Fertilization Characteristics of Spermatozoa Collected from Bulls Grazing Tall Fescue Pastures

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Jessica Pegan Harris

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Dedication

This thesis is dedicated to my twin sister and best friend, Josey Megan Harris. Without my “womb mate” I would never have made it this far. Thank you Josey for always being there for me, and continuously pushing me to do my best.

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Abstract

Consumption of toxic endophyte-infected (E+) tall fescue pastures is known to have a negative impact on bull reproductive performance. Since decreased cleavage rates of embryos fertilized with spermatozoa from bulls grazing E+ tall fescue pastures have been observed in several studies using differing sets of bulls, technicians, pastures, and other methods of inducing tall fescue toxicosis (ergotamine tartrate), it is hypothesized that spermatozoa function from bulls grazing E+ is impaired in ways undetectable by gross semen examination.

During a three-month grazing study, 6 Angus bulls were utilized to determine the effects of grazing E+ tall fescue pastures on growth performance and spermatozoa function. Bulls were appointed to graze Kentucky 31 tall fescue (*Festuca arundinacea* Schreb.) infected with *Neotyphodium coenophialum*, an ergot alkaloid producing endophyte (n=3) or Jesup tall fescue infected with non-ergot alkaloid producing endophyte (NTE) MaxQ^{TM} (n=3). Bulls were grouped by body weight (BW) and scrotal circumference (SC) to graze pastures from April 18-June 26, 2007. Blood samples, BW, SC, semen, and rectal temperatures (RT) were collected every 7 d. Scrotal temperatures (ST) were obtained before semen collection each week in June. Semen was evaluated for gross motility, morphology, and Computer Assisted Semen Analysis (CASA) parameters. Semen from a subset of bulls (n=2 per treatment) was used to assess spermatozoa ability to function utilizing *in vitro* assays.

Growth performance was decreased in E+ bulls compared to bulls grazing NTE tall fescue pastures (P = 0.002). Concentrations of prolactin were reduced in bulls

grazing E+ compared to bulls grazing NTE tall fescue pastures (P = 0.055). Motility post-thaw and during a 3-hour stress test were decreased (P = 0.024 and P < 0.0001, respectively), in addition to altered CASA parameters for spermatozoa. Penetration was reduced in oocytes fertilized with spermatozoa from bulls grazing E+ (64.54 ± 3.28%) compared to NTE tall fescue pastures (87.42 ± 1.63%, P < 0.0001) coupled with hastened meiotic completion, and reduced intracellular calcium parameters. These findings indicate impaired spermatozoa function in bulls grazing E+ tall fescue pastures that extends beyond gross semen characteristics, and may provide direction for future studies.

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Chapter 1

Introduction

Improving economic returns for beef cattle producers and lowering expenses to consumers is of utmost importance for sustainability of animal agriculture. In Tennessee, only 76% of beef cows wean a calf annually (Neel 2004). Most of this inefficiency is due to reproduction failure and loss during establishment of pregnancy. A potential cause of this reproductive failure may be due to the primary forage base in Tennessee, namely Kentucky 31 endophyte-infected tall fescue (E+).

Consumption of E+ tall fescue pastures causes severe economic and production losses for beef operations (Hoveland 1993). The endophyte *(Neotyphodium coenaphialum)* that resides within tall fescue grass contributes both positive (drought and insect tolerance of host plant) and negative (decreased performance of grazing animals) components in beef cattle production systems in the midsouth eastern United States. The endophyte is responsible for numerous declines in production traits such as weight gains (Paterson *et al.* 1995) and reproductive efficiency in both males and females (Browning *et al.* 1998; Burke *et al.* 2001a; Schuenemann *et al.* 2004; Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b; Schuenemann *et al.* 2005c; Seals *et al.* 2005).

In cows and heifers, reductions in pregnancy rates (Seals *et al.* 2005), embryo quality and development (Schuenemann *et al.* 2005c), calving rates, milk yield, and circulating hormones (Burke *et al.* 2001a; Burke *et al.* 2001b) have been observed. However, follicular dynamics, luteal function, and uterine environment have been shown

not to be affected during simulation of fescue toxicosis (feeding ergotamine tartrate) (Schuenemann *et al.* 2005c; Seals *et al.* 2005).

In bulls grazing E+ tall fescue pastures, core testicular temperatures were reduced (Schuenemann *et al.* 2005b), but gross semen morphology and motility were similar to bulls grazing non-toxic endophyte-infected (Max-Q) tall fescue. However, cleavage rates of oocytes fertilized with spermatozoa from bulls grazing E+ tall fescue were reduced (Schuenemann *et al.* 2005b). Simulation of tall fescue toxicosis by feeding ergotamine tartrate resulted in identical patterns of semen quality and cleavage rates as observed in the above grazing trial (Schuenemann *et al.* 2005a). Furthermore, tall fescue toxicosis is a syndrome that is worsened by high environmental temperatures, making associated symptoms more obvious during the hot summer months (Porter and Thompson 1992).

The effects of reduced testicular core temperatures (possibly restricted blood supply) on the function of sperm have yet to be examined in cattle. It is known that maintenance of testicular temperature between 30 and 33 ºC is critical for effective spermatogenesis in the bull (Harrison and Weiner 1948). Spermatogenesis is an essential process by which spermatozoa are produced in the testes (Johnson *et al.* 2000) and matured in the epididymis (Amann and Hammerstedt 1993). If this process is altered, consequences would be detrimental to fertility. Spermatozoa from bulls grazing E+ tall fescue pastures appear normal in terms of gross motility and morphology, but it is not known if sperm from these bulls are being altered in a way that is not detectible with a microscope. Thermoregulation is important for bull fertility since complex

processes occur in spermatozoa; and if these are impaired, then decreases in fertility would be seen (Kastelic 1999).

The objectives of the current study are to evaluate penetration rates (i.e. fertilization), intracellular calcium oscillations, and embryo development in oocytes fertilized with semen from bulls grazing toxic endophyte infected (E+) and non-toxic endophyte infected (NTE) tall fescue pastures. The following review of literature will discuss attributes of tall fescue, effects on male and female reproductive performance, spermatogenesis, and the remodeling that occurs in order for an oocyte and spermatozoa to become an embryo.

Chapter 2

Literature Review

Consumption of endophyte-infected (E+) tall fescue pastures is estimated to be responsible for losses totaling more than $609 million annually for the United States beef industry (Hoveland 1993). In the United States, over 14 million ha of tall fescue grass exists, most of which is contained in the eastern half of the country (Paterson *et al.* 1995). Many studies have been conducted on the use of novel strains of endophyte tall fescue, endophyte-free varieties of tall fescue, and other options to minimize effects of tall fescue on animal performance (Hoveland 1993; Gunter and Beck 2004). However, the endophyte and tall fescue plant have a symbiotic relationship, and plants do not survive well without the endophyte (Hoveland 1993). The effects on female reproductive performance have been heavily studied, but the male's contribution in reproductive performance is currently of interest. The female is typically blamed as the cause of reproductive failure with the bull being overlooked as a potential source. This is why spermatozoa function from bulls grazing toxic (E+) and non-toxic (NTE) endophyte-infected tall fescue pastures is of interest to our laboratory and to beef cattle producers who depend heavily on tall fescue pastures.

2.1 Tall Fescue Toxicosis

Tall fescue toxicosis is a major burden to producers, and the economic effects are well known (Hoveland 1993). It has been estimated that greater than 95% of tall fescue pastures in the United States are infected with the endophyte *Neotyphodium coenophialum* (Shelby and Dalrymple 1993). As mentioned above, tall fescue grass benefits from its relationship with the endophyte. These benefits to the E+ tall fescue

plant include pest resistance as well as persistence and drought tolerance. The endophyte provides defensive qualities to the tall fescue plant, and the plant provides the endophyte with protection, nutrients, and the means to proliferate (Bacon 1995). This beneficial relationship is due to the ergot alkaloids (n > 40) that are produced by the plant in response to the fungus (Clay 1988). Strains of tall fescue grass absent of the endophyte have been developed but these have not proven to be hardy and therefore impractical for use over an extended period of time (Jones *et al.* 2004).

Cattle exposed to E+ tall fescue experience symptoms such as decreased feed intake and overall performance, elevated body temperatures, photosensitization (Thompson *et al.* 1993), elevated respiration rates, rough hair coats, and loss of circulation due to vasoconstriction of capillaries that results in necrosis of extremities (Paterson *et al.* 1995; Burke *et al.* 2001a; Klotz *et al.* 2010). Reproductive effects of endophyte consumption and resulting tall fescue toxicosis include a decrease in pregnancy rates (Seals *et al.* 2005), calving rates, serum prolactin (Seals *et al.* 2005), serum progesterone, estradiol and cholesterol (Bond *et al.* 1984), serum luteinizing hormone (Browning *et al.* 1998), and milk yield (Thompson and Stuedemann 1993).

2.2 Effects of Tall Fescue Toxicosis

2. 2. 1. Issues Associated with Female Reproduction

Female reproduction has been a heavily studied topic in reproductive physiology. The female is an essential component of the developing offspring and shares responsibility in establishing the embryo, maintaining a healthy fetus, and carrying that fetus to term. However, without the bull or semen these events will not occur (except in the extreme case of cloning). Fescue toxicosis in females is reported to be responsible

for many detrimental side effects as mentioned previously. Barth (2007) estimated that for every 21 days of the breeding season that a female remains open, it will cost the producer a loss of 20-30 kg of weaning weight for the calf in the following year. This could cost producers an economic loss of $65 per calf based on current market prices. This value depends on how severe sub-fertility is, what the bull-female ratios are and current market prices for weaned calves (Barth 2007).

Burke *et al.* (2001b) reported that the diameter of the pre-ovulatory follicle was reduced in heifers fed E+ tall fescue seed and exposed to heat stress conditions in an environmental chamber. It has also been reported that pregnancy rates and embryonic losses do not differ between cows consuming non-toxic endophyte infected fescue seed (NTE) and cows consuming toxic endophyte infected fescue seed (E+) (Burke *et al.* 2001a). However, exposure to elevated environmental temperatures seems to exacerbate tall fescue toxicosis symptoms (Aldrich *et al.* 1993).

In contrast, Seals *et al.* (2005) demonstrated that follicular dynamics of heifers fed ergotamine tartrate to simulate tall fescue toxicosis were similar to control animals. Average daily gains, concentrations of progesterone, estradiol and follicle stimulating hormone did not differ between treatment groups. Heifers fed ergotamine tartrate displayed longer estrous cycles than did control animals, but follicular parameters (total number of follicles, age and diameter of the F_1 and F_2 follicles, age and size of the ovulatory follicle) were similar between treatments. Serum prolactin was reduced for heifers consuming ergotamine tartrate indicating simulation of tall fescue toxicosis was achieved. Furthermore, pregnancy rates of ergotamine tartrate fed heifers were reduced

compared to controls. These results suggest a possible issue with the oocyte within what would appear to be a normal growing follicle.

Subsequently, Schuenemann *et al.* (2005c) reported decreased embryo recovery and percentage of transferable embryos from heifers being fed ergotamine tartrate to simulate fescue toxicosis. However, the uterine environment appears capable of establishing and maintaining pregnancy in heifers receiving ergotamine tartrate and transfer of a good quality embryo day 7 post-estrus (Schuenemann *et al.* 2005c). Burke *et al.* (2001b) reported that there was a greater frequency of dysfunctional corpora lutea (*dysfunctional* meaning that the corpus luteum produced lower levels of progesterone) using heat-stressed heifers that were fed E+ seed. Decreased serum concentrations of total cholesterol were also observed, which could possibly lead to decreased steroidogenesis. Reduced levels of luteinizing hormone have also been reported when heifers were given acute injections of ergotamine tartrate or ergonovine maleate (Browning *et al.* 1998).

2. 2. 2. Reproduction Issues Associated with Bulls Grazing Tall Fescue

Function of spermatozoa from bulls ingesting ergotamine tartrate or grazing E+ tall fescue pastures is known to negatively impact the ability of oocytes to cleave after fertilization (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b). Reduced cleavage rates have been seen in multiple studies with multiple sets of animals in our lab (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b), and resulted in further interest in spermatozoa function from bulls grazing E+ tall fescue pastures. Scrotal circumference, gross sperm morphology and motility, and blastocyst development of cleaved embyros are all normal when fertilized with spermatozoa from bulls grazing E+

tall fescue pastures. However, scrotal temperatures and serum prolactin concentrations are reduced. These differences could be partially responsible for reductions in fertility.

Many factors contribute to a bull's success in terms of reproductive performance. A bull must possess the following characteristics in order to be considered fertile: adequate libido, physical soundness, and good semen quality (Barth 2007). The most practical and straightforward way to evaluate a bull's worth in terms of reproductive soundness is through a breeding soundness evaluation (Kennedy *et al.* 2002). This examination evaluates parameters such as physical conformation, scrotal circumference, status of the accessory sex glands and penis, and gross sperm motility and morphology. However, a bull can pass this evaluation and still not be able to settle a group of females. The bull is the most important part of the beef herd when using a natural breeding scenario; and if he is unable to impregnate females, then genetic progress will be impeded. The breeding soundness evaluation does not test the bull's ability to produce pregnancies, it only predicts reproductive performance on the day the evaluation was performed. Therefore, problems associated with actually fertilizing an oocyte and contributing to the development of an embryo cannot be addressed with this examination and may be overlooked until excessive non-pregnant females result from the bull's continued breeding efforts. After which it is usually too late to salvage the breeding season.

As discussed above, a breeding soundness exam can only evaluate a bull's predicted performance in terms of sperm quality (gross motility and morphology). These measurements are often performed on farm and with the use of a microscope. In the laboratory, additional means exist for testing certain parameters associated with

spermatozoa quality. Computer assisted spermatozoa analysis (CASA) using an integrated visual optic system (IVOS) can be used to provide more objective evaluations of spermatozoa characteristics than visual appraisals using a microscope. Although this is not a completely valid method for on-farm determination of motility (mobile units do exist with significant cost), it is certainly useful in a laboratory setting and is highly repeatable (Farrell *et al.* 1998). CASA is capable of measuring many variables that can be used to assess spermatozoa characteristics and path velocities (Table 1).

Table 1. Spermatozoa parameters measured by Hamilton-Thorne Sperm Analyzer- CASA (Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA)

Variable	Description
Motile	% of total spermatozoa moving at path velocity $\geq$ 30 µm/sec and progressive velocity $\geq$ 15 µm/sec
Progressive	% of total spermatozoa moving at path velocity > 50 µm/sec and straightness $\geq$ 70%
Rapid	Progressive % with path velocity > 50 µm/sec
Medium	Progressive % with path velocity < 50 µm/sec but > 30 µm/sec
Slow	Progressive % with path velocity < 30 µm/sec and progressive velocity < 15 µm/sec
Static	Immobile sperm
Path velocity (VAP)	Mean velocity of the smoothed cell path (µm/sec)
Progressive velocity (VSL)	Mean velocity measured in a straight line from the beginning to end of the track
Track speed (VCL)	Mean velocity measured over the actual point-to-point track
Lateral amplitude (ALH)	Average width of the head oscillation as the sperm swims
Beat frequency	Frequency of spermatozoa head intersecting the sperm average path in either direction
Straightness	Measures departure of mean sperm path from straight line (ratio of VSL/VAP)
Linearity	Measures departure of actual sperm track from straight line (ratio of VSL/VCL)
Elongation	Ratio (%) of head width to head length
Area	Mean size of sperm heads (μ^2)

2. 3. Spermatogenesis

Spermatogenesis is an important factor when considering male fertility. In the bull, it is a 61-day process that requires specific temperature control and adequate hormone production. If alterations occur in the testis such as temperature increase/decrease, trauma or disease, then spermatogenesis will be affected (Kastelic 1999). Increased testicular temperatures have been linked to increased production of abnormal spermatozoa and unstable chromatin (Saacke *et al.* 2000). Integrity of spermatozoa chromatin is essential for normal pronuclear formation once the sperm has gained entrance into the oocyte. If chromatin is altered or otherwise compromised, then reductions in embryo development could be seen. Sperm chromatin abnormalities are an uncompensable deficiency, where increasing insemination dose will not increase fertility (Saacke *et al.* 2000).

Spermatogenesis is the process by which mature spermatozoa are produced in the testes of the male (Johnson *et al.* 2000). This process involves levels of cellular differentiation and division that are critical for production of spermatozoa capable of fertilizing an oocyte (Johnson *et al.* 2000). The seminiferous tubules in the testicle are the location for the production of spermatozoa. Before birth, primordial germ cells populate at the urogenital ridge. The primordial germ cells divide and form undifferentiated gonocytes (Parker *et al.* 1999). The first step in the production of spermatozoa is for spermatogonia (differentiated primordial germ cells) to undergo mitosis and divide into primary spermatocytes. These primary spermatocytes undergo meiosis to produce secondary spermatocytes, which go through a second meiotic division to produce spermatids (Johnson *et al.* 2000). Spermatids undergo cellular

differentiation to form spermatozoa cells but do not continue dividing. Spermatids must undergo further processing to become spermatozoa cells that can actually fertilize an oocyte. This process is termed spermiogenesis.

During spermiogenesis, spermatozoa are formed from spermatids that have undergone a conformational change in their nuclei to resemble germ cells shaped appropriately for their respective species. A tail is developed from the flagellum, and the head forms from the nucleus (Johnson *et al.* 2000). Along with these morphological changes, the chromatin condenses and the acrosomal cap develops, which is the responsibility of the golgi apparatus (Johnson 1995). The acrosome is important since it contains hydrolytic enzymes necessary for the spermatozoa to fertilize an oocyte successfully (Johnson 1995). These enzymes include hyaluronidase, which is responsible for aiding the sperm's entrance into the oocyte by dissolving cumulus cells. Without the acrosome a sperm will not be successful at fertilization attempts.

Spermiation occurrence is coincident with spermatozoa being considered mature and is characterized by spermatozoa being released into the lumen of the seminiferous tubules. Spermiation can be physiologically compared to ovulation in the female (Johnson 1995). Amann (1970) discovered that spermatocytogenesis occurs in 21 days, meiosis takes 23 days, and spermatocytogenesis occurs during a 17-day time period. Therefore the entire process of spermatozoa formation takes 61 days in the bovine (Amann 1970), with sperm moving into the epididymis.

The epididymis plays a critical role in the process of sperm maturation and storage. According to thermography images acquired by Schuenemann *et al.* (2005b), testicular temperatures were lowest in the region of the epididymis. Due to the inherent

nature of the epididymis and mixing occurring therein, the population of sperm cells in this region are heterogeneous in nature. Sperm in the epididymis and sperm that have been ejaculated have no ability to synthesize molecules such as proteins and lipids (Amann *et al.* 1993). Spermatozoa have other attributes that help them to survive in the female reproductive tract and the epididymis is critical for these processes. This is why epididymal maturation and bathing in seminal fluids through the process of emission (travel through the male reproductive tract) is important for spermatozoa to acquire the ability to fertilize an oocyte (Amann *et al.* 1993). The epididymis and efferent ducts are storage sites for spermatozoa. Amann and co-workers (1993) described four processes that the epididymis and efferent ducts are responsible which include: removal of water, transport via smooth muscle contractions, maturation, and storage/maintenance. If proper spermatozoa numbers are not maintained in these regions, then fertility could be negatively altered. Consequently, spermatozoa must go through the entire epididymis to obtain maximal fertilization potential (Robaire *et al.* 2006). Therefore, the epididymis is an important organ that is very complex in nature.

