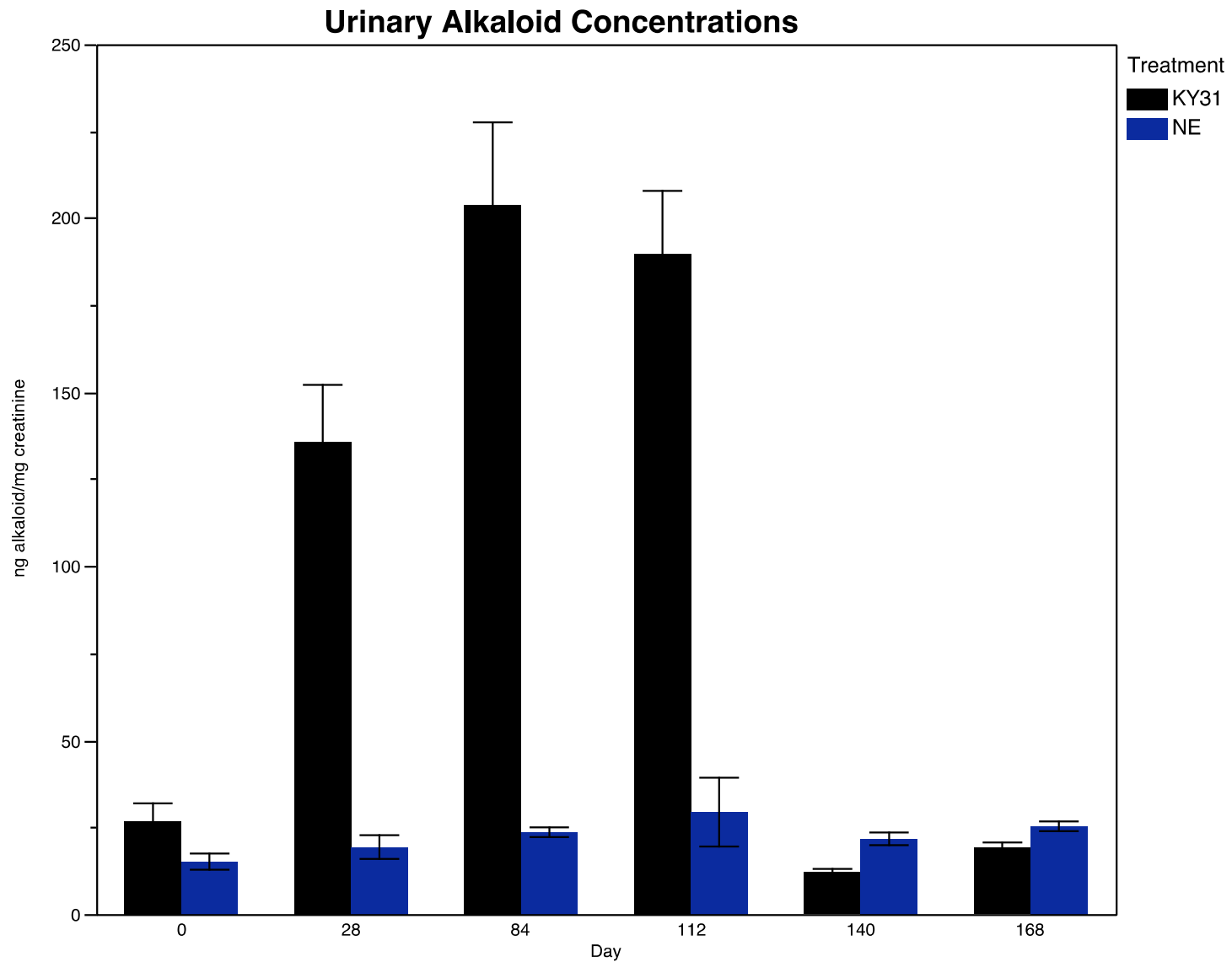
Semen Cryopreservation

There was a significant TRT effect for progressive motility post-thaw, in which the KY31 TRT was significantly less than the NE TRT ($P < 0.05$). There were significant TRT x d interactions for concentration, percent motility, total motile sperm per dose, and

total progressive motile sperm per dose ($P < 0.05$). The KY31 TRT was significantly less than NE for concentration on d 84; for percent motility on d 28, 84, and 168; for total motile sperm per dose on d 28, 84, and 168; and for total progressive motile sperm per dose on d 28 and 84 ($P < 0.05$).