2. 3. 1. Alterations in Spermatozoa Production

Spermatogenesis is a critical process that requires specific temperatures and hormone concentrations. If either of these is altered, then significant decreases in fertility would be observed. Increases in testicular temperature have been shown to increase the amount of abnormal spermatozoa in the ejaculate (Vogler *et al.* 1993). However, with fescue toxicosis, a decrease in scrotal temperature is seen due to vasoconstriction and subsequent decreased blood flow to the testicles (Schuenemann *et al.* 2005b). Bulls grazing E+ tall fescue pastures have been shown to have decreased

blood flow to the testicles when compared to bulls grazing NTE tall fescue pastures due to vasoconstriction of the testicular artery (Glen Aiken, personal communication). Decreased blood flow to the testicles could mean detrimental effects on spermatozoa.

Seminal plasma, secreted from the accessory sex glands in the bull is very important to spermatozoa in an ejaculate. These fluids, which are the sum of secretions from the testis, epididymis and accessory sex glands, comprise the major fluid portion in semen (Vishwanath and Shannon 1997). Seminal plasma is credited with several important roles including sperm motility (Vishwanath and Shannon 1997), capacitation (Miller *et al.* 1990), antibacterial properties, and providing antioxidant activity to protect spermatozoa (Jones *et al.* 1979). However, seminal plasma can also be detrimental to sperm under the appropriate conditions. Seminal plasma protects spermatozoa during emission and transit through the female reproductive tract. This is necessary because the vagina is very acidic and seminal plasma protects spermatozoa from this hostile environment (Harper 1994). Seminal plasma has to be removed prior to cryopreservation since prolonged exposure can alter spermatozoa membranes by increasing permeability, leading to cryogenic injury and decreased survival (Pangawkar *et al.* 1988). Nonetheless, alterations in seminal plasma can have deleterious effects on spermatozoa since they are in close contact with one another for a period of time.

2. 4. The Process of Fertilization

Fertilization is the process by which the sperm binds to the oocyte to gain entrance and eventually form a zygote. After ejaculation from the male reproductive tract, spermatozoa face the challenge of traversing the female reproductive tract and going through two processes in order to successfully fertilize an oocyte. They are motile

once ejaculated, but have not undergone capacitation or the acrosome reaction at this point.

Capacitation is the process by which the glycoprotein layer surrounding the spermatozoa head is removed (Hunter and Rodriguez Martinez 2004). This exposes the acrosome and its receptors that are necessary for zona pellucida binding. Capacitation occurs in the female's reproductive tract ideally, and is also associated with increasing spermatozoa motility by hyperactivation (Yanagimachi 1994). Once the spermatozoon is capacitated, the plasma membrane changes characteristics and becomes more fluid-like. Additionally, de-capacitation factor is removed (this anchors zona receptors on the spermatozoa). These changes are primarily due to cholesterol efflux (Yanagimachi 1994).

Hyperactivation facilitates spermatozoa transport to the site of fertilization. Spermatozoa must be morphologically normal in order to successfully complete the journey from the site of insemination to this region of the oviduct (Scott 2000). In the mouse, spermatozoa with head abnormalities will likely not be able to pass through the utero-tubal junction (Scott 2000) hence, the female's reproductive tract serves as a type of filter for sperm. The occurrence and timing of the acrosome reaction are very important for spermatozoa. If the acrosome reaction occurs too soon, then the sperm will be excluded from entering the cumulus cells surrounding the oocyte. If it occurs too late, sperm will also be excluded. Only capacitated, acrosome-intact sperm are allowed to proceed to the oocyte (Saling 1991).

There are two extracellular matrices that the spermatozoa must negotiate before it can access the oocyte (Saling 1991). First is the cumulus matrix which is thought to

serve as a filter for sperm that possess adequate fertilizing potential. Cumulus cell navigation is accomplished via acrosomal enzymes that assist the sperm by softening the cumulus cells and allowing access to the second extracellular matrix surrounding the oocyte, the zona pellucida. The zona pellucida contains specific glycoproteins that are important in the process of spermatozoa-oocyte binding (Saling 1991).

These glycoproteins are thought to have separate functions, but are very similar to one another. For example, in most mammals, zona pellucida protein 3 (ZP3) is a specific receptor for spermatozoa and is thought to induce the acrosome reaction whereas zona pellucida protein 2 (ZP2) binds exclusively to acrosome-reacted spermatozoa and is a secondary receptor (Hinsch *et al.* 2005). Zona pellucida receptor 1 (ZP1) does not participate in gamete interaction but is more responsible for structural stability (Saling 1991). The spermatozoon must first bind to the ZP3 receptor, undergo acrosome reaction, bind to the ZP2 receptor, and gain access to the perivitilline space (Howes and Jones 2002). More specifically, sperm are challenged to interact with the oocyte in such a way as to deliver genetic material to the egg cytoplasm successfully. This requires sperm motility and certain enzymes from the spermatozoon relating to zona-adhesion (Primakoff and Myles 2002).

The acrosome is a structure that resembles a cap and is bound by a membrane. It covers the anterior portion of the spermatozooon's nucleus (Yanagimachi 1994). It contains many hydrolytic enzymes which include hyaluronidase and acrosin. These enzymes aid in the process of spermatozoa entrance through the zona pellucida. The acrosome reaction occurs upon binding to the zona pellucida receptor 3, and can be considered a process of controlled cell lysis (Yanagimachi 1994; Primakoff and Myles

2002). This process is important for entrance into the zona pellucida and subsequent fertilization of the oocyte. However, if the zona pellucida receptor 3 peptide chain is not intact, then it cannot exert its ligand effects (Yanagimachi 1994). Interestingly, spermatozoa possess certain receptors (R_{ZP}) that are specific for the zona pellucida 3 receptor. Once the spermatozoa have completed the acrosome reaction, another series of events must occur for successful oocyte entrance.

The zona pellucida is penetrated only by acrosome-reacted spermatozoa (Saling 1991; Yanagimachi 1994). Once the spermatozoa penetrates the zona pellucida, then it must pass through the perivitilline space and interact with the oocyte plasma membrane (Ben-Yosef and Shalgi 1998). The spermatozoa and the oocyte must navigate the process of remodeling to become a viable embryo beginning with oocyte activation.

2. 5. The Remodeling of an Oocyte

The oocyte is ovulated while arrested at metaphase of the second meiotic division, or metaphase II (Yanagimachi 1994; Yoon and Fissore 2007). The spermatozoon must penetrate the zona pellucida in order for the oocyte to resume meiosis and become activated. The spermatozoon is essential because it causes an increase in intracellular free calcium ions (Ca^{2+}) in the oocyte (Malcuit *et al.* 2006). This intracellular rise in free calcium allows the ovum to undergo activation events that are essential for continued progression (Schultz and Kopf 1995). These events include cortical granule exocytosis for prevention of polyspermy, completion of the meiotic cell cycle, and pronuclear formation (Malcuit *et al.* 2006). These activation events are initiated within the hour of the first onset of Ca^{2+} oscillations.

The mechanism by which spermatozoa perform the event of activation and induce intracellular Ca^{2+} oscillations is not specifically known but is thought to be the result of a certain sperm factor. This could be a specific phospholipase C (PLC-ζ) as reviewed by Malcuit *et al.* (2006). It is not known if this is actually the sperm factor or if it simply is activated by the sperm factor that is released. Nonetheless, a soluble sperm factor that is delivered to the oocyte by the spermatozoa activates the phosphoinositide pathway, which triggers Ca^{2+} release and oocyte activation (Swann 1990). This factor is a sperm specific protein, since it is heat and protease sensitive. Nor is this protein species specific since spermatozoa extracts have been injected into mouse, human, cow, hamster, and nemertean worm eggs resulting in subsequent Ca^{2+} oscillations (Swann 1990). Additionally, it has been reported that all sperm factor activity was released into the ooplasm within a time period of 2 hours following sperm entry (Yoon and Fissore 2007).

Once the soluble sperm factor is released into the oocyte, an increase in inositol 1,4,5-triphosphate (IP3) has been shown (Wu *et al.* 2001). This is the result of the hydrolysis of phosphoinositol 4,5-bisphosphate (PIP2) by a PLC. Inositol triphosphate is responsible for stimulating Ca^{2+} release from the endoplasmic reticulum (Yoon and Fissore 2007). The patterns of Ca^{2+} release (i.e. duration, frequency and amplitude) are species dependent, and not just a single rise in Ca^{2+} concentration. Instead, Ca^{2+} release consists of patterns that are synchronized with the cell cycle (Ducibella *et al.* 2006). Fissore and co-workers (1992) showed that the frequency of intracellular Ca^{2+} oscillations depend on how far the maternal and paternal chromatin have progressed through the cell cycle. Furthermore, a zygote with one Ca^{2+} peak displayed a larger

increase in intracellular Ca^{2+} concentration than a zygote with multiple Ca^{2+} peaks. It is thought that the duration of the interval between Ca^{2+} peaks is dependent upon how quickly the cell can regain its Ca^{2+} stores and oscillate again (Fissore *et al.* 1992). This is why different zygotes have altered responses to sperm-induced Ca^{2+} oscillations. Individual sperm from bulls could also have the ability to alter the patterns of intracellular Ca^{2+} concentrations.

2. 6. Summary of Literature

In conclusion, tall fescue toxicosis results in numerous economic declines for producers in the eastern midsouth. Function of spermatozoa from bulls grazing toxic endophyte infected (E+) tall fescue pastures and non-toxic endophyte infected (NTE) tall fescue pastures is of interest due to repeated findings in our laboratory. Decreased cleavage rates of oocytes fertilized with semen from bulls grazing E+ pastures have been observed multiple times and the experiments in this present study will address mechanisms of this discovery. Penetration will be assessed to determine if the spermatozoa's ability to penetrate the zona pellucida and gain entrance into the oocyte is sufficient. Intracellular calcium oscillations are of interest because an increase in cytosolic calcium concentrations is important for completion and initiation of embryonic cell cycles, thus marking the beginning of development (Fissore *et al.* 1992). Embryo development will be assessed in order to repeat previous findings of decreased cleavage rates (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b).

In addition to *in vitro* assessment of spermatozoa, other data were collected on the bulls in each respective treatment. These data include body weights, rectal

temperatures, scrotal circumference measurements, blood samples, thermography scans of the testicles, and gross motility and morphology.

Chapter 3

Materials and Methods

3.1 Animals and Treatments

During a three-month grazing period, sexually mature, phenotypically similar, Angus bulls (n=6, mean age=15.1 ± 0.042 months) were used to determine effects of grazing toxic (E+) and non-toxic endophyte (NTE) infected tall fescue on growth performance, semen attributes, endocrine profiles and spermatozoa function. These animals were obtained from the East Tennessee Research and Education Center (Knoxville, TN) and were managed under station standard operating procedures (SOPs) for nutrition and care. Animals were allowed *ad libitum* access to water, mineral, and artificial shade (west fence line of paddocks was also lined with trees) in each 1.2 ha pasture. All experimental procedures were reviewed and approved by the University Institutional Animal Care and Use Committee. Beginning on April 18, 2007, bulls (n=3 per treatment; mean weight at start of experiment = 656.06 ± 10.86 kg; mean scrotal circumference at start of experiment = 39.29 ± 1.23 cm), were placed on 'Kentucky 31' endophyte-infected tall fescue pastures (E+), or 'Jesup' Max-Q^{TM} pastures (NTE) both without clover. Ergovaline content of E+ pasture was 410 ppb with 93-95% infestation rate, and NTE pasture had non-detectible ergovaline concentrations with 93-95% infestation rate (Dr. J.C. Waller, personal communication).

Beginning in mid-May, semen and blood samples were collected once weekly from bulls. Collection of blood, semen, scrotal circumference, body weight, rectal temperature, and scrotal temperatures were collected as described by Schuenemann *et al.* (2005b). Briefly, body weights (scales: Moly Manufacturing, Lorraine, KS, USA),

scrotal circumference (scrotal tape: Lane Manufacturing, Denver, CO, USA), and rectal temperature (RT; thermometer: GLA Agricultural Electronics, San Luis Obispo, CA, USA) were measured each week. Blood samples (Monovette; Sarstadt, Newton, NC, USA) were obtained for prolactin and testosterone assays via caudal venipuncture and placed on ice until centrifugation. Scrotal temperatures (ST) were obtained before semen collection for each date in June using thermography (Harrison and Weiner 1948). The camera utilized had an opaque chopper and internal calibration which allowed absolute temperatures to be obtained (eMerge Vision DTIS 500, eMerge International Inc., Sebastian, FL, USA). EResearch software allowed analysis of images recorded. Images were imported to the software and a region of interest (ROI) was drawn on each testis. Each ROI contained the same number of pixels and the same region was drawn on each testis. The program calculated absolute temperatures for each ROI based on this information.

3.2 Semen Collection and Cryopreservation

Semen was collected once weekly beginning in mid-May and ending the last week of June between 0600-0800 each morning. Semen was immediately evaluated for gross morphology and motility by a board certified theriogenologist (Dr. F. M. Hopkins or Dr. T. M. Prado) with no knowledge of treatments. Semen was collected using an electroejaculator (Lane Manufacturing Co.; Denver, CO) and was placed in a 10 mL sterile conical tube.

Estimation of progressively motile spermatozoa was obtained using a light microscope at 400X. Twenty-five microliters of semen were added to a pre-warmed glass slide and covered with a cover slip to assess motility. A separate sample of

semen was placed on a glass slide, mixed with eosin-nigrosin stain, smeared using another glass slide and allowed to dry. Evaluation of morphology was performed under oil immersion at 1000X using a light microscope (Spitzer *et al.* 1988).

Semen was extended according to procedures described by Schuenemann *et al.* (2005b). After estimations of progressive motility and gross morphology were obtained, semen was extended using Bioxcell® animal protein-free formula (IMV, Aigle, France) extender solution. Total ejaculate volume, concentration, and motility were recorded at collection and this information was used to determine the amount of Bioxcell® extender solution to add. Antibiotics (CSS 1000) were added to the raw semen at 2% of total volume. This antibiotic solution was comprised of 11.5 mL distilled water and 0.5 mL antibiotics. The solution was pre-warmed to 32-34°C, added to semen, mixed and allowed to stand for 5 minutes. After completion of the antibiotic treatment, semen was extended with Bioxcell® CSS1 (400 mL of distilled water and 100 mL concentrated extender pre-warmed to 32-34°C) at a 1:1 ratio of extender to semen and allowed to stand for 10 minutes at 32-34°C. Extended semen was then packaged in a cooler with cold packs for transport back to the laboratory.

Upon return to the laboratory, extended semen concentration and motility were determined using a computer assisted semen analyzer (CASA Version 12 Integrated Visual Optic System-IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) and the final dilution was determined. To achieve the desired spermatozoa concentration of 20 million sperm/straw, a final dilution was obtained by adding CSS2 (400 mL of distilled water and 100 mL of concentrated extender pre-cooled to 4°C in a cold room) over a period of 1-2 minutes at 4°C and swirled gently to mix. The extended semen was

allowed to equilibrate over a period of 3 hours at 4℃ in a cold room, then packaged in straws, sealed, and allowed to sit for 30 minutes. Straws of semen were then frozen for 7 minutes in static vapor of liquid nitrogen and plunged into liquid nitrogen filled goblets, and stored at –196ºC until further analysis.

Post-thaw characteristics of semen were analyzed using computer assisted semen analysis (CASA). Semen was thawed in water at 36.7℃ for 45 seconds and then decanted into black micro-centrifuge tubes for protection from light and maintained at 36.7℃. A 3.5 µl sample of semen was analyzed using CASA at 0 and 3 hours post-thaw.

3.2 In Vitro Evaluation of Spermatozoa

In vitro production of bovine embryos was performed as previously described by Edwards *et al.* (2009) with certain modifications. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise noted. Medium-199 (M199) containing Earle's salts was purchased from Invitrogen (Division of Life Technologies; Carlsbad, CA, USA) while M199 with Hank's salts was purchased from Mediatech (Manassas, VA, USA). Penicillin-streptomycin was purchased from Millipore (Billerica, MA, USA). Standard fetal bovine serum (S-FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA) while premium fetal bovine serum (P-FBS) and gentamicin were purchased from BioWhittaker (Walkersville, MD). Oocyte maturation medium (OMM) consisted of M199 (with Earle's salts), 10% P-FBS, 50 µl/mL gentamicin, 5 µl/mL FSH, 0.2 mM sodium pyruvate, and 2 mM L-glutamine (Schrock *et al.* 2007). Modified Tyrodes' Albumin Lactate Pyruvate media was prepared in the laboratory (HEPES-TALP, IVF-TALP, and Sperm-TALP) as per Parrish et al. (1988).

Potassium simplex optimized medium was prepared as previously described (Biggers *et al.* 2000) but modified to contain 0.5% BSA, 10 mM glycine, 1 mM L-glutamine, 1x nonessential amino acids, 50 U/mL penicillin, and 50 µl/mL streptomycin (KSOM). Dulbecco's Phosphate Buffered Saline (DPBS) without $MgCl_2$ and $CaCl_2$ was purchased and prepared in the laboratory.

Ovaries were obtained from a local abbatoir (Southeastern Provision LLC., Bean Station, TN, USA) and placed in thermoses before transport in a cooler to the laboratory. Upon arrival, ovaries were quickly rinsed in warm water that was equilibrated to the arrival temperature of the ovaries (between 28 to 30℃). Tissue surrounding the ovary was removed with scissors and ovaries were washed once more with pre-equilibrated water.

Ovaries were processed for oocyte recovery within ~4 hours of collection at the abbatoir. Briefly, cumulus oocyte complexes (COC) were harvested from antral follicles (~3-10 mm) on ovaries selected for COC collection. Ovaries were held firmly by a pair of hemostats that was clamped onto the base of the ovary. Small, lengthwise incisions were made across follicles and were followed by crosswise incisions to create a checkerboard pattern on the surface of the ovary. Ovaries were washed thoroughly in oocyte collection medium (OCM) supplemented with 1% fetal bovine serum, penicillin-streptomycin, and L-glutamine to liberate cumulus oocyte complexes. Oocyte collection medium was filtered and rinsed using an Emcon filter (Vet Concepts, Spring Valley, WI, USA) until medium was clear of debris. This medium was then poured into a gridded plastic culture dish for oocyte retrieval. Upon recovery, COC were then placed into a plate of 2% oocyte collection medium supplemented with 2% fetal bovine serum,

penicillin-streptomycin, and L-glutamine, where they were washed four times to eliminate cell debris, and evaluated for quality. Only cumulus oocyte complexes with a dark, evenly distributed ooplasm and compact cumulus were matured in groups of 30-45 in 500 µl of oocyte maturation medium (OMM). Maturation was performed in an incubator at 5.5% CO_2 in humidified air at 38.5°C until time of fertilization. Oocyte maturation medium was pre-equilibrated in the incubator (5.5% CO_2 in air at 38.5°C) for a minimum of 5 hours before oocyte collection was performed.

Approximately 24 hours after placement into OMM, maturation medium was aspirated from each well containing oocytes using a small bore glass pipette and 500 µl of fertilization medium (IVF-TALP) and 25 µl of penicillamine/hypotaurine/epinephrine (PHE) were added back to each well. Fertilization was performed as per Edwards *et al.* (2003) with certain modifications. Briefly, approximately two straws of semen from each bull collected and cryopreserved on June 12 and 26 were used for in vitro fertilization. One bull from each treatment group (toxic endophyte infected tall fescue-E+, and non-toxic endophyte infected fescue-NTE) was used to fertilize oocytes from each collection date. Straws of semen were removed from the liquid nitrogen dewar by an independent observer to keep bull identification number and treatments unknown by evaluators. Straws were placed in water at 36.7°C for 45 seconds and decanted onto a 10 mL column of DPBS (without $MgCl_2$ and $CaCl_2$) in a sterile 15 mL conical tube. Only one straw was placed onto each column of DPBS in order to adequately wash the spermatozoa contained in each straw. Each column was then centrifuged at 800 x g for 3 minutes in order to remove excess extender and debris. Upon completion of centrifugation, almost all DPBS was aspirated from columns and the spermatozoa

pellets from two different columns were combined. This gave one sample of DPBS purified spermatozoa for each bull. Once the pellets were combined, 50 ul of IVF-TL containing heparin (8.75 units / µl) was added to each spermatozoa sample. A 3.5 µl spermatozoa sample was then placed into a chamber in the CASA for motility and concentration assessment. The spermatozoa to be used for fertilization were placed into the incubator for 15 minutes (5.5% CO_2 in air at 38.5°C). IVF-TALP and IVF-TL containing heparin were both pre-equilibrated in the incubator (5.5% CO_2 in air at 38.5°C) for a minimum of 5 hours prior to in vitro fertilization. Following 15 minute incubation, another 3.5 µl sample of spermatozoa was evaluated by CASA. Then motility and concentration values were used to calculate volume of motile spermatozoa to add to each 500 µl well containing oocytes (750,000 motile spermatozoa).

Approximately 6.5-7.5 hours post fertilization, presumptive zygotes (PZs) were denuded of cumulus cells by vortexing for 4.5 minutes in a sterile 15 mL conical tube containing 500 µl of a solution comprised of 700 µl of HEPES-TALP and 300 µl of hyaluronidase. Recovered presumptive zygotes were washed four times in HEPES-TALP and evaluated. After evaluation, presumptive zygotes were used for the penetration assay, intracellular calcium assay, or were placed into KSOM to assess development to blastocyst stage (Figure 3.1).

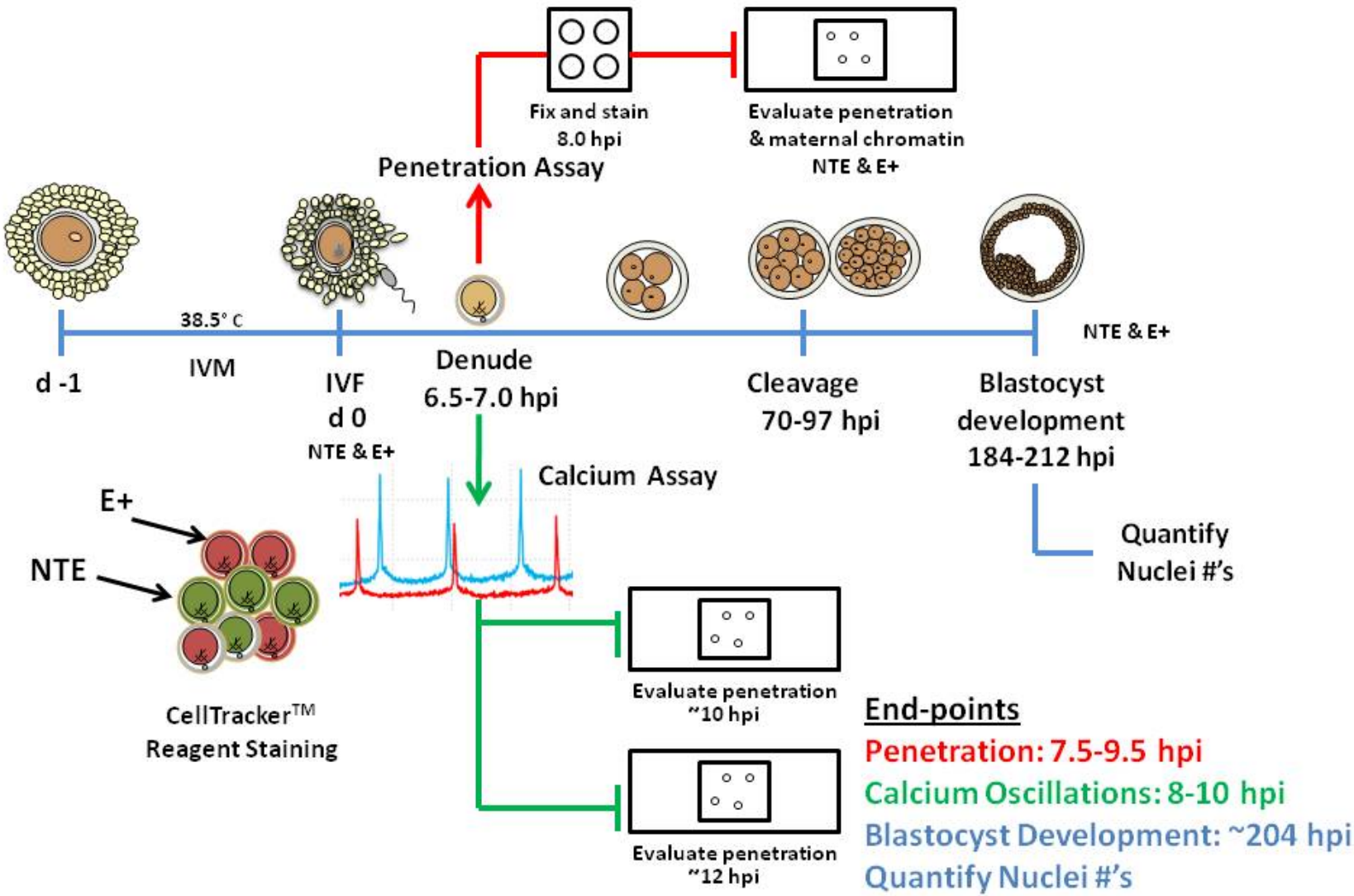


Figure 3.1 Experimental Schematic of In Vitro Evaluation of Spermatozoa

3.2.1 Penetration Assay

Penetration rates were assessed as described by Payton *et al.* (2004). In brief, between 7.5-9.5 hours after IVF, presumptive zygotes were denuded and then placed sequentially in 0.5% pronase for zona pellucida removal, 10% paraformaldehyde (paraformaldehyde diluted in Dulbecco's phosphate buffered saline) for fixation, and a 10 µg/mL concentration of Hoechst 33342 for chromatin staining respectively. Presumptive zygotes were then mounted on a glass slide, covered with a cover slip, sealed and chromatin status assessed using a Nikon TE300 inverted optical microscope at a minimum of 20X magnification using fluorescence imaging. This experiment was replicated on 33 different occasions evaluating a total of 2,547 presumptive zygotes.

3.2.2 Intracellular Calcium Assay

Intracellular calcium measurements were performed as described by Fissore *et al.* (1995) and Takahashi *et al.* (2000) with minor modifications. Briefly, presumptive zygotes (PZs) were completely denuded of cumulus cells approximately 6.5-7.5 hours post insemination, then incubated in IVF-TALP (pre-equilibrated at 38.5°C and 5.5% CO_2) containing 3 µM Fura-PE3/AM (Calbiochem, La Jolla, CA, USA), 0.06% Pluronic F127 (Invitrogen, Carlsbad, CA, USA) and 2.5 µM CellTracker™ Orange CMTMR or 2.5 µM CellTracker™ Green CMFDA for a minimum of 45 minutes at 38.5°C. Two different CellTracker™ reagents were used so that groups of PZs fertilized by two different bulls could be measured simultaneously (Figure 3.2). The dye-loaded presumptive zygotes were washed three times in HEPES-TALP, and transferred to pre-warmed HEPES-TL (BSA-free). Intracellular calcium was monitored in each presumptive zygote using a

Nikon Ti-E microscope with Super Fluor 10 X 0.5 NA objective and heated stage adaptor (Delta T® Open Dish System, Bioptechs, Butler, PA, USA). Presumptive zygotes were measured in a group of ~30 (15 from each treatment group) in a 30 µl droplet of HEPES-TL overlaid with mineral oil pre-warmed to 38.5°C on a Delta T® dish (Bioptechs) pre-coated with poly-L-lysine. Nikon Elements Advanced Research Software was utilized to obtain fluorescence values (340 and 380 nm excitations with 510 nm emission) every 5 seconds for 90 minutes. Intracellular calcium concentrations were determined from fluorescence ratio ($R=F_{340}/F_{380}$) and calibration solutions according to Grynkiewicz *et al.* (1985), $K_d * \beta * [(R-R_{min})/(R_{max}-R)]$, where K_d is the dissociation constant for Fura-PE3 for calcium binding, β is the ratio of fluorescence at 380 nm for free and bound Fura-PE3, and R_{min} and R_{max} are the fluorescent ratios at zero and saturating, respectively. Calibration solution of 10 µM Fura-3 salt in HEPES-TL with a 60% sucrose (w/v) supplement to correct for intracellular viscosity (Poenie 1990) was used to determine 340 to 380 nm fluorescence ratio at zero (R_{min}; containing 5 mM EDTA) and saturating calcium (R_{max}; containing 8 mM $CaCl_2$) concentrations. Value of β was determined using ratio of fluorescence readings at 380 nm wavelength for zero calcium to saturating calcium solutions. Background values for subtraction were acquired by measuring calibration solution without Fura-3 salt. This experiment was replicated on 4 different occasions with a total of 192 oocytes.

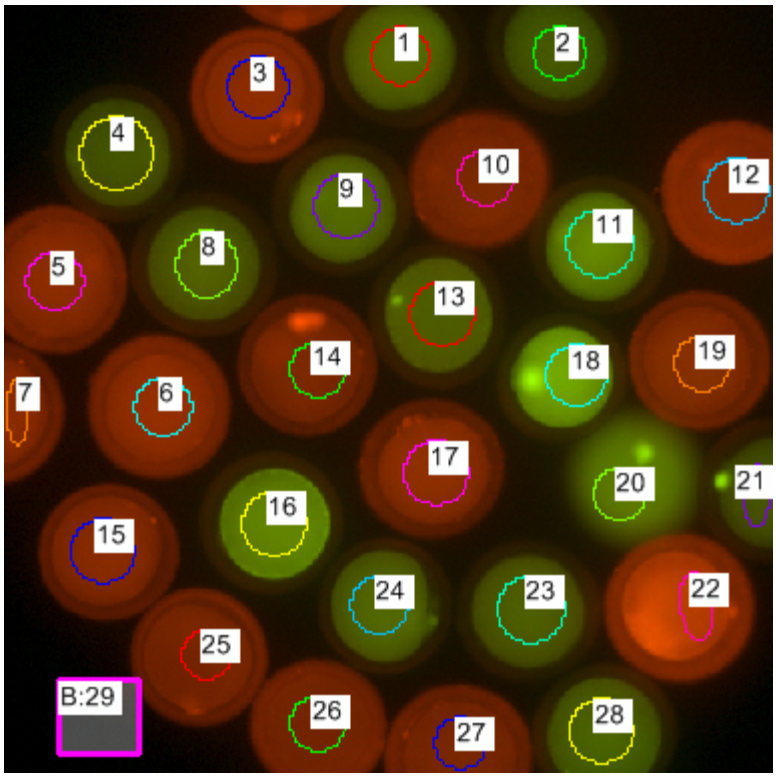


Figure 3.2 SNAP Image of Putative Zygotes using CellTracker™ reagents

3.3. Statistical Analysis

The experiment was divided into three separate analyses. Analysis I evaluated performance of bulls and gross characteristics of semen in each treatment. Analysis II evaluated CASA parameters for sperm characteristics post-thaw and after a three-hour stress test, in addition to sperm utilized for IVF assessment. Analysis III assessed IVF function of spermatozoa: penetration, intracellular calcium oscillations, and embryo development.

3. 3. 1. Analysis I

Bull performance and gross semen characteristics were analyzed for bulls grazing toxic endophyte-infected (E+) and non-toxic endophyte-infected tall fescue pastures (NTE). In general, data were analyzed as a randomized block design (RBD) with fixed effects of treatment, random effects of date and date*treatment, and blocking on date of semen collection using the mixed models procedure (SAS 9.2, SAS Inst., Inc, Cary, NC, USA). Data were tested for normality (Shapiro-Wilk W ≥ 0.90), and treatment differences were determined using F-protected least significant differences, reported as least squares means ± SEM.

3. 3. 2. Analysis II

Semen parameters as measured by Computer Assisted Sperm Analysis (CASA) were evaluated for bulls grazing E+ tall fescue pastures and NTE tall fescue pastures. Data were analyzed as a randomized block design with fixed effects of treatment, blocking on semen collection date, utilizing the mixed models procedure (SAS 9.2, SAS Inst., Inc, Cary, NC). Data were tested for normality (Shapiro-Wilk W ≥ 0.90), and

treatment differences were determined using F-protected least significant differences, reported as least squares means ± SEM.

3. 3. 3. Analysis III

In general, data were analyzed as a randomized block design with fixed effects of treatment, semen collection date, and hours post-insemination at time of denuding, blocking on replicate (date of oocyte collection), with use of binomial or normal distributions as appropriate and generalized linear mixed models (PROC GLIMMIX; SAS 9.2, SAS Inst., Cary, NC, USA) unless otherwise noted.

Initiation and termination of intracellular increase in calcium were determined by fitting a four straight-line segmented nonlinear model (PROC NLIN, SAS 9.2), with segments consisting of an initial flat baseline, then a rise to the peak, followed by a fall to the termination of release, and then a final plateau which was in general not flat. Join points provided estimates for initiation and termination. Once these were estimated variables of interest were calculated. Variables included basal calcium concentration (baseline), time of rise, amplitude, time of decline, duration of the calcium peak, area under the curve (total calcium oscillation) and post-release plateau. Data were tested for normality (Shapiro-Wilk W ≥ 0.90) and analyzed using the mixed models procedure (SAS 9.2, SAS Inst., Inc, Cary, NC) with a main effect of treatment (spermatozoa from E+ or NTE tall fescue bulls) and hours post insemination (HPI), while blocking on replicate. Data were tested for normality (Shapiro-Wilk W ≥ 0.90), and treatment differences were determined using F-protected least significant differences, reported as least squares means ± SEM.

Chapter 4

Results

4.1. Analysis 1: Bull Performance

4. 1. 1. Animal Parameters

Likely attributable to severe drought and higher than normal ambient temperatures in Knoxville, TN during the experimental period in 2007, bulls in both treatment groups had decreased weight gain over time. However, bulls grazing E+ tall fescue pastures lost more weight weekly on average (-11.14 ± 1.34 kg) during the study compared to bulls grazing NTE tall fescue pastures (-4.65 ± 1.34 kg, $P = 0.002$, Figure 4.1). Serum concentrations of prolactin were reduced in bulls grazing E+ tall fescue pastures (97.13 ± 9.23 ng/ml) compared to bulls grazing NTE (123.43 ± 9.23 ng/ml, $P = 0.055$). Concentrations of testosterone from bulls grazing E+ and NTE tall fescue pastures were not affected by treatment (1263.22 ± 169.89 vs. 1444.61 ± 169.89 dL/mL respectively, $P = 0.47$). Treatment did not affect how much scrotal circumference (cm) was reduced over the three month grazing period as all bulls had decreasing scrotal circumference measurements throughout the study ($P = 0.447$, Figure 4.2). Rectal temperatures were not different in bulls grazing E+ tall fescue pastures (38.6 ± 0.08 ºC) and NTE tall fescue pastures (38.6 ± 0.08℃, $P = 0.8708$). Scrotal temperatures were obtained on June 12, 19, and 26. Scrotal temperatures were not altered in bulls grazing E+ and NTE tall fescue pastures (29.4 ± 0.75 vs. 29.7 ± 0.75 ℃, $P = 0.54$).

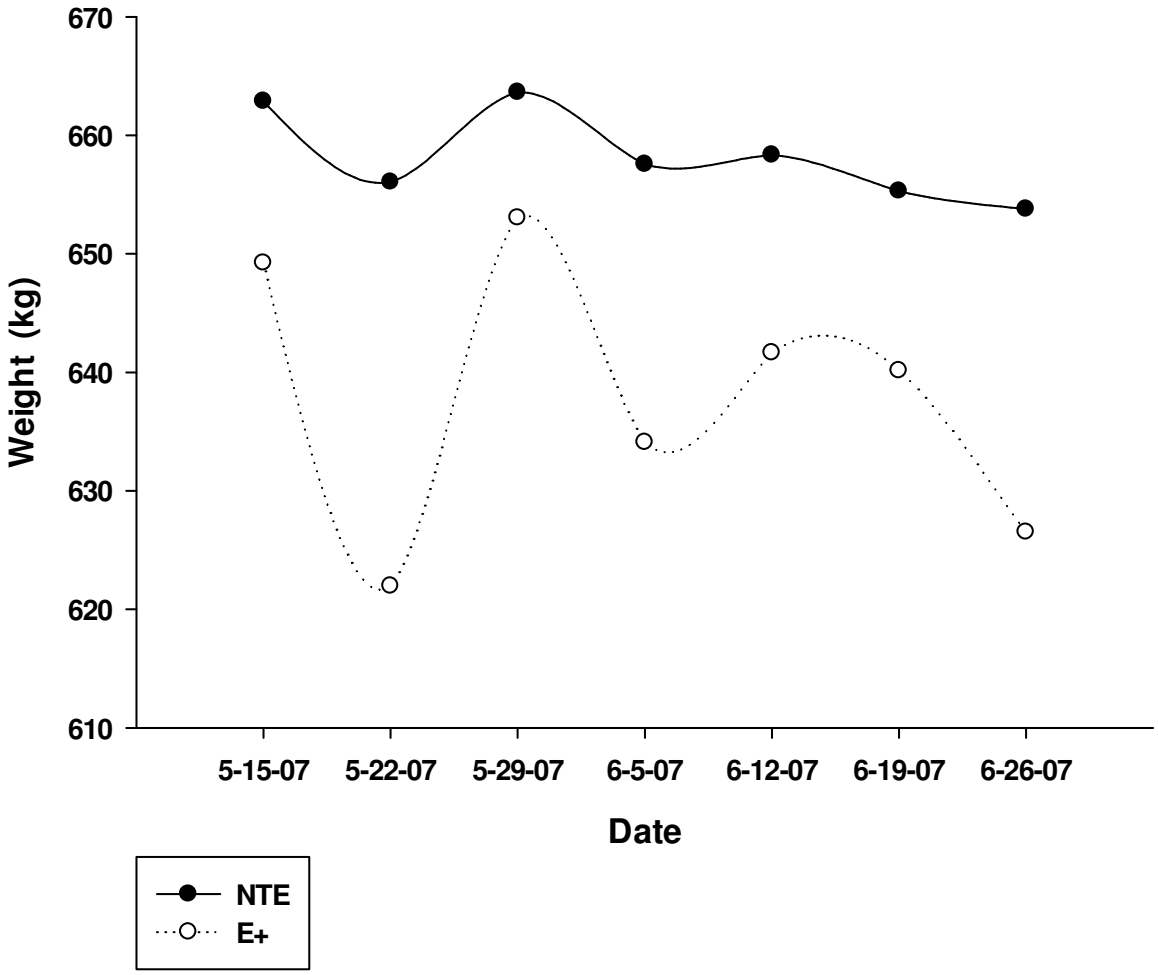


Figure 4.1 Bulls grazing E+ tall fescue pastures lost more weight on average during the experimental period when compared to bulls grazing NTE tall fescue pastures. (*P* = 0.002).