. Mean serum prolactin concentrations are given on the y-axis and day of treatment is on the x-axis. Significant TRT x d interactions were detected for serum prolactin concentrations ($P < 0.05$). Specific days with TRT x d interactions ($P < 0.05$) are indicated by an asterisk (*).



Mean urinary alkaloid concentrations are given on the y-axis and day of treatment is on the x-axis. Significant TRT x d interactions were detected for urinary alkaloid concentrations ($P < 0.05$). Specific days with TRT x d interactions ($P < 0.05$) are indicated by an asterisk (*).

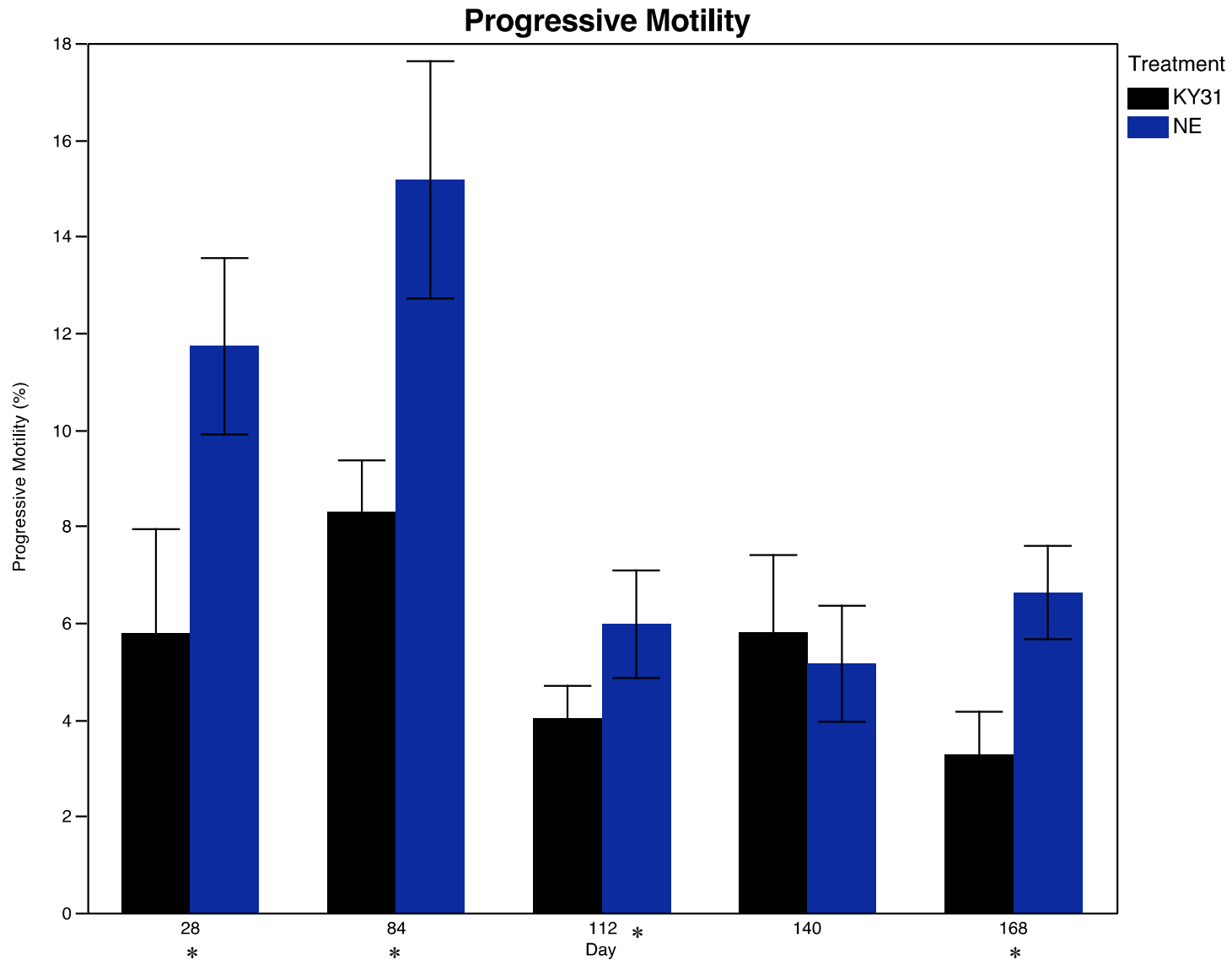


Figure 3.3. Means for post-thaw sperm progressive motility are given on the y-axis and day of treatment is on the x-axis. An overall TRT effect was detected for progressive motility ($P < 0.05$). Specific days with a TRT effect ($P < 0.05$) are indicated by an asterisk (*).