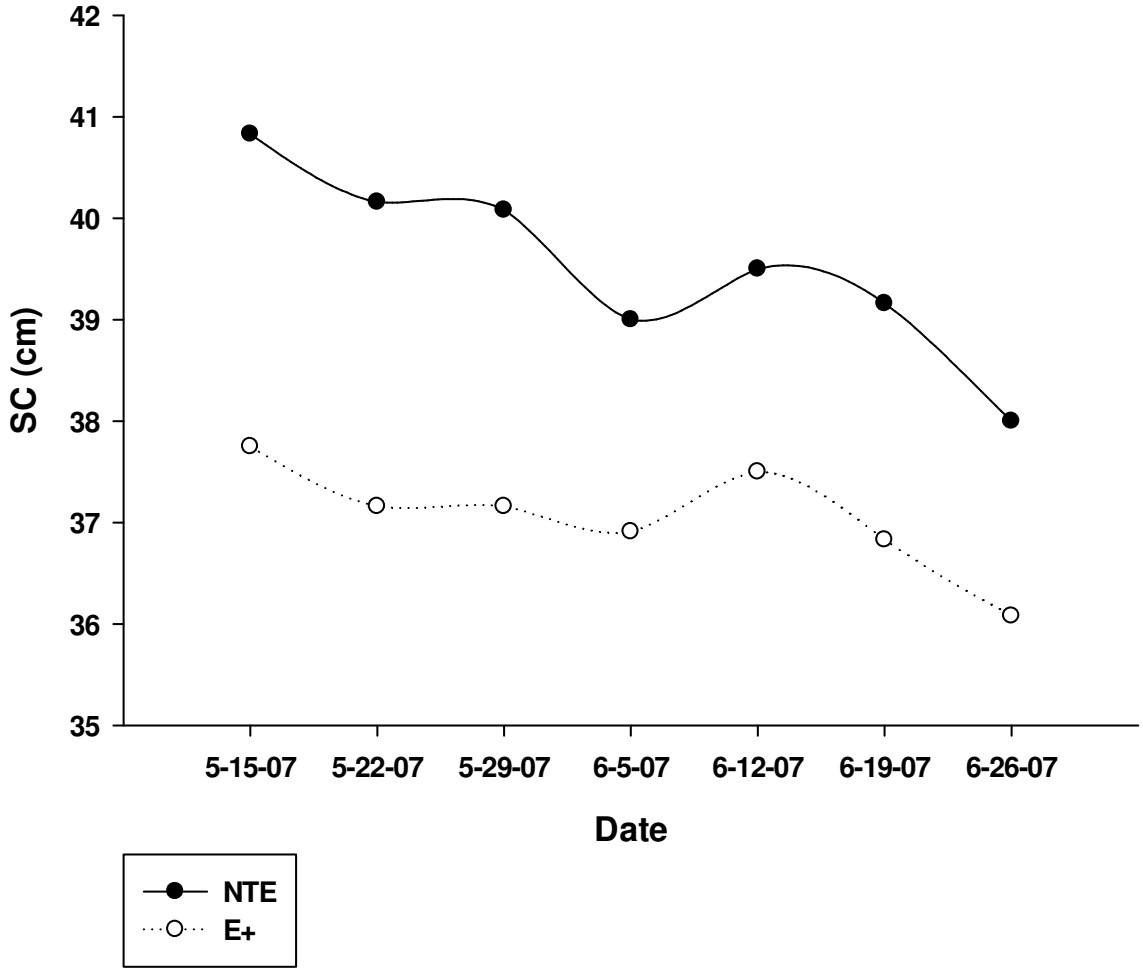


Figure 4.2 There were no treatment differences in scrotal circumference measurements. Each treatment group decreased in scrotal circumference throughout the duration of the experimental period. ($P = 0.45$).

4. 1. 4. Semen: Gross Motility, Morphology, and Concentration After Collection

Gross motility and morphology were assessed immediately after semen collection on specific dates in May and June. Gross semen motility was not different between bulls grazing E+ and NTE tall fescue pastures (P = 0.17, Table 2). Percent morphologically normal spermatozoa were similar between bulls grazing E+ (77.61 ± 1.93%) and NTE tall fescue pastures (77.14 ± 1.93%, P = 0.87). Percentage of primary and secondary abnormalities were also similar between treatments (18.61 ± 1.67 and 4.00 ± 0.65% vs. 17.71 ± 1.67 and 4.71 ± 0.65%, P = 0.71 and P = 0.40, for primary and secondary, respectively). Finally, concentration of raw ejaculate was similar between bulls grazing E+ and NTE tall fescue pastures (544.20 ± 108.88 vs. 469 ± 108.88 million/ml, respectively, P = 0.50).

Table 2. Motility means from semen collection to post-thaw CASA assessment

	NTE	E+	*P*-value
Gross Motility[1]	90.95 ± 2.67[A]	85.62 ± 2.67[A]	0.17
Pre-Freeze Motility[2]	94.88 ± 0.98[A]	94.23 ± 1.01[A]	0.65
Post-Thaw 0 hr[2]	58.27 ± 2.81[A]	43.84 ± 5.30[B]	0.02
Post-Thaw 3 hr[2]	51.13 ± 3.88[A]	23.33 ± 3.23[B]	< 0.0001
0 hr. – 3 hr. Difference	-5.99 ± 3.81[A]	-18.50 ± 4.12[B]	0.05

[1] Motility assessed .visually by individuals at farm uninformed of treatment origin

[2] Motility assessed using Computer Assisted Semen Analysis (CASA)

[A,B] Values with different superscripts within a row differ.

4. 2. Analysis 2: CASA Evaluation of Semen

4. 2. 1. Post-Thaw CASA Semen: Stress Evaluation

4. 2. 1. 1. Spermatozoa Concentration

Concentration of semen loaded into individual straws (i.e. breeding units) did not differ between treatments or time of assessment (0 vs. 3 hours) post-thaw (P = 0.08 vs P = 0.55, respectively, data not shown). Motility of extended semen prior to cryopreservation (i.e. pre-freeze) was similar between bulls grazing E+ and NTE tall fescue pastures (P = 0.17, Table 2). Independent of time of assessment post-thaw, the percentage of motile sperm was less when derived from bulls grazing E+ (32.77 ± 3.7%) versus those for bulls grazing NTE tall fescue pastures (54.72 ± 2.34%, P < 0.0001). Additionally, at 0 hours and 3 hours post-thaw, percent motility was decreased for bulls grazing E+ when compared to NTE tall fescue pastures (P = 0.02 and P < 0.0001 for 0 and 3 hours post-thaw respectively, Table 2). Bulls grazing E+ experienced a greater decrease in percentage of motile spermatozoa (-18.50 ± 4.12%) during the 3 hour stress test versus bulls grazing NTE tall fescue pastures (-5.99 ± 3.81, P = 0.05, Table 2). Independent of treatment, there were more percent motile spermatozoa at 0 hours (51.08 ± 2.85%) than at 3 hours post-thaw (36.07 ± 3.59%, P = 0.0025). Treatment had a significant effect on percent progressively motile spermatozoa with bulls grazing E+ having lower percent progressive motile spermatozoa (10.42 ± 0.99%) when compared to bulls grazing NTE tall fescue pastures (22.44 ± 1.38%, P < 0.0001). Independent of treatment and time of assessment post-thaw, date of semen collection had a significant effect on percent progressively motile spermatozoa (15.27 ± 1.42% vs.18.84 ± 1.48% vs. 12.85 ± 1.50% for June 12, 19, and 26, respectively, P = 0.02).

4. 2. 1. 2. CASA Semen Parameters

The impact of grazing E+ and NTE tall fescue pastures on VAP (i.e. path velocity or mean velocity of the smoothed sperm path, P = 0.001, Figure 4.3), VSL (i.e. progressive velocity or mean velocity measured in a straight line from the beginning to the end of the track, P = 0.003, Figure 4.4), and VCL (i.e. track speed or mean velocity measured over the actual point-to-point track, P = 0.002; Figure 4.5) depended on date of semen collection.

In evaluation of ALH (lateral amplitude or average width of the sperm head oscillation as the sperm swims), treatment differences depended on the date semen was collected (P = 0.01, Figure 4.6). Treatment differences in BCF (i.e. beat frequency or frequency of the sperm head intersecting the sperm average path in either direction) depended on the time of assessment post-thaw (0 vs. 3 hours) (P = 0.05; Figure 4.7). Independent of treatment, an interaction was also noted for BCF between time of assessment post-thaw (0 vs. 3 hours) and date of semen collection (P = 0.04; Figure 4.8).

Treatment differences for straightness (departure of the mean sperm path from a straight line VSL/VAP) depended on time of assessment post-thaw (0 vs. 3 hours) (P = 0.04; Figure 4.9). The impact of grazing E+ and NTE tall fescue pastures on linearity (i.e. departure of the actual sperm track from a straight line, VSL/VCL), differed depending on date of semen collection (P = 0.01; Figure 4.10). No effect of treatment, time of assessment post-thaw (0 vs. 3 hours), or date of semen collection was observed on elongation (ratio of spermatozoa head width to length). Differences between

treatments in area of the spermatozoa head depended on time of assessment (0 vs. 3 hours) ($P = 0.04$; Figure 4.11).

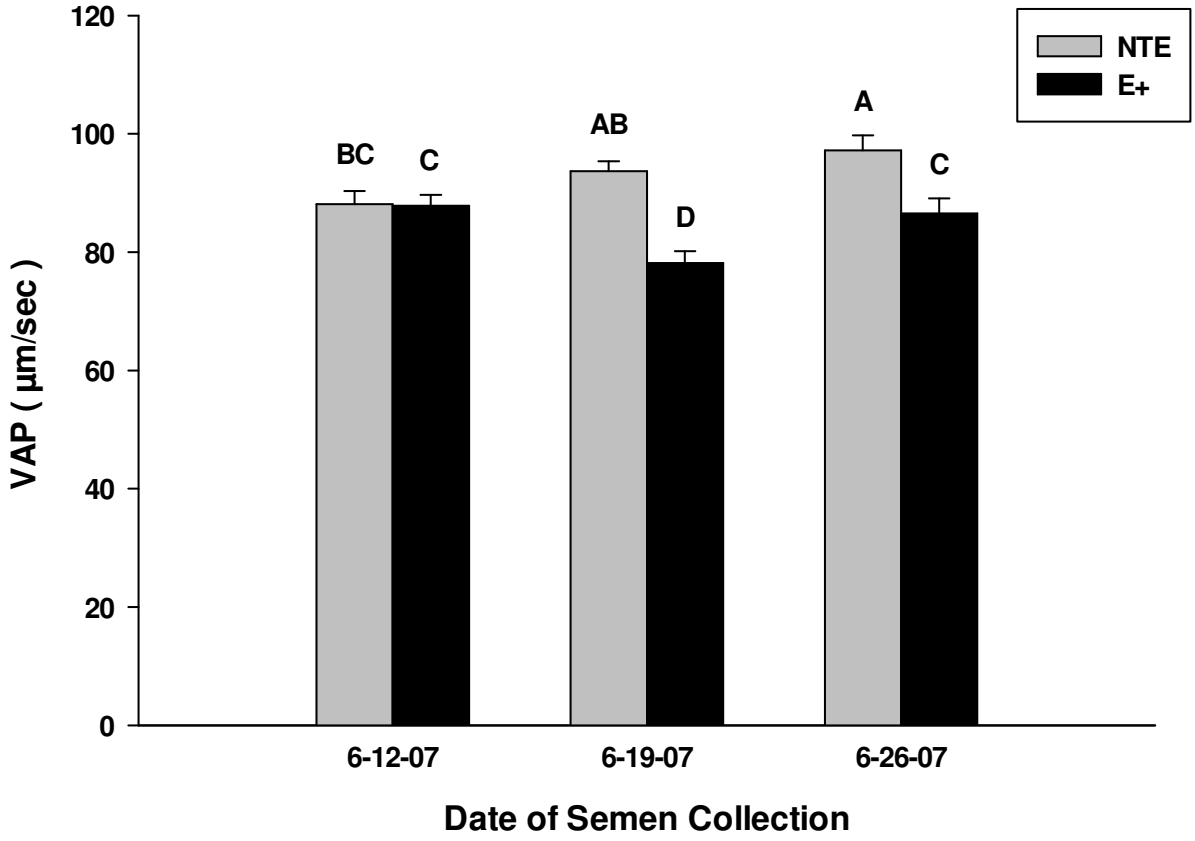


Figure 4.3 Treatment differences in VAP: path velocity or mean velocity of the smoother sperm path, differed depending on date of semen collection. Path velocity was lower in general for bulls grazing E+ tall fescue pastures when compared to bulls grazing NTE tall fescue pastures. (^{A,B,C,D} Least squares means differ between treatments and between date of semen collection; $P = 0.001$).

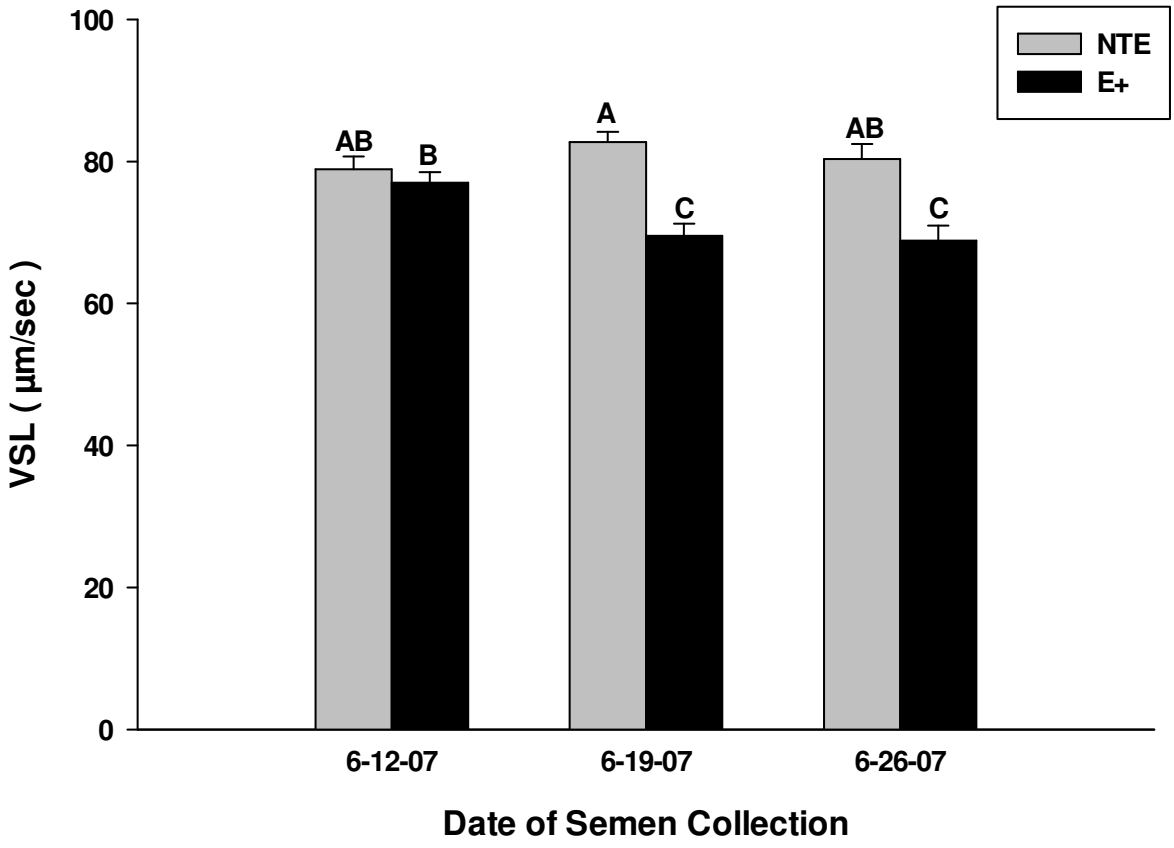


Figure 4.4 Treatment differences in VSL: progressive velocity or mean velocity measured in a straight line from the beginning to the end of the track, differed depending on date of semen collection. (^{A,B,C} Least squares means differ between treatments and between date of semen collection; $P = 0.003$).

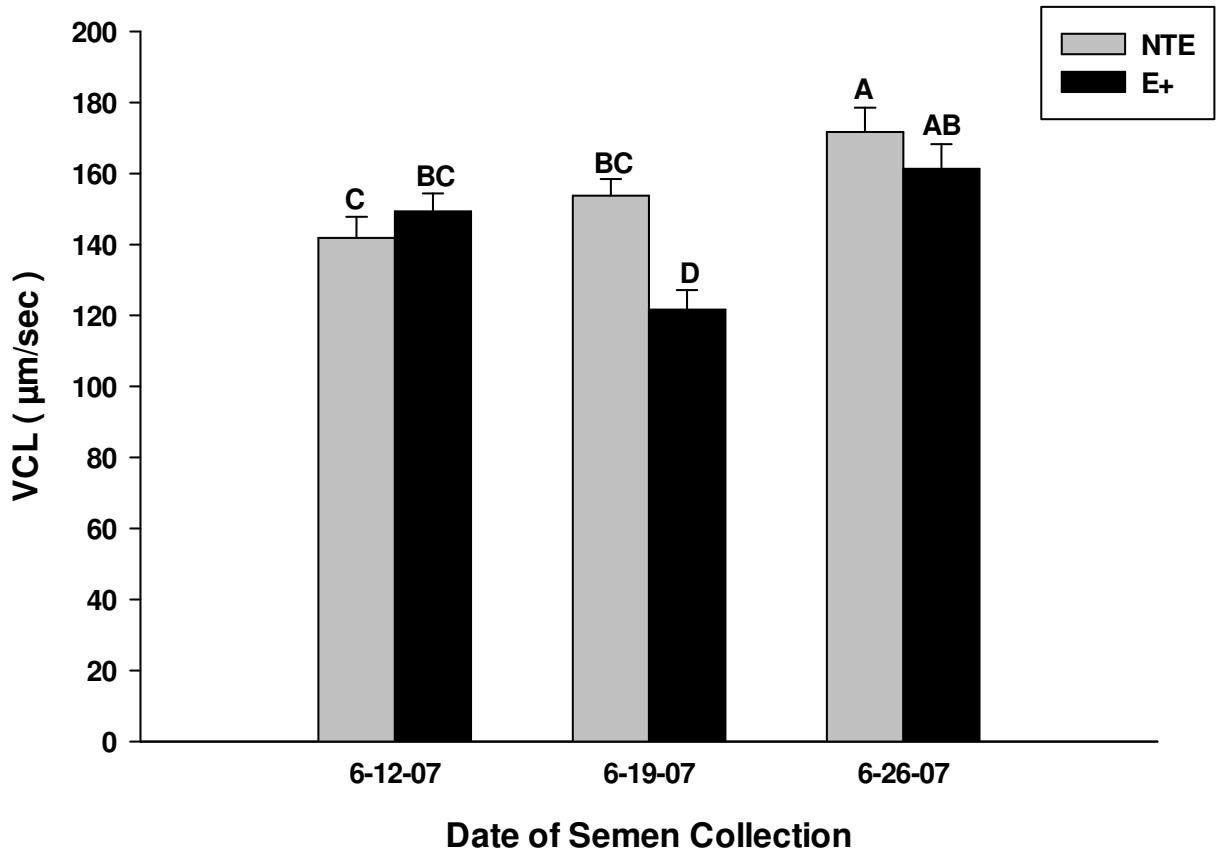


Figure 4.5 Treatment differences in VCL: track speed or mean velocity measured over the actual point-to-point track, differed depending on date of semen collection. (^{A,B,C,D} Least squares means differ between treatments and between date of semen collection; $P = 0.002$).

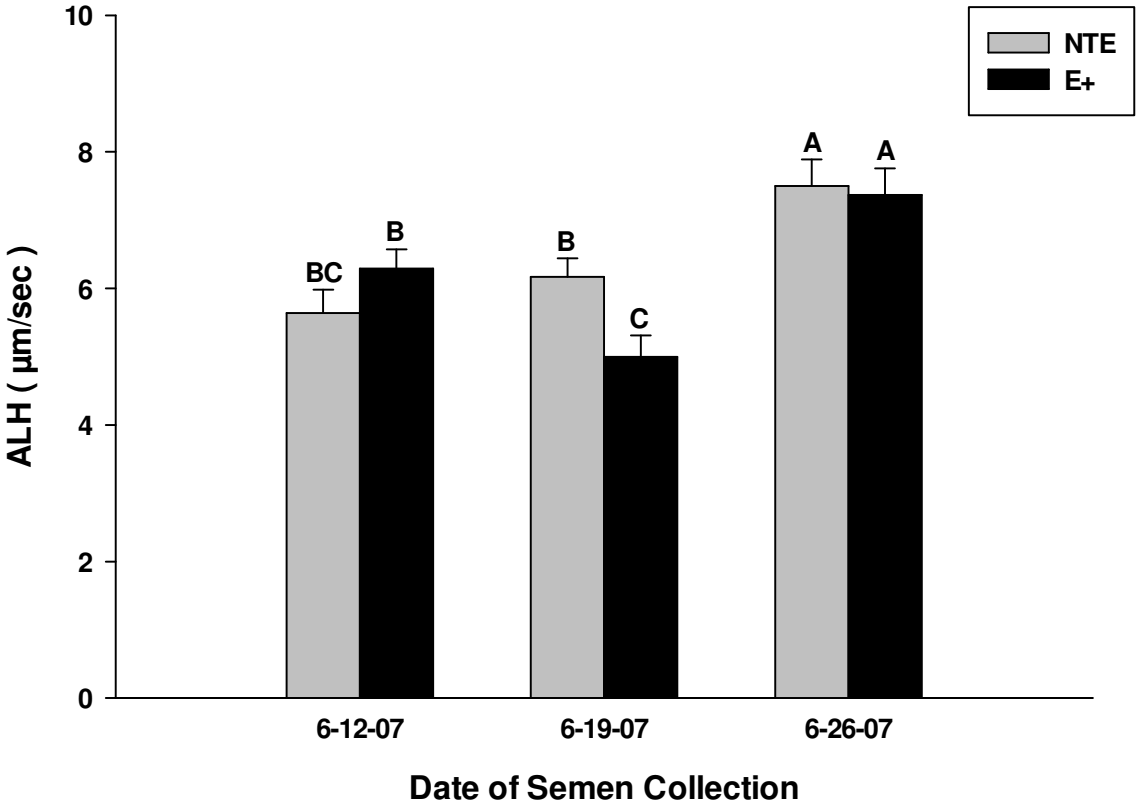


Figure 4.6 Treatment differences in ALH: lateral amplitude or average width of head oscillation as the sperm swims, depended on date of semen collection. On June 19, 2007 spermatozoa from bulls grazing E+ tall fescue pastures showed decreased lateral amplitude when compared to spermatozoa from bulls grazing NTE tall fescue pastures (^{A,B,C} Least squares means differ between treatments and between date of semen collection; $P = 0.01$).

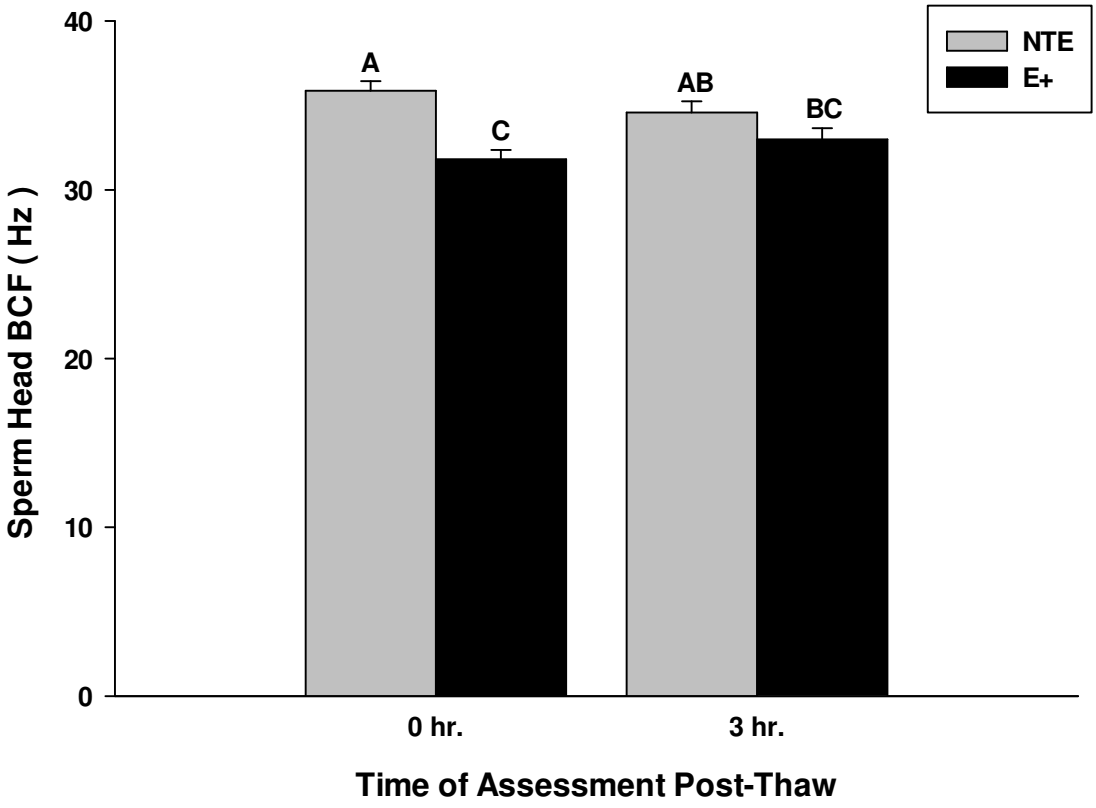


Figure 4.7 Treatment differences in BCF: beat frequency or frequency of the sperm head intersecting the sperm average path in either direction, depended on time of CASA assessment post-thaw (0 vs. 3 hours). At 0 hours post-thaw beat cross frequency of the spermatozoa head was decreased for bulls grazing E+ tall fescue pastures. There was no difference in beat cross frequency at 3 hours post-thaw. (^{A,B,C} Least squares means differ between treatments and between time of assessment post-thaw; $P = 0.05$).

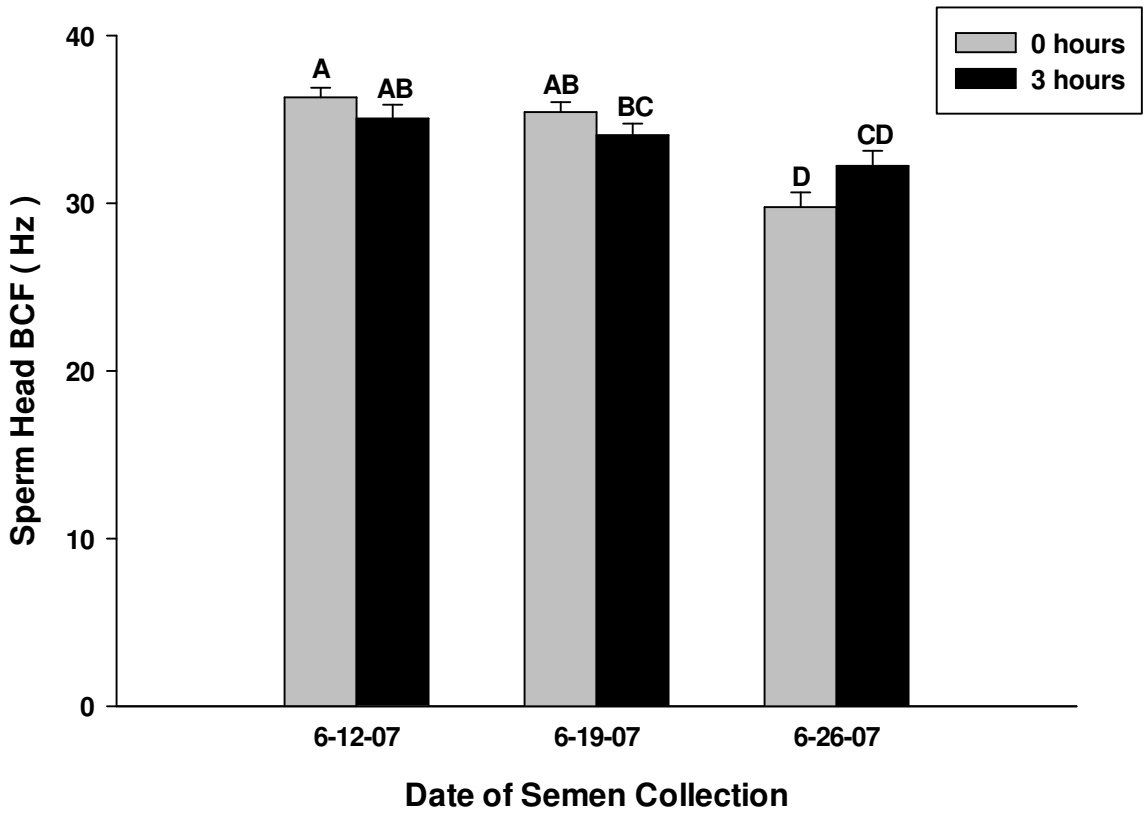


Figure 4.8 Date of semen collection differences in BCF: beat frequency or frequency of the sperm head intersecting the sperm average path in either direction, depended on time of CASA assessment post-thaw (0 vs. 3 hours). (^{A,B,C,D} Least squares means differ between time of assessment post-thaw and between date of semen collection; P = 0.04).

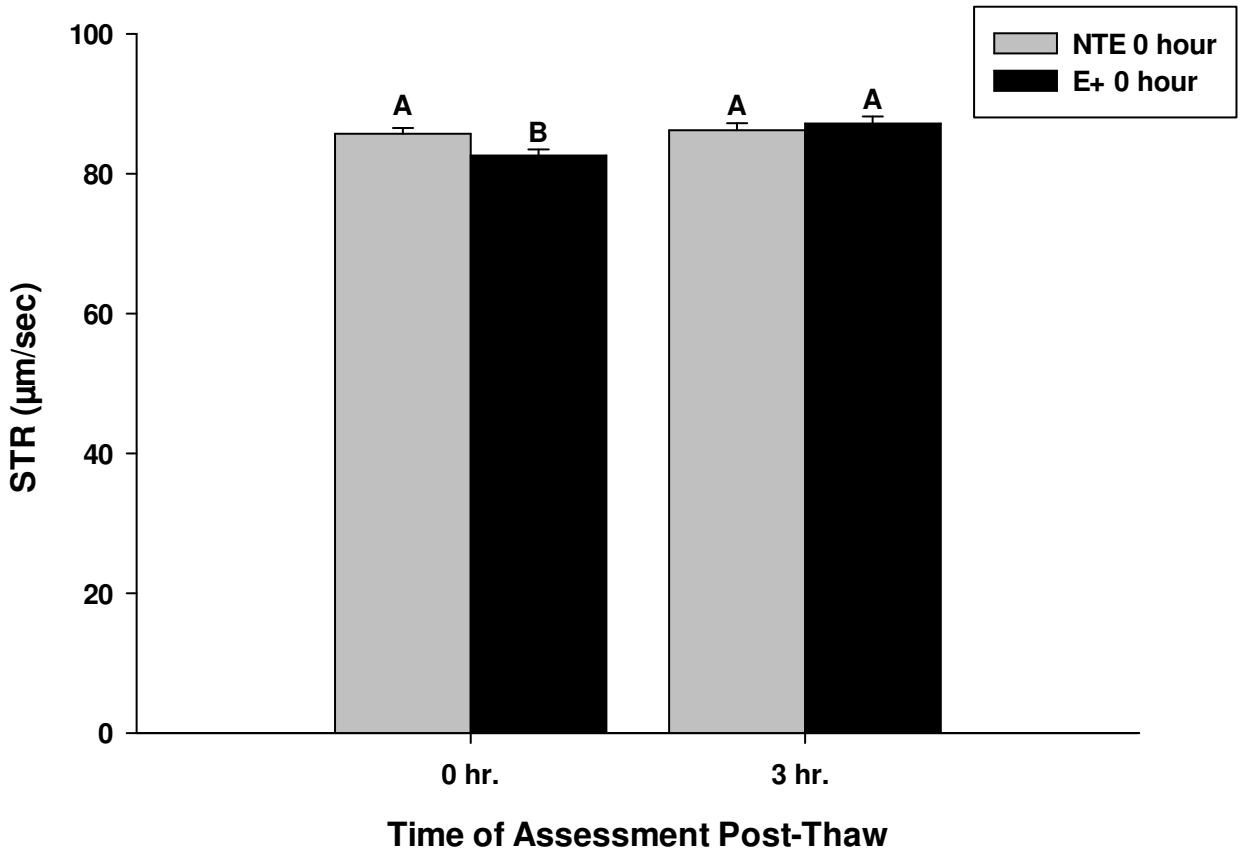


Figure 4.9 Treatment differences in straightness: departure of the mean spermatozoa path from a straight line (VSL/VAP), depended on time of assessment post-thaw (0 vs. 3 hours). (^{A,B,C} Least squares means differ between treatments and between date of semen collection; $P = 0.04$).

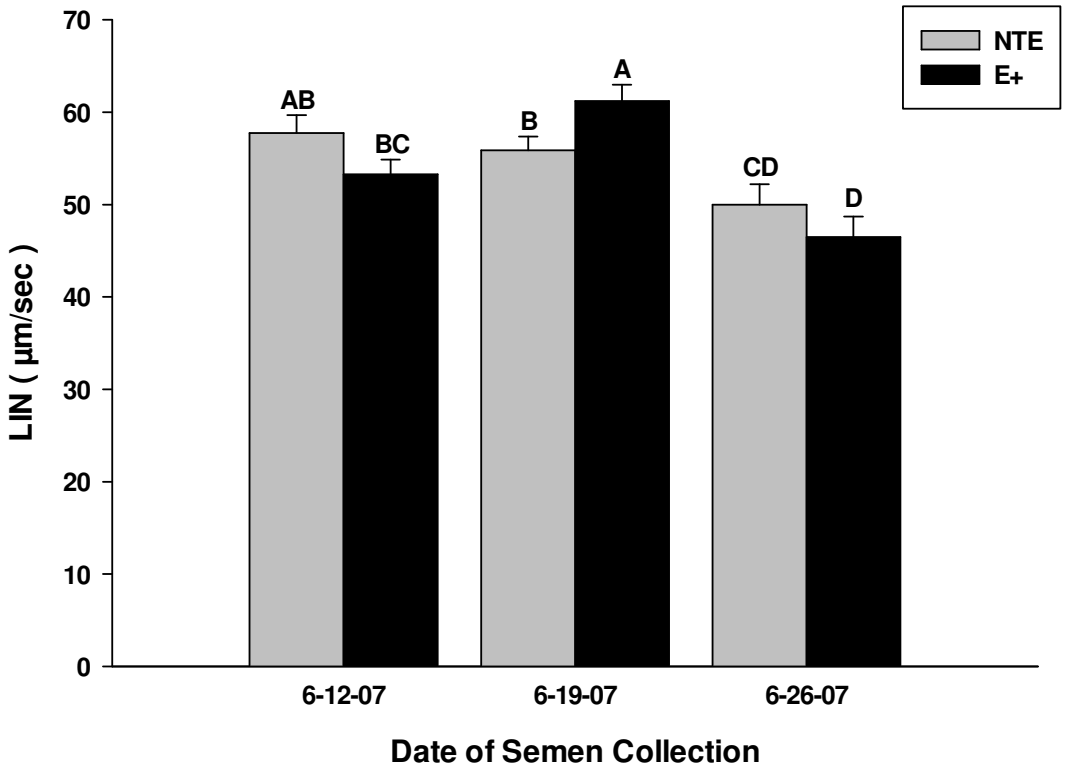


Figure 4.10 Treatment differences in linearity: departure of the actual sperm track from a straight line (VSL/VCL), depended on date of semen collection. (^{A,B,C,D} Least squares means differ between treatments and between date of semen collection; $P = 0.01$).