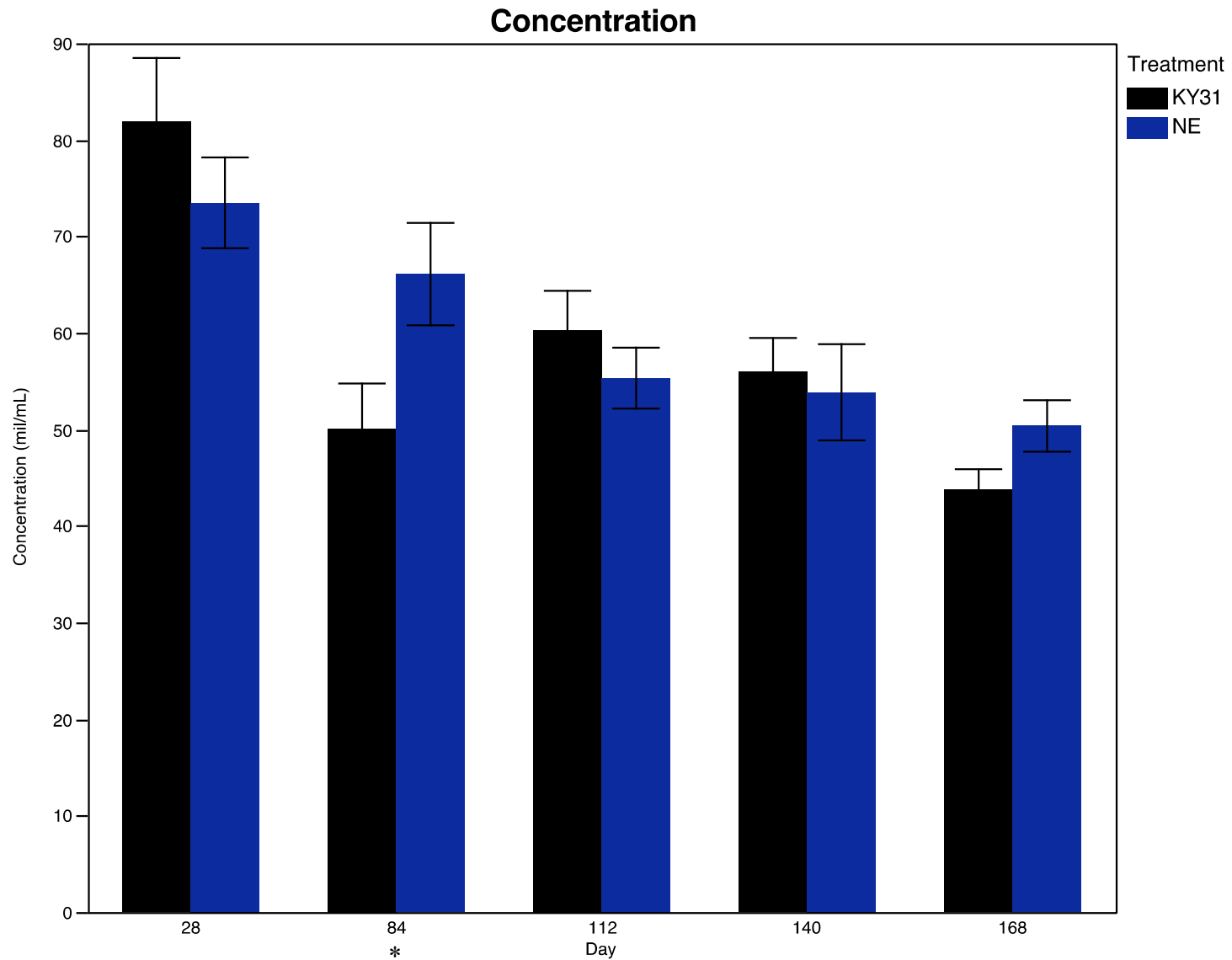
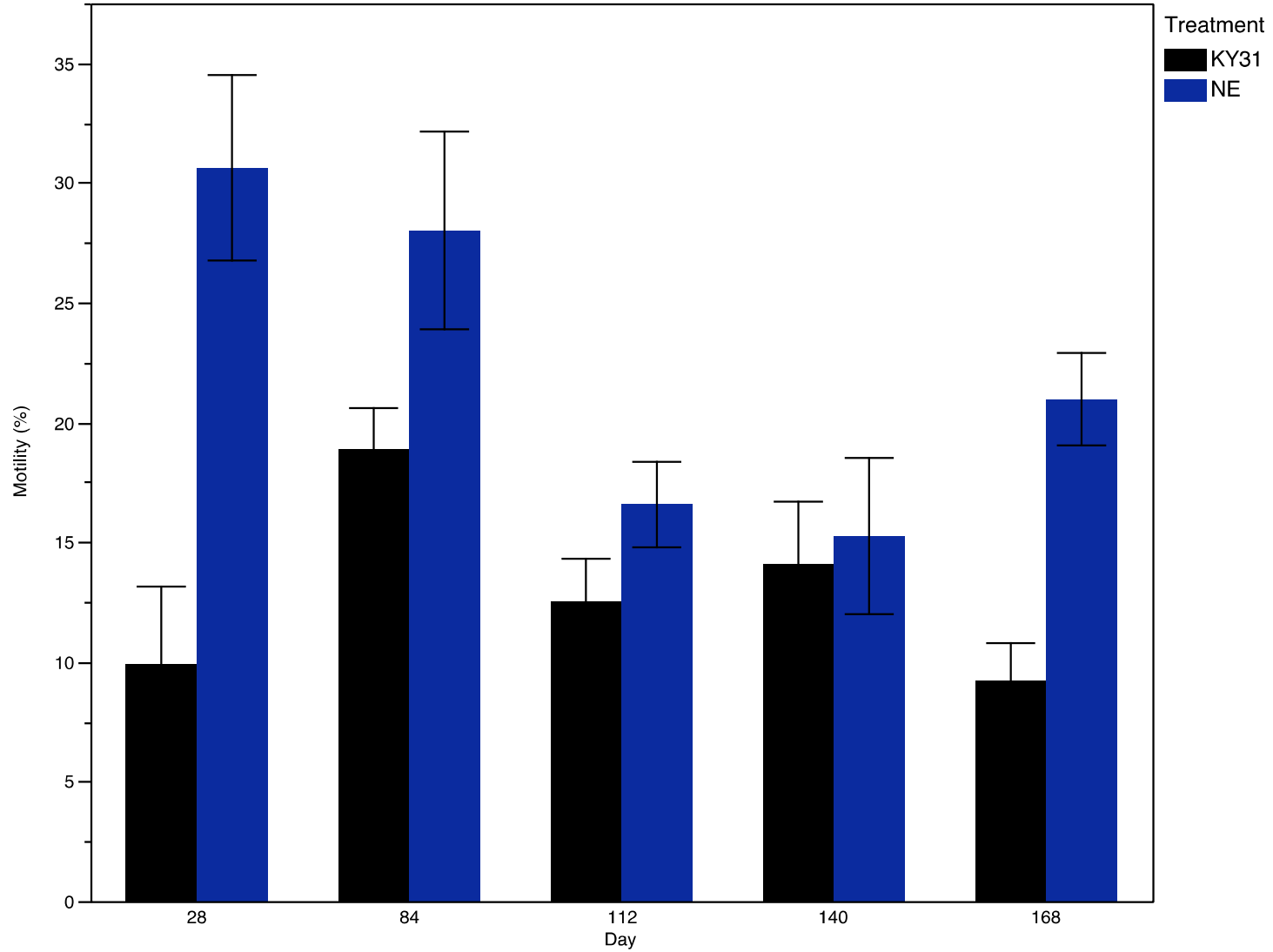