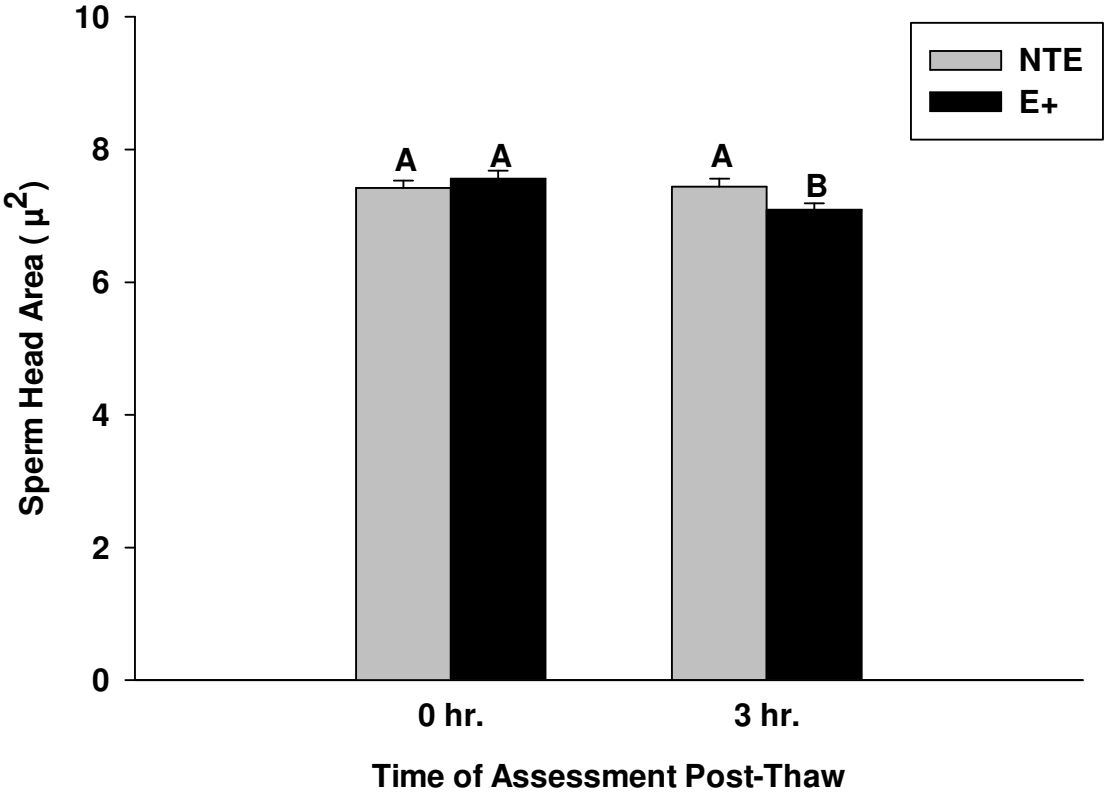


Figure 4.11 Area treatment differences depended on time of CASA assessment post-Thaw (0 vs. 3 hours). Between treatment groups there were no differences in area. However, spermatozoa from bulls grazing E+ tall fescue pastures decreased in size over time($^{A, B}$ Least squares means differ between treatments and time of assessment post-thaw; $P = 0.04$).

4. 2. 1. 3. Velocity Distribution

The effect of bulls grazing E+ and NTE tall fescue pastures on percentage of "rapid" spermatozoa was significant, whereas, bulls grazing E+ had less percentage of "rapid" spermatozoa when compared to bulls grazing NTE ($P < 0.0001$, Figure 4.12). Time of assessment post-thaw (0 vs. 3 hours) had an impact on percentage of "rapid" spermatozoa independent of treatment. Mean percentage of "rapid" spermatozoa 0 hours post-thaw was higher (21.48 ± 1.33%) than percentage of "rapid" spermatozoa 3 hours post-thaw (13.68 ± 1.14%, $P < 0.0001$).

Spermatozoa from bulls grazing E+ tall fescue pastures showed less percent "medium" spermatozoa when compared to bulls grazing NTE tall fescue pastures ($P = 0.004$, Figure 4.12). Time of assessment post-thaw (0 vs. 3 hours) had an impact on percent "medium" spermatozoa independent of treatment. Mean percentage of "medium" spermatozoa 0 hours post-thaw (5.31 ± 0.37%) was higher than percentage of "medium" spermatozoa 3 hours post-thaw (3.04 ± 0.25%, $P = 0.0003$). Bulls grazing E+ tall fescue pastures had an increased percentage of "static" spermatozoa when compared to bulls grazing NTE tall fescue pastures ($P < 0.0001$.Figure 4.12). Treatment differences in percentage of "slow" spermatozoa depended on time of assessment (0 vs. 3 hours) post-thaw ($P = 0.02$; Figure 4.13). Independent of treatment, time of assessment (0 vs. 3 hours) post-thaw had an impact on percentage of "static" spermatozoa (48.92 ± 2.85% vs. 63.92 ± 3.59%, 0 and 3-hours post-thaw, respectively, $P = 0.002$).

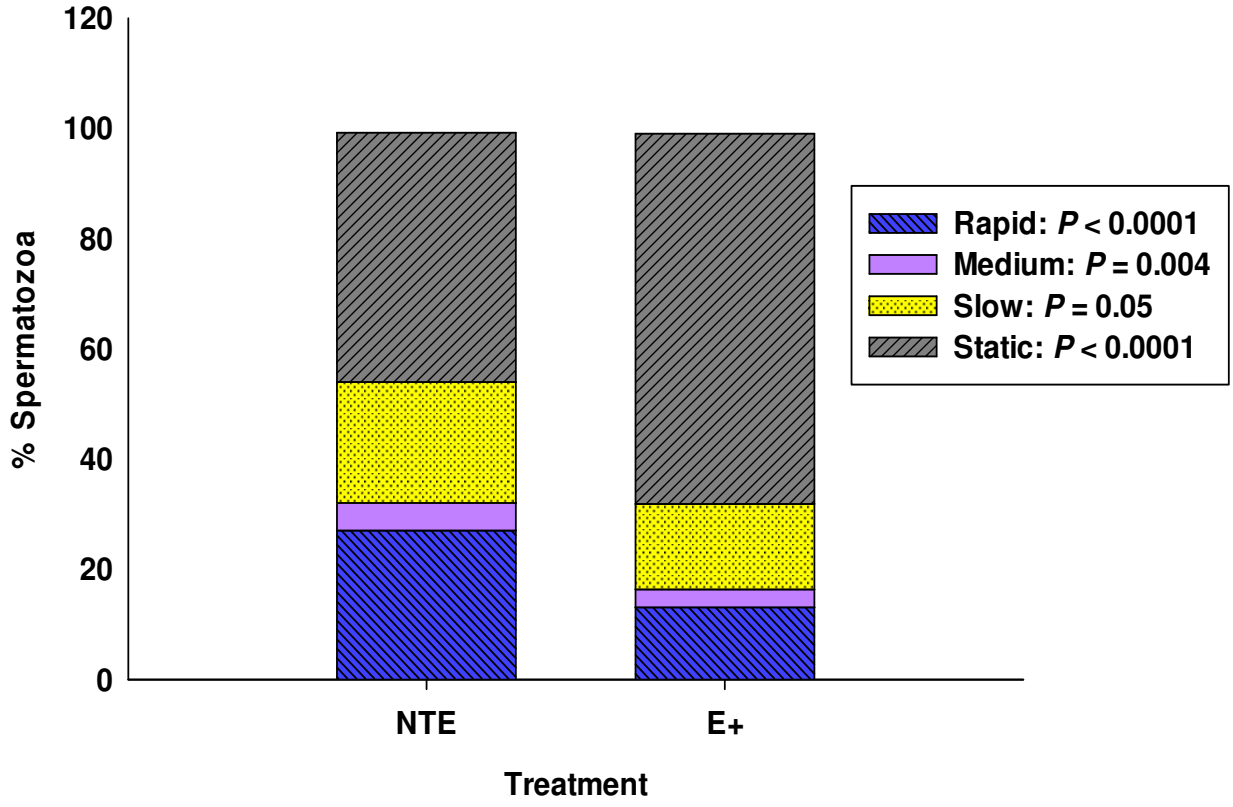


Figure 4.12 Bulls grazing E+ and NTE tall fescue pastures had a significant impact on all measurements of spermatozoa velocity distribution (rapid, medium, slow, and static).

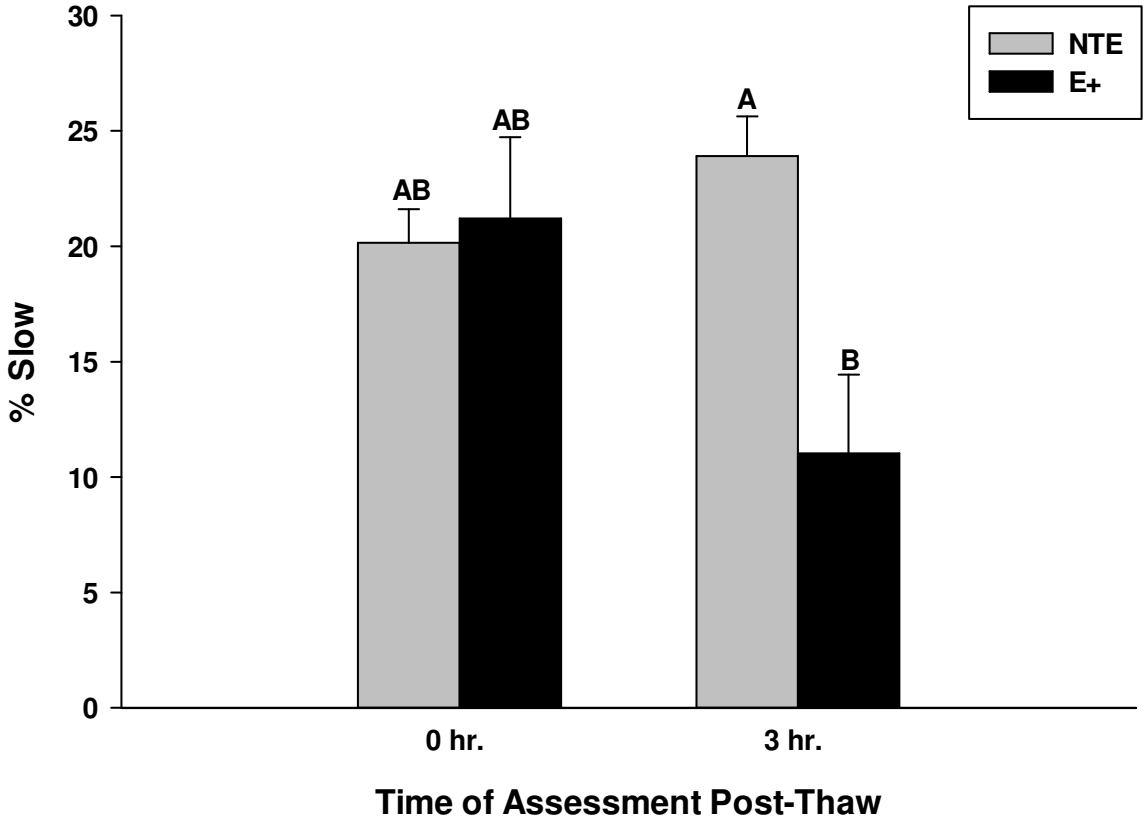


Figure 4.13 Percentage of "slow" spermatozoa decreased significantly at 3 hours post thaw for bulls grazing E+ compared to bulls grazing NTE tall fescue pastures (^{A,B} Least squares means differ between treatments and between time of assessment post-thaw; $P = 0.02$).

4. 2. 2. CASA: Characteristics of Semen Utilized in IVF Protocols

4. 2. 2. 1. Spermatozoa Concentration

Total concentration of semen loaded into straws (i.e. breeding units) did not differ for bulls grazing E+ tall fescue pastures when compared to semen from bulls grazing NTE tall fescue pastures (data not shown). As expected, concentration of semen before heparin incubation was higher (63.45 ± 2.63 million/mL) when compared to semen total concentration after heparin incubation (53.47 ± 2.62 million/mL, respectively, P = 0.0001). Furthermore, concentration of semen for straws dated 6-12-07 was more concentrated (64.45 ± 3.85 million/mL) when compared to straws labeled 6-26-07 (52.48 ± 2.53 million/mL respectively, P = 0.01). Percent motile spermatozoa for bulls grazing E+ tall fescue pastures was significantly decreased (26.64 ± 1.33%) when compared to bulls grazing NTE tall fescue pastures (51.97 ± 1.84% respectively, P < 0.0001). Independent of treatment, percent motile spermatozoa decreased from semen collection dates of 6-12-07 to 6-26-07 (46.60 ± 2.00 vs. 31.05 ± 1.28% respectively, P < 0.0001). Treatment differences in percent progressively motile spermatozoa depended on date of semen collection (P = 0.009; Figure 4.14).

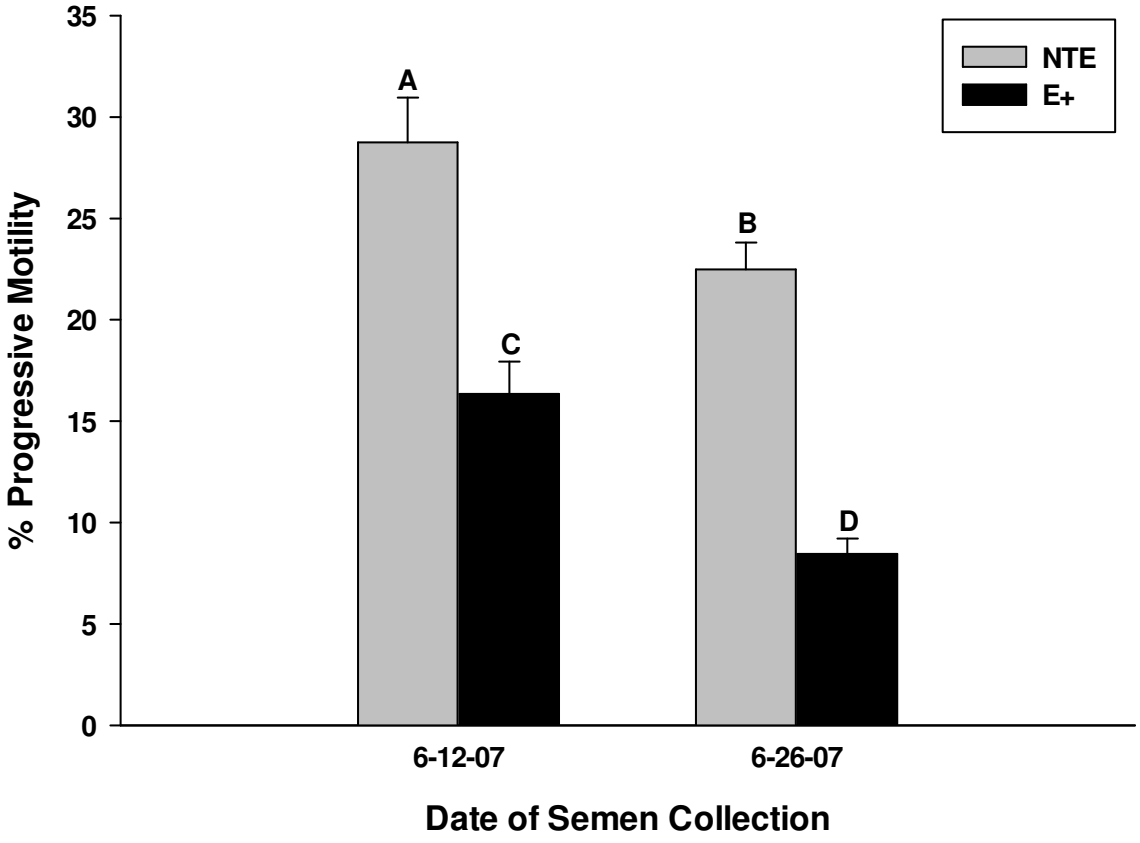


Figure 4.14 Treatment differences in percent progressively motile spermatozoa depended on date of semen collection. In general, spermatozoa from bulls grazing E+ tall fescue pastures had decreased percent progressive motility when compared to bulls grazing NTE tall fescue pastures. (^{A,B,C,D} Least squares means differ between treatments and date of semen collection; $P = 0.009$).

4. 2. 2. 2. CASA Parameters for IVF Semen

Spermatozoa from bulls grazing E+ had decreased VAP (i.e. path velocity or mean velocity of the smoothed cell path, 96.09 ± 1.79 µm/sec), when compared to bulls grazing NTE tall fescue pastures (109.70 ± 1.86 µm/sec, $P < 0.0001$). Before heparin incubation, sperm had higher VAP than sperm assessed after heparin incubation (104.77 ± 1.81 µm/sec vs. 101.03 ± 1.80 µm/sec, respectively, $P = 0.05$). Independent of heparin incubation time, sperm from bulls grazing E+ had reduced VSL (i.e. progressive velocity or mean velocity measured in a straight line from the beginning to the end of the track, 77.05 ± 1.73 µm/sec) than bulls grazing NTE tall fescue pastures (88.83 ± 1.80 µm/sec, $P < 0.0001$). Bulls grazing E+ had decreased VCL (i.e. track speed or average velocity measured over the actual point-to-point track, 171.58 ± 2.85 µm/sec) than sperm from bulls grazing NTE tall fescue pastures (192.82 ± 2.94 µm/sec, $P < 0.0001$). Mean VCL for sperm was higher before heparin incubation when compared to after heparin incubation values (192.24 ± 2.87 vs. 172.16 ± 2.86 µm/sec, respectively, $P < 0.0001$).

Lateral amplitude (i.e. average width of the head oscillation as the sperm swims or ALH) was decreased for sperm from bulls grazing E+ (7.83 ± 0.12 µm) when compared to sperm from bulls grazing NTE tall fescue pastures (8.22 ± 0.11 µm, $P = 0.004$). Independent of treatment, ALH was decreased for sperm before heparin incubation versus after heparin incubation values (7.48 ± 0.12 vs. 8.57 ± 0.12 µm, respectively, $P < 0.0001$). Treatment differences in BCF depended on before and after heparin incubation values ($P = 0.01$, Figure 4.15). No treatment differences were detected in straightness (i.e. departure of the mean sperm path from a straight line,

VSL/VAP). Straightness was greater for sperm after heparin incubation than sperm before heparin incubation (80.77 ± 0.58 µm/sec vs. 76.76 ± 0.58 µm/sec respectively, *P* < 0.0001). The same was true for linearity of sperm (i.e. departure of the actual sperm track from a straight line, VSL/VCL) after heparin incubation as compared to sperm before heparin incubation values. Linearity was increased in sperm after heparin incubation (49.45 ± 0.62 µm/sec) when compared to sperm before heparin incubation (44.21 ± 0.63 µm/sec, *P* < 0.0001). There were no differences for elongation between treatment groups, before and after heparin incubation or date of semen collection (P = 0.2030, data not shown).