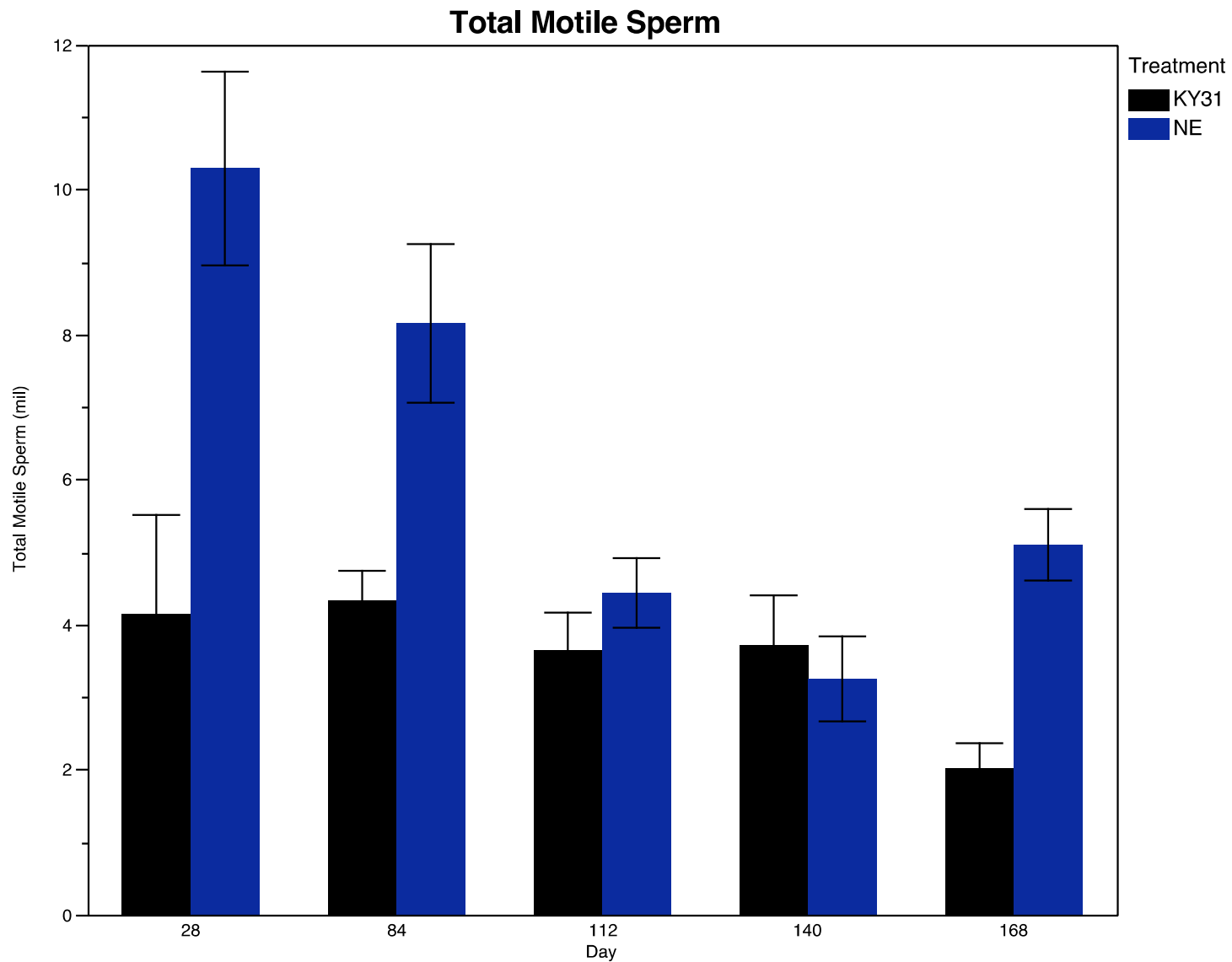


Figure 3.4. Means for post-thaw sperm concentration are given on the y-axis and day of treatment is on the x-axis. Significant TRT x d interactions were detected for concentration ($P < 0.05$). Specific days with TRT x d interactions ($P < 0.05$) are indicated by an asterisk (*).

Motility



Means for post-thaw sperm motility are given on the y-axis and day of treatment is on the x-axis. Significant TRT x d interactions were detected for motility ($P < 0.05$). Specific days with TRT x d interactions ($P < 0.05$) are indicated by an asterisk (*).



Means for post-thaw total motile sperm are given on the y-axis and day of treatment is on the x-axis. Significant TRT x d interactions were detected for total motile sperm ($P < 0.05$). Specific days with TRT x d interactions ($P < 0.05$) are indicated by an asterisk (*).

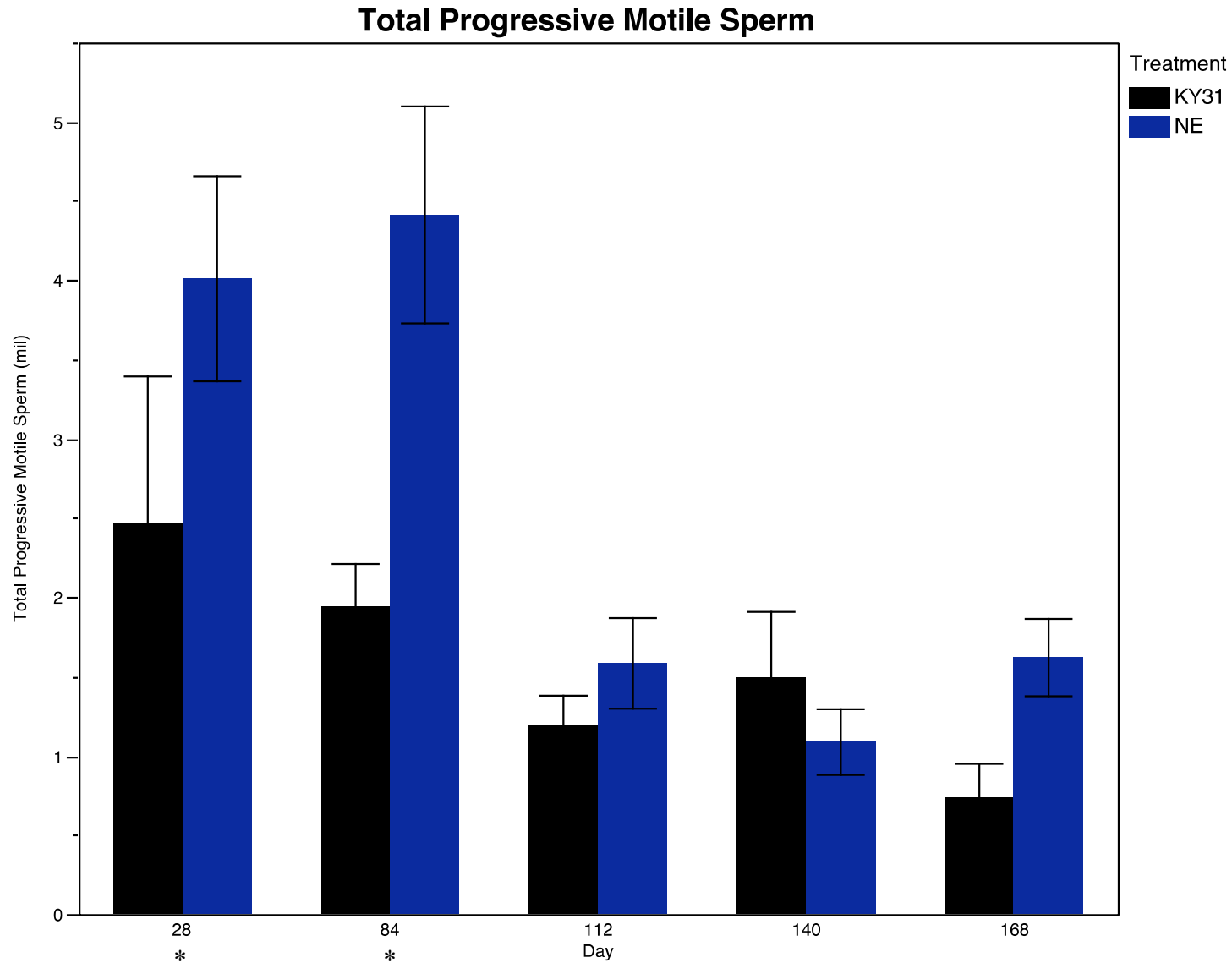


Figure 3.7. Means for post-thaw total progressive motile sperm are given on the y-axis and day of treatment is given on the x-axis. Specific TRT x d interactions were detected for total progressive motile sperm ($P < 0.05$). Specific days with TRT x d interactions ($P < 0.05$) are indicated by an asterisk (*).

DISCUSSION

Serum PRL concentrations observed in this study are in agreement with previous data reported from our lab (Pratt et al., 2015; Stowe et al., 2013) and from others (Looper et al., 2009; Schuenemann et al., 2005a,b), confirming the induction of fescue toxicosis in the KY31 treatment. To further verify the effectiveness of treatment, differences were also observed in urinary alkaloid excretions, which have not been previously reported.