4. 2. 2. 3. Velocity Distribution

Differences in percent "rapid" spermatozoa depended on date of semen collection (*P* = 0.0287; Figure 4.16). Spermatozoa from bulls grazing E+ tall fescue pastures had reduced percent "medium" spermatozoa (2.76 ± 0.24%) when compared to bulls grazing NTE tall fescue pastures (5.36 ± 0.36%, *P* < 0.0001). Date of semen collection also had an influence on percent "medium" spermatozoa. Percent of "medium" spermatozoa decreased from 6-12-07 to 6-26-07 (4.6 ± 0.40% vs. 3.1 ± 0.23% respectively, *P* = 0.0010). Percent "slow" spermatozoa was decreased for spermatozoa from bulls grazing E+ (7.4 ± 0.61%) in comparison to bulls grazing NTE tall fescue pastures (10.90 ± 0.82%, *P* < 0.0001). After heparin incubation, percent "slow" spermatozoa increased when compared to before heparin incubation values (9.97 ± 0.77% vs. 8.1 ± 0.65%, respectively, *P* = 0.0042). Bulls grazing E+ tall fescue pastures had a greater percentage of "static" spermatozoa (73.36 ± 1.3%) when compared to bulls grazing NTE tall fescue pastures (48.03 ± 1.84%, *P* < 0.0001).

Semen collection date influenced percent “static” spermatozoa as well. Percent of “static” spermatozoa increased from 6-12-07 to 6-26-07 (53.40 ± 2.0% vs. 68.95 ± 1.28%, respectively, $P < 0.0001$).

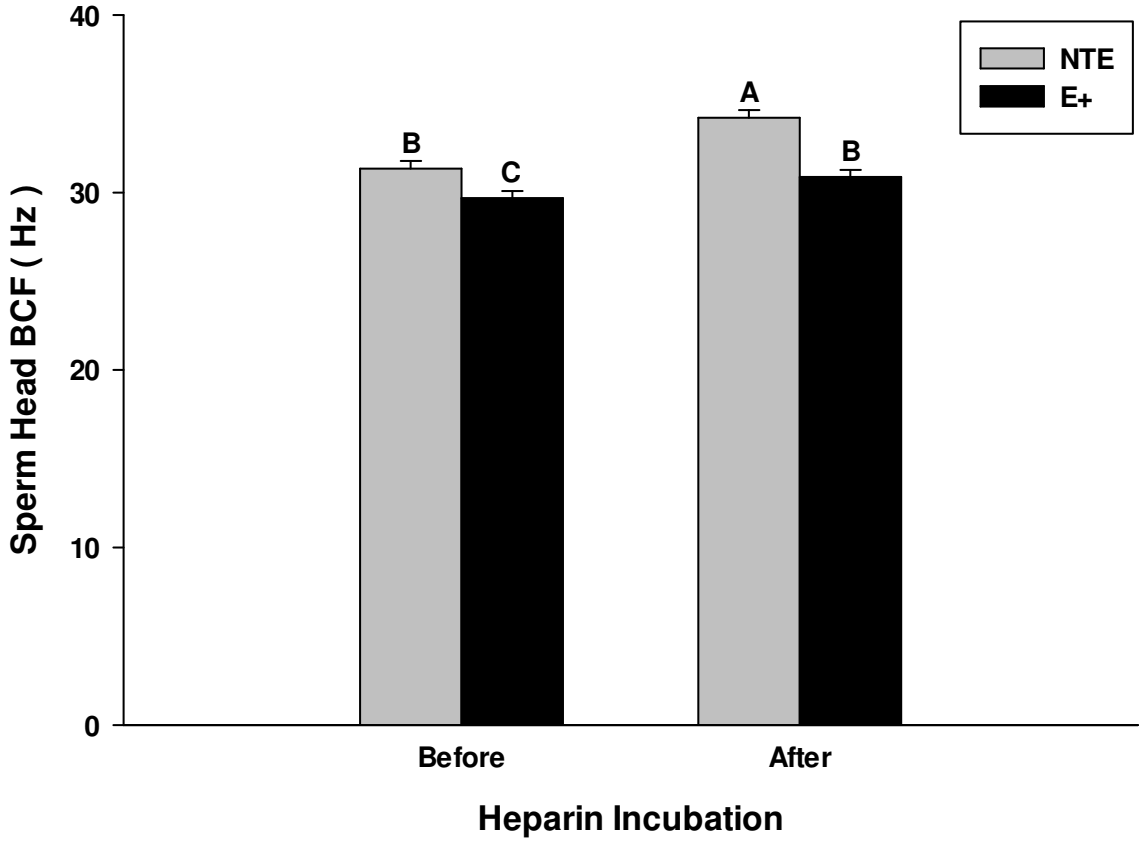


Figure 4.15 Treatment differences in BCF: beat frequency or frequency of the sperm head crossing the sperm average path in either direction, depended on before and after heparin incubation values. (^{A,B,C} Least squares means differ between treatments and before and after heparin incubation values; $P = 0.0108$).

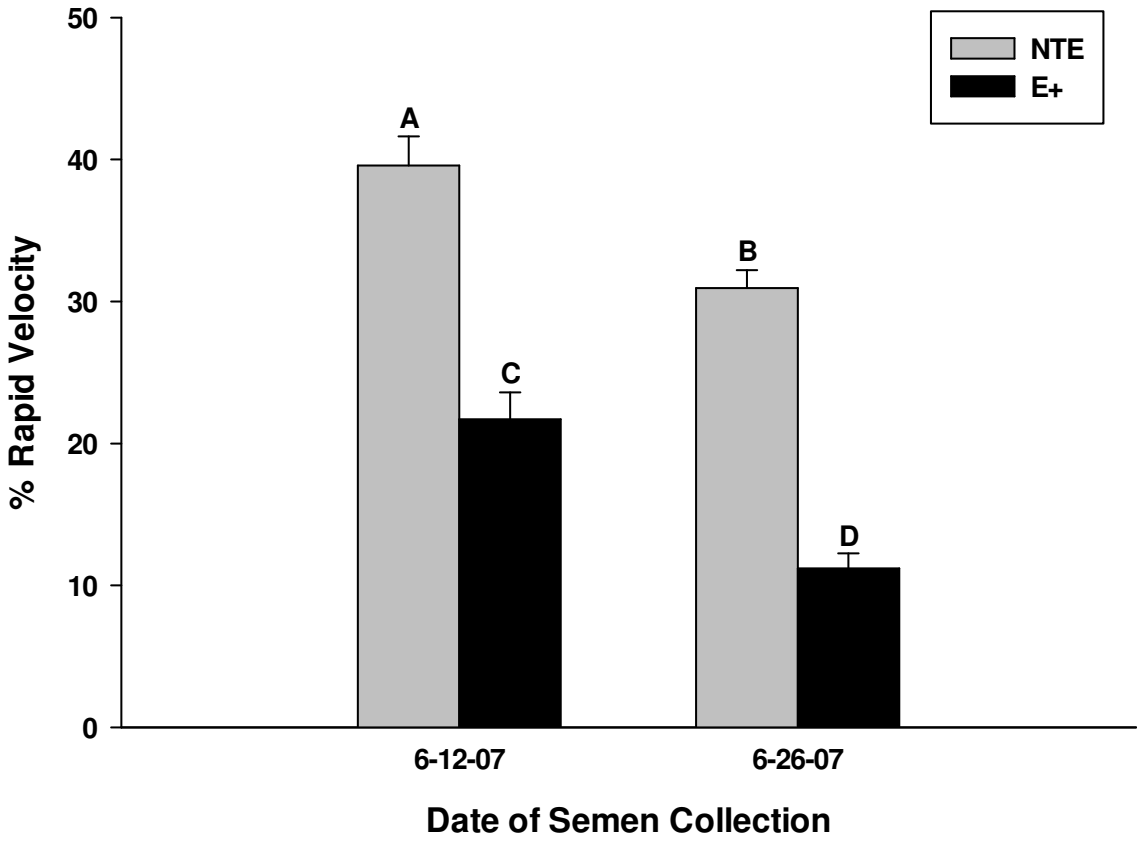


Figure 4.16 Treatment differences in percent "rapid" spermatozoa depended on date of semen collection. In general, percent "rapid" spermatozoa for bulls grazing E+ tall fescue pastures were decreased between treatment groups and over time when compared to bulls grazing NTE tall fescue pastures. (^{A,B,C,D} Least squares means differ between treatments and date of semen collection; $P = 0.0287$).

4.3 Analysis 3: In Vitro Evaluation of Semen

4. 3. 1. Embryo Development

Percent of presumptive zygotes recovered and percent of presumptive zygotes lysed did not differ between treatments (P = 0.48 and 0.15, respectively, Table 3). Percent 2-cell (P = 0.21), 4-cell (P = 0.84), and 8-16 cell (P = 0.34) relative to proportion of embryos cleaved at time of cleavage evaluation were not different for oocytes fertilized with spermatozoa from bulls grazing E+ tall fescue when compared to NTE tall fescue pastures (Table 3). However, percent of embryos that cleaved was significantly lower for oocytes when fertilized by spermatozoa from bulls grazing E+ tall fescue pastures when compared to NTE tall fescue pastures (P = 0.007, Table 3). However, percent blastocyst development relative to proportion of embryos cleaved was not different in presumptive zygotes fertilized with spermatozoa from bulls grazing E+ tall fescue pastures when compared to NTE (P = 0.7041, Table 2). Percent blastocyst development relative to proportion of presumptive zygotes placed into culture tended to differ between treatments (P = 0.0666, Table 3). Average blastocyst nuclei counts did not differ between treatments (163.27 ± 7.31 vs. 170.88 ± 7.10, E+ and NTE, respectively, P = 0.23).

Table 3. Embryo development after otherwise developmentally competent ova underwent in vitro maturation (IVM) at 38.5°C

		6.5-8.5 hpi		Cleavage, 70 to 97 hpi					Blastocyst, 184 to 212 hpi		
Treatment	Total COC	Rcd (%)[1]	Lysed (%)[2]	No. PZ	Cleaved (%)	2-Cell[3] (%)	4-Cell[3] (%)	8 to 16-Cell[3] (%)	No. PZ	Blast of PZ (%)	Blast of Cleaved (%)
NTE	2032	94.56	0.70	789	76.30[A]	10.39	26.28	63.36	853	39.77	51.69
E+	1999	95.15	0.32	750	58.92[B]	13.07	26.86	60.27	856	29.63	50.45
SEM		0.9150	0.2277		3.933	1.452	2.920	3.087		3.044	2.561
P-Value		0.48	0.15		0.007	0.21	0.84	0.34		0.07	0.70

Abbreviations: NTE = non-toxic endophyte infected tall fescue; E+ = toxic endophyte infected tall fescue; Rcd = presumptive zygotes recovered after removal of cumulus; PZ = presumptive zygote; COC = cumulus oocyte complex

[1]PZ recovered after denuding as a proportion of total number of cumulus-oocyte complexes matured, [2]PZ without an intact plasma membrane after denuding as a proportion of recovered, [3]Relative to proportion of embryos cleaved

For all useable reps n=10

[A,B] Values differ between row

4. 3. 2. Penetration Assay

Percent of presumptive zygotes recovered and percent of presumptive zygotes lysed did not differ between oocytes fertilized with sperm from bulls grazing E+ or NTE tall fescue pastures (data not shown). Rate of oocyte penetration by at least one sperm was significantly reduced for bulls grazing E+ tall fescue pastures (64.54 ± 3.28) when compared to bulls grazing NTE tall fescue pastures (87.42 ± 1.63%, $P < 0.0001$). Of all oocytes that were penetrated, there was no difference in percent polyspermic (8.20 ± 1.23% vs. 10.74 ± 1.35%, E+ and NTE, respectively, $P = 0.17$) or monospermic fertilization (91.83 ± 1.25% vs. 89.37 ± 1.36%, E+ and NTE respectively, $P = 0.19$) between bulls grazing E+ and NTE tall fescue pastures.

When evaluating maternal chromatin after penetration by spermatozoa from bulls grazing E+ tall fescue pastures more oocytes remained arrested at metaphase II when evaluated at 7.0-9.5 hours post insemination (12.43 ± 1.29% vs. 9.36 ± 0.96%, for E+ and NTE, respectively, $P = 0.057$). However, penetration by spermatozoa from bulls grazing NTE resulted in a higher percentage of oocytes whose maternal chromatin had progressed to the condensed stage of the meiotic cell cycle (58.24 ± 2.63%) compared to spermatozoa from bulls grazing E+ tall fescue pastures (36.31 ± 2.75%, $P < 0.0001$). Penetration by spermatozoa from bulls grazing E+ resulted in an increased percentage of oocytes whose maternal chromatin had progressed to anaphase II of the meiotic cell cycle (4.27 ± 0.64%) compared to spermatozoa from bulls grazing NTE tall fescue pastures (1.40 ± 0.31%, $P < 0.0001$). Additionally, spermatozoa from bulls grazing E+ tall fescue pastures resulted in an increased percentage of oocytes whose maternal chromatin had progressed to telophase II of the meiotic cell cycle (46.54 ± 2.88%)

compared to spermatozoa from bulls grazing NTE tall fescue pastures (30.10 ± 2.29%, $P < 0.0001$). Percentage of presumptive zygotes whose maternal chromatin had progressed to the pronuclear stage with a visible spermatozoa head, single pronuclear stage with no visible spermatozoa head, and two visible pronuclei were not different between treatment groups (data not shown). Percentages of maternal chromatin in metaphase II, condensed, anaphase II, and telophase II of the meiotic cell cycle are summarized in Figure 4.17.

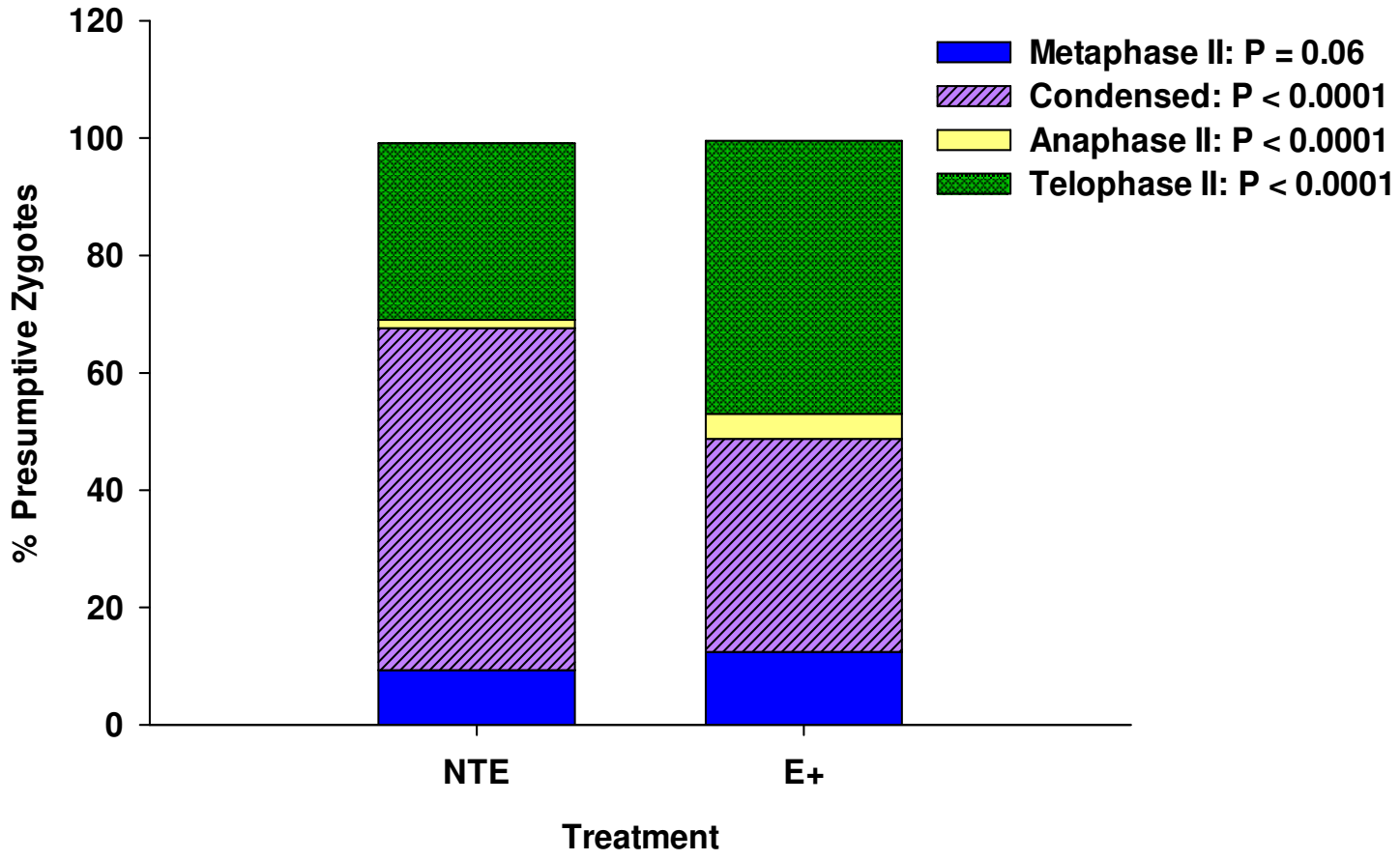


Figure 4.17 Percentage of maternal chromatin in metaphase II, condensed, anaphase II, and telophase II of meiotic cell cycle 7.0-9.5 hours post insemination for oocytes penetrated by sperm from bulls grazing E+ and NTE tall fescue pastures differed for each stage.

4. 3. 3. Intracellular Calcium Parameters

Baseline calcium concentrations were lower for presumptive zygotes fertilized with spermatozoa from bulls grazing E+ tall fescue pastures (268.95 ± 26.60 nM) compared to presumptive zygotes fertilized with spermatozoa from bulls grazing NTE tall fescue pastures (304.62 ± 26.47 nM, P = 0.01). Mean duration of calcium oscillations (time from start to end of peak) was not different between treatment groups (86.62 ± 10.53 ms vs. 88.42 ± 10.48 ms, E+ and NTE, respectively, P = 0.71). Amplitude of calcium oscillations was significantly decreased for presumptive zygotes fertilized with spermatozoa from bulls grazing E+ tall fescue pastures (383.49 ± 52.36 nM) compared to presumptive zygotes fertilized with spermatozoa from bulls grazing NTE tall fescue pastures (520.72 ± 52.01 nM, P = 0.0002). Area under the curve, or total calcium response, was significantly reduced for presumptive zygotes fertilized with spermatozoa from bulls grazing E+ tall fescue pastures (15,784 ± 1,933.45 nM) compared to presumptive zygotes fertilized with spermatozoa from bulls grazing NTE tall fescue pastures (19,859 ± 1,917.71 nM, P = 0.006). Interval between oscillations for presumptive zygotes that had more than one calcium oscillation was not altered between treatments (33.46 ± 1.78 ms vs. 30.12 ±1.71 ms, E+ and NTE, respectively, P = 0.17).