To the authors' knowledge, this is only the second study examining the influence of toxic tall fescue consumption on spermatozoa physiology as measured by survival of spermatozoa following cryopreservation. Post-thaw computerized sperm quality analysis showed that spermatozoa from bulls grazing toxic tall fescue exhibited decreased progressive motility, percent motility, total motile sperm per dose, and total progressive motile sperm per dose when compared to bulls grazing nontoxic fescue. These data are consistent with the previous study by our lab evaluating post-thaw spermatozoa physiology on day 50 or greater after the start of treatment (Pratt et al., 2015). However, in the present study, we observed a difference in concentration post-thaw on day 84, which was perhaps due to an error in semen extension.

With the exception of concentration, it is likely that these differences in sperm physiology are due to fescue toxicosis; however, the mechanisms by which ergot alkaloids negatively affect spermatozoa are unknown at this time. Previous semen cryopreservation studies have shown that incubating motile spermatozoa with ergot alkaloids results in reduced sperm motility (Gallagher and Senger, 1989; Wang et al., 2009). It has also been confirmed that adrenergic (Wang et al., 2009), serotonin (Pratt et

al., unpublished data), and dopamine (Pratt et al., unpublished data) receptors are present on bovine sperm cells. Moreover, because ergot alkaloids have structural similarities to receptors of neurotransmitters, such as serotonin and dopamine, it is not difficult to postulate that ergot alkaloids could bind these receptors and elicit undesirable biological activity (Berde, 1980; Weber, 1980). Further, there could be ergot alkaloids present in seminal fluid; however, there are no assays available for their detection or quantitation.

These data demonstrate that grazing toxic tall fescue negatively impacts semen cryopreservation. Further, these data suggest that there is a residual negative impact from grazing Kentucky 31 following removal from toxic pasture. This information supports the idea that toxins may be gradually released months after grazing animals have been removed from toxic tall fescue pasture (Roberts and Andrae, 2004).

Additional replicate studies are warranted in order to establish a timeline for determining the earliest toxic effects of Kentucky 31 on semen cryopreservation. Furthermore, additional research is necessary for establishing a recovery period from grazing toxic tall fescue.

ACKNOWLEDGEMENTS

The author would like to thank Marcy Dimmick for her assistance throughout this experiment.

CHAPTER FOUR

CONCLUSION

Tall fescue is used extensively in livestock production systems and is well known for its wide range of adaptation, ease of establishment, and tolerance to poor management. Unfortunately, the same endophyte that gives tall fescue its desirable agronomic traits poses a threat to animals that consume the grass. Consumption of toxic tall fescue and subsequent accumulation of ergot alkaloids in animal systems results in a syndrome known as fescue toxicosis. Research efforts concerning the effects of fescue toxicosis on bovine male reproduction have been minimal. Furthermore, there is variability between studies and inconsistent reports make it difficult to pinpoint the reproductive parameters affected.

Our studies showed that there were few, if any, effects of fescue toxicosis on acrosomal integrity of spermatozoa from bulls consuming ergot alkaloid-producing Kentucky 31. However, our data from a semen cryopreservation study showed that grazing Kentucky 31 fescue was detrimental to spermatozoa physiology post-thaw. Furthermore, the data suggest that there is a residual negative impact from grazing tall fescue following removal from toxic pasture. Additional replicate studies are necessary to determine the negative effects of toxic tall fescue consumption on bovine male fertility. Moreover, more research is required to establish a timeline for determining the earliest

effects of toxic tall fescue consumption on semen cryopreservation. Also, more research is warranted for establishing a recovery period from grazing Kentucky 31.

APPENDICES

Appendix A

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December 14, 2015

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Sincerely,

A handwritten signature in black ink that reads 'P.L. Senger'.

Phil Senger, PhD
President
Current Conceptions, Inc.

Appendix B

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Callie Burnett <crburne@g.clemson.edu>

Re: Photos from Tall Fescue Toxicosis and Management Paper

2 messages

Callie Burnett <crburne@g.clemson.edu>
To: jandrae@clemson.edu

Tue, Nov 3, 2015 at 4:08 PM

Dr. Andrae,

I would like to use the fescue foot and fat necrosis photos in the Tall Fescue Toxicosis Management paper in my literature review. I am unsure as to how I should go about getting permission to use them. Do I contact them directly or get permission from you and/or Dr. Roberts?

Thank you,

Callie R. Burnett
91 Poole Agricultural Center
Department of Animal & Veterinary Sciences
Clemson University
crburne@clemson.edu

On Thu, Sep 24, 2015 at 6:28 PM, Callie Burnett <crburne@g.clemson.edu> wrote:
Dr. Andrae,

I would like to include the fescue foot (George Garner) and fat necrosis (John Stuedemann) photos in the 2004 Tall Fescue Toxicosis and Management paper in my literature review. I am unsure as to how I should I go about getting permission to use them. Do I contact them directly or get permission from you and/or Dr. Roberts?

Thank you for your assistance.

Callie R. Burnett
91 Poole Agricultural Center
Department of Animal & Veterinary Sciences
Clemson University
crburne@clemson.edu

John Andrae <jandrae@clemson.edu>
To: Callie Burnett <crburne@g.clemson.edu>

Tue, Nov 3, 2015 at 4:26 PM

Callie,
I think George Garner is dead and John Stuedemann has been retired almost 10 years. I'd suggest just listing them in the photo credit or you could cite Roberts and Andrae. Your choice.
John

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