Chapter 5

Discussion

5. 1. General Comments

Previous research has shown that consumption of endophyte-infected (E+) tall fescue decreases performance and reproductive efficiency in males (Jones *et al.* 2004; Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b). Examination of semen from E+ bulls indicated that motility post-thaw and during a 3 hour stress test was reduced. When semen was added at the same concentration as bulls grazing non-toxic tall fescue (NTE) in an IVP system, cleavage rates of oocytes were reduced when fertilized with semen from E+ bulls. Subsequent efforts observed lower penetration (i.e. fertilization) rates of oocytes, and altered intracellular calcium oscillations in those that were fertilized with sperm from E+ bulls. Collectively, these described results support altered spermatozoa function in bulls consuming E+ tall fescue pastures by inducing non compensatory effects.

5. 2. Analysis 1: Bull Performance

During the three-month grazing period, average daily gains were lower for all bulls regardless of treatment group, likely due to severe drought conditions and higher than normal ambient temperatures. Bulls grazing E+ tall fescue pastures had reduced serum concentrations of prolactin compared to bulls grazing NTE tall fescue pastures. This is a common indicator of fescue toxicosis seen consistently throughout the literature (Evans *et al.* 1988; Porter and Thompson 1992; Paterson *et al.* 1995; Waller 2009).

However, another common indicator, rectal temperatures, were not different between treatment groups. This was due to collection of rectal temperatures between 0600-0800 each morning, a time period when one would expect a “minimal” difference. Collection of semen and other experimental variables was performed at this time to reduce effects of environmental stress on animals that would also be incurring electroejaculation. Typically, increased body temperatures as observed during fescue toxicosis for animals grazing E+ tall fescue pastures are seen when measurements are made during the heat of the day or under heat stress conditions (Burke *et al.* 2001b; Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b). Scrotal temperatures were also collected between 0600-0800 each morning and were not different due to pasture type. Again, scrotal temperature may have been different if scrotal thermography had been performed during the heat of the day, as observed by Schuenemann *et al.* (2005a; 2005b). Furthermore, scrotal temperatures may have been altered if exposure to E+ tall fescue had been longer as seen previously (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b). All bulls had a decrease in scrotal circumference over the experimental period, most likely due to weight loss. Scrotal circumference and body weight are positively correlated with one another so it is not surprising that a decrease in scrotal circumference was seen for all bulls regardless of treatment (Pratt *et al.* 1991). Not surprisingly, testosterone concentration was similar between treatments in agreement with previous studies in our laboratory (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b).

Motility of semen immediately after collection (gross motility) and in fresh extended semen was similar between treatment groups at time of semen collection.

Furthermore, semen morphology at collection was also not different between bulls grazing E+ and NTE tall fescue pastures. This agrees with previous research in our laboratory, and others, where differences in gross motility and morphology have not been seen in semen from bulls grazing E+ and NTE tall fescue pastures (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b) or consuming endophytic tall fescue seed (Jones *et al.* 2004). These are considered subjective measurements of motility and morphology, but they were assessed by board certified theriogenologists with many years of experience in the field (Drs. F.M. Hopkins and T. M. Prado) and motility assessment of fresh extended semen was performed using CASA in this study.

5. 3. Analysis 2: Post-Thaw Characteristics of Semen

Evaluation of semen immediately post-thaw (0 h) revealed decreased motility for spermatozoa from bulls grazing E+ tall fescue. This would be the same thaw time used to prepare semen for artificial insemination. As mentioned previously, gross motility immediately after collection was not different between treatment groups, nor was motility of extended fresh semen (immediately pre-freeze) when evaluated with CASA. Thus, consumption of E+ tall fescue causes a reduced ability to withstand damages due to cryopreservation. Furthermore, motility at 3 hours post-thaw was markedly decreased for sperm from bulls consuming E+ when compared to bulls consuming NTE tall fescue. This decrease in motility seen after 3 hour incubation further indicates that E+ tall fescue consumption impairs spermatozoa in a way that is not readily detectible via gross assessments of semen motility and morphology.

Decreases in gross motility and CASA motility are supported by other semen evaluation parameters including VAP (i.e. path velocity or mean velocity of the

smoothed sperm path), VSL (i.e. progressive velocity or velocity of the sperm measured in a straight line from the beginning to the end of the track), and VCL (i.e. track speed or mean velocity measured over the actual point-to-point track). These are velocities that are highly correlated with fertility (Budworth *et al.* 1988), and were all lowered for sperm from bulls consuming E+ tall fescue. Differences in VAP, VSL, and VCL depended on date of semen collection, indicating that prolonged exposure to E+ tall fescue pastures further enhances problems in spermatozoa velocities. These findings indicate spermatozoa from E+ bulls are impaired, which is supported by other velocity estimates (rapid, slow and medium) and could be a reason for reductions in fertility. Bulls grazing E+ tall fescue had reduced velocities, indicating that sperm from these bulls are “slower” than control bulls. The same was true for progressive velocity of the smoothed sperm path. This further indicates not only “slower” spermatozoa, but also spermatozoa that are not progressive in their movement (i.e. moving forward in a straight line). Wang *et al.* (2009) reported that sperm motility was altered by addition of ergot alkaloids (ergotamine or dihydroergotamine) directly to semen. This alteration in motility occurred in a concentration dependent manner, and agrees that ergot alkaloids have an adverse effect on spermatozoa function after prolonged exposure in fed animals.

In addition to altered velocity parameters (VAP, VCL, and VSL) for spermatozoa from bulls grazing E+ tall fescue pastures, a decrease in ALH (i.e. lateral amplitude or average width of the head oscillation as the sperm swims) and BCF (i.e. beat frequency or frequency of the sperm head intersecting the sperm average path in either direction) were also observed. Lateral amplitude was influenced by date of semen collection. Alterations in spermatozoa velocity parameters mentioned previously could be

explained by reduced ALH and BCF. If head oscillations are not as fast or as frequent as normal, then impairments in motility could be seen that would alter the ability of the spermatozoa to fertilize oocytes. Looper *et al.* (2009) reported similar results for spermatozoa from Brahman-influenced bulls grazing E+ tall fescue (Kentucky 31); whereas, these bulls produced spermatozoa that were less motile and less progressive. In addition, spermatozoa from bulls grazing E+ tall fescue in the same study had reduced VAP, VSL, and ALH compared to controls (Looper *et al.* 2009).

Additional information about sperm function obtained from CASA indicated a higher percentage of “rapid” sperm from bulls grazing NTE tall fescue pastures. Bulls grazing E+ tall fescue pastures had a lower percentage of “medium” sperm independent of time of assessment post-thaw, a lower percentage of “slow” sperm at 3-hours post-thaw, and a higher percentage of “static” spermatozoa independent of time of assessment post-thaw. These results are supportive of previous reports that showed alterations in velocity distributions for sperm from bulls grazing E+ tall fescue pastures (Looper *et al.* 2009) and also indicative of impaired spermatozoa function due to consumption of E+ tall fescue. Studies have also shown that ergot alkaloids alter post-thaw motility of spermatozoa (Gallagher and Senger 1989; Wang *et al.* 2009).

Area of the sperm head was reduced in E+ grazing bulls after the 3-hour stress test. This finding indicates “shrinkage” of the sperm head or possibly compromised membranes. Wang *et al.* (2009) suggested that ergot alkaloids could be interacting with sperm membranes and their receptors in such a way as to directly affect intracellular signaling paths and sperm function in a negative way. Cryopreservation itself is known to have detrimental effects on sperm membrane structure (Parks and Graham 1992),

but it is not known if consumption of E+ tall fescue pastures is exaggerating this effect in the current study. Therefore, further research is need to fully elucidate the mechanisms by which ergot alkaloids are exerting their effects on spermatozoa from bulls grazing E+ tall fescue pastures.

Results reported for CASA characteristics of semen were similar for both the stress test and semen utilized in IVF protocols. Each of which are supportive of impaired spermatozoa function as a result of consumption of E+ tall fescue pastures.

5. 4. Analysis 3: In Vitro Evaluation of Semen

5. 4. 1. Embryo Development

In the present study, cleavage rates were significantly reduced for oocytes fertilized with spermatozoa from bulls grazing E+ tall fescue pastures. This is in support of several studies conducted in our laboratory over numerous years, different sets of bulls, and multiple technicians (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b). This repeatability indicates the importance of the present study, and aids in greater understanding of the problem: decreased reproductive efficiency of spermatozoa function due to E+ tall fescue consumption. While oocytes fertilized with sperm from bulls grazing E+ tall fescue pastures experienced a decreased ability to cleave, of those embryos that cleaved, there was not a significant difference in blastocyst development between treatments. This indicates cleavage as the primary problem in addition to possible alterations in sperm integrity as suggested by previous studies (Schuenemann *et al.* 2005a; Schuenemann *et al.* 2005b). The findings in the current study indicate that additional effort is needed to fully explain reduction in cleavage rates of oocytes fertilized with sperm from bulls grazing E+ tall fescue pastures.

5. 4. 2. Penetration Assay

Previous research in our laboratory has reported a consistent decrease in cleavage rates of oocytes fertilized with sperm from bulls grazing E+ tall fescue pastures (Schuenemann *et al.* 2005b) or ergotamine tartrate to simulate fescue toxicosis (Schuenemann *et al.* 2005a). However, of those that cleaved, blastocyst development does not differ significantly between bulls experiencing and those not

experiencing tall fescue toxicosis. These findings indicate that a problem exists prior to cleavage that may be responsible for a reduction in cleavage rates.

Males are often not blamed for cases of infertility in a beef herd, but sperm from the male contributes half of the genetic material needed to produce offspring. Therefore, sperm motility, morphology, and even membrane structure are critical for success: gaining entrance into the oocyte and producing a viable embryo. In order for an embryo to cleave, a multitude of events must occur in sequence. Spermatozoa have to be able to arrive at the oocyte located in the oviduct, and have the capability of gaining entrance.

In the current study, an equal amount of motile spermatozoa (375,000 motile) was added to oocytes (n = 30-45) during *in vitro* fertilization (in a ~500 µl well), but a reduction in penetration rates was still observed for bulls grazing E+ tall fescue pastures. Thus, indicating that no matter how many motile sperm are present, penetration rates are reduced. Even with motility reductions mentioned previously, spermatozoa do not have to traverse the female reproductive tract because of the *in vitro* nature of this study, and therefore are at an advantage even with lower motility. If spermatozoa cannot gain entrance into the oocyte, therein lies a significant problem that cannot be compensated for with increased spermatozoa numbers; therefore, becoming uncompensable (Saacke *et al.* 1994). In other words, increased spermatozoa numbers delivered to the female in cases such as this will not result in an increase in fertility. Saacke *et al.* (1994) explains compensable and uncompensable in terms of semen quality. That which is compensable can be compensated for by increasing sperm dosage to the female, and is thought to be associated with the sperm's incompetence

with regards to making it to the site of fertilization or initiating the block to polyspermy. As mentioned previously, semen quality which is uncompensable will not result in an increase in fertility with increased sperm dosage to the female. This type of trait is thought to be associated with the fertilizing sperm's deficiency in maintaining the fertilization process or possibly sustaining early embryo development (Saacke *et al.* 1994).

Thundathil *et al.* (2000) reported that bulls whose spermatozoa had a high percentage of knobbed acrosome defect displayed a decreased ability to bind to and penetrate the zona pellucida, and the severity of the defect influenced the sperm's zona pellucida binding capability. Spermatozoa with knobbed acrosome defects in the same study also produced lower cleavage, morulae, and blastocyst rates than the control bull. The alteration caused by consuing E+ tall fescue may not be severe enough to be detected via gross semen evaluation or affect subsequent blastocyst development, since spermatozoa in the present study were morphologically normal at collection but still displayed decreased cleavage rates of oocytes (with similar blastocyst development).

Spermatozoa are highly organized and contain a specific set of "safety nets" designed to aid in their journey from ejaculation to the site of fertilization, due to the fact that spermatozoa have limited biosynthetic capability at this point (Amann *et al.* 1993). If this structure is altered or compromised, then reductions in fertility would be seen. It is known that cryopreservation has a negative impact on sperm membrane structures (Parks and Graham 1992), but spermatozoa from bulls grazing NTE tall fescue pastures were cryopreserved identically to spermatozoa from E+ bulls. This indicates that

spermatozoa from E+ bulls have a decreased ability to penetrate oocytes, and maybe a decreased ability to even reach the two extracellular matrices that surround an oocyte because of ultrastructural damage. These matrices are the cumulus matrix and the zona pellucida (Saling 1991); both of which provide ultra-structural support and protection to the ova contained therein. If spermatozoa from bulls grazing E+ pastures are compromised in a way that is undetectable with a microscope (i.e. altered plasma membrane composition leading to weakness, premature acrosome reaction), then this could help explain the decreased penetrating ability seen in this study. It is possible that a deficiency in sperm's ability to bind to the zona pellucida receptors (ZP2 and ZP3) is responsible for penetration failure as well as due to the fact that premature acrosome reaction could exclude spermatozoa from the oocyte (Saling 1991). Gallagher and Senger (1989) reported a reduced percentage of intact acrosomes prior to and after cryopreservation for sperm from semen that was extended with intermediate and high concentrations of ergonovine. These data, coupled with the current study, support a possible problem pertaining to acrosome reaction or plasma membrane structure possibly due to effect of ergot alkaloids. This suggests the need for further examination of molecular components unique to sperm membranes from E+ grazing bulls both before and after cryopreservation.

Purdy and Graham (2004) discovered that addition of cholesterol to spermatozoa prior to cryopreservation led to increased survival of the cryogenic process, and maintenance of fertilization potential. If spermatozoa from E+ grazing bulls have intact but compromised membranes, the addition of cholesterol prior to cryopreservation could lead to increased survival, and it may help to improve

penetrating capability. Cholesterol efflux from the spermatozoon plasma membrane is a first step towards capacitation (Ehrenwald *et al.* 1988). Decreased cholesterol content could be to blame for the instability of associated membranes in sperm from E+ grazing bulls. Thus, further research is essential to understand the questions generated as a result of the present study.

Maternal chromatin status was assessed for presumptive zygotes that were penetrated by at least one sperm. Sperm from bulls grazing E+ tall fescue pastures appeared to hasten meiotic progression in presumptive zygotes. A great percentage of maternal chromatin in presumptive zygotes fertilized with spermatozoa from E+ bulls were in telophase II of the meiotic cell cycle compared to presumptive zygotes fertilized with spermatozoa from NTE bulls, whose maternal chromatin were mostly in the condensed stage at time of evaluation. A previous study with a different sperm preparation indicated that by 8-12 hours post insemination, 80.5% maternal chromatin in monospermic zygotes were in MII-TII (Long *et al.* 1993). This indicates that spermatozoa from E+ bulls accelerated meiotic progression since a larger percentage were in TII as early as 7.5-9.5 hours post insemination. However, it cannot be assumed that all spermatozoa from both treatment groups fertilized oocytes at the same time. It is possible that spermatozoa from bulls grazing E+ that penetrated an oocyte did so more quickly, and therefore were closer to the completion of meiosis at the time of evaluation than bulls grazing NTE tall fescue pastures.

Interestingly, intracellular calcium oscillation parameters (baseline, amplitude, duration, and area under the curve) were all reduced for oocytes fertilized with spermatozoa from bulls grazing E+ tall fescue pastures. However, calcium oscillations

are necessary for all oocyte activation events, including resumption of meiosis (Malcuit *et al.* 2006). It appears that spermatozoa in the current study, while displaying altered intracellular calcium oscillations, were still perfectly capable of inducing meiotic resumption. Furthermore, E+ spermatozoa did this at an accelerated rate compared to NTE spermatozoa. This indicates that hastened meiotic progression may not be as detrimental to early embryo development. However, additional research is required to completely understand the effects that hastened meiotic progression could have on cleavage of presumptive zygotes.

These findings indicate that not only are spermatozoa from E+ bulls less capable of penetrating an oocyte (altered membrane structure, possible decrease in cholesterol content) but these sperm are accelerated in their ability to navigate the meiotic path of an early embryo; hastened meiotic progress may or may not be a negative aspect. Future studies should closely examine sperm membranes, membrane receptors, and their interaction with the zona pellucida of the oocyte to help explain what this study has shown with regards to spermatozoa from bulls grazing E+ tall fescue pastures.

5. 4. 3. Intracellular Calcium Assay

As mentioned previously, oocytes fertilized with spermatozoa from bulls grazing E+ displayed altered intracellular calcium parameters when compared to spermatozoa from bulls grazing NTE tall fescue pastures. Intracellular calcium oscillations are critical for activation success of the oocyte, and the sperm initiates these increases in cytosolic calcium (Malcuit *et al.* 2006). Typically, if an oocyte is fertilized it will exhibit calcium oscillations (Fissore *et al.* 1992) via the phosphoinositide pathway. Altered calcium parameters could compound the problems that an oocyte experiences when faced with

the challenge of making a viable embryo with sperm from bulls grazing E+ tall fescue pastures. The findings in the present study show reduced baseline calcium levels, amplitude of calcium oscillations, and total calcium response in presumptive zygotes fertilized with spermatozoa from bulls grazing E+ tall fescue pastures. All of which are indicative of altered oocyte activation that could further explain decreased cleavage rates.

5.5 Summary and Conclusion

These data suggest that bulls grazing E+ tall fescue pastures exhibited tall fescue toxicosis, which subsequently decreased overall performance and spermatozoa function. Serum prolactin concentrations decreased, gross motility and morphology of semen remained uncompromised, and altered spermatozoa function was observed post-thaw in four instances: CASA semen parameters, penetration assessment, intracellular calcium parameters, and embryo development. These data further suggest that not only are spermatozoa from E+ grazing bulls compromised in terms of characteristics measured by CASA (i.e. path velocities, motility, area of the sperm head), but they also displayed a reduced ability to penetrate oocytes, and an apparent hastening of meiotic progression in fertilized oocytes. Moreover, a deficiency in intracellular calcium oscillations and a reduction in cleavage rates of presumptive zygotes were also seen. Therefore, while gross motility and morphology of spermatozoa from bulls grazing E+ tall fescue pastures do not differ, spermatozoa function post-thaw is impaired in ways undetectable by normal methodologies.

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Vita

Jessica Pegan Harris was born January 1, 1987 in Knoxville, Tennessee. She attended Lenoir City High School and graduated in 2005, after which she attended the University of Tennessee, Knoxville, to obtain her B.S. degree in Animal Science. She graduated from the University of Tennessee, Knoxville in 2009 and began graduate school at the University of Tennessee under the direction of Dr. F. Neal Schrick. In August of 2011, Jessica graduated with a M.S. degree in Animal Science with a concentration in Reproductive Physiology